On the biosynthesis and evolution of apocarotenoid plant growth regulators.

On the Biosynthesis and Evolution of Apocarotenoid Plant Growth Regulators

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Abstract

Carotenoids are an important source of metabolites with regulatory function, which include the plant hormones abscisic acid (ABA) and strigolactones (SLs), and several recently identified growth regulators and signaling molecules. These carotenoid-derivatives originate from oxidative breakdown of double bonds in the carotenoid polyene, a common metabolic process that gives rise to diverse carbonyl cleavage-products known as apocarotenoids. Apocarotenoids exert biologically important functions in all taxa. In plants, they are a major regulator of plant growth, development and response to biotic and abiotic environmental stimuli, and mediate plant's communication with surrounding organisms. In this article, we provide a general overview on the biology of plant apocarotenoids, focusing on ABA, SLs, and recently identified apocarotenoid growth regulators. Following an introduction on carotenoids, we describe plant apocarotenoid biosynthesis, signal transduction, and evolution and summarize their biological functions. Moreover, we discuss the evolution of these intriguing metabolites, which has not been adequately addressed in the literature.

Keywords: carotenoid, apocarotenoid, strigolactone, abscisic acid, zaxinone, β-cyclocitral, anchorene, plant growth regulators, plant hormones

1. Introduction

Carotenoids are common lipophilic isoprenoid pigments produced by all photosynthetic organisms and numerous non-photosynthetic microorganisms [1, 2]. They are indispensable components of the photosynthetic apparatus, as they protect it and other cellular components from photo-oxidation, and contribute to the light-harvesting process by absorbing light in the blue-green region [1, 3]. Carotenoids are important antioxidants also in non-photosynthetic organisms, including humans. In addition, they stabilize membranes, and are responsible for the bright colors of many fruits, flowers, birds, fishes, and crustaceans [1, 2]. Animals do not synthesize carotenoids *de novo* but obtain them from their diet [1, 3].

Generally, carotenoids are defined by a common C_{40} polyene backbone carrying 3 to 11 conjugated double bonds with different stereo-configurations. Introduction of end-ring structures, hydroxylation, oxygenation, and further derivatization give rise – together with the polyene geometry – to the large structural diversity of the more than 700 known carotenoids [3]. Plants utilize two routes for isopentenyl diphosphate (IPP, C_5) synthesis: the cytosolic mevalonate and the plastid methylerythritol phosphate pathway (MEP) pathway [3], which form the precursor for cytosolic (e.g. sterols), and plastid isoprenoids (e.g. carotenoids), respectively. The colorless carotenoid, 15-*cis*-phytoene (C_{40}), which contains three conjugated double bonds, results from condensation of two molecules of geranylgeranyl diphosphate (GGPP, C_{20}). The latter is formed through condensation of IPP and its isomer dimethylallyl diphosphate (DMAPP) [3, 4]. Desaturation and isomerization reactions, catalyzed in plants and cyanobacteria by four different enzymes but only by one in fungi and non-photosynthetic bacteria, transform phytoene into the red all-*trans*-lycopene, a linear C_{40} polyene with eleven conjugated double bonds. There is some indication that *cis*-configured lycopene precursors, which can accumulate in non-photosynthetic tissues or in leafs of corresponding mutants in prolonged darkness, produce signaling molecules determining different aspects of plant development and regulating the carotenoid biosynthesis

pathway itself, which explains why plants produce them and require the activity of four enzymes, instead of one [1, 5, 6, 7].

Lycopene is the substrate of cyclases that introduce terminal ring structures, giving rise to β- (Fig.1) and α-carotene and the corresponding two branches in plant carotenogenesis. Hydroxylation of β- and α-carotene leads to zeaxanthin and lutein, respectively. Repeated epoxidation of the two β-rings in zeaxanthin yields violaxanthin via the mono-epoxy carotenoid antheraxanthin and builds together with the reverse, de-epoxidation reactions the xanthophyll cycle, a protection mechanism that regulates the level of the particularly photoprotective zeaxanthin [8]. Finally, violaxanthin is converted into neoxanthin that carries an allenic double bond (Fig.2). The carotenoid pattern in different plant cells and tissues is determined by the type of plastids. Chloroplasts contain high levels of carotenoids composed of around 45% lutein, 25–30% β-carotene, and 10–15% each of neoxanthin and violaxanthin, while root leucoplasts harbor only small amounts with a higher ratio of β-branch carotenoids [8].

The polyene structure of carotenoids responsible for their role in photosynthesis, anti-oxidation capacity, and color makes them vulnerable to oxidation that splits double bonds, forming carbonyl products called apocarotenoids (designated based on the C-atom number at the cleavage site, Fig. 1). This cleavage can occur at any of the polyene double bonds, which explains, together with subsequent modifications, the structural diversity and different physicochemical properties of natural apocarotenoids ranging from the tomato fragrance 6-methyl-5-hepten-2-one (C_8) to colorful pigments [9], such as β-citraurin in citrus fruits (C_{30}) or neurosporaxanthin (C_{35}) in the fungus *Neurospora crassa* [10]. Apocarotenoids are an important class of compounds [8, 11], which includes retinal, vitamin A, signaling molecules, e.g. the vertebrate morphogen retinoic acid, the fungal pheromone trisporic acid, the plant hormones abscisic acid (ABA) and strigolactones (SLs), and known or postulated plant growth regulators [12, 13, 14]. Examples for the latter are β-cyclocitral [15], zaxinone [16], and anchorene [17].

Carotenoid cleavage can be catalyzed enzymatically or by reactive oxygen species (ROS). These pigments are continuously oxidized and cleaved in chloroplasts, particularly under high light that increases the formation of ROS, leading to different products, including the stress signal β-cyclocitral [15]. It may be hypothesized that susceptibility to oxidation makes carotenoids an excellent sensor for oxidative stress, which may have been the reason for recruiting apocarotenoids as signals during evolution. The "spontaneous" formation of apocarotenoid signals may have become tightly regulated by evolving cleavage enzymes, mainly members of the carotenoid cleavage dioxygenase family, which target specific double bonds in defined carotenoid or apocarotenoid substrate [18, 19]. Involving enzyme-catalyzed, regulated cleavage may have increased the sensitivity of the system and allowed using the concept of carotenoid cleavage as a sensor for other stimuli, paving the way for the emergence of apocarotenoid plant hormones. Although there is currently no experimental evidence for this scenario, several studies show an increase in CCD-catalyzed apocarotenoid formation in cyanobacteria under stress conditions, e.g. high-light stress [20, 21]. Apocarotenoid volatiles, including β-ionone, released by cyanobacteria, are also allelopathic agents affecting the growth of several cyanobacterial species [22, 23].

In this article, we provide an overview on the function, biosynthesis, and signaling of the known carotenoid-derived hormones ABA and SLs, and describe the role of the recently identified apocarotenoid growth regulators zaxinone, cyclocitral, and anchorene in plant growth and development. In addition, we shed light on the evolution of plant apocarotenoid signaling molecules, which has not been covered in recent literature.

2. Abscisic acid

2.1 Functions of ABA in plants

ABA was discovered in the early 1960s, due to its role in regulating senescence and stress responses in abscising organs. Since then, ABA has been shown to determine many aspects of plant growth and development, which include seed maturation, germination and dormancy, seedling

growth, bud dormancy, shoot branching, and leaf senescence [24], and to be key in stress response and regulating water loss through the control of stomatal closure [25]. This regulation was probably a major factor in the evolution of land plants, ensuring the survival under fluctuating water supply [26, 27]. Indeed, ABA is intricately involved in the adaptation of terrestrial plants to abiotic stress conditions, such as water scarcity, increased salinity and extreme temperatures [24, 28], which also includes modulation of plant architucture, e.g. establishing the appropriate root architecture under drought conditions [24] (Fig.1). Moreover, ABA plays a role in the establishment of fungal symbiosis [29, 30] and is a major component in plant-pathogen response [31].

2.2 ABA biosynthesis and signaling

ABA is a sesquiterpene (C_{15}) that occurs in a defined stereo-configuration in nature [(+)*cis*, *trans*-ABA] (Fig.1). It can be synthesized either "directly" from farnesyl diphosphate (C_{15}) that arises from the condensation of IPP with geranyldiphosphate (C_{10}), or "indirectly" by cleaving carotenoid precursors [25, 32]. Plants utilize the latter route starting as yet poorly understood isomerization of all-*trans*-violaxanthin/-neoxanthin into the corresponding 9-*cis*-/9'-*cis*-isomer, respectively, which are cleaved by nine-*cis*-epoxycarotenoid cleavage dioxygenases (NCEDs) at the C11'-C12' or C11-C12, respectively, to form the ABA precursor xanthoxin (C_{15}) and the corresponding apo-12'-/apo12-carotenoid (C_{25}) [11, 25]. Xanthoxin is then translocated into the cytoplasm, where it is converted by short-chain dehydrogenase reductase (SDR) into abscisic aldehyde. Oxidation of the latter by abscisic aldehyde oxidase (AAO), an enzyme requiring a molybdenum cofactor provided by the sulfurase ABA3, leads to ABA (Fig.2) [25]. A number of phytopathogenic fungi, e.g. *Botrytis cinerea* [32], synthesize ABA – that might affect the immune response in plants - in the cytosol through the cyclization of farnesyl diphosphate and subsequent oxidation steps in a "direct" pathway [33]. This cytosolic route is likely used by ABA producing animal cells, including human cells, which lack carotenoid biosynthesis (see ABA occurrence and evolution).

Besides biosynthesis, plant ABA levels are determined by conjugation and hydroxylation [28]. The conjugation of ABA by the uridine diphosphate glucosyltransferases (UGTs) leads to inactive ABA-glucose ester (ABA-GE) that can be considered as a storage or long-distance, root-to-shoot transport form [25]. Under stress conditions, ABA-GE can be rapidly hydrolyzed into ABA by β-glucosidase and released from vacuoles, providing a fast response to environment changes [34, 35]. ABA can be deactivated by cytochrome P450 707A enzymes (CYP 707), forming unstable 8'-OH-ABA that spontaneously isomerizes to phaseic acid (PA). PA is further catalyzed to the fully inactive dihydrophaseic acid (DPA) and DPA-4-O-β-D-glucoside (DPAG) by the soluble PA reductase and glycosyltransferase, respectively [36]. Interestingly, PA can still act as a low activity hormone and be recognized by some ABA receptors in seed plants [36].

In angiosperms, the core of ABA perception and signaling pathway comprises three major components: the receptor proteins PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) (hereafter referred to as PYL), TYPE 2C PROTEIN PHOSPHATASE (PP2C), and SNF1-RELATED PROTEIN KINASE 2 (SnRK2) [25, 37, 38]. In the absence of ABA, PP2C prevents the SnRK2 kinase activity by dephosphorylating the kinase-activating loop; while in ABA presence, PYL recruits PP2C to form a complex and release SnRK2 kinase activity that stimulates the transcription factors AREB/ABF [ABA-RESPONSIVE ELEMENT (ABRE)-BINDING PROTEIN/ABRE-BINDING FACTOR], including a basic-domain leucine zipper (bZIP) family and the B3-type transcription factor ABA-INSENSITIVE 3 (ABI3), triggering the expression of ABA-responsive genes [39, 40, 41, 42] (Fig.3). This signaling pathway is highly conserved in plants employing ABA for regulating stress response and development [43]. Stomatal closure is caused by the increase in ABA concentration in guard cells, which leads to the formation of the PYL/PP2C complex and the release of the OPEN STOMATA 1 (OST1; SnRK-III) kinase, resulting in the phosphorylation of several target proteins and the activation of plasma membrane anion channels. The latter triggers membrane depolarization

158 followed by K^+ efflux, which decreases turgor pressure, causing stomatal closure (for review, see
159 [44]).

160 The translocation of ABA between tissues and organs is required for systemic stress response [25].
161 ABA exists as both an anionic ABA^- and a protonated ABAH that can passively diffuse across the
162 cell membrane in a limited amount [45]. Due to a p*Ka* of 4.7, most ABA in the cytosol (pH≈7.3)
163 occurs in the non-diffusible ABA^-, which creates a need for active transport. The ATP-BINDING
164 CASSETTE G (ABCG), ABA-IMPORTING TRANSPORTER 1 (AIT1), MULTIDRUG AND
165 TOXIC COMPOUND EXTRUSION (MATE)-type/DTX transporters (DTX50), and AWPM-19
166 family proteins (e.g. the rice OsPM1) transporters [45, 46, 47] can actively deliver ABA to specific
167 tissues in response to developmental and environmental cues (reviewed in [45]). ABA transporters
168 play a key role in plant seed germination, root development, and transpiration [25, 45].
169

170 **2.3 Occurrence and evolution of ABA**

171 ABA has been found among almost all clades of organisms, including cyanobacteria, algae, lichens,
172 sponges, plants, and mammals [48, 49]. However, our knowledge about the biological function,
173 biosynthesis, and possible signaling pathways in lower photosynthetic and non-photosynthetic
174 organisms is quite limited. In lower photosynthetic organisms, several studies connect ABA to
175 abiotic stress [48, 50]. It was also shown that exogenous ABA modulates *Chamydomonas*
176 *reinhardtii* HCO_3^- uptake and that ABA concentration determines the position of this aquatic algae
177 in the water column depending on light intensities, which could be one of the original functions of
178 this hormone in regulating tropisms [50].
179 In plant ABA biosynthesis, the most crucial step is the carotenoid cleavage reaction catalyzed by
180 NCEDs, which are absent in green algae, but present together with their epoxy-substrates (Fig. 2) in
181 bryophytes, such as *Physcomitrella patens* [48, 51, 52]. Hence, it has been supposed that the
182 NCED-mediated-"indirect" pathway evolved during land colonization [52]. The comparative

genomic analysis and functional divergence analysis of NCEDs revealed that duplication and divergence have occurred at the base of lycophytes to angiosperms, and tissue-specific functional divergence of NCED subfamilies was developed [53]. This evolutionary event was probably followed by the CYP707A-mediated ABA degradation, as no encoded *CYP707A* orthologues have been found in *P. patens* [48, 54].

Despite the presence of ABA, plant ABA-mediated PYL receptors are absent in most algae, except *Zygnema circumcarinatum* that contains one *PYL* orthologue named *ZcPYL8* that can inhibit Arabidopsis PP2C independent of ABA [27]. In bryophytes, PYL orthologues of liverwort *Marchantia polymorpha* and moss *P. patens* demonstrate both ABA-independent and ABA-induced activities, while in vascular plants, PYL orthologues of lycophyte *Selaginella moellendorffii* and Arabidopsis have diverged into two groups, exerting both activities or merely an ABA-induced one [27]. The phylogenetic analysis demonstrated that *PYLs* have diverged into two major clades in vascular plants, suggesting a duplication after the separation from bryophytes [55]. Briefly, ABA receptor PYL originated from an ABA-independent algal ancestor diversified and duplicated into two major clades with ABA-independent or ABA-induced activity in the tracheophytes [27].

The number of *PP2C* genes increased from one in the green algae *C. reinhardtii* to 80 in Arabidopsis, suggesting repeated gene duplication events that accompanied the emergence of the PP2C-SnRK2 regulation during land colonization [56, 57]. Arabidopsis *PP2C* genes are classified into ten subgroups (A–J) with diverse functions [58]. For example, subgroup A contains genes involved in ABA signaling, such as *ABI1*, while subgroup B mediates stress-induced MAPK pathway [58]. Subgroup A is already present in green algae, but not in prokaryotes or non-plant eukaryotes, while subgroup B exists in lycophytes and higher plants, indicating both subgroups are specifically evolved *in planta* [56]. The disruption of both subgroup A *PP2CA* homologs in the moss *P. patens* led to ABA hypersensitivity; however, ABA-dependent kinases were slightly activated, indicating moss PP2CA is not the primary factor in releasing their activity, in contrast to the angiosperm PP2CA-SnRK2 signaling [59].

In angiosperm, SnRK2 proteins are presented by three subfamilies (I–III) that are responsible for ABA signaling pathway. Subfamily III is the ancestral one and exists in early-diverging bryophytes [60, 61]. Interestingly, a single copy gene of subclass III *SnRK2* is already present in the charophyte algae *K. nitens*, indicating that subfamily III SnRK2 signaling might have emerged before ABA response during land colonization [60, 61].

Promotors of ABA-inducible genes contain a conserved ABRE motif allowing the binding of ABA-dependent transcription factors, such as bZIP transcription factors [43]. A phylogenetic analysis of bZIPs clustered Arabidopsis ABA-regulated bZIPs together with putative orthologues in the charophyte algae *K. nitens* and the moss *P. patens*, revealing that ABA-regulatory functions appeared early during land colonization that was accompanied by an expansion of bZIP families during terrestrialization [60]. Similarly, B3 family transcription factors, such as ABI3, continuously expanded from 2 members in chlorophyte *C. reinhardtii* to 66 in angiosperm Arabidopsis [60]. Although multiple ABI3-like members emerged during this expansion, phylogenetic analysis demonstrates that only a single ABI3 is present in tracheophytes, indicating that a purge of ABI3-like has occurred but a single ancient ABI3 has been maintained during evolution [60].

3. Strigolactones

3.1 Biological functions of SLs

SLs were firstly discovered in root exudates as germination stimulants of root parasitic plants of the *Orobanchacea* family, which infest many crops, including cereals and *Solanaceae* species, causing tremendous yield losses in temperate and warm zones [62, 63]. Afterward, SLs were shown to play an important role in establishing arbuscular mycorrhizal (AM) symbiosis, by inducing fungal spore germination, hyphal branching and elongation, and hyphopodia formation [64, 65], and to contribute to further interactions with microorganisms, including symbiotic rhizobia [65]. The role of SLs in establishing AM symbiosis explains their release by plants [65] (Fig.1).

Characterization of high-branching/-tillering mutants in different species, including *more axillary growth* (*max*) in Arabidopsis, *ramosus* (*rms*) in pea, *dwarf (d)/high-tillering dwarf* (*htd*) in rice, and *decreased apical dominance* (*dad*) in petunia, unraveled the hormonal function of SLs [4, 66]. Indeed, SLs are a major regulator of plant architecture. This hormone inhibits axillary bud outgrowth through either an auxin-dependent or auxin-independent pathway [66, 67, 68], triggers internode elongation through induction of cell division instead of cell elongation [69, 70, 71], and regulates lateral root growth, root hair elongation, and adventitious root formation [24]. Moreover, SLs are a positive regulator of leaf and floral organ senescence and contribute to biotic and abiotic stress response [66] (Fig.1).

3.2 SL biosynthesis and signaling

Natural SLs are characterized by a methylbutenolide ring (D-ring) linked by an enol ether bridge [(*R*)-configuration] to a tricyclic lactone (ABC-ring) in canonical SLs, including orobanchol-type (C-ring in α-orientation/down) and strigol-type SLs (C-ring in β-orientation/up), or to a different structure in non-canonical ones (Fig.4) [4, 66]. The biosynthesis of SLs starts with the D27-catalyzed isomerization of all-*trans*-β-carotene into 9-*cis*-β-carotene that is cleaved by the carotenoid cleavage enzyme CCD7 to produce 9-*cis*-β-apo-10'-carotenal and β-ionone [72, 73]. Next, CCD8 converts 9-*cis*-β-apo-10'-carotenal through a combination of reactions into the central intermediate carlactone (CL) (Fig.4) that already contains the D-ring characteristic for SLs [74]. CL biosynthesis is conserved in land plants, as shown for *P. patens* [75]. CCD8 enzymes can also produce a C3-hydroxylated CL, which might be a precursor of yet unidentified SLs, from the corresponding precursor [76]. CL is transported into the cytosol where it is converted by CYP enzymes, in particular the Arabidopsis MORE AXILLARY GROWTH1 (MAX1) and its homologs that belong to the 711 clade [4, 77, 78] (Fig.4). MAX1 homologs can be classified into three types (A1, A2, and A3). Type A1 enzymes, including the Arabidopsis AtMAX1 and its orthologues, convert CL into carlactonoic acid (CLA) [79]. Type A2 enzymes, represented by the rice Os900,

transform CL into the 4-deoxyorobanchol (4DO), which involves the generation of CLA and additional oxygenation followed by B/C lactone ring closure [78]. Enzymes of the CYP711A3 type, represented by the rice Os1400, conduct the hydroxylation of 4DO to produce orobanchol [79] (Fig.4). In Arabidopsis, CLA is converted by an unidentified methyltransferase [4, 77, 80] (Fig.4) into methyl carlactonate (MeCLA) that was shown to be oxygenated *in vitro* by LATERAL BRANCHING OXIDOREDUCTASE (LBO), an enzyme required for normal shoot branching in Arabidopsis and a member of the 2-oxoglutarate and Fe-(II)-dependent dioxygenase family, into unidentified product [81]. Recently, it was shown that members of the CYP722C clade from *Solanum lycopersicum*/*Vigna unguiculata* and *Gossypium arboreum* mediate the direct conversion of CLA into orobanchol and 5-deoxystrigol, respectively [82, 83] (Fig.4).

Despite structural diversity, SLs are perceived by the same receptor, DWARF14 (D14), an α/β-hydrolase containing a conserved Ser-His-Asp catalytic triad [84]. Following binding, D14 hydrolyzes the SL ligand into the D-ring that remains covalently bound to the catalytic His residue, forming a covalently linked receptor molecule (CLIM) and releasing the second moiety [85]. CLIM formation is accompanied by changing D14 conformation, which stimulates its interaction with MAX2/DWARF3 (D3), a component of Skp1/Cullin/F-box (SCF)-type E3 ubiquitin ligase [85]. The D14-SCF$^{MAX2/D3}$ complex recruits a subset of transcription factors, such as the Arabidopsis SUPPRESSOR OF MAX2 1-LIKE (SMXL), and triggers their poly-ubiquitination and proteasomal degradation [86, 87, 88] (Fig.5). Thus, the SL signaling cascade relies on regulated protein turnover through the ubiquitin-proteasome pathway, similar to auxin, jasmonic acid, and gibberellic acid [84, 89].

3.3. Evolution of SL biosynthesis and perception

During the evolution, an ancestor of the enzyme D27, which is already present in some cyanobacteria, chlorophyte, and charophyte algae, encountered gene duplication and differentiation into three types, i.e. *D27*, *D27-like1* (*D27L1*) and *D27-like2* (*D27L2*) [90, 91, 92]. Phylogenetic

286 analyses of *D27*, *D27L1*, and *D27L2* sequences from major land plant groups, charophyte algae,
287 and appropriate chlorophyte algae demonstrated that duplication of the ancestral *D27L1* lineage at
288 the basal land plants gave rise to *D27* that represents a neo-functional lineage in embryophytes [91].
289 CCD7 homologs are also already present in algae. However, sequence and substrate specificity of
290 algal and land plants' CCD7 are presumably quite different. Indeed, the essential amino acids for
291 substrate specificity are absent or inconsistent in algal CCD7s [93]. Nevertheless, phylogenetic
292 analysis of *CCD7-like* sequences from chlorophytes, charophytes, and major land plants did not
293 reveal significant gene duplications [91]. Phylogenetic analysis of *CCD8* and *CCD8-like* sequences
294 from major land plants and chlorophyte algae demonstrated that *CCD8* is ancestral in terrestrial
295 plants [93]. The moss *P. patens ccd8* mutant, which showed striking phenotypes, such as more
296 caulonema filament branching, is the first genetic evidence for the production of a strigolactone-like
297 compound in bryophytes [92, 94]. *Ppccd8* phenotypes were restored by a synthetic SL analog *rac*-
298 GR24 and by complementation with pea *CCD8*, identifying *PpCCD*8 as the functional orthologue
299 of CCD8 from the land plants [92, 94]. This conclusion was confirmed by the *in vitro* study of *P.*
300 *patens* CCD8 enzymatic activity [75].
301 Similar to *CCD7* and *CCD8*, *MAX1* is present in a single copy in most of the species, indicating a
302 low frequency of gene duplication during evolution [92]. While *P. patens* and *M. polymorpha*
303 genomes do not encode any CYP711 enzyme, most of the other mosses and liverworts possess
304 *MAX1* mainly as a single copy gene [92, 95]. Unlike the other SL biosynthesis genes, *LBO* may
305 have originated from gene duplication in the ancestral proto-LBO lineage at the base of
306 spermatophytes, leading to separation of Related to Strigolactone Synthesis (RSS) and LBO clades,
307 which appear in both angiosperms and gymnosperms [91]. The phylogenetic studies, which suggest
308 that SL biosynthesis enzymes are ancestral in land plants, support the hypothesis that SL production
309 is specific to terrestrial plants [93].
310 The core SL signaling pathway shares high similarity and common components with that of
311 karrikins, smoke derived compounds that mimic a yet unidentified plant signaling molecule and

trigger seed germination in non-parasitic plants [96]. Indeed, the karrikin receptor, KARRIKIN-INSENSITIVE2 (KAI2)/HYPOSENSITIVE TO LIGHT (HTL)/D14-LIKE (D14L), is a close homolog of D14 [97, 98]. Interestingly, KAI2 is present among all the land plants, including bryophytes, while true D14 is absent in mosses and liverworts [92, 93]. This suggests that the emergence of D14 in seed plants maybe a result of gene duplication of *KAI2* during the evolution of land plants [84, 92]. KAI2 contains the conserved α/β-hydrolase catalytic triad [99] and a lid domain, responsible for its interaction with MAX2 [85]. Interestingly, both *kai2* and *max2* mutants show some identical phenotypes, including increased seed dormancy and lower light sensitivity [100]. Furthermore, it has been reported that KAI2 is essential for the establishment of AM symbiosis in rice [101]. These findings suggest that the D14-dependent signal transduction is likely derived from the karrikin signaling pathway, which is supported by the presence of *KAI2-LIKE* genes in charophytes [93, 102]. Indeed, *MAX2* is present – as a single-copy gene – across more than 50 species of land plants and charophyte algae [102]. On the contrary, SMXL proteins, the proteolytic target of SL and karrikin response, show more diversification and high complexity in the seed plant lineage [91]. Most of the non-seed plants, including liverworts, mosses, hornworts, lycophytes, and monilophytes, possess one SMXL clade most closely to SMXL1, while gymnosperms and angiosperms contain two or four distinct clades of SMXL proteins, respectively [91]. The two, distinct gymnosperm SMXL clades show around 50% identity with SMXL proteins from non-seed plants, indicating equal conservation of the ancestral SMXL sequence in both of them [91]. In contrast, the four angiosperm SMXL clades show more diversity among each other [91]. Further duplication at the base of eudicots gave rise to two further sub-clades: SMXL78, including AtSMXL7/OsD53 and SMXL8, and SMXL39, including SMXL3 and SMXL9, leading to the basal set of six SMXL proteins in eudicots [91]. The late occurrence of SMXL7/D53 protein corroborates the late origin of canonical SL signaling, particularly in angiosperms, which supports the independent origin of SL perception in mosses [91].

4. Novel apocarotenoid signaling molecules

4.1 Zaxinone, a candidate for a novel plant hormone

The biosynthesis of ABA and SLs demonstrates the importance of CCD enzymes and their role in plant responses to environmental and developmental stimuli. Recently, Wang *et al.* conducted a comprehensive survey of *CCD* genes from 69 plant genomes from mosses, ferns to seed plants, which unraveled a new subfamily common in plant species. In *in vitro* studies, a rice representative of this subfamily produced the C_{18} ketone 3-OH-β-apo-13-carotenone (apo-13-zeaxanthinal) from 3-OH-β-apo-10′ -carotenal. The product and the enzyme were designated as zaxinone and ZAXINONE SYNTHASE (ZAS), respectively [16] (Fig.1). ZAS orthologues are widely conserved in land plants, distributed in a taxon-dependent manner. Rice and other cereals contain two sub-clusters, indicating that ZAS may have acquired a new function in *Poaceae* [16, 103]. Interestingly, ZAS orthologues are absent in non-mycorrhizal species, such as *Brassicales* species, including Arabidopsis and *Camelina sativa* [16]. Although the loss of AM symbiosis apparently occurred independently in a wide range of plant taxa [16], the disappearance of *ZAS* from their genomes might be a common feature of this evolutionary event. Indeed, a *Tos17*-insertion *zas* mutant showed decreased mycorrhization, retarded growth, less root zaxinone content, and higher SL content and release [16], which indicates an essential role of zaxinone and ZAS in plant growth, development, biotic interactions and SL homeostasis (Fig.1). Accordingly, exogenous application of zaxinone restored several phenotypes of *zas* mutant, promoted root growth in wild-type seedlings, and decreased SL biosynthesis by reducing transcript levels of SL biosynthetic genes [16]. Zaxinone is a common plant metabolite occurring also in plants lacking *ZAS* genes [16, 104]. Interestingly, zaxinone is also a regulatory metabolite in Arabidopsis; however, it promotes there SL and ABA biosynthesis, in contrast to its activity in rice [105]. Indeed, we are at the very beginning of understanding the biology of zaxinone that may be a new carotenoid-derived plant hormone or a precursor thereof.

4.2 β-cyclocitral, a novel abiotic stress signal and growth regulator

β-cyclocitral (C_{10}) is a volatile originating from β-carotene either through singlet oxygen $^1O_2$ attack in photosynthetic tissues or by lipooxygenases, which utilize carotenoids as co-substrates during fatty acid oxidation, and some CCD enzymes, such as CCD4b in citrus fruits [106, 107, 108, 109] (Fig.1). The formation of cyclocitral in plastids by $^1O_2$ makes it a suitable candidate for sensing high-light/oxidative stress and communicating it to the nucleus. Indeed, β-cyclocitral acts in Arabidopsis as a retrograde signal modulating the transcription of $^1O_2$-regulated genes, via a small zinc finger protein called METHYLENE BLUE SENSITIVITY ~~protein~~, which enables acclimation to photo-oxidative stress [110, 111, 112]. Very recently, it has been shown that the conversion of β-cyclocitral into β-cyclocitric acid, a reaction that occurs spontaneously in water, is an initial step in β-cyclocitral signaling and that exogenous application of β-cyclocitric acid triggers the expression of β-cyclocitral induced and $^1O_2$-regulated genes [113]. Moreover, β-cyclocitric acid application promoted the expression of several water stress-responsive genes under sufficient water supply and increased Arabidopsis tolerance to drought, indicating a potential for application in agriculture [113]. However, the mechanism underlying this effect is still elusive. Interestingly, β-cyclocitral acts also as a growth regulator, increasing root growth and branching by promoting stem cell divisions. It can be speculated that the growth-regulating activity of β-cyclocitral is linked to its general function in abiotic stress response. Supporting this hypothesis, the exogenous application of β-cyclocitral under high-salinity conditions increased shoot and root growth in plants [15].

4.3 Anchorene, a representative of overlooked diapocarotenoid signaling molecules?

Carotenoids can be repeatedly cleaved at different double bonds, which generates dialdehyde products (diapocarotenoids) [9, 21]. However, instability and low abundance impeded the exploration of possible regulatory functions of diapocarotenoids that have been mainly studied as precursors of natural pigments, such as crocin in saffron stigma [114]. Recently, screening for bioactive, presumably carotenoid-derived dialdehydes, using synthetic compounds and under light

conditions that increase diapocarotenoid stability, revealed a C_{10}-dialdehyde, anchorene, as a specific regulator of the growth of anchor roots in Arabidopsis [17]. Anchor roots are a less investigated type of Arabidopsis secondary roots that emerge in the collet region located at the root hypocotyl junction [17] (Fig.1). Confirming its specificity, modifications of anchorene structure significantly diminished the ability to induce anchor root formation. Thus, the application of iso-anchorene, a structural isomer of anchorene, which differs in the position of a methyl group, led to a significant decrease of primary root growth and a slightly increase in anchor root formation (unpublished data). Mutant and chemical inhibitor studies demonstrated that carotenoid biosynthesis is required for anchor root growth and that anchorene can replace this pathway in exerting this function [17]. Although the biosynthesis of anchorene is still elusive, LC-MS analysis confirmed its being a natural metabolite and indicated its carotenogenic origin [17]. Studies employing auxin reporter lines and an auxin transport inhibitor, together with transcriptome analysis, demonstrated that anchorene promotes anchor root formation by altering auxin homeostasis [17]. Interestingly, anchor root formation as well as anchorene content increase under nitrogen deficiency [17], indicating a role in the regulation of nitrogen uptake in Arabidopsis. The discovery of anchorene demonstrates that diapocarotenoids may also have growth regulatory functions, similar to their apocarotenoid counterparts.

Conclusion and Future Perspective

The carotenoid biosynthesis pathway is an essential source of signaling molecules and hormones involved in virtually all aspects of the plant's life. These metabolites arise through a common metabolic process, i.e. oxidative cleavage of carotenoids, which yields apocarotenoids. Further conversion of the primary cleavage products increases the structural and functional diversity of this class of compounds. Non-enzymatic formation of apocarotenoids, which accompanies photosynthesis, may explain why they were recruited as signaling molecules and modified them later to hormones with the corresponding perception and signal transduction machinery. Recently

discovered apocarotenoid signaling molecules/hormone candidates will unravel overlooked aspects in plant development, resilience, and biotic interactions, emphasizing the importance of apocarotenoids for basic and applied plant science.

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Conflict of Interest Statement

The authors declare no any competing financial interests.

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Legends to Figures

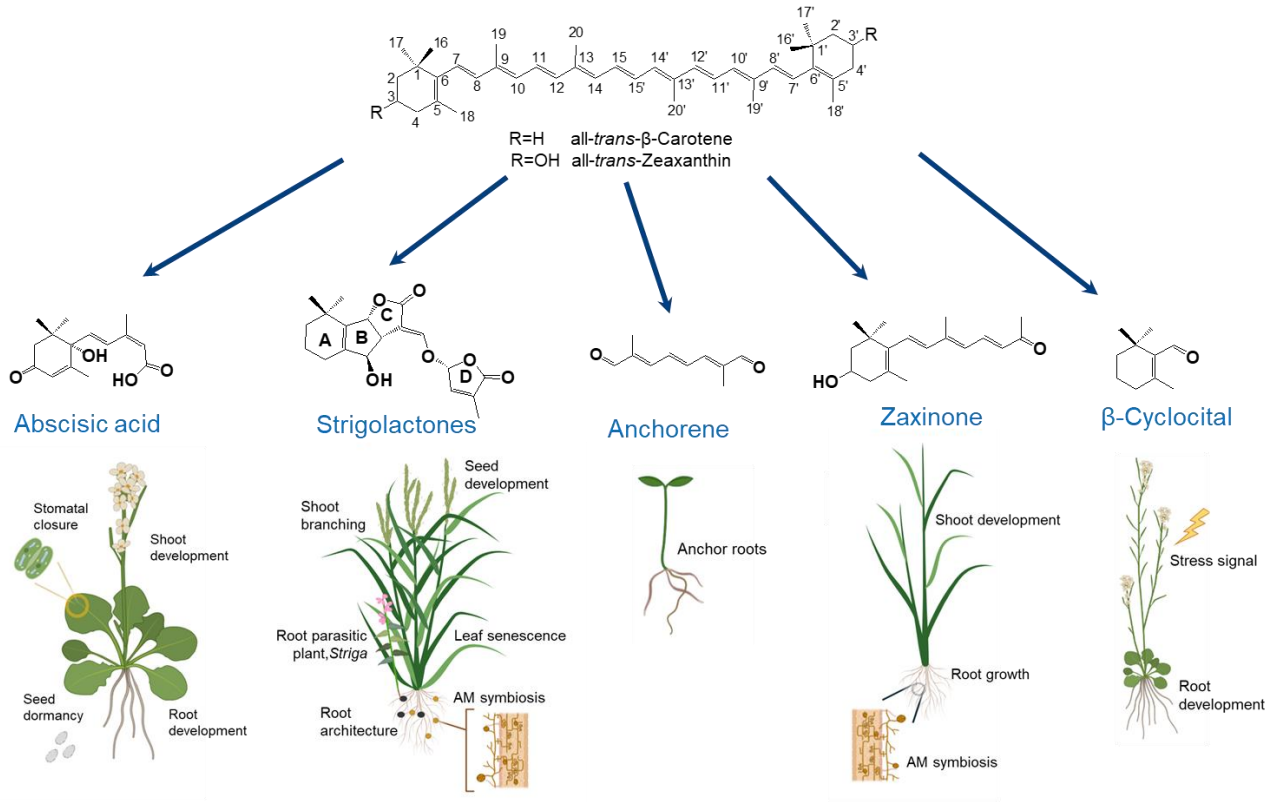

Figure 1. Biological functions of plant apocarotenoids

The upper structure shows all-*trans*-β-carotene with C-atom numbering. Depicted signaling molecules are formed through oxidative cleavage of double bonds, frequently followed by enzymatic conversions. ABA regulates seed dormancy, stomatal closure, root and shoot development, and biotic/abiotic stress responses. SLs, e.g. orobanchol, determine plant architecture and mediate rhizospheric interactions. Zaxinone, formed by zaxinone synthase (ZAS), is required for normal rice growth and development and is involved in AM symbiosis. β-cyclocitral, formed by ROS ($^1O_2$) attack, CsCCD4 or lipoxygenases, mediates high-light stress response, abiotic stress response and promotes root growth. Anchorene, a diapocarotenoid, stimulates anchor root formation in Arabidopsis. Created with 'Biorender'.

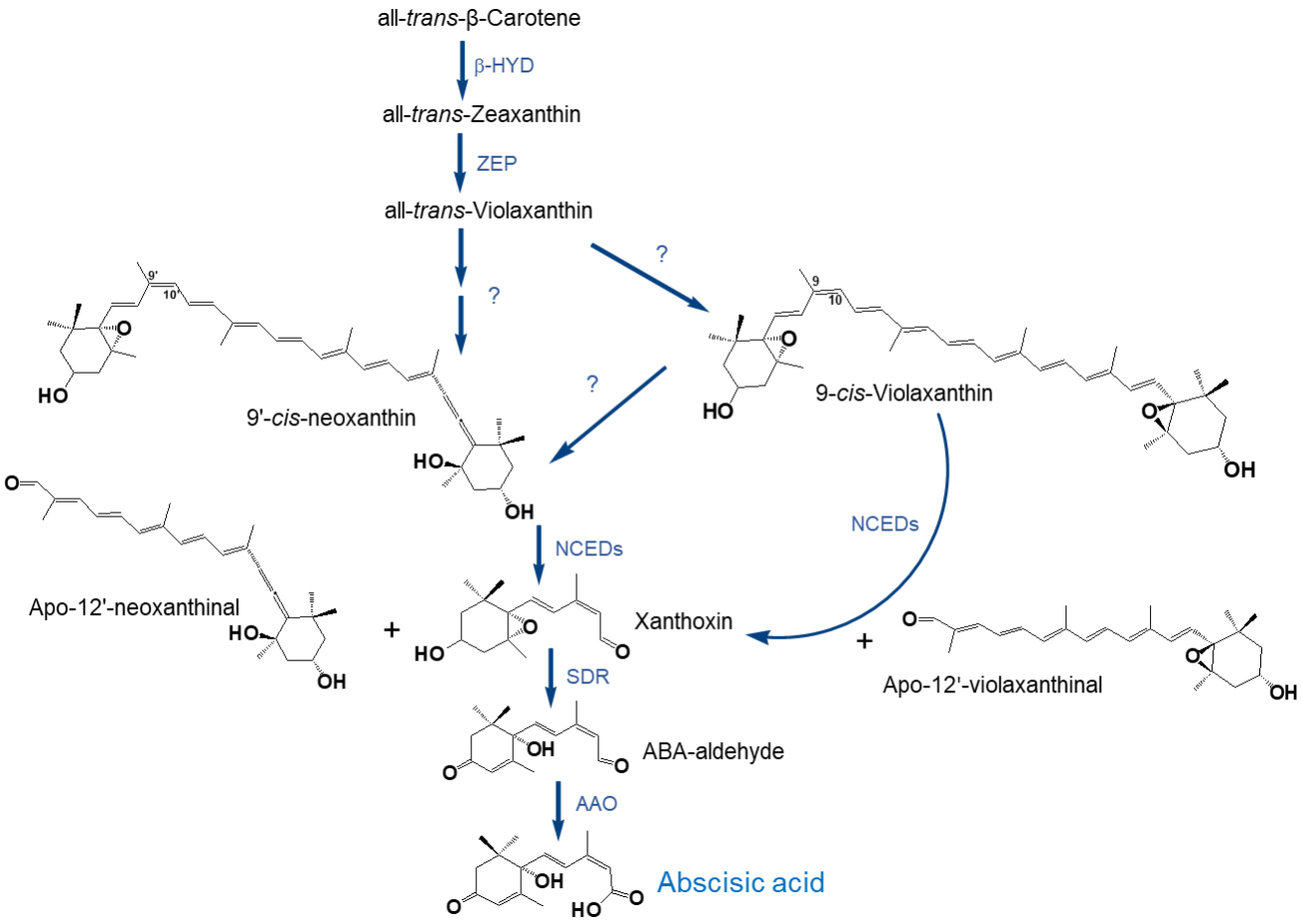

Figure 2. ABA biosynthesis

β-hydroxylases convert all-*trans*-β-carotene into all-*trans*-zeaxanthin. Two epoxidation reactions, catalyzed by ZEP, transform the latter into all-*trans*-violaxanthin. Nine-*cis*-Epoxycarotenoid Dioxygenases cleave 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin, which formation is still elusive, yielding the ABA precursor xanthoxin (C_{15}) and the corresponding apo-12-carotenoid (C_{25}). Xanthoxin is then converted to ABA by SDR and AAO via ABA-aldehyde in the cytosol. Abbreviations: β-HYD, β-hydroxylase; ZEP, zeaxanthin epoxidase; SDR: short-chain dehydrogenase reductase; AAO: Abscisic aldehyde oxidase.

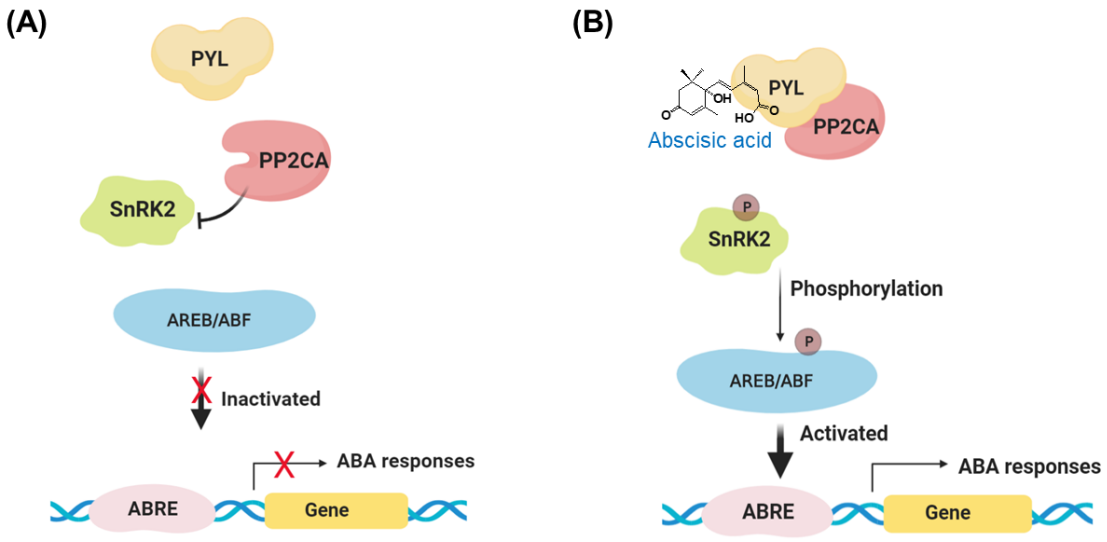

Figure 3. Scheme of ABA signaling

(A) PP2C represses SnRK2 activity by dephosphorylating its kinase-activating loop. **(B)** Binding of ABA leads to the formation of a complex containing the receptor (PYR/PYL/RCAR) and PP2C, which inhibits PP2C and releases SnRK2 kinase activity. The latter triggers downstream ABA-responsive element (ABRE)-binding protein/ABRE-binding factor (AREB/ABF) transcription factors that induce the ABA response. PP2C, protein phosphatase 2C; SnRK2, Snf1-related protein kinase 2; PYR, pyrabactin resistance; PYL, PYR1-LIKE; RCAR, regulatory component of aba receptor. Created with 'Biorender'.

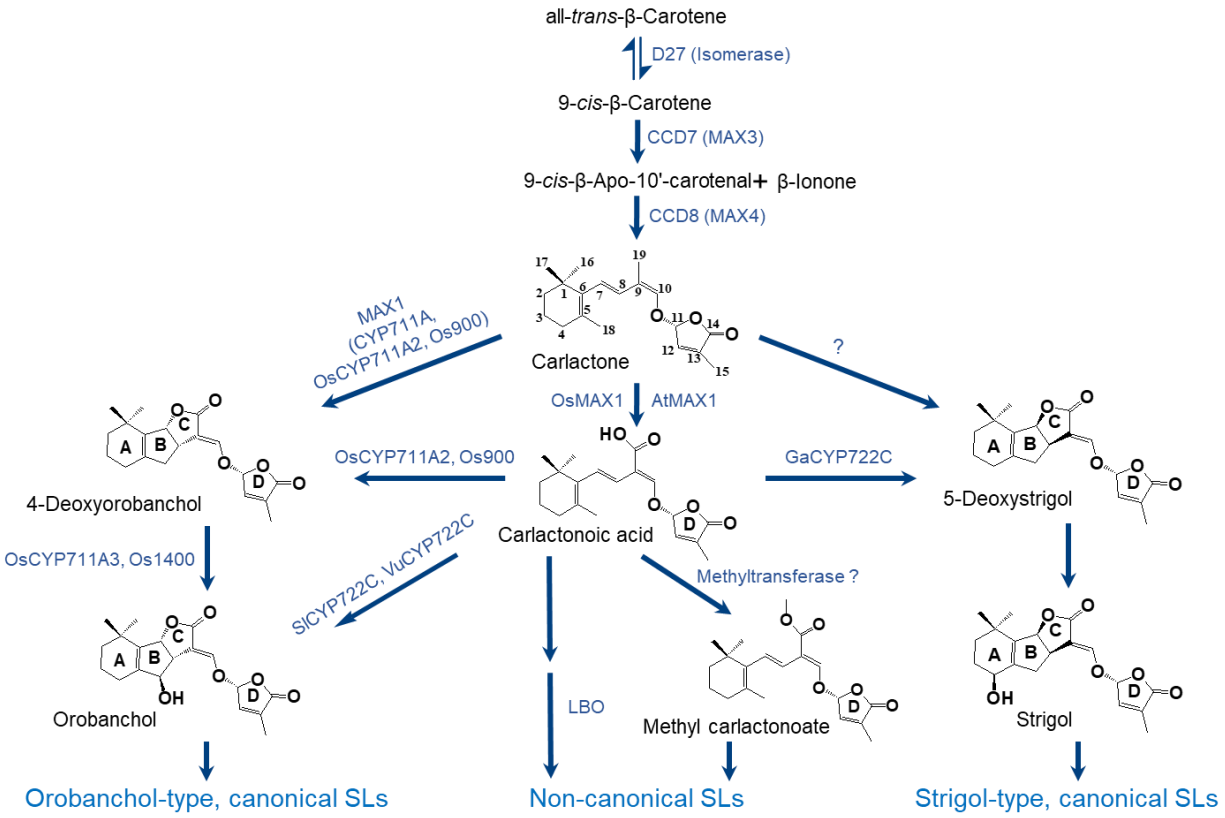

Figure 4. SL biosynthesis

D27 isomerizes all-*trans*- to 9-*cis*-β-carotene that is cleaved by CCD7 to produce 9-*cis*-β-apo-10'-carotenal and β-ionone. Through a combination of reactions, CCD8 transforms 9-*cis*-β-apo-10'-carotenal to carlactone (CL). CL is further modified by cytochrome P450 enzymes to form different types of canonical, e.g. 4-deoxyorobanchol, and non-canonical, e.g. carlactonoic acid, SLs. SL biosynthesis involves further enzymes, such as LBO and a methyl transferase. Abbreviations: MAX: more axillary growth; LBO, lateral branching oxidoreductase; Os, *Oryza sativa*; Sl, *Solanum lycopersicum*; Vu, *Vigna unguiculata*; Ga, *Gossypium arboreum*; At, *Arabidopsis thaliana.*

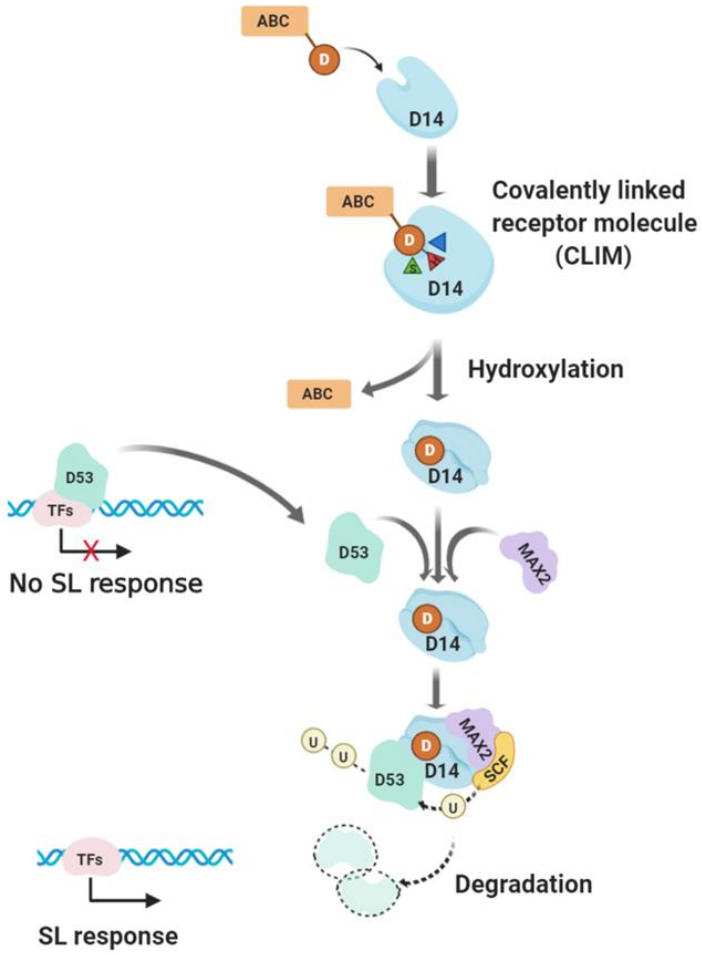

Figure 5. Scheme of SL signaling

The receptor D14 binds and hydrolyzes SLs, forming a covalently linked receptor molecule (CLIM) that contains the SL D-ring (linked to the catalytic His-residue), and releasing the second SL moiety. These steps are accompanied by a conformational change of D14, which promotes the interaction with MAX2 and repressor proteins, e.g. the Arabidopsis SMXL or the rice D53. The recruitment into the SCF complex leads to polyubiquitination and proteasome-mediated degradation of repressor proteins. Abbreviations: MAX: more axillary growth. D14, DWARF14; SMXL, SUPPRESSOR OF MAX2 1-LIKE; D53, DWARF53. Created with 'Biorender'.