Strigolactones - Biology and Applications

Chapter 1: Strigolactone biosynthesis and signal transduction

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Abstract

Strigolactones (SLs) are a group of carotenoid-derivatives that act as a hormone regulating plant development and response to environmental stimuli. SLs are also released into soil as a signal indicating the presence of a host for symbiotic arbuscular mycorrhizal fungi and root parasitic weeds. In this chapter, we provide an overview on the enormous progress that has been recently made in elucidating SL biosynthesis and signal transduction. We describe the tailoring pathway from the carotenoid precursor to the central intermediate carlactone, highlighting the stereo-specificity of the involved enzymes, the all-*trans*/9-*cis*- β -carotene isomerase (D27), the 9-*cis*-specific CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7) as well as CCD8 and its unusual catalytic activity. We then outline the oxidation of carlactone by cytochrome P450 enzymes, such as the Arabidopsis MORE AXILLARY GROWTH 1 (MAX1), into different SLs and the role of other enzymes in generating this diversity, and discuss why plants produce many different SLs. This is followed by depicting hormonal and nutritional factors that regulate SL biosynthesis and release, and by a description of transport mechanisms. In the second part of our chapter, we focus on SL perception and signal transduction, describing the SL receptor DECREASED APICAL DOMINANCE 2 (DAD2)/DWARF14 (D14) and its unique features, the central function of protein degradation mediated by the F-box protein MAX2 and its homologs. We also discuss the latest advances in understanding how SLs regulate the transcription of target genes and the role of SMXL/D53 transcription inhibitors.

Key Words: Strigolactone biosynthesis, Carotenoids, D27, CCD7, CCD8, Carlactone, MAX1, Strigolactone signaling, Strigolactone perception

Glossary

ABC transporter: ATP-binding cassette transporters, consisting of multi-subunits including transmembrane proteins and membrane-associated ATPases, with especially important roles in transport of plant secondary metabolites and hormones.

α/β -fold Hydrolase: a large, diverse superfamily of hydrolytic enzymes characterized by a core alpha/beta-sheet, which contains eight beta strands connected by six alpha helices and a catalytic triad.

Apocarotenoids: the oxidative cleavage products of carotenoids by CCDs or spontaneous oxidation.

Arbuscular mycorrhizal (AM) fungi: a class of symbiotic fungi of the phylum Glomeromycota, characterized by the formation of unique intracellular structures called arbuscules that receive organic carbon from the host and assist the plant in the acquisition of mineral nutrients through their associations with roots.

Butenolide: a lactone with a four-carbon heterocyclic ring structure. It is a common moiety in all SLs.

Canonical SLs: a subfamily of SLs characterized by the presence of a tricyclic lactone (ABC ring) connected to a conserved butenolide ring (D-ring) via an enol ether bridge in *R*-configuration.

Carlactone: a core intermediate in the biosynthesis of SLs, generated by the sequential action of D27, CCD7 and CCD8 from all-*trans*- β -carotene.

Carotenoids: a class of terpenoid pigments produced in plants, algae and some bacterial. They fulfill essential functions in photosynthesis and serve as precursors of hormones and signaling molecules.

Carotenoid cleavage dioxygenases (CCDs): a large family of non-heme iron (II)-dependent enzymes which break C=C double bonds in carotenoid or apocarotenoid backbone, leading to two carbonyl products.

Catalytic triad: a set of three coordinated amino acids comprising an acid, a base, and a nucleophile (often Asp, His, and Ser, respectively) found in the active site of hydrolases.

F-box protein: a component of the SCF type E3 ubiquitin-protein ligase complexes, which is responsible for substrate recognition, polyubiquitination and eventually protein degradation.

HPLC: High-performance liquid chromatography, an analytical chemistry technique used to separate, identify, and quantify different compounds in a sample mixture, which relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Due to the slightly different interaction of each substance in the sample with the adsorbent material, different substances have different flow rates when flowing out of the column therefore leading to their separation.

LC-MS: Liquid chromatography-mass spectrometry, a commonly used technique in analytical chemistry to identify a chemical by combining liquid chromatography (LC) or High-performance liquid chromatography (HPLC) with the mass analysis capabilities of mass spectrometry (MS).

MEP pathway: 2-C-Methyl-D-erythritol 4-phosphate pathway, a route for the biosynthesis of the isoprenoid precursor isopentenyl pyrophosphate (IPP), which starts with the condensation of pyruvate with D-glyceraldehyde phosphate. The MEP pathway is responsible for the synthesis of the isoprenoid building block IPP in bacteria and plastids.

Mevalonate pathway: a pathway for the synthesis of isopentenyl pyrophosphate (IPP) in the cytoplasm of eukaryotic cells, archaea and some bacteria. The mevalonate pathway is initiated by the condensation of two molecules acetyl-CoA and is the source of IPP in the cytoplasm of eukaryotic cells.

MS: mass spectrometry, an analytical technique that ionizes chemical species by electrons, ions or photons, energetic neutral atoms, or heavy cluster ions, and sorts the ions based on their mass-to-charge ratio (m/z) and to detect them qualitatively and quantitatively by their respective m/z and abundance.

Rhizosphere: the region of soil that surrounding the roots, which is directly affected by root secretions and is enriched in soil microorganisms.

Rootstock: the lower part of the combined grafted plant.

Scion: the upper part of the combined grafted plant.

Sesquiterpenes: a class of terpenes formed by the condensation of three isoprene units and consisting of a C_{15} skeleton.

Non-canonical SLs: subfamily of SLs that contain a variable second moiety instead of the tricyclic lactone connected to a conserved butenolide ring (D-ring) via an enol ether bridge in *R*-configuration.

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1.1 INTRODUCTION

1.1.1 Strigolactones - an Overview

Plants use chemical signals, such as hormones, to coordinate growth and developmental processes as well as responses to environmental stimuli. In addition, chemical signals are the major means in the communication of plants with other organisms in their environment. Strigolactones (SLs) are an exceptional example because of their dual activity as plant hormone with various developmental and stress-related functions and as communication signal in the rhizosphere (Akiyama et al., 2005; Cook et al., 1966; Gomez-Roldan et al., 2008; Siame et al., 1993; Umehara et al., 2008). Interestingly, it was the latter activity that led to the discovery of SLs. Strigol, the first identified SL, was isolated as a $C_{19}H_{22}O_6$ compound in 1966 from root exudates of cotton as seed germination stimulant of the root parasitic weed *Striga lutea* (Cook et al., 1966), but its structure was only elucidated six years later (Cook et al. 1972). Since then, a series of structurally related compounds have been isolated from root exudates of different plant species, based on their capability to trigger seed germination in root parasitic plants, i.e. *Striga*, *Orobancha*, *Alectra*, and *Phelipanche* spp. (Yoneyama et al., 2013; Xie et al., 2010). The collective name strigolactone was coined almost thirty years after the discovery of strigol to designate this group of intriguing compounds (Butler, 1995). Fig. 1 briefly introduces a timeline of discovery of SLs and of their various biological functions.

Root parasitic plants are obligate parasites that have partially or completely lost their photosynthetic capacity during the evolution towards parasitism (Bouwmeester et al., 2003). These weeds produce enormous numbers of tiny seeds containing few reserves that can ensure survival of the seedlings after germination for only few days. Hence, seed germination in *Striga* and related root parasitic species is tightly regulated and takes place only if a host root is present in close vicinity. This synchronization is brought about by the strict germination dependency on host released chemical signals, in most cases SLs. Following germination, seedlings develop a haustorium to connect to the host root, and siphon off water, minerals and assimilates (Bouwmeester et al., 2003; Delavault et al., 2002; Xie et al., 2010). This parasitism stunts the growth of the host. Thus, infestation of crops by root parasitic plants is a severe problem for

agriculture in warm and temperate zones of Europe, Asia and Africa, causing enormous yield losses in crops, such as cereals, legumes, rapeseed, tomato and sunflower (Parker, 2009).

The question why plants release SLs into the rhizosphere, though they provoke thereby the attack of parasitic weeds, had been bothering plant scientists for decades. In 2005, Akiyama *et al.* provided the answer to this problem by showing that SLs are a crucial factor in establishing the symbiosis of roots with beneficial arbuscular mycorrhizal (AM) fungi (Akiyama *et al.*, 2005). SLs induce branching of hyphae (thread-like filaments) in AM fungi, allowing them to grow towards and colonize the host root. AM symbiosis is an important support for the growth and survival of land plants, as evidenced by its presence in around 80% of them (Bonfante and Genre, 2015; Gutjahr and Parniske, 2013). This symbiosis plays a key role in supplying the host plant with minerals absorbed by the fungal hyphae that extend the plant's roots system, enabling the exploitation of a much larger soil volume. In return, the heterotrophic fungal partner obtains reduced carbon in the form of sugars that are produced by photosynthesis and which are used as energy and carbon source (Bonfante and Genre, 2015; Gutjahr and Parniske, 2013). The tremendous benefits of AM symbiosis in natural ecosystems explain why plants, particularly under phosphorus deficiency, release SLs into soil (Gutjahr, 2014; Khosla and Nelson, 2016). It can be assumed that root parasitic weeds, which evolved much later than the AM symbiosis, have coopted the SL signal as reliable indicator for host presence, by evolving a highly sensitive SL detection system coupled with the induction of seed germination.

The function of SLs as plant hormone was reported three years after revealing their role in AM symbiosis (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). This discovery became possible due to the availability of increased shoot branching and high-tillering mutants from several plant species. Genetic analysis and grafting studies enabled the classification of these mutants into two groups. The first one is deficient in the synthesis of a postulated mobile shoot branching-inhibitory signal, while the second one is affected in the perception of this signal. The lack of SLs in mutants from the first group and the capability of the SL analog GR24 to rescue their high-branching/-tillering phenotype suggested that SLs are the long sought-after shoot branching inhibitory signal. This conclusion was further confirmed by the lack of response to GR24 in the supposed perception mutants (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Nowadays, SLs

are recognized as a plant hormone that mediates the adaptation to nutrient deficiency and is involved (beyond attracting AM fungi as helpers in nutrient acquisition) in different aspects of plant growth and development, such as root development, stem secondary growth and senescence (Al-Babili and Bouwmeester, 2015; Brewer et al., 2013; Jia et al., 2018; Koltai, 2011; Ruyter-Spira et al., 2013; Waters et al., 2017). In addition, several lines of evidence suggest a role of SLs in plant response to biotic and abiotic stresses (Decker et al., 2017; Liu et al., 2015; Torres-Vera et al., 2014; Van Ha et al., 2014) (Fig. 2 illustrates the major biological functions of SLs).

1.1.2 SLs Examples, Structures and Nomenclature

So far, there are around 25 characterized natural SLs, and it can be expected that this number will increase in the future (Xie, 2016). The structure of strigol as the first-discovered SL, was also the first-elucidated (Cook et al. 1972, Fig. 3). This compound was also later identified as the major *Striga* seed germination stimulant in maize root exudates (Siame et al., 1993) and shown to be present in moonseed root culture filtrate (Yasuda et al., 2003). Interestingly, maize also produces 5-deoxystrigol, sorgomol (Fig. 3), and, as recently shown, two novel SLs that were designated as zealactone and zeapyranolactone (see chapter 1.2.5) (Yoneyama et al., 2015; Charnikhova et al., 2017; Xie et al., 2017). Similarly, sorghum produces and exudes various structurally different SLs, including strigol, sorgolactone, 5-deoxystrigol, and sorgomol that were identified in root exudates (Siame et al., 1993; Awad et al., 2006; Xie et al., 2008; Jamil et al., 2013; Motonami et al., 2013). Heliolactone (Fig. 3) is a further example for a natural SL, which was isolated as seed germination stimulant for both *Orobancha cumana* and *Striga hermonthica* from root exudates of sunflower (Ueno et al., 2014). Orobanchol (Fig. 3), the first discovered seed germination stimulant for *Orobancha minor*, was isolated together with its acetate, alectrol, from red clover (Yokota et al., 1998). Fabacyl acetate, identified in pea root exudates, is the first identified SL containing an epoxide group (Xie et al., 2009). Solanacol, which was identified in tobacco and tomato, is the first natural strigolactone equipped with a benzene ring (Xie et al., 2007).

These and other natural SLs are defined as carotenoid-derivatives characterized by a structure consisting of a conserved butenolide ring (D-ring, Fig. 3) that is linked in a defined stereo-chemical configuration (*R*-configuration) to a second, variable moiety (Jia et al., 2018). Based on the

structure of the second part, SLs are considered as canonical if they contain a conserved tricyclic lactone (ABC-ring, Fig. 3) or non-canonical if they have a different structure instead. Canonical SLs are divided based on the stereochemistry of the BC-ring junction into the orobanchol-type SLs with the C-ring in α -orientation (8b*R*-configuration) and the strigol-type SLs, with the C-ring in β -orientation (up; 8b*S*-configuration) (Jia et al., 2018, Fig. 3). Strigol and orobanchol were named according to their activity in triggering seed germination in *Striga* and *Orobanche* species, and derive from the corresponding precursors 5-deoxy-strigol and ent-2'-*epi*-5-deoxystrigol (4-deoxyorobanchol, see 1.2.5), respectively (Cook et al., 1972; Siame et al., 1993; Yokota et al., 1998). Strigol and orobanchol act as references to designate other, structurally related SLs that differ by substitution (s), or in the stereo-configuration of chiral centers at the C2'-atom and/or the B/C junction. The stereo-configuration is usually described by the abbreviations *ent*- and *epi*-referring to enantiomer (a mirror image of the reference) and epimer (opposite orientation at a single C-atom), respectively. However, the usage of these abbreviations may in some cases be confusing. Therefore, there is a tendency to replace complicated names by simple ones, e.g. 4-deoxyorobanchol instead of *ent*-2'-*epi*-5-deoxystrigol. Non-canonical SLs are distinguished by the presence of different structures as a second moiety instead of the ABC-ring characteristic for all canonical SLs (Al-Babili and Bouwmeester, 2015; Jia et al., 2018). Methyl carlactonoate (Abe et al., 2014), heliolactone (Ueno et al., 2014), zealactone and zeapyranolactone (Charnikhova et al., 2017; Charnikhova et al., 2018; Xie et al., 2017) and avenaol (Kim et al., 2014) are examples for this type of SLs (Fig. 3). The variability of non-canonical SLs and modifications of the A and B ring in canonical SLs, which include hydroxylation, methylation, epoxidation and ketolation, give rise to the diversity of natural SLs (Jia et al., 2018; Wang et al., 2018).

Synthesis of natural SLs is laborious because of their complex structure and the presence of chiral centers. In addition, plants release SLs in minute concentrations, which make the isolation of large quantities of these compounds from natural sources almost impossible. Therefore, SL research and application have been depending on synthetic analogs, such as the widely used GR24 (Fig. 3) that contains an ABC-ring (with a phenolic A-ring) coupled to the characteristic D-ring. However, in the meanwhile there are more simple analogs with considerable biological

activity. CN-debranone (Fukui et al., 2011), nitro-phenlactone (Jia et al., 2016) and methyl phenlactonoates (MP3, Jamil et al., 2017) are examples for such compounds (Fig. 3).

There is a different class of signaling molecules, the karrikins that share the butenolide ring as a conserved structural element with SLs (Fig. 3). They are perceived by a homologous signal transduction pathway with some common signal transduction components (Waters et al., 2013). Karrikins were first isolated from the smoke of burning plant material as compounds triggering seed germination in pioneer plants that emerge after forest and bush fires (Nelson et al., 2009). However, karrikins do not induce seed germination in root parasitic plants, in which the karrikin receptor gene has been presumably subjected to repeated duplication during the evolution of parasitism, accompanied by changing the ligand specificity towards SLs (Conn et al., 2015; Tsuchiya et al., 2015). Karrikins are supposed to mimic a yet unidentified signaling molecule that regulates developmental processes, such as seed dormancy and photomorphogenesis different from those governed by SLs (Flematti et al., 2015; Nelson et al., 2010). It should be mentioned here that the common SL-analog GR24 is usually available as a racemic mixture of the two enantiomers GR24 and *ent*-GR24, which binds to the SL receptor D14 and the karrikin receptor D14-like 1 (KAI2), respectively (Scaffidi et al., 2014). Therefore, *rac*-GR24 activates both SL and karrikin signaling pathways.

1.2 SL BIOSYNTHESIS

1.2.1 Sesquiterpenes or Carotenoid-derivatives?

For almost 40 years after the discovery of the first SL, there was no progress in elucidating SL biosynthesis because of the uncertainty regarding the metabolic origin of these compounds. SLs were supposed to be sesquiterpene lactones (Fig. 4) (Butler, 1995; Yokota et al., 1998), a class of secondary metabolites with medical importance, which originate from the cytosolic C₁₅ compound farnesyl diphosphate (de Kraker et al., 1998). However, the structural similarity of the A-ring of SLs with the ionone rings in carotenoids led to the hypothesis that SLs may originate from carotenoids, like the plant hormone abscisic acid (ABA) (Fig. 4, Bouwmeester et al., 2003; Parry and Horgan, 1992; Tan et al., 1997). Both carotenoids and sesquiterpenes are isoprenoids, i.e. they derive from the universal C₅ building block isopentenyl diphosphate (IPP) (Fig. 4).

However, plants utilize two different pathways for IPP synthesis: the mevalonate pathway that leads to sesqui- (C_{15}), triterpenes (C_{30}), steroids and other cytosolic isoprenoids, and the plastid 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway that provides the IPP precursor for the biosynthesis of plastid isoprenoids, such as mono- and diterpenes (C_{20}), chlorophylls, carotenoids and tocopherols. To answer the question on the SL metabolic origin, Matusova *et al.*, 2005 applied mevastatin and fosmidomycin to maize seedlings, which are isoprenoid biosynthesis inhibitors specific for the mevalonate and MEP pathway, respectively (Fig. 4, Matusova *et al.*, 2005). In addition, they used fluridone, an inhibitor of carotenoid biosynthesis (Fig. 4). After treatment, Matusova *et al.* 2005 investigated the *Striga hermonthica* seed germination stimulating activity of root exudates collected from treated seedlings, as proxy for the content of SLs. Intriguingly, the *Striga* seeds germination stimulating activity was greatly decreased in root exudates of fluridone-treated maize (Matusova *et al.*, 2005). Similar results were also obtained for fluridone-treated cowpea and sorghum root exudates that induced much lower germination of seeds of *Orobancha crenata* and *Striga hermonthica*, respectively. Because fluridone inhibits the enzymatic activity of phytoene desaturase, which catalyzes an early step in carotenoid biosynthesis, root exudates of several maize carotenoid biosynthesis deficient mutants (*lw1*, *y10*, *al1y3*, *vp5*, *y9* and *vp14-2274*) were tested for their parasitic plant seed germination stimulating activity. Indeed, root exudates of all these mutants induced lower germination of *Striga* seeds than those of wild-type seedlings (Matusova *et al.*, 2005). Taken together, these results indicated that SLs likely derive from carotenoids or apocarotenoids (carotenoid cleavage products) rather than sesquiterpene lactones.

1.2.2 Carotenoids - an Overview

The attractive colors of many fruits and flowers are the result of carotenoid accumulation. However, these widespread isoprenoid pigments also fulfill more important and vital functions in plants and other photosynthetic organisms (DellaPenna and Pogson, 2006; Walter and Strack, 2011). Carotenoids are indispensable constituents of the photosynthetic apparatus, protecting chlorophyll and other cellular components from photo-oxidation and contributing to the light-harvesting process (Hashimoto H, 2016). Carotenoids are split into two classes: the oxygen-free carotenes, and the xanthophylls that carry oxygen-containing functional groups (Fig. 5). Besides

this classification, carotenoids are distinguished by the type of their end groups (acyclic or linear, monocyclic and bicyclic) and by the stereo-configuration of their double bonds. In plants, carotenoid biosynthesis takes place in plastids and is initiated by the condensation of two molecules of geranylgeranyl diphosphate (C₂₀) to 15-*cis*-phytoene (C₄₀), which is catalyzed by the key enzyme phytoene synthase. Phytoene contains only three conjugated double bonds and is, hence, colorless. Phytoene is transformed in the next steps by a sequence of desaturation and *cis/trans* isomerization reactions into the red linear all-*trans*-lycopene that is equipped with 11 conjugated double bonds (Fig. 5). Cyclization reactions convert all-*trans*-lycopene into all-*trans*- β -carotene (Fig. 5), which carries two β -ionone rings, and all-*trans*- α -carotene (Fig. 5) equipped with an ϵ - and a β -ionone ring, dividing the pathway into the α - and β -branches. Hydroxylation of the two ionone rings in α - and β -carotene leads to all-*trans*-lutein and all-*trans*-zeaxanthin, respectively (Fig. 5). Epoxidation of the ionone rings in zeaxanthin forms violaxanthin (Fig. 5) via the mono-epoxidated antheraxanthin. All-*trans*-violaxanthin is the precursor of all-*trans*-neoxanthin (Fig. 5), the final product of the β -branch in plant carotenoid biosynthesis, which contains an allenic double bond (DellaPenna and Pogson, 2006; Fraser and Bramley, 2004; Moise et al., 2014; Walter and Strack, 2011). The composition and amounts of carotenoids in different tissues are variable and depend on the type of plastids (Howitt and Pogson, 2006; Ruiz-Sola and Rodríguez-Concepción, 2012). Chromoplasts, which contain specialized structures to accommodate high amounts of carotenoids, accumulate certain types of carotenoids, such as lutein in daffodil flowers or lycopene in tomato fruits (DellaPenna and Pogson, 2006; Fraser and Bramley, 2004), while the photosynthetically active chloroplasts have more defined carotenoid composition consisting of about 45% lutein, 25–30% β -carotene, and 10–15% of each of violaxanthin and neoxanthin (Goodwin, 1988; Lakshminarayana et al., 2005). Root leucoplasts contain only low amounts of carotenoids composed of around 30% lutein, 25% β -carotene, and 45% β -xanthophylls, as was shown for Arabidopsis (Britton, 1995; Maass et al., 2009).

The conjugated double bond system of carotenoids, which is responsible for their color and functions in photosynthesis, makes them prone to oxidation (Fig. 6). This process leads to cleavage of carotenoids into smaller molecules that carry carbonyl groups and which are generally called apocarotenoids (Giuliano et al., 2003; Nisar et al., 2015; Walter and Strack, 2011).

Carotenoid cleavage can occur at each double bond in the carotenoid backbone, triggered by reactive oxygen species (ROSs) that arise particularly under stress conditions. However, plants and other organisms, including humans, utilize an enzymatic cleavage of defined double bonds in specific carotenoids to generate particular apocarotenoids that exert important biological functions. Prominent examples of such apocarotenoids are the vision chromophore and vitamin A precursor retinal (Moise et al., 2005) and precursors for the fungal pheromone trisporic acid as well as the plant hormones ABA and SLs (Alder et al., 2012; Medina et al., 2011; Schwartz et al., 1997, Fig. 6). The synthesis of these compounds is catalyzed by a ubiquitous family of non-heme iron enzymes called carotenoid cleavage dioxygenases (CCDs). CCDs utilize molecular oxygen to break C - C double bonds, splitting their substrates into two products that carry either an aldehyde or a ketone functional group. Members of this enzyme family show different substrate specificities with regard to the type and stereo-configuration (*cis/trans*) of the carotenoid substrate and the position of the double bond that is cleaved. In addition, some CCDs convert apocarotenoids instead of intact carotenoids. Carotenoid cleavage leads to a plentitude of products with different physicochemical properties, ranging from volatiles and scents with short chain lengths, such as citral (C₁₀) in citrus fruits or β -ionone (C₁₃) in roses (Chikara et al., 2018; Huang et al., 2009), to long-chain, lipophilic pigments, such as the fungal neurosporaxanthin and the citrus fruit pigment citraurin (C₃₀) (Estrada et al., 2008; Rodrigo et. al., 2013).

There are five major plant CCD subfamilies: CCD1, CCD4, CCD7, CCD8, and the nine-*cis*-epoxy-carotenoid dioxygenases (NCEDs) (Auldridge et al., 2006; Walter and Strack, 2011). NCEDs are responsible for ABA biosynthesis, catalyzing the stereospecific cleavage of 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin at the C11-C12 (for carbon numbering of carotenoids, see Fig. 6) double bond to produce the ABA precursor xanthoxin (C₁₅) (Giuliano et al., 2003; Schwartz et al., 1997). CCD1 enzymes cleave different cyclic and acyclic all-*trans*-carotenoids as well as apocarotenoids at several positions, leading to a wide range of products. It has been assumed that the primary function of CCD1 is the scavenging of damaged carotenoids, allowing their replacement by intact ones (Ilg et al., 2010; Scherzinger and Al-Babili, 2008). However, CCD1 has also been implicated in the synthesis of special pigments formed upon colonization of plant roots by symbiotic mycorrhizal fungi (Floss et al., 2008; Walter, 2013). CCD4 enzymes catalyze the cleavage of the

C7'-C8' or the C9'-C10' double bond in bicyclic all-*trans* carotenoids, leading to C₁₀- or C₁₃-volatiles and C₃₀ or C₂₇ apocarotenoids (Bruno et al., 2015; Bruno et al., 2016; Ma et al., 2013; Rodrigo et al., 2013). The CCD4-mediated cleavage of the C9'-C10' double bond in all-*trans*-bicyclic carotenoids determines the carotenoid content in different plant tissues, such as Arabidopsis seeds (Gonzalez-Jorge et al., 2013) Chrysanthemum flowers (Ohmiya et al., 2006), and potato tubers (Campbell, et al., 2010; Bruno et al., 2015). In addition, the Arabidopsis CCD4 is supposed to mediate the formation of a hitherto unidentified signal from acyclic *cis*-configured desaturation intermediates, which regulates leaf and early chloroplast development (Avendano-Vazquez et al., 2014). CCD7 and CCD8 (Alder et al., 2012) are responsible for SL biosynthesis and will be discussed below.

1.2.3 Genetic Identification of Key SL Biosynthetic Genes

Matusova *et al.* (2005) demonstrated that carotenoids are the precursor of SLs. This conclusion was drawn from lack of SL activity in root exudates obtained from carotenoid deficient maize mutants and from maize, cowpea, and sorghum plants treated with carotenoid biosynthesis inhibitors. The presence of two moieties in SLs and their connection by an enol ether bridge led to the assumption that each of these moieties (ABC- and D-ring in canonical SLs; non-canonical SLs were unknown at that time) is synthesized separately, followed by a coupling reaction to form a parent SL molecule (5-deoxystrigol or 4-deoxyorobanchol). Because of lack of knowledge about the involved enzymes, Matusova *et al.* (2005) proposed that the ABC-ring, consisting of a C₁₄ skeleton, arises from a C₁₅ apocarotenal (it is not possible to generate a C₁₄-apocarotenal from direct cleavage of a C=C double bond in carotenoids), which would subsequently be converted by a series of hypothetical reactions into a tricyclic lactone (ABC-ring) that is then linked in a final step to a D-ring of unknown origin. The C₁₅-apocarotenal was supposed to be xanthoxin, the precursor of ABA, or 9-*cis*- β -apo-11-carotenal that can be formed by cleaving 9-*cis*- β -carotene. The proposed biosynthesis route consisted of nine steps, implying a quite complicated biosynthetic pathway involving many enzymes.

At about the same time, plant developmental biologists from different research groups were trying to identify a putative plant growth regulator that is transported from roots to the shoot to

limit the number of branches by inhibiting the outgrowth of axillar buds. The presence of this inhibitory signal was proposed upon the discovery of mutants with increased numbers of shoot branches/tillers, and was supported by grafting studies showing that a wild type rootstock can rescue the more branching/high tillering phenotype of mutant scions. These mutants were called *more axillary growth (max)* in Arabidopsis (Booker et al., 2004; Booker et al., 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; Arite et al., 2009; Ishikawa et al., 2005; Lin et al., 2009; Zhou et al., 2013), and *decreased apical dominance (dad)* in petunia (Drummond et al., 2009; Drummond et al., 2011; Hamiaux et al., 2012; Simons et al., 2007; Snowden et al., 2005). Table 1 gives an overview of these mutants and the function of the corresponding genes. Mapping of the two mutants groups *max3*, *rms5*, *dad3*, and *d17/htd1* on the one hand, and *max4*, *rms1*, *dad1* and *d10* on the other, identified *CCD7* and *CCD8* as the respective corresponding genes, suggesting a carotenoid origin of the branching inhibitory signal. Identification of the loci corresponding to further mutants demonstrated that the synthesis of the inhibitory signal also required a cytochrome P450 of the clade 711 (MAX1 in Arabidopsis), a small iron-containing protein (DWARF27 in rice) and an oxoglutarate-dependent oxidoreductase (Lateral branching Oxidoreductase; (LBO) in Arabidopsis) (Booker et al., 2005; Brewer et al., 2016; Lin et al., 2009). The breakthrough discovery that SLs are identical with the postulated inhibitory signal (Gomez-Roldan et al., 2008; Umehara et al., 2008) enabled deciphering the SL biosynthesis step by step, leading, in a relatively short time, to an almost complete picture of the core of this pathway, and showing that it needs much less enzymes than initially assumed.

1.2.4 From β -Carotene to Carlactone

Engineered *E. coli* strains that accumulate carotenoids are powerful tools to investigate the activity of carotenoid biosynthesis and modifying enzymes. These strains are usually generated through introducing carotenoid gene clusters from the bacteria *Pantoea agglomerans* and *Pantoea ananas* (formerly known as *Erwinia herbicola* and *Erwinia uredovora*, respectively), which confer *E. coli* cells with colors of accumulated all-*trans*-carotenoids. Transformation of these strains with CCDs leads to de-colorization due to shortening of the chromophore

(conjugated double bond system) upon cleavage, given that the CCD can cleave the substrate produced by the particular strain. HPLC, GC-MS, and LC-MS analysis of carotenoids and apocarotenoids can subsequently identify substrate and product(s) of the introduced CCDs. Booker *et al.* (2004) used this *in vivo* system to characterize the activity of the Arabidopsis AtCCD7. The authors transformed the corresponding cDNA in phytoene, ζ -carotene, lycopene, δ -carotene (a monocyclic carotene that carries a ϵ -ionone ring), β -carotene, and zeaxanthin (for structures, see Fig. 5) accumulating *E. coli* cells. GC-MS analysis detected C_{13} volatiles as AtCCD7 products produced in β -, ζ -, δ -carotene and zeaxanthin strains. The formation of these volatiles implies that AtCCD7 cleaves the C9-C10 and/or C9'-C10' double bond(s). This activity must also lead to a second product, a C_{27} -apocarotenal (plant carotenoids are C_{40} compounds), in case of a single cleavage reaction, and a C_{14} -diapocarotenal, in case of double cleavage. However, the authors did not provide data on the nature of the second product. Therefore, the question whether AtCCD7 catalyzes a single or double cleavage reaction remained unanswered. In the same year, Schwartz *et al.*, (2004) published a study on the enzymatic activity of AtCCD7 and AtCCD8. The authors confirmed the AtCCD7 activity, in β -carotene- and lycopene-accumulating *E. coli* cells, which leads to C_{13} -volatiles. Moreover, they showed that the second product is a C_{27} -apocarotenal (β -apo-10'-carotenal in case of β -carotene accumulating *E. coli* cells), suggesting a single cleavage reaction. In addition, Schwartz *et al.* (2004) investigated the activity of AtCCD7 *in vitro*. They expressed the enzyme in normal *E. coli* cells, purified and incubated it with different carotenoid substrates. Assays were then analyzed by HPLC. The *in vitro* incubation with β -carotene isolated from spinach leaves led to the formation of a C_{27} -apocarotenal. In a second experiment, AtCCD7 and AtCCD8 were simultaneously introduced in β -carotene accumulating cells, followed by HPLC analysis of the apocarotenoid content. This analysis unraveled a C_{18} -apocarotenoid (β -apo-13-carotenone; also called d'orenone (Schlicht *et al.*, 2008)) as a product of combined AtCCD7/AtCCD8 activity (Schwartz *et al.*, 2004). This result indicated that AtCCD7 and AtCCD8 catalyze a sequential cleavage of all-*trans*- β -carotene into β -apo-13-carotenone and that AtCCD8 cleaves the AtCCD7 product β -apo-10'-carotenal at the C13-C14 double bond (for carbon numbering of carotenoids, see Fig. 6). Indeed, an *in vitro* study by Alder *et al.*, (2008) showed that CCD8 enzymes from Arabidopsis and pea catalyze the conversion of all-*trans*- β -apo-

10'-carotenal (C₂₇) into β -apo-13-carotenone (C₁₈). However, the authors did not identify the second product, a presumed C₉-dialdehyde that is expected to arise together with β -apo-13-carotenone (C₁₈). These results led to the assumption that β -apo-13-carotenone is the precursor of the shoot inhibitory signal that was later demonstrated to be SL. However, there were two reasons that raised doubts in this biosynthesis scheme. The first one is the many structural differences between β -apo-13-carotenone (C₁₈) and SLs, which would require an even more complicated pathway than the one proposed to start with a C₁₅-apocarotenal (see 1.2.3). The second reason was the failure of β -apo-13-carotenone to rescue the high-tillering phenotype of the rice *ccd8* mutant (*d10*) (Alder et al., 2012), which contradicts the presumed role of this compound as SL precursor. Hence, there was a need for revisiting CCD7 and CCD8 enzymatic activities.

1.2.4.1 Stereo-specificity of CCD7

cis/trans-Isomerization is a major feature of carotenoids and apocarotenoids, which plays a crucial role in biological processes, such as light perception (also in vision!) and the formation of the plant hormone ABA that arises from a specific, *cis*-configured precursor (9-*cis*-violaxanthin or 9'-*cis*-neoxanthin) (Schwartz et al., 1997). However, this aspect had been overlooked in the course of investigating AtCCD7 and AtCCD8 activity. By virtue of conjugated double bond systems, carotenoids and apocarotenoids are prone to photo- and thermo-induced *cis/trans*-isomerization. This sensitivity impedes conclusions about the isomeric state of CCD substrates and products, if suitable analytical methods that can distinguish the *cis/trans*-isomers are missing. For instance, carotenoid-accumulating *E. coli* strains contain traces of *cis*-configured carotenoids that arise by physical (photo- or thermo-) isomerization of the all-*trans*-isomers formed by the introduced biosynthetic genes. In addition, β -carotene, isolated from plant material without further separation, is a mixture of different isomers, though all-*trans*- β -carotene is the major constituent. Taking these considerations into account, Alder *et al.*, (2012) revisited the proposed AtCCD7 activity, by employing *in vitro* assays and using pure β -carotene stereoisomers. Analysis of incubations with CCD7 from Arabidopsis, rice and pea demonstrated a clear substrate stereo-specificity of these enzymes. They cleaved 9-*cis*- β -carotene, but not all-*trans*-, 13-*cis*-, or 15-*cis*-

β -carotene. The cleavage reaction led to β -ionone and a β -apo-10'-carotenal isomer different from the all-*trans*- one, as demonstrated by HPLC-analysis (Fig. 7). Hence, it was concluded that the β -apo-10'-carotenal produced by CCD7 is 9-*cis*-configured, which was supported by comparison to a tentative 9-*cis*- β -apo-10'-carotenal and later by NMR-analysis (Alder et al., 2012; Bruno et al., 2014).

In vitro incubation with further 9-*cis*-configured carotenoids confirmed the strict stereo- and regio-specificity of the CCD7 enzymes. CCDs from Arabidopsis, pea and the moss *Physcomitrella patens* 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; Decker et al., 2017). An *in vitro* study of AtCCD7 activity on acyclic carotenoids led to 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). AtCCD7 and PsCCD7 showed higher affinity to 9-*cis*- β -carotene than to other substrates, such as 9-*cis*-zeaxanthin, which is consistent with the assumption that 9-*cis*- β -carotene is the precursor of SLs (Bruno et al., 2016). However, the cleavage of other carotenoids points to the possibility that some SLs may have a different origin. Indeed, a very recent study on CCD8 from different plant species showed the formation of 3-OH-carlactone from 9-*cis*-3-OH- β -apo-10'-carotenal and confirmed the presence of this product in plants (Baz et al., 2018).

1.2.4.2 CCD8, an Unusual CCD Producing Carlactone from a *cis*-Substrate

The identification of 9-*cis*- β -apo-10'-carotenal as the product of CCD7 raised the question on whether this apocarotenal can be converted by CCD8 and, if yes, into which product. To answer this question, Alder et al., (2012) incubated heterologously generated CCD8 enzymes from Arabidopsis, rice and pea with CCD7-produced 9-*cis*- β -apo-10'-carotenal. This incubation led to an unexpected product that was called carlactone and which differed from known carotenoid/apocarotenoid cleavage products (Fig. 7). Carlactone showed surprisingly common structural features with SLs and is identical with them in the number of C-atoms (C₁₉). It is a non-carbonyl, tri-oxygenated compound that already contains a lactone ring identical to the D-ring of SLs. Moreover, the two moieties of carlactone are connected by an enol ether bridge that is

present in all SLs. Carlactone also shares with natural SLs the same stereo-configuration (*R*-configuration) at the C2' atom, as shown later by Seto et al. (2014). Moreover, application of carlactone restored the wild-type tillering phenotype of the rice *ccd8* (*d10*) mutant and induced the germination of *Striga* seeds. These results suggested that carlactone is the intermediate of SL biosynthesis, which is formed by CCD8, although this compound was initially identified only as an *in vitro* product. Indeed, the presence of carlactone in rice and Arabidopsis was later demonstrated by a different group (Seto et al., 2014).

The formation of carlactone by CCD8 from 9-*cis*- β -apo-10'-carotenal and the previously reported cleavage of all-*trans*- β -apo-10'-carotenal into β -apo-13-carotenone suggest that this enzyme is an unusual CCD that catalyzes different types of reactions, depending on the stereochemistry of the substrate. When forming β -apo-13-carotenone, it is assumed that CCD8 utilizes a common dioxygenase mechanism, i.e. introduction of molecular oxygen into a C=C double bond, which leads to the formation of a dioxetane that breaks down into two carbonyl products (Fig. 8A), while the structure of carlactone implies different types of reactions and repeated oxygenations (Fig. 8B). To get insight into the reaction mechanism used by CCD8 to form carlactone, Bruno et al. (2017) applied a derivatization reagent to catch the second product expected to arise simultaneously with carlactone (C₁₉) from 9-*cis*- β -apo-10'-carotenal (C₂₇) and which had not been detected before. This approach identified ω -OH-(4-CH₃)-hepta-2,4,6-trien-al as a further, unusual CCD-product formed by CCD8, which carries an aldehyde and an alcohol functional group (Fig. 8B). The authors also used ¹⁸O₂ and ¹³C-labeling, which demonstrated that the three O-atoms of carlactone derive from atmospheric oxygen and that the C11-atom in carlactone (C2'-atom in SLs) corresponds to the C11 atom in 9-*cis*- β -apo-10'-carotenal. Based on these results, Bruno et al., (2017) developed a proposal for the mechanism of carlactone formation, which includes a series of reactions composed of isomerization, repeated oxygenation and intramolecular rearrangements. Furthermore, it is proposed that the stereo-configuration 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 to confirm this proposed mechanism.

1.2.4.3 Dwarf27, a 9-*cis*/all-*trans* β -Carotene Isomerase

The stereo-specificity of CCD7 and CCD8 suggests that SL biosynthesis relies on 9-*cis*- β -carotene as precursor. 9-*Cis*- β -carotene is a natural compound that may be formed from all-*trans*- β -carotene, the isomer produced by the plant carotenoid pathway, by photo- or thermo-isomerization. However, Alder et al. (2012) assumed that hormone synthesis would need a more regulated precursor supply and, hence, that 9-*cis*- β -carotene is formed from the all-*trans*-isomer by an enzymatic activity. Some years before, the iron-containing OsDWARF27 (OsD27) rice protein was identified as a plastidial enzyme required for SL biosynthesis, but with unknown enzymatic activity (Lin et al., 2009). The role of D27 in SL biosynthesis was also further confirmed by investigating the corresponding Arabidopsis mutant (Waters et al., 2012a). Hence, it was tempting to hypothesize that OsD27 might be the first described enzyme with an all-*trans*/9-*cis*- β -carotene isomerase activity. To test this hypothesis, Alder et al., (2012) expressed OsD27 in a β -carotene accumulating *E. coli* strain and analyzed the β -carotene pattern. This experiment showed that the introduction of OsD27 resulted in an increase in the 9-*cis*-/all-*trans*- β -carotene ratio, indicating an isomerase activity. *In vitro* assays performed with heterologously expressed and purified OsD27 confirmed this activity and showed that OsD27 catalyzes the isomerization of all-*trans*- into 9-*cis*- β -carotene and *vice versa*, resulting in an isomer equilibