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REVIEW PAPER

From carotenoids to strigolactones

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Abstract

Strigolactones are phytohormones that regulate various plant developmental and adaptation processes. When released into soil, strigolactones act as chemical signals, attracting symbiotic arbuscular mycorrhizal fungi and inducing seed germination in root-parasitic weeds. Strigolactones are carotenoid derivatives, characterized by the presence of a butenolide ring that is connected by an enol ether bridge to a less conserved second moiety. Carotenoids are isopenoid pigments that differ in structure, number of conjugated double bonds, and stereoconfiguration. Genetic analysis and enzymatic studies have demonstrated that strigolactones originate from all-*trans*-β-carotene in a pathway that involves the all-*trans*-/9-*cis*-β-carotene isomerase DWARF27 and carotenoid cleavage dioxygenase 7 and 8 (CCD7, 8). The CCD7-mediated, regiospecific and stereospecific double-bond cleavage of 9-*cis*-β-carotene leads to a 9-*cis*-configured intermediate that is converted by CCD8 via a combination of reactions into the central metabolite carlactone. By catalyzing repeated oxygenation reactions that can be coupled to ring closure, CYP711 enzymes convert carlactone into tricyclic-ring-containing canonical and non-canonical strigolactones. Modifying enzymes, which are mostly unknown, further increase the diversity of strigolactones. This review explores carotenogenesis, provides an update on strigolactone biosynthesis, with emphasis on the substrate specificity and reactions catalyzed by the different enzymes, and describes the regulation of the biosynthetic pathway.

Keywords: Carlactone, carotenoid cleavage dioxygenase, carotenoids, CCD7, CCD8, DWARF27, MAX1, strigolactone biosynthesis.

Introduction

Strigolactones (SLs), which were named after their activity as germination stimulants of root-parasitic plants of the genus *Striga* (from the Latin for 'witch'), are intriguing compounds that act as plant hormones (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008) and as inter-specific communication signals (Akiyama *et al.*, 2005; Butler, 1994; Cook *et al.*, 1966). SLs regulate various aspects of plant development, such as inhibiting shoot branching (the best-known SL hormonal function), shaping root architecture, promoting leaf senescence, and regulating secondary growth (reviewed by Al-Babili and Bouwmeester, 2015; Brewer *et al.*, 2013; de Saint Germain *et al.*, 2013; Flematti *et al.*, 2016; Koltai, 2011; Ruyter-Spira *et al.*, 2013; Seto and Yamaguchi, 2014; Smith and Li, 2014; Waldie *et al.*, 2014; Waters *et al.*, 2017). In addition, SLs may contribute to biotic and abiotic stress responses (Decker *et al.*, 2017; Liu *et al.*, 2015; Torres-Vera *et al.*, 2014; Van Ha *et al.*, 2014). SLs are also released by plant roots to attract arbuscular mycorrhizal fungi for building a symbiosis that provides the plants with minerals and water and supplies the fungal partner with carbohydrates (Akiyama *et al.*, 2005; Bonfante and Genre, 2015; Gutjahr and Parniske, 2013). In the course of evolution, SLs have been co-opted by root-parasitic plants to recognize their hosts. Seeds of these obligate parasites germinate only upon perceiving SLs released into

the substrate, which indicate the presence of a host needed for their survival (Khosla and Nelson, 2016; Xie *et al.*, 2010; Zwanenburg *et al.*, 2016). *Striga* and related parasitic weeds infest many crops, such as cereals, tomato, and sunflower, negatively affecting yields and representing a severe agricultural problem in sub-Saharan Africa, southern Europe, the Middle East, and Asia (Parker, 2009).

Natural SLs (Fig. 1) are carotenoid derivatives characterized by the presence of a butenolide ring (D ring) in the *R* configuration, which is connected via an enol ether bridge to a structurally variable second moiety (Al-Babili and Bouwmeester, 2015). SLs are classified according to their variable moiety into canonical, tricyclic lactone (ABC ring)-containing SLs, such as strigol, the first SL to be identified (Cook *et al.*, 1966), and non-canonical SLs, such as methyl carlactonoate (Abe *et al.*, 2014), heliolactone (Ueno *et al.*, 2014), and zealactone (Charnikhova *et al.*, 2017; Xie *et al.*, 2017) which have different structural elements (Fig. 1). Canonical SLs are divided into the strigol- and orobanchol-like SLs, which differ in the stereochemistry of the BC ring junction (Ueno *et al.*, 2011; Xie *et al.*, 2013). Orobanchol-like SLs contain the C ring in α orientation (Fig. 1, type I compounds, 8b*R* configuration), while the C ring in strigol-like SLs is in β orientation (Fig. 1, type II compounds, 8b*S* configuration). Modifications of the AB ring in canonical SLs and the presence of different structures coupled to the conserved D ring in non-canonical

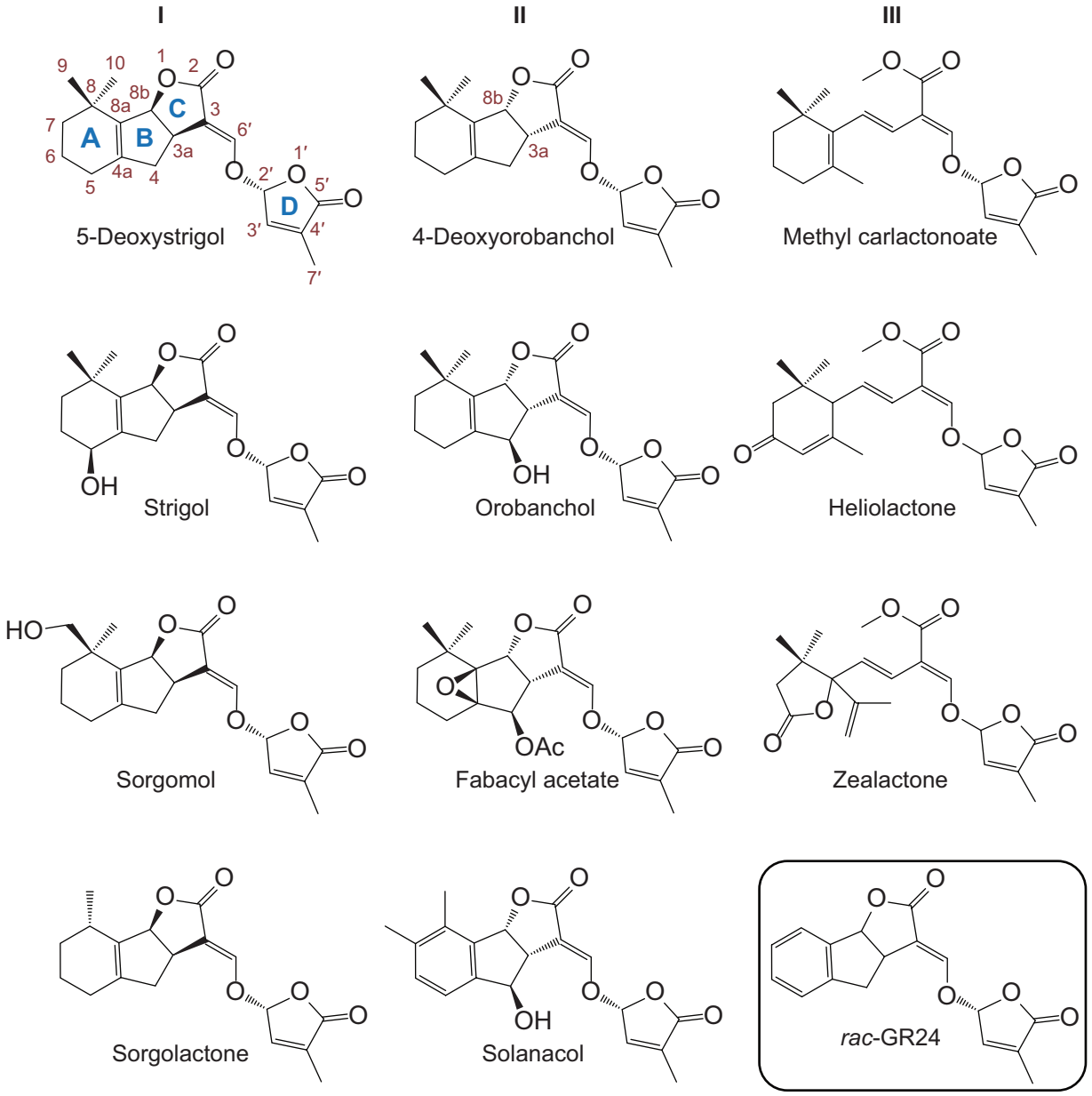

Fig. 1. Structures of natural strigolactones (SLs). Natural SLs are classified into canonical SLs (I and II), which contain the tricyclic ABC-lactone, and non-canonical SLs (III) which possess a variable corresponding moiety. Depending on the stereochemistry of the BC ring junction at the chiral centers C8b and C8a, canonical SLs are divided into strigol-like (I) and orobanchol-like (II) SLs. Modifications of the ABC-lactone in canonical SLs and the presence of different structures in non-canonical SLs give rise to the diversity of natural SLs. The structure of the commonly used synthetic SL analog GR24 is shown in the inset. (This figure is available in colour at *JXB* online.)

SLs give rise to the diversity of this group of plant hormones. The two canonical SLs, strigol (Cook *et al.*, 1966), which can be structurally derived from 5-deoxystrigol, and orobanchol (Yokota *et al.*, 1998), which is synthesized from *ent*-2′-*epi*-5-deoxystrigol (4-deoxyorobanchol, see below) were designated on the basis of their activity in inducing seed germination in *Striga* and *Orobanche* spp., respectively. For historical reasons, it is common to use these two SLs as references in the nomenclature of other SLs that are either structural derivatives, such as 7-hydroxyorobanchol or 5-deoxystrigol, or stereoisomers, that is, enantiomers that are a mirror image of the reference, or epimers that have an opposite orientation at a single C atom. The abbreviations *ent* and *epi* are usually used to designate these two types of stereoisomers, respectively. However, these abbreviations can be quite confusing, as in the case of *ent*-2′-*epi*-5-deoxystrigol. Therefore, here we use the name 4-deoxyorobanchol instead of *ent*-2′-*epi*-5-deoxystrigol, as suggested previously (Al-Babili and Bouwmeester, 2015; Scaffidi *et al.*, 2014). In addition, we refer to 5-deoxystrigol and 4-deoxyorobanchol as the parent molecules of the two families of canonical SLs.

Recently, great progress has been made in elucidating key steps of SL biosynthesis and signal transduction, enabled by the availability of corresponding mutants from different plant species. These mutants were isolated owing to their more branching/high tillering phenotype and designated as more axillary growth (*max*) in Arabidopsis (Booker *et al.*, 2004, 2005; Sorefan *et al.*, 2003; Stirnberg *et al.*, 2007), *ramosus* (*rms*) in pea (Beveridge *et al.*, 1996; Foo *et al.*, 2005; Morris *et al.*, 2001; Sorefan *et al.*, 2003), *dwarf* (*d*)/high tillering dwarf (*htd*) in rice (Arite *et al.*, 2007, 2009; Ishikawa *et al.*, 2005; Jiang *et al.*, 2013; Lin *et al.*, 2009; Zhou *et al.*, 2013), and decreased apical dominance (*dad*) in petunia (Drummond *et al.*, 2009, 2011; Hamiaux *et al.*, 2012; Simons *et al.*, 2007; Snowden *et al.*, 2005). The more branching/high tillering phenotype is caused by a deficiency in the biosynthesis or perception of a proposed shoot branching inhibitory signal that was later identified as SL. Table 1 gives an overview of the identified SL-related mutants and the function of the corresponding genes.

SLs are perceived by an α/β-hydrolase [DWARF14 (D14) in rice] that conveys the signal to a leucine-rich-repeat F-box protein (MAX2 in Arabidopsis; D3 in rice), which acts as the recognition subunit in an SKP1-CUL1-F-box-protein (SCF)-type ubiquitin ligase complex and initiates the 26*S* proteosomal degradation of transcription repressors, such as SMXLs in Arabidopsis and D53 in rice (Jiang *et al.*, 2013; Soundappan *et al.*, 2015; Stanga *et al.*, 2013; Wang *et al.*, 2015; Zhou *et al.*, 2013). D14 contains a conserved catalytic serine-histidine-aspartic acid triad that is required for the hydrolytic activity (Hamiaux *et al.*, 2012). It was shown that the petunia D14 (DAD2) binds and hydrolyzes the SL analog GR24 (Fig. 1), and that this hydrolytic activity is indispensable for the protein–protein interaction of DAD2 with PhMAX2 (Hamiaux *et al.*, 2012). The crystal structure of the SL-induced AtD14-D3-ASK1 complex reveals that, upon SL hydrolysis, AtD14 covalently binds the SL D ring and undergoes an open-to-closed state conformational change to facilitate interaction with D3 (Yao *et al.*, 2016). Covalent binding of the D ring by D14 and orthologs has also been shown for the pea D14 (RMS3) (de Saint Germain *et al.*, 2016) and the *Striga* ShHTL7 (Yao *et al.*, 2017). For recent reviews about

Table 1. *Genes involved in strigolactone biosynthesis and signaling*

Role	Protein identity/function	Gene name			
		Arabidopsis	**Pea**	**Petunia**	**Rice**
SL biosynthesis	9-*cis*/all-*trans*-β-carotene isomerase	*AtD27*	–	–	*D27*
	Carotenoid Cleavage Dioxygenase 7 (CCD7)	*MAX3*	*RMS5*	*DAD3*	*HTD1/D17*
	Carotenoid Cleavage Dioxygenase 8 (CCD8)	*MAX4*	*RMS1*	*DAD1*	*D10*
	Cytochrome P450, 711 (CYP711)	*MAX1*	–	*PhMAX1*	*Carlactone Oxidase* (*Os01g0700900*), *Orobanchol Synthase* (*Os01g0701400*), *Os01g0701500*, *Os02g0221900*, *Os06g0565100*
	Lateral Branching Oxidoreductase	*LBO*	–	–	–
SL perception/signaling	α/β-hydrolase	*AtD14*	*RMS3*	*DAD2*	*D14/D88/HTD2*
	F-box protein	*MAX2*	*RMS4*	*PhMAX2A*, *PhMAX2B*	*D3*
	Repressor of SL signaling	*SMXLs*	–	–	*D53*
	Corepressors	*TPR2*	–	–	*TPL/TPR2*
	Transcription factors	*BRC1, BRC2*	–	–	*IPA1*

BRC, Branching; D, Dwarf; DAD, Decreased apical dominance; HTD, High tillering dwarf; IPA, Ideal plant architecture; LBO, Lateral branching oxidoreductase; MAX, More axillary growth; RMS, Ramosus; SL, strigolactone; SMXL, SMAX1-LIKE; TPL/TPR, Topless/Topless-related protein.
– indicates that the corresponding gene has not yet been identified.

SL signaling, see Flematti *et al.*, (2016). Lumba *et al.* (2017), and Waters *et al.* (2017).

Owing to structural similarities, SLs were originally considered to belong to the sesquiterpene lactones (Akiyama *et al.*, 2005; Butler, 1994), a widespread family of compounds formed from the cytosolic compound farnesyl diphosphate. However, analysis of root exudates from maize carotenoid biosynthesis mutants and the application of fluridone, an inhibitor of the phytoene desaturation step in the plant carotenoid biosynthesis pathway (see later), suggested that SLs derive from carotenoids (Matusova *et al.*, 2005). In the absence of knowledge about the enzymes involved, it was proposed that SLs arise from a carotenoid cleavage product converted via a complicated series of reactions into the ABC ring, which is linked in a final reaction to a D ring of a different origin (Matusova *et al.*, 2005). It is now known that the entire C skeleton of SLs derives from carotenoids and that the pathway from carotenoids to SLs requires fewer enzymes than originally supposed (Fig. 2), a result of the unusual activity of carotenoid cleavage dioxygenase 8 (CCD8) in simultaneously catalyzing a combination of reactions that lead to the production of carlactone, a compound that already contains the SL D ring (Al-Babili and Bouwmeester, 2015; Alder *et al.*, 2012). The SL biosynthetic pathway (Fig. 2) is also characterized by strict stereospecificity, involving a novel *cis*/*trans*-isomerase, the all-*trans*/9-*cis*-β-carotene isomerase DWARF27 (D27), and CCD7, which supplies the next enzyme in the pathway, CCD8, with the substrate in the required stereoconfiguration. The activity of D27, CCD7, and CCD8 forms a core pathway that branches, leading to different types of SLs synthesized by cytochrome P450 (CYP) enzymes of the 711 clade (Abe *et al.*, 2014; Alder *et al.*, 2012; Zhang *et al.*, 2014). The recently identified Lateral Branching Oxidoreductase (LBO), which may catalyze the final step in SL biosynthesis in Arabidopsis, is an example of other enzymes that contribute to the diversity of SLs (Brewer *et al.*, 2016).

In this review, we provide an update on SL biosynthesis. We briefly describe plant carotenoid biosynthesis and the carotenoid cleavage enzymes and focus on the reactions catalyzed by the enzymes D27, CCD7, CCD8, and MAX1. We also report recent advances in knowledge about the regulation of SL formation.

Carotenogenesis in plants

Carotenoids are widespread terpenoid pigments. They are equipped with a system of conjugated double bonds that is responsible for their colors. Carotenoids are divided into the oxygen-free carotenes and their oxygenated derivatives, the xanthophylls. In addition, they are distinguished by the structure of their end groups (acyclic or linear, monocyclic, and bicyclic) and by the stereoconfiguration (i.e. *cis*/*trans*-configuration) of their double bonds. Carotenoids are formed by all photosynthetic organisms and by many fungi and heterotrophic bacteria (DellaPenna and Pogson, 2006; Walter and Strack, 2011), and are indispensable for photosynthetic organisms, as they play a vital role in protecting chlorophylls from photo-oxidation and as constituents of the light-harvesting complexes (Hashimoto *et al.*, 2016). In plants, carotenoid biosynthesis (Fig. 3) takes place in the plastids. It is initiated by the condensation of two molecules of geranylgeranyl diphosphate (C_{20}) to 15-*cis*-phytoene (C_{40}); for reviews, see (DellaPenna and Pogson, 2006; Fraser and Bramley, 2004; Moise *et al.*, 2014; Walter and Strack, 2011). A sequence of desaturation reactions increases the number of conjugated double bonds from 3 in the colorless 15-*cis*-phytoene to 11 in the red all-*trans*-lycopene, accompanied by *cis*/*trans*-isomerization steps. Cyclization reactions convert all-*trans*-lycopene into all-*trans*-β-carotene and all-*trans*-α-carotene, dividing the pathway into the α- and β-branches. Hydroxylation of the two ionone rings in α-carotene and β-carotene leads to all-*trans*-lutein and all-*trans*-zeaxanthin, respectively. All-*trans*-zeaxanthin is interconverted into all-*trans*-violaxanthin, which is formed by the reversible epoxidation of the β-ionone rings. All-*trans*-violaxanthin is the precursor of all-*trans*-neoxanthin, the final product of the β-branch. Cyclic carotenoids can also occur in different *cis*-configurations, such as the abscisic acid (ABA) precursor 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin; however, knowledge of the formation of such isomers is in most cases still elusive (Schwartz *et al.*, 1997).

In plants, all types of plastids can synthesize carotenoids (Howitt and Pogson, 2006). However, the composition and amounts of accumulated carotenoids depend on the type of plastid (Howitt and Pogson, 2006; Ruiz-Sola and Rodríguez-Concepción, 2012). Chromoplasts contain high amounts of a certain carotenoid, for example, lutein in daffodil flowers or lycopene in tomato fruits (DellaPenna and Pogson, 2006; Fraser and Bramley, 2004), while the highly accumulated chloroplast carotenoids comprise 45% lutein, 25–30% β-carotene, and 10–15% each of violaxanthin and neoxanthin (Goodwin, 1988; Lakshminarayana *et al.*, 2005). Root leucoplasts contain only low amounts of carotenoids, consisting of around 30% lutein, 25% β-carotene, and 45% β-xanthophylls, as was shown for Arabidopsis (Britton, 1995; Maass *et al.*, 2009).

Carotenoid cleavage dioxygenases

Owing to its susceptibility to oxidative cleavage, the polyene system of carotenoids gives rise to their role as precursors of a large number of compounds that are generally known as apocarotenoids (Giuliano *et al.*, 2003; Nisar *et al.*, 2015; Walter and Strack, 2011). These cleavage products are usually designated based on the C-atom number at the cleavage site that leads to their formation (for C-atom numbering, see Fig. 3). Apocarotenoids and their derivatives include biologically important molecules such as the vision chromophore retinal (Moise *et al.*, 2005), the vertebrate morphogen retinoic acid (Rhinn and Dollé, 2012), the phytohormones ABA (Schwartz *et al.*, 1997) and SLs (Alder *et al.*, 2012), and the fungal morphogen trisporic acid (Medina *et al.*, 2011). Carotenoid cleavage can be triggered by reactive oxygen species, which arise particularly under high-light stress. It can

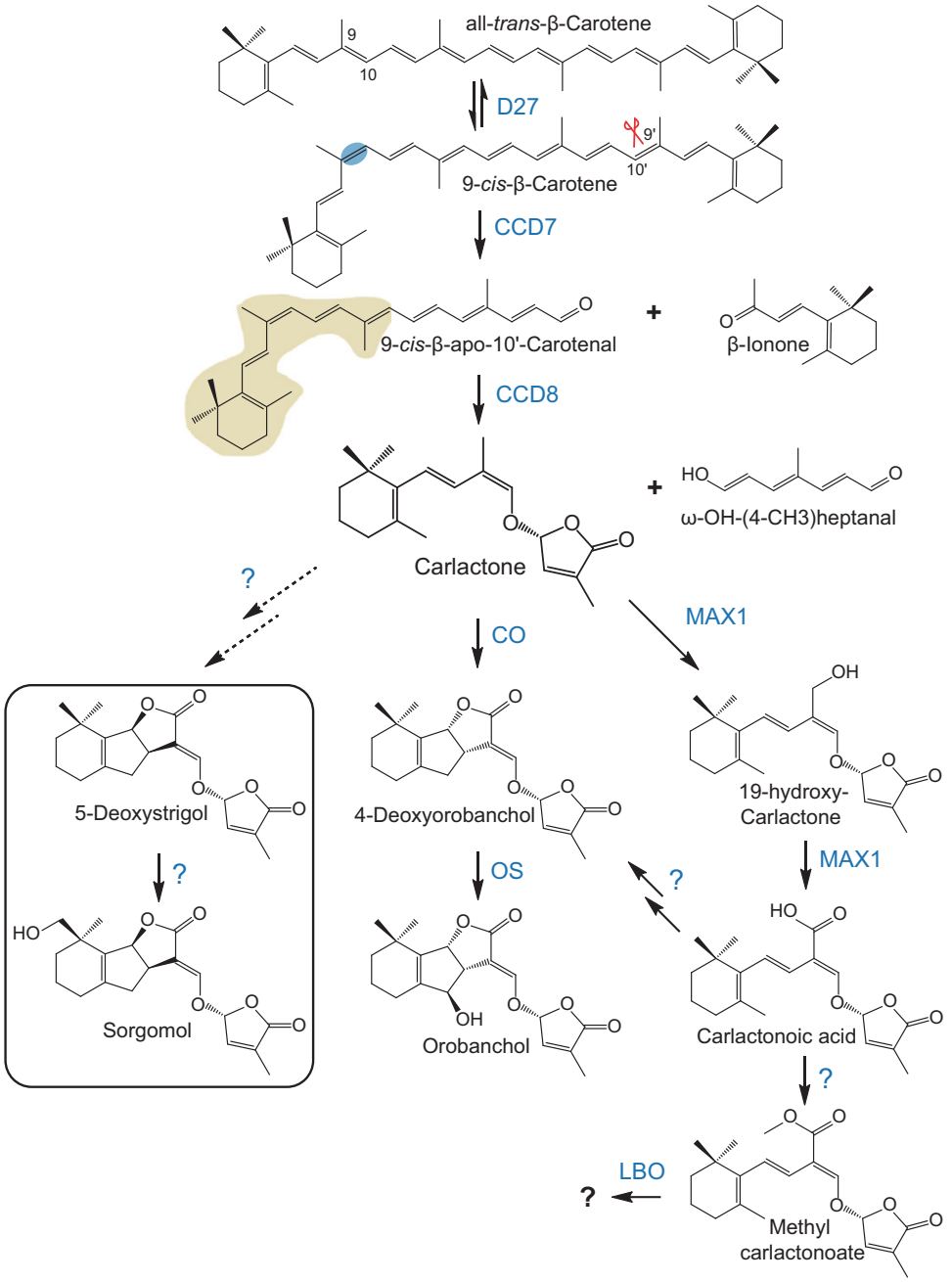

Fig. 2. Strigolactone (SL) biosynthesis pathway. The carotene isomerase D27 catalyzes the reversible conversion of all-*trans*-β-carotene into 9-*cis*-β-carotene, which is cleaved by the stereospecific carotenoid cleavage enzyme CCD7 at the C9′-C10′ double bond, yielding 9-*cis*-β-apo-10′-carotenal (C_{27}) and β-ionone (C_{13}). In the third step, CCD8 converts 9-*cis*-β-apo-10′-carotenal into carlactone (C_{19}) and the C_8 compound ω-OH-(4-CH_3)heptanal. The C skeleton of carlactone corresponds to the shaded part of 9-*cis*-β-apo-10′-carotenal. Carlactone is the central metabolite of the pathway and precursor of canonical and non-canonical SLs. In Arabidopsis, MAX1 catalyzes the successive oxygenation of carlactone, leading to carlactonoic acid via 19-hydroxy-carlactone and, likely, 19-oxo-carlactone (structure not shown). Carlactonoic acid is further converted by an unknown methyl transferase into methyl carlactonoate, the substrate of Lateral Branching Oxidoreductase (LBO), which forms an as yet unidentified hydroxylated methyl carlactonoate. In rice, the MAX1 homolog carlactone oxidase (CO) catalyzes repeated oxygenation of carlactone and a stereospecific BC ring closure to form 4-deoxyorobanchol, the parent molecule of orobanchol-like SLs. By introducing a hydroxyl group at the C4 position, a second rice MAX1 homolog, orobanchol synthase (OS), converts 4-deoxyorobanchol into orobanchol. Experiments involving feeding rice with labeled carlactonoic acid suggested that it can be converted into 4-deoxyorobanchol, but the enzyme responsible for this conversion is unknown. It is assumed that carlactone is also the precursor of 5-deoxystrigol, the parent molecule of the strigol-like SLs. However, experimental evidence is still needed. In sorghum, 5-deoxystrigol is converted by an as yet unidentified CYP into sorgomol, a major sorghum SL.

therefore take place without enzymatic catalysis, as shown for the formation of the volatile cyclocitral, which acts as a retrograde signal specifically regulating the expression of singlet oxygen ($^1O_2$)-responsive genes (Ramel *et al.*, 2012). However, in most cases, apocarotenoid formation requires the activity of carotenoid cleaving enzymes that break C=C double bonds, leading to two carbonyl products, and which generally belong to the ubiquitous family of carotenoid cleavage

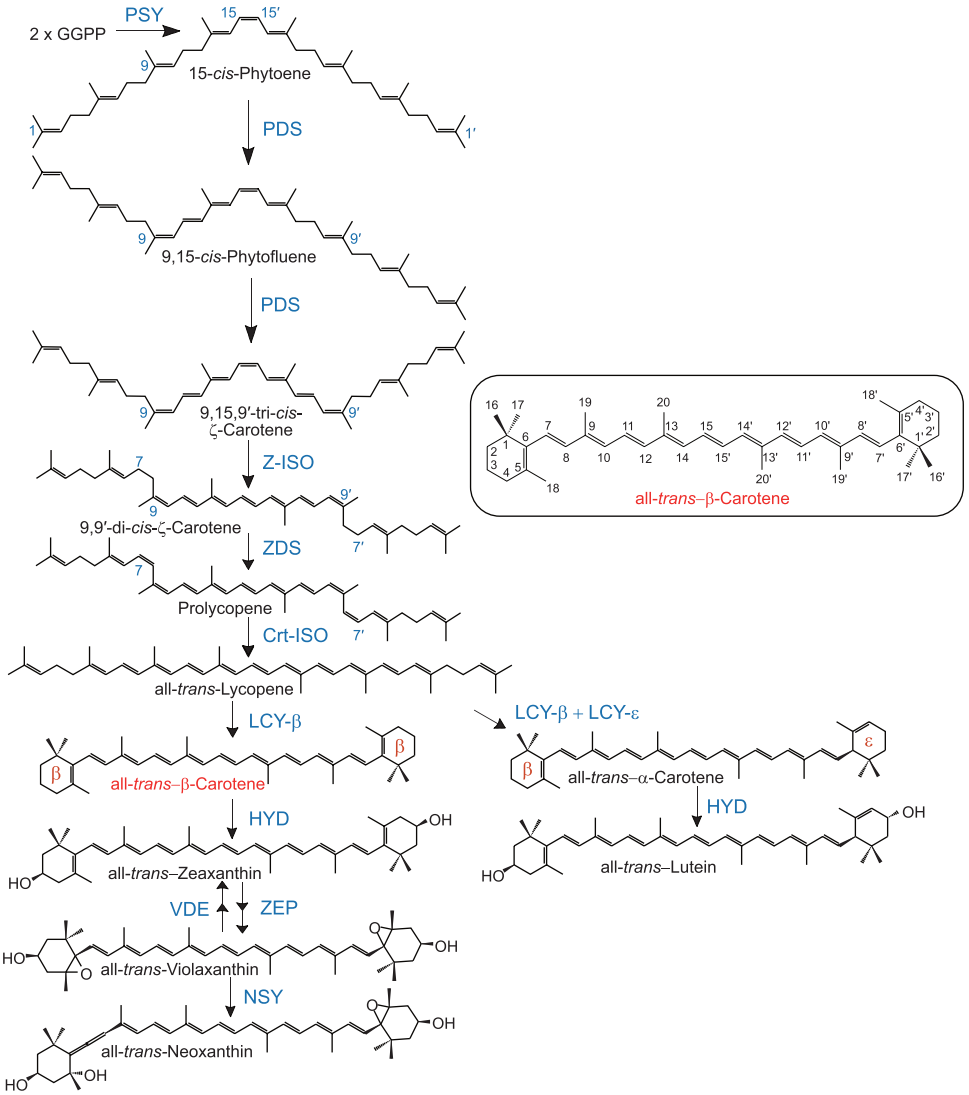

Fig. 3. Carotenoid biosynthesis in plants. Phytoene synthase (PSY) mediates the first committed step in carotenogenesis by catalyzing the condensation of two geranylgeranyl diphosphate (GGPP; C_{20}) molecules into the C_{40} product 15-*cis*-phytoene. Phytoene desaturase (PDS) introduces two double bonds at C11 and C11′, which is concomitantly accompanied by *trans*- to *cis*-isomerization of the neighboring double bonds at C9 and C9′. These reactions lead to 9,15,9′-tri-*cis*-ζ-carotene via 9,15-*cis*-phytofluene. ζ-carotene isomerase (Z-ISO) converts 9,15,9′-tri-*cis*-ζ-carotene into 9,9′-di-*cis*-ζ-carotene, the substrate of ζ-carotene desaturase (ZDS), which introduces two double bonds at C7 and C7′, increasing the number of conjugated double bonds to 11 and forming 7,7′,9,9′-tetra-*cis*-lycopene (prolycopene). Carotene isomerase (Crt-ISO) catalyzes the isomerization of the four *cis* double bonds in prolycopene, leading to all-*trans*-lycopene. Lycopene-β-cyclase (LCY-β) introduces two β-ionone rings into all-*trans*-lycopene, leading to all-*trans*-β-carotene. The formation of α-carotene requires LCY-β and lycopene-ε-cyclase (LCY-ε), which introduce a β- and a ε-ionone ring, respectively. Different hydroxylases (HYD) convert the cyclic carotenes α-carotene and β-carotene into all-*trans*-lutein and all-*trans*-zeaxanthin, respectively. All-*trans*-zeaxanthin can be reversibly epoxidated into all-*trans*-violaxanthin. The epoxidation and de-epoxidation are catalyzed by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), respectively. Neoxanthin synthase (NSY) produces the final product of the β-branch, all-*trans*-neoxanthin, from all-*trans*-violaxanthin. The inset shows the numbering of C atoms in the carotenoid skeleton of all-*trans*-β-carotene, as an example.

oxygenases (CCOs). CCOs are non-heme Fe^{II}-dependent enzymes that differ in their substrate specificity, regiospecificity (double bond), and stereospecificity (*cis*/*trans*). Moreover, several CCOs catalyze a secondary cleavage reaction by cleaving apocarotenoids instead of carotenoids (for reviews, see Ahrazem *et al.*, 2016; Auldridge *et al.*, 2006; Giuliano *et al.*, 2003; Moise *et al.*, 2005; Walter and Strack, 2011). As shown by the crystal structure of three members of this enzyme family, the cyanobacterial, retinal-forming enzyme SynACO (Kloer *et al.*, 2005; Ruch *et al.*, 2005), maize VP14 (Messing *et al.*, 2010), and retinal pigment epithelium protein of 65 kDa (RPE65) from *Bos taurus* (Kiser *et al.*, 2009), CCOs consist of a rigid seven-bladed β-propeller capped by extended loops with short helices building a large dome. A substrate-binding tunnel perpendicular to the propeller axis extends from the outside of the protein to the active center harboring the ferrous iron that is strictly required to activate the triplet oxygen and is coordinated by four conserved histidine residues. Three conserved glutamine residues build a second coordination sphere. The active center is located on the top side of the propeller, near its axis. Hydrophobic patches surrounding the active site tunnels allow membrane insertion and enable the

uptake of the lipophilic substrates. In VP14 and SynACO, the cleavage products are likely released via an exit tunnel (Kloer *et al.*, 2005; Messing *et al.*, 2010; Sui *et al.*, 2013).

The mechanism underlying the cleavage reaction of the CCOs is still under debate, although several lines of evidence indicate that these enzymes act as dioxygenases rather than as monooxygenases (Sui *et al.*, 2016). Dioxygenases incorporate both O-atoms of atmospheric oxygen (O_2) into the substrate, leading to a dioxetane intermediate that spontaneously disintegrates into two carbonyl products (Fig. 4). In contrast, monooxygenases reduce one atom of atmospheric O_2 to water and introduce the other atom into the substrate; in the case of carotenoid cleavage, this would lead to an epoxide intermediate, which can, after the addition of water, be split into two carbonyl compounds (Sui *et al.*, 2013). In plants, CCOs are generally considered to be dioxygenases (i.e., CCDs) (Auldridge *et al.*, 2006; Giuliano *et al.*, 2003), although experimental proof of the mechanism has not been provided for many of them. There are five major CCD subfamilies in plants: CCD1, CCD4, CCD7, CCD8, and 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) (Auldridge *et al.*, 2006; Walter and Strack, 2011). NCEDs are responsible for ABA biosynthesis, catalyzing the stereospecific cleavage of 9-*cis*-epoxycarotenoids at the C11-C12 site into the ABA precursor xanthoxin (C_{15}) and the corresponding apo-12′-epoxcarotenal (C_{25}) (Giuliano *et al.*, 2003; Schwartz *et al.*, 1997). CCD1 enzymes are characterized by their wide substrate and double-bond specificity. They cleave different cyclic and acyclic all-*trans*-carotenoids as well as apocarotenoids at several double-bond positions, leading to a large number of volatiles and dialdehyde products; this finding indicates a role for these enzymes in scavenging destructed carotenoids (Ilg *et al.*, 2009, 2014). CCD4 enzymes catalyze the cleavage of the C9′-C10′ or C7′-C8′ position in bicyclic carotenoids, leading to C_{10} or C_{13}- volatiles, such as cyclocitral or β-ionone, and C_{30} or C_{27} apocarotenal, respectively (Bruno *et al.*, 2015, 2016; Ma *et al.*, 2013; Rodrigo *et al.*, 2013). The carotenoid cleavage activity of CCD4 enzymes regulates carotenoid levels in different plant tissues, such as Arabidopsis seeds (Gonzalez-Jorge *et al.*, 2013) and *Chrysanthemum* flowers (Ohmiya *et al.*, 2006), or forms new pigments, as shown for *Citrus* CCD4s that produce the deep red β-apo-8′-carotenal and β-citraurin (Ma *et al.*, 2013; Rodrigo *et al.*, 2013). In addition, Arabidopsis CCD4 has been assumed to mediate the formation of a hitherto unidentified signal from acyclic *cis*-configured desaturation intermediates, which regulates leaf and chloroplast development (Avendaño-Vazquez *et al.*, 2014). However, AtCCD4 does not cleave acyclic carotenoids *in vitro* (Bruno *et al.*, 2016). The remaining two subfamilies of plant CCDs, CCD7 and CCD8, are responsible for SL biosynthesis (Alder *et al.*, 2012).

Cis/*trans* isomerization requires the breaking of the target double bond, rotation around the remaining single bond, and reintroducing the double bond (Stoker, 2015). In special cases, CCOs can combine *cis*/*trans*-isomerase and double-bond cleavage activity (isomerooxygenase). This was shown for the insect enzyme NinaB, which catalyzes the cleavage of the central C15-C15′ double bond and the isomerization of all-*trans*-β-carotene into the corresponding 11-*cis*-isomer, leading to one molecule of all-*trans*-retinal and one molecule of 11-*cis*-retinal with the stereoconfiguration required for the visual chromophore (Oberhauser *et al.*, 2008). Other unique members of the CCO family are the SL biosynthetic enzyme CCD8 (see below) and the vertebrate RPE65, which does not catalyze oxidative cleavage of C=C double bonds but combines ester hydrolase and isomerase activity, acting as an all-*trans* retinyl ester isomerohydrolase that generates 11-*cis*-retinal required for visual pigment formation from all-*trans*-retinyl ester during the visual cycle (Kiser *et al.*, 2009). The isomerization of double bonds in the carotenoid backbone can also be catalyzed by other types of enzymes, such as the FAD(RED)-dependent flavoprotein CrtISO that forms all-*trans*-lycopene (Yu *et al.*, 2011) and the heme-dependent ζ-carotene isomerase, which isomerizes the central C15-C15′ double bond in the intermediate 9,15,9′-tri-*cis*-ζ-carotene to generate 9,9′-di-*cis*-ζ-carotene, the substrate of ζ-carotene desaturase (Isaacson *et al.*, 2004; Park *et al.*, 2002) (see Fig. 3). A further example of a carotene isomerase is the SL biosynthetic enzyme D27.

Strigolactone biosynthetic enzymes

D27

D27 is a small polypeptide that was identified as a result of the SL-deficient phenotype of the corresponding rice mutant (Lin *et al.*, 2009). D27 is an iron-binding protein that does not show homology to any known enzyme. However, the strict stereospecificity of CCD7 (see below) led Alder *et al.* (2012) to hypothesize that D27 may act as the all-*trans*/9-*cis*-β-carotene isomerase required to supply CCD7 with β-carotene in the right stereoconfiguration. Indeed, expression of OsD27 in *Escherichia coli* cells engineered to accumulate all-*trans*-β-carotene substrate led to a clear increase in the 9-*cis*/all-*trans* ratio, suggesting that OsD27 is the first β-carotene *cis*/*trans* isomerase to be reported so far (Alder *et al.*, 2012). *In vitro* assays confirmed this activity and showed that OsD27 catalyzes the reversible isomerization of all-*trans*-β-carotene into 9-*cis*-β-carotene, resulting in an equilibrium that is in favor of all-*trans*-β-carotene (Alder *et al.*, 2012; Bruno and Al-Babili, 2016; Harrison *et al.*, 2015) (Fig. 2). Besides the enhanced accumulation of 9-*cis*-β-carotene, the expression of OsD27 in β-carotene-accumulating *E. coli* led to a detectable increase in the 15-*cis*/all-*trans*-β-carotene ratio, indicating that the enzyme may also isomerize the central C15-C15′ double bond. However, OsD27 did not show isomerization activity with 15-*cis*- β-carotene or 13-*cis*-β-carotene, a further common isomer, in *in vitro* assays, suggesting the specificity of OsD27 for the C9-C10 double bond (Bruno and Al-Babili, 2016). The mechanism of D27-catalyzed β-carotene isomerization is still unknown. However, OsD27 activity is inhibited in the presence of silver acetate, indicating the involvement of an iron-sulfur cluster in the catalysis (Harrison *et al.*, 2015).

Like the SLs, the biosynthesis of ABA requires a carotenoid precursor with *cis*-configuration at the C9-C10 (9-*cis*-violaxanthin) or C9′-C10′ (9′-*cis*-neoxanthin) double bond. Hence,

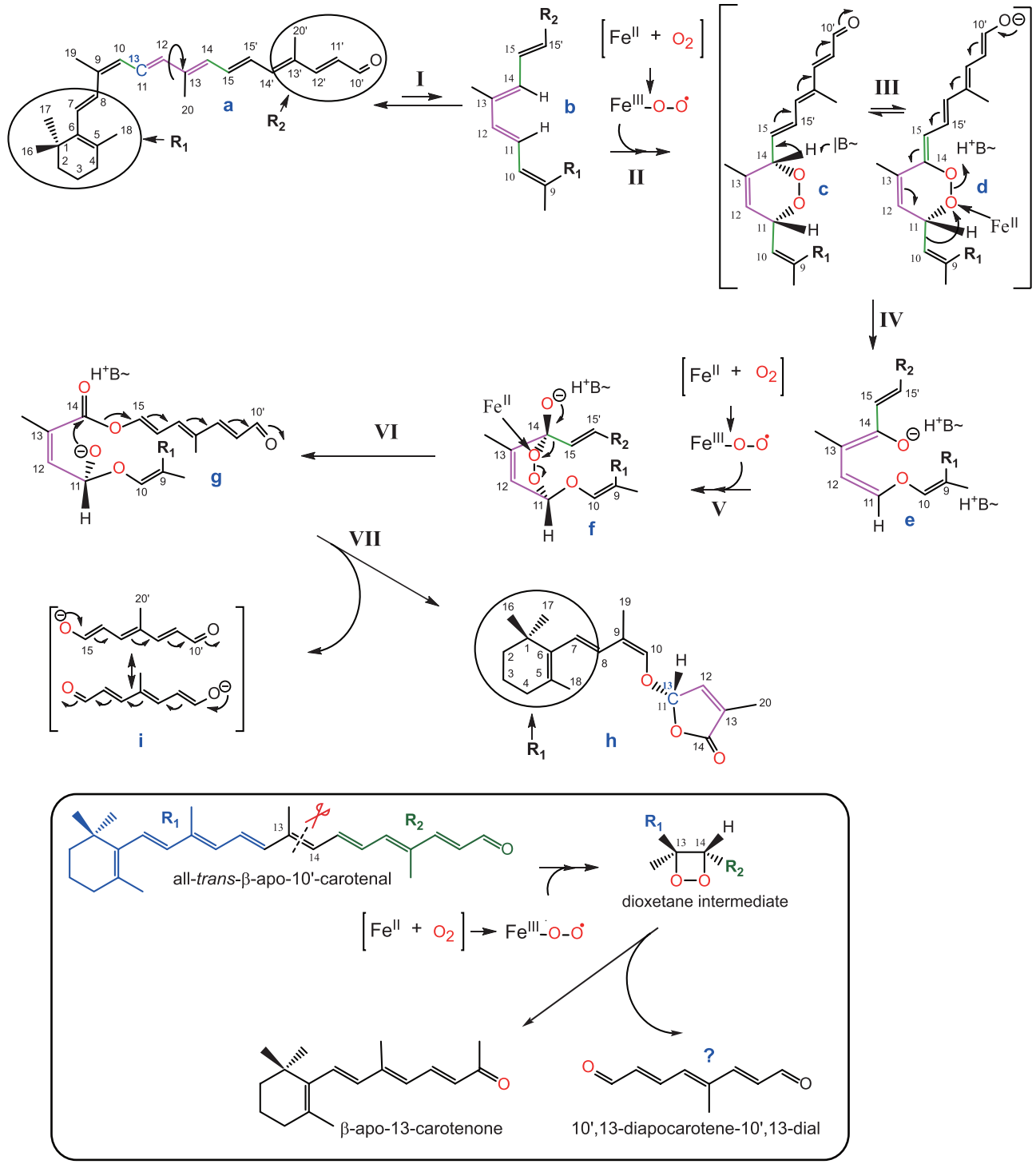

Fig. 4. The catalytic mechanisms of CCD8. A proposed pathway for the formation of carlactone (h) from 9-*cis*-β-apo-10′-carotenal (a). Under this proposal, CCD8 first catalyzes a reversible rotation around the C12-C13 bond in the substrate (a), changing the configuration of the C11-C12, C12-C13, and C13-C14 bonds (shown in magenta) from *transoid* in (a) into *cisoid* in (b). Activation of atmospheric O_2 by enzyme-bound ferrous iron leads to the Fe^{III}-O-O• species that attacks C14, the site where the *trans*-substrate is also cleaved (see below), producing an endoperoxide (c). The delocalization of the formed negative charge increases the 'acidification' of the C14-H (c, d). Abstraction of this proton by an active center base (|B~) initiates the first rearrangement, which involves the migration of the C10-C11 bond (depicted in green) and is assisted by the interaction of the endoperoxide with the $Fe^{II/III+}$ center or an acid catalyst (d). The mechanisms of the intramolecular rearrangements leading to (e) are thought to be analogous to the Baeyer–Villiger/Criegee ones. A second deoxygenation reaction is initiated by the repeated attack of the Fe^{III}-O-O• species at C14, leading to a second endoperoxide in (f). The second rearrangements are thought to proceed analogously to the first and involve migration of the C14-C15 bond (depicted in green), leading to (g). The formation of carlactone (h) requires a transesterification reaction that builds the butenolide ring (step VII), which is triggered by the release of the second product (i), an extensively conjugated ω-OH-aldehyde thought to be a good leaving group. Shown are mesomeric forms of the second product (i), which stabilize the negative charge. As shown by ^{13}C labeling, C11 (depicted in green) in the substrate (a) corresponds to C11 (depicted in green) in carlactone (h), which corresponds to C2′ in SLs. The inset shows a proposal for the formation of β-apo-13-carotenone by CCD8 using all-*trans*-β-carotene as a substrate. The reactive Fe^{III}-O-O• species attacks all-*trans*-β-apo-10′-carotenal at C14, replacing the C13-C14 double bond by an unstable dioxetane intermediate that breaks into two carbonyl products. The dialdehyde that is thought to arise concomitantly with β-apo-13-carotenone has not been detected yet.

it is tempting to speculate that D27 may act as the still elusive epoxycarotenoid 9-*cis*/all-*trans* isomerase that provides the ABA carotenoid precursor in the required stereoconfiguration. Such an activity would place D27 at a central point in the formation of both carotenoid-derived plant hormones and implies that the biosynthetic pathways of ABA and SLs might interfere with each other and underlie a common regulatory system at the precursor level. However, results of *in vitro* incubations of OsD27 with all-*trans*-violaxanthin and all-*trans*-neoxanthin did not support this hypothesis, making a direct involvement of this enzyme in ABA biosynthesis unlikely (Bruno and Al-Babili, 2016). Nevertheless, D27 may contribute to ABA biosynthesis by an unknown mechanism, as was suggested by analysis of the corresponding rice mutant (Imran Haider, personal communication).

As discussed below, CCD7 enzymes from pea, Arabidopsis, and *Physcomitrella patens* cleave 9-*cis*-zeaxanthin and 9-*cis*-lutein *in vitro* (Bruno *et al.*, 2014; Decker *et al.*, 2017). This finding raised the question whether these substrates can be also produced by D27. Further analysis of the substrate specificity *in vitro* showed that OsD27 substrates are bicyclic carotenoids that have at least one unmodified β-ionone ring, which includes β-carotene, its mono-hydroxylated derivative β,β-cryptoxanthin, and α-carotene (leading to 9-*cis*-α-carotene and 9′-*cis*-α-carotene) (Bruno and Al-Babili, 2016). While 9-*cis*-β,β-cryptoxanthin and 9-*cis*-α-carotene can be converted by CCD7 into the SL biosynthesis intermediate 9-*cis*-β-apo-10′-carotenal, the question about the biological significance of the *in vitro* formation of 9′-*cis*-α-carotene remains open. However, it could be speculated that this activity initiates the synthesis of SLs with a ε-ionone ring, such as heliolactone (Ueno *et al.*, 2014), if such SLs are not derived from the β-ionone ring-containing carlactone.

In both rice and Arabidopsis, the high tillering/more branching phenotype of *d27* mutants is less severe than that of other SL-deficient mutants (Lin *et al.*, 2009; Waters *et al.*, 2012). It could be assumed that lack of D27 activity can be partially compensated for by photoisomerization. However, it is also possible that the two D27 homologs (D27-LIKE 1 and D27-LIKE 2) present in Arabidopsis, rice, and other plants also contribute to the formation of 9-*cis*-β-carotene. It should be also mentioned that 9-*cis*-β-carotene is not only the precursor of SLs but also a structural component of the photosynthetic cytochrome *b6f* complex (Cramer *et al.*, 2006; Yan *et al.*, 2001).

CCD7

The first report on the biological function of AtCCD7 (MAX3) demonstrated its key role in the synthesis of a supposed shoot branching inhibitor that was later identified as SL, and confirmed its activity as a carotenoid cleavage enzyme. To determine the substrates of AtCCD7, Booker *et al.* (2004) expressed the enzyme in *E. coli* strains that accumulate all-*trans*-configured phytoene, ζ-carotene, lycopene, δ-carotene, β-carotene, and zeaxanthin. This study identified β-carotene, ζ-carotene, and potentially δ-carotene and zeaxanthin as substrates of CCD7. Based on the detection of C_{13}-volatile products, it was assumed that AtCCD7 catalyzes a single or double cleavage reaction targeting the C9-C10 and/or C9′-C10′ double bond, which would produce apo-10′-carotenal (C_{27}) and/or 10,10′-diapocarotene-10,10′-dial (C_{14}) as a second product. A further study on the enzymatic activity of AtCCD7 in carotenoid-accumulating *E. coli* strains confirmed its role in the cleavage of β-carotene and lycopene, but excluded zeaxanthin as a substrate (Schwartz *et al.*, 2004). Moreover, the authors showed that AtCCD7 catalyzes a single cleavage reaction at the C9-C10 or C9′-C10′ double bond, which leads to a C_{13}-volatile and β-apo-10-carotenol and apo-10′-lycopenol (C_{27}), respectively, the alcohols formed by non-specific *E. coli* aldehyde dehydrogenase activity from the corresponding primary aldehyde cleavage products. This cleavage activity was also shown for the tomato enzyme LeCCD7 (Vogel *et al.*, 2010). *In vitro* assays with heterologously expressed and purified AtCCD7 confirmed the cleavage of β-carotene, indicating that AtCCD7 likely catalyzes the first step in sequential cleavage reactions that involve AtCCD8 as another biosynthetic enzyme (Schwartz *et al.*, 2004). Indeed, co-expression of both carotenoid cleavage enzymes in β-carotene-accumulating *E. coli* cells led to the production of the C_{18} compound β-apo-13-carotenol, a C_{18} alcohol formed by reduction of the primary cleavage product β-apo-13-carotenone (Schwartz *et al.*, 2004), also called d'orenone, which regulates root hair growth in Arabidopsis (Schlicht *et al.*, 2008). This result indicated that AtCCD8 cleaves the AtCCD7 product β-apo-10′-carotenal at the C13-C14 double bond, which was later confirmed by an *in vitro* study with CCD8 enzymes from Arabidopsis, pea, and rice and the substrate all-*trans*-β-apo-10′-carotenal (Alder *et al.*, 2008). On the basis of these results, it was assumed that β-apo-13-carotenone is the precursor of the shoot branching inhibitor. However, the application of this compound did not rescue the high tillering phenotype of the rice *ccd8* mutant (Alder *et al.*, 2012), a finding that did not support its presumed role as an SL precursor.

Carotenoids and apocarotenoids are susceptible to photo- and thermo-*cis*/*trans*-isomerization. This may complicate the elucidation of carotenoid metabolic pathways by causing possible stereospecificity of involved enzymes to be overlooked, leading to incorrect conclusions about precursors and intermediates. Indeed, *in vitro* incubation of CCD7 enzymes from different plant species with pure β-carotene stereoisomers demonstrated that CCD7 is a stereospecific enzyme that cleaves only 9-*cis*-β-carotene, not the all-*trans*-, 13-*cis*-, or 15-*cis*- isomers (Alder *et al.*, 2012; Bruno *et al.*, 2014). CCD7 activity leads to the generation of β-ionone and 9-*cis*-configured β-apo-10′-carotenal, as suggested by HPLC, LC-MS, and NMR analysis (Alder *et al.*, 2012; Bruno *et al.*, 2014). The stereospecificity of CCD7 was the key for unraveling the enzymatic function of D27 and for the discovery of carlactone, the central metabolite in the SL biosynthesis pathway.

In vitro incubation with different carotenoids confirmed the wide substrate specificity and strict stereospecificity- and regiospecificity of CCD7 enzymes from different species. AtCCD7, PsCCD7, and PpCCD7 converted 9-*cis*-zeaxanthin and 9-*cis*-lutein into 9-*cis*-3-OH-β-apo-10′-carotenal

and 9-*cis*-3-OH-ε-apo-10′-carotenal, respectively (Bruno *et al.*, 2014). An *in vitro* study of AtCCD7 activity on acyclic carotenoids provided similar results (Bruno *et al.*, 2016). The enzyme converted 9-*cis*-ζ-carotene, 9′-*cis*-neurosporene, and 9-*cis*-lycopene, producing the corresponding 9-*cis*-configured products 9-*cis*-ζ-apo-10′-carotenal and 9-*cis*-apo-10′-lycopenal, respectively (Bruno *et al.*, 2016). However, CCD7 enzymes do not act on 9-*cis*-violaxanthin *in vitro*, indicating that this enzyme does not directly compete with NCEDs that cleave 9-*cis*-epoxycarotenoids as a first step in ABA biosynthesis.

CCD7 enzymes such as AtCCD7 and PsCCD7 showed higher affinity for 9-*cis*-β-carotene than to 9-*cis*-zeaxanthin *in vitro* (Bruno *et al.*, 2014). Moreover, the structures of the majority of known SLs point to β-carotene as the likely precursor. Nevertheless, the activity observed with 9-*cis*-zeaxanthin, 9-*cis*-lutein, and correspondingly configured acyclic carotenoids raises the question of whether these carotenoids are a source of as yet unidentified SLs in *planta*. In other words, it remains to be determined whether CCD8 can convert other 9-*cis*-configured apo-10′-carotenoids into carlactone-like structures. However, it can also be assumed that CCD7 enzymes are involved in biological processes other than SL biosynthesis. Several pieces of evidence indicate that CCD7 action is required for the production of glycosylated C_{13}-cyclohexenone and linear C_{14}-mycorradicin, the two apocarotenoids that accumulate in roots upon mycorrhizal colonization (Walter, 2013). Cyclohexenones and mycorradicin are likely derived from a hydroxylated C_{40} carotenoid that is cleaved at C9-C10 and C9′-C10′, leading to two C_{13} (α- or β-ionone) and one C_{14} (rosafluene dialdehyde) products. Based on the reduction in the content of these apocarotenoids in tomato knock-down *slccd7* and pea *rms5* mutants and on previous studies confirming the role of CCD7 enzymes in this process (Vogel *et al.*, 2010), it has been shown that CCD7 catalyzes the first cleavage reaction, leading to a C_{13} ketone (α-ionone) and a hydroxylated apo-10′-carotenal that is further cleaved by CCD1 into a second C_{13} ketone and a C_{14} dialdehyde (Floss *et al.*, 2008). Modifications of the primary cleavage products give rise to the accumulated mycorrhizal pigments (Walter, 2013). This activity suggests that CCD7 likely plays a central role in the mycorrhiza-related apocarotenoid metabolism.

CCD8

The cleavage of C_{40} carotenoids by CCD7 enzymes suggested that CCD8 catalyzes a following secondary cleavage reaction on an apocarotenoid substrate. Indeed, CCD8 enzymes cleaved all-*trans*-β-apo-10′-carotenal (Fig. 4), which was initially supposed to be a CCD7 product, into β-apo-13-carotenone (Schwartz *et al.*, 2004). However, the strict stereospecificity of CCD7 activity suggested that 9-*cis*-β-apo-10′-carotenal, rather than the corresponding all-*trans*-isomer, is the true intermediate in the SL biosynthetic pathway (Alder *et al.*, 2012; Bruno *et al.*, 2014). Confirming this assumption, CCD8 enzymes converted 9-*cis*-β-apo-10′-carotenal *in vitro* into a novel compound with surprising similarities to known SLs, which has been named carlactone (Alder *et al.*, 2012) (Figs 2 and 4). In contrast to other CCD products, carlactone is a non-carbonyl, trioxygenated compound that contains a lactone ring identical to the D ring of SLs. Moreover, carlactone is identical to 5-deoxystrigol and 4-deoxyorobanchol, the parent compounds of canonical SLs, in terms of the number of C-atoms, and resembles all SLs in the presence of the enol ether bridge that connects its two moieties. Moreover, carlactone and natural SLs share the same stereochemistry (*R* configuration) at the C2′ atom (corresponding to C11 in carlactone; see Fig. 4), as shown by different research groups (Seto *et al.*, 2014; Zhang *et al.*, 2014).

CCD8 converts 9-*cis*-β-apo-10′-carotenal at higher rates than the corresponding all-*trans*-isomer, indicating that the formation of carlactone is the preferred reaction (Alder *et al.*, 2012). Nevertheless, the structure of the two products, β-apo-13-carotenone and carlactone, suggests that CCD8 is an unusual carotenoid cleavage enzyme that can catalyze different types of reactions, depending on the stereochemistry of the substrates. It can be assumed that β-apo-13-carotenone is formed by a classical carotenoid cleavage reaction that likely occurs via a dioxetane intermediate, as is supposed for the CCDs (Fig. 4). In contrast, the synthesis of carlactone requires a unique combination of different reactions, as can be deduced from the structure of this product. The formation of carlactone must also simultaneously lead to a second product, proposed to be a C_8 compound with an aldehyde and alcohol group (ω-OH-(4-CH_3)-hepta-2,4,6-trien-al; Figs 2 and 4) (Bruno *et al.*, 2017), which was not identified in previous studies, likely due to its instability. However, the recent usage of a ketone/aldehyde-selective derivatization reagent allowed the identification of this second product and confirmed the initially proposed structure (Bruno *et al.*, 2017).

The reaction mechanism underlying carlactone generation is still elusive. Nevertheless, labeling experiments with $^{18}O_2$ and $H_2{}^{18}O$, which demonstrated that CCD8 acts as a dioxygenase; the use of ^{13}C-labeled 9-*cis*-β-apo-10′-carotenal (Fig. 4), which showed that the C11 atom in the substrate corresponds to the C11 atom in carlactone (C2′ atom in SLs); the identification of the second product; and, finally, the assumption that CCD8 acts also as a dioxygenase when cleaving the C13-C14 double bond in its all-*trans*-substrate, allowed the formulation of a new minimal reaction mechanism (Bruno *et al.*, 2017). As shown in Fig. 4, the formation of carlactone is proposed to occur via a sequence of reactions, including the conversion of the *transoid* configuration of the C12-C13 double bond of 9-*cis*-β-apo-10′-carotenal into a *cisoid* one, repeated oxygenation at the C14 atom, repeated formation of a cyclic endoperoxide at C14, and migration of the C10-C11 and C14-C15 bonds. It is proposed that the stereoconfiguration of the C9-C10 double bond determines whether the enzymes build a dioxetane intermediate, leading to β-apo-13-carotenone, or a cyclic endoperoxide intermediate, to form carlactone (Bruno *et al.*, 2017). However, further experimental and structural data are still needed in order to understand this process. It remains to be determined whether CCD8 can also produce carlactone-like molecules from different substrates, which might contribute to the diversity of natural SLs. As

outlined above, CCD7 enzymes can provide suitable precursors for such compounds. Indeed, we have recently identified 3-OH-carlactone as a further CCD8 *in vitro* product that may also be produced in planta (L Baz, personal communication).

MAX1 and other downstream enzymes

Carlactone was originally discovered as an *in vitro* product of CCD8. It is now recognized as a central metabolite in SL biosynthesis, which has also been identified in the early diverging moss lineage (Decker *et al.*, 2017), and a precursor of canonical and non-canonical SLs (Seto *et al.*, 2014; Zhang *et al.*, 2014). Indeed, the structure of carlactone indicates that only a few steps are required to convert it into canonical SLs, as suggested by Alder *et al.*, (2012). Seto *et al.* (2014) confirmed the presence of carlactone *in planta* and showed that feeding the SL-deficient rice *d10*/*ccd8* mutant with ^{13}C-labeled carlactone resulted in the formation of ^{13}C-labeled 4-deoxyorobanchol ((−)-[^{13}C]-2′-*epi*-5-deoxystrigol) and orobanchol. The same feeding experiment performed with the corresponding Arabidopsis mutant (*max4*) led to the formation of a labeled SL-like compound (SL-like1) that was later identified as the non-canonical SL methyl carlactonoate (Abe *et al.*, 2014; Seto *et al.*, 2014). In the absence of further candidate enzymes, it was assumed that Arabidopsis MAX1 and homologs are the enzymes that follow CCD8 in the SL biosynthesis pathway, and that they may directly convert carlactone. Supporting this assumption, the content of carlactone in the Arabidopsis *max1* mutant is approximately 700-fold higher than that in the wild type (Seto *et al.*, 2014).

Zhang *et al.* (2014) investigated the function of the four functional rice MAX1 homologs by incubating carlactone with enzyme-containing *Saccharomyces* microsomes and by transient co-expression with carlactone-forming enzymes in *Nicotiana benthamiana* leaves. This study demonstrated that rice Os900 [to which we refer here as carlactone oxidase (CO), following Zhang *et al.* (2014)] converts carlactone into 4-deoxyorobanchol (*ent*-2′-*epi*-5-deoxystrigol) (Figs 2 and 5), suggesting that four enzymes (D27, CCD7, CCD8, and CO) are sufficient to produce the parent molecule of orobanchol-like SLs from the precursor all-*trans*-β-carotene (Zhang *et al.*, 2014). CO catalyzes the triple oxygenation of carlactone and stereospecific formation of the BC ring (Fig. 5), which is proposed to proceed via the addition of a proton to the hydroxyl group and the subtraction of another proton from the carboxyl group of the intermediate, accompanied by the loss of a water molecule (Alder *et al.*, 2012; Zhang *et al.*, 2014). *In vitro* study of the activity of Arabidopsis MAX1, the only CYP711 in this plant, led to the discovery of a novel compound, carlactonoic acid, in which the C19 methyl group of carlactone is replaced by a carboxylic acid (Abe *et al.*, 2014). Carlactonoic acid is formed by successive oxidation via 19-hydroxy-carlactone, which was detected as an intermediate (Fig. 2). Carlactonoic acid is an endogenous compound, as shown for Arabidopsis and rice, and its formation from carlactone *in planta* has been confirmed by feeding experiments (Abe *et al.*, 2014). Interestingly, carlactonoic acid also can be converted into 4-deoxyorobanchol and orobanchol in rice (Abe *et al.*, 2014). However, it is not clear whether this conversion is catalyzed by the rice CO or not. It can be speculated that carlactonoic acid is an intermediate produced by the rice CO in the course of 4-deoxyorobanchol formation.

Zhang *et al.* (2014) identified a second MAX1 homolog, Os1400, as orobanchol synthase, mediating the hydroxylation at C4 of 4-deoxyorobanchol to yield orobanchol. This suggests that MAX1 and its homologs are not only responsible for the BC ring closure of SLs, but also probably contribute to the modifications at A, B or C rings to produce various SLs.

Recently, a transcriptomics approach that detects changes in the transcript levels of SL biosynthetic enzymes under different conditions, combined with reverse genetics, has led to the identification of LBO, an oxidoreductase-like enzyme of the 2-oxoglutarate and Fe^{II}-dependent dioxygenase family, which acts downstream of MAX1 (Fig. 2) and is required for maintaining normal shoot branching in Arabidopsis (Brewer *et al.*, 2016). LBO catalyzes the hydroxylation of methyl carlactonoate, leading to an as yet unidentified compound that may be the final product of SL biosynthesis in Arabidopsis. In sorghum, feeding with ^{2}H-labeled 4-deoxyorobanchol (*ent*-2′-*epi*-5-deoxystrigol) and 5-deoxystrigol led to the production of ^{2}H-labeled *ent*-2′-*epi*-sorgomol and sorgomol (Fig. 2), respectively. This study confirmed that sorgomol derives from 5-deoxystrigol, and also demonstrated the involvement of a CYP in this conversion as it could be inhibited by the CYP inhibitor uniconazole-P (Motonami *et al.*, 2013).

Our knowledge about the formation of 5-deoxystrigol, the parent of the strigol-like canonical SLs, is still very limited. However, a recent study unraveled the involvement of *LOW*

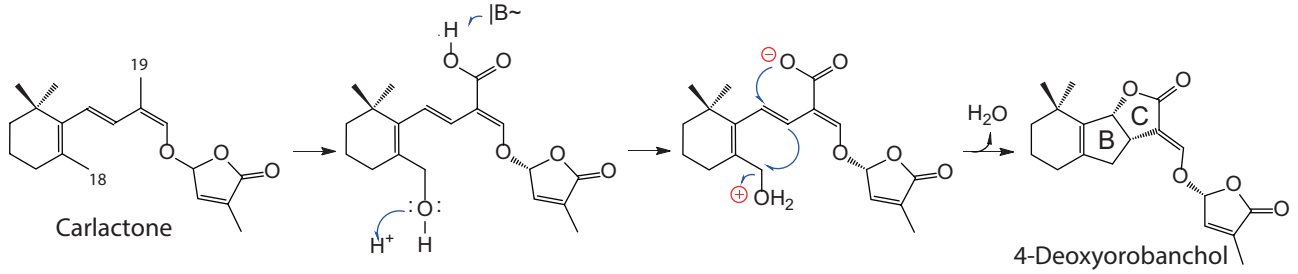

Fig. 5. Proposed mechanism for the formation of 4-deoxyorobanchol from carlactone by carlactone oxidase (CO). The enzyme catalyzes three oxygenation reactions, starting with carboxylation of C19, which is followed by hydroxylation of the C18 atom. Abstraction of a proton from the carboxyl group by an active center base (|B~) and the addition of a proton to the hydroxyl group at C18 triggers the stereospecific BC ring closure and the release of a water molecule as a leaving group. (This figure is available in colour at *JXB* online.)

GERMINATION STIMULANT 1 (*LGS1*), which encodes an enzyme annotated as a sulfotransferase, in determining the stereochemistry of canonical SLs released by sorghum roots (Gobena *et al.*, 2017). Functional loss of *LGS1* alters the pattern of released SLs, replacing the major SL 5-deoxystrigol by orobanchol. This alteration led to increased resistance to *Striga*, as 5-deoxystrigol is a more potent inducer of *Striga* seed germination than orobanchol (Gobena *et al.*, 2017). Although the mechanism underlying the effects of *LGS1* is still elusive, this discovery demonstrates the possibility of changing the pattern of SLs and opens up a new possibility for generating resistance to *Striga*.

Regulation of SL biosynthesis

SL biosynthesis is autoregulated by a negative feedback mechanism and is also regulated by other plant hormones. The availability of nutrients, particularly phosphorus, is a further important determinant of SL production. In the following section, we briefly describe factors regulating SL biosynthesis.

Hormonal control

Similar to auxin and gibberellins (GAs) (Brumos *et al.*, 2014; Hedden and Thomas, 2012), SL biosynthesis is regulated at transcript level via a negative feedback mechanism that determines hormone homeostasis. The application of GR24 to Arabidopsis plants led to a decrease in levels of *CCD7* and *CCD8* transcripts (Mashiguchi *et al.*, 2009). Consistent with this observation, Arabidopsis, pea, rice, and petunia SL biosynthesis and perception mutants show elevated transcript levels of genes encoding SL biosynthetic enzymes. This increase was observed with *CCD8* transcripts in pea, rice, and petunia, and with *MAX1*, *CCD7*, *CCD8*, and *LBO* transcripts in Arabidopsis (Arite *et al.*, 2007; Brewer *et al.*, 2016; Hayward *et al.*, 2009; Simons *et al.*, 2007; Snowden *et al.*, 2005). In rice, the negative feedback signal is transduced via the SL signaling repressor D53, as shown by the gain-of-function *d53* mutant, which displays increased transcript levels of SL biosynthetic genes and elevated SL contents (Jiang *et al.*, 2013; Zhou *et al.*, 2013).

SL content and polar auxin transport (PAT) affect each other to maintain the hormone balance needed to determine shoot architecture. In Arabidopsis, the inhibitory effect of SLs on shoot branching is hypothesized to be mediated by regulation of the gene expression of *BRC1*/*BRC2* transcription factor and/or by reducing PAT (Seale *et al.*, 2017; for reviews, see Domagalska and Leyser, 2011; Waldie *et al.*, 2014). On the other hand, auxin is a major regulator of SL biosynthesis at transcriptional level, as shown for pea, rice, Arabidopsis, *Chrysanthemum*, and tomato. In pea, removing the source of auxin by decapitation or reducing PAT by treatment with 1-*N*-naphthylphthalamic acid, an auxin transport inhibitor, significantly decreased the transcript levels of *RMS5* and, particularly, *RMS1* in the upper part of stems, which could be restored by auxin application (Foo *et al.*, 2005; Johnson *et al.*, 2006). Similar results were reported for the transcript levels of *DgD27* in *Chrysanthemum* and *MAX3* in Arabidopsis (Hayward *et al.*, 2009; Wen *et al.*, 2016). Auxin also positively regulates the expression of *MAX4* and *D10* in Arabidopsis and rice, respectively (Arite *et al.*, 2007; Hayward *et al.*, 2009). In Arabidopsis, the induction of *MAX3* and *MAX4* transcription by auxin is dependent on AUXIN RESISTANT 1 (AXR1), a subunit of the RUB1 activating enzyme that regulates the auxin receptor complex (Hayward *et al.*, 2009). In tomato, silencing of the gene encoding the auxin signaling component AUX/IAA protein Sl-IAA27 resulted in down-regulation of the SL biosynthetic genes *D27* and *MAX1* (Guillotin *et al.*, 2017).

ABA is an important plant hormone that regulates various processes and is mainly known for its role in the response to abiotic stresses (Sah *et al.*, 2016; Vishwakarma *et al.*, 2017). It was shown that osmotic stress decreased the SL content of tissues and root exudates of *Lotus* by suppressing transcript levels of SL biosynthetic and transporter genes. Moreover, pre-treatment with exogenous SLs inhibited the osmotic-stress-induced ABA increase, suggesting that SLs might interact with ABA to regulate abiotic stress response (Liu *et al.*, 2015). However, it is still controversial whether SLs are involved in the regulation of drought and salt stress responses in Arabidopsis (Bu *et al.*, 2014; López-Ráez, 2016; Van Ha *et al.*, 2014).

Several lines of evidence indicate that ABA regulates SL biosynthesis. In tomato, it was shown that inhibition of NCED (a key enzyme in ABA biosynthesis) by applying AbaminSG decreased the levels of SLs, indicating that ABA may be a positive regulator of SL biosynthesis (López-Ráez *et al.*, 2010). In accordance with this hypothesis, ABA-deficient mutants, such as *notabilis*, showed reduced transcript levels of the SL biosynthetic genes *LeCCD7* and *LeCCD8* and released smaller amounts of SLs (López-Ráez *et al.*, 2010; Schwartz *et al.*, 1997). A positive role for ABA in regulating SL biosynthesis in maize is indicated by the decreased SL content in root exudate of the ABA-deficient mutant *viviparous14* (López-Ráez *et al.*, 2010).

A recent investigation of the effect of GAs on SL biosynthesis in rice suggested a role as a negative regulator. Treatment with GA1, GA3, or GA4 resulted in decreased 4-deoxyorobanchol levels in both root tissues and exudates, which was dependent on the GA receptor GID1 and F-box protein GID2 (Ito *et al.*, 2017). This decrease in SL biosynthesis is caused by down-regulation of *D27*, *D10*, *D17*, *Os900*, and *Os1400* transcript levels.

Regulation by phosphate availability

The vital element phosphorus is very common in soil; however, it mainly exists as inorganic phosphate that cannot be directly accessed by plants (Péret *et al.*, 2011). To increase the soil volume available for phosphate uptake, plants modulate their root architecture and recruit symbiotic partners, such as arbuscular mycorrhizal fungi (for a review, see Gutjahr, 2014). In Arabidopsis, lack of phosphate leads to shorter primary roots, more and longer lateral roots, and denser root

hairs (Péret *et al.*, 2011). This shallow root system enables better 'foraging' of the upper part of the soil, where phosphate is present at higher concentrations. However, the impact of phosphate starvation on root architecture is not uniform, and depends on the species and even the ecotype (Niu *et al.*, 2013; Sun *et al.*, 2014). For instance, rice responds to this type of stress by prolonging primary, lateral, and crown roots and decreasing lateral root density (Arite *et al.*, 2012; Sun *et al.*, 2014). In both rice and Arabidopsis, modulation of root architecture in response to phosphate deficiency depends on SLs (Koltai, 2011; Kumar *et al.*, 2015).

SLs are also crucial for establishing mycorrhizal symbiosis, which is particularly important under phosphate starvation conditions (Akiyama *et al.*, 2005; Gutjahr, 2014; Bonfante and Genre, 2015). Consistent with the role of SLs in modulating plant architecture and attracting mycorrhizal symbiotic partners, phosphate deficiency triggers SL release and leads to an increase in SL root content, which can be reversed by re-supply of phosphate (Jamil *et al.*, 2011, 2012; Yoneyama *et al.*, 2013). Indeed, under normal conditions SLs are present at very low concentrations, and they are usually detected and measured under phosphate starvation conditions. Inorganic phosphate availability regulates the transcript levels of genes encoding SL biosynthetic enzymes and the SL transporter, as shown for petunia *DAD1* (Breuillin *et al.*, 2010) and the SL transporter gene *PDR1* (Kretzschmar *et al.*, 2012), rice *D10*, *D27*, and *D17*/*HDT1* (Sun *et al.*, 2014), chrysanthemum *DgD27*, *DgCCD7*, and *DgCCD8*, and *Medicago truncatula* *MtCCD7*, *MtCCD8*, *MtD27*, and *MtMAX1* (Bonneau *et al.*, 2013; Wen *et al.*, 2016). In *M truncatula*, SL transcript levels are also induced by nitrogen deficiency. In addition, the expression of *MtD27* and *MtMAX1* is regulated by the *NODULATION SIGNALING PATHWAY 1* (*NSP1*) and *NSP2* transcription factors, which are required for the establishment of rhizobial as well as arbuscular mycorrhizal symbiosis (Liu *et al.*, 2011). The expression of the SL biosynthetic gene MtD27 is also induced by a rhizobial signal, dependent on *NSP1* and *NSP2* (van Zeijl *et al.*, 2015). Recently, it was shown that SL production in the moss *Physcomitrella patens* is also induced by phosphate deficiency, indicating that the role of SLs in coordinating adaptation to nutrient availability is conserved and may be an original function of this class of plant hormones (Decker *et al.*, 2017).

Outlook

Recently, enormous progress has been made in understanding key steps of SL biosynthesis. However, several intriguing questions still need to be answered; for example, regarding the biological relevance of the formation of β-apo-13-carotenone by CCD8 enzymes, and whether these enzymes produce carlactone-like compounds, such as α-carotene-derived carlactone. We also still do not know the identity of the product of the enzyme LBO, or whether there are further, as yet unidentified, enzymes involved in SL metabolism. The pathway by which 5-deoxystrigol, the parent molecule of strigol-like SLs, is formed is another major area for which there are questions that have not been answered yet. It remains to be determined whether the conversion of carlactone into 5-deoxystrigol also involves a CYP711 enzyme; what the mechanism of LGS1 action is; and what determines the stereochemistry of the BC ring closure. A final and major question is why plants produce so many different SLs. With the increasing interest of the scientific community and awareness of the importance of SLs for basic science and agricultural applications, it is very likely that we will get exciting answers for these and other open questions in the near future.

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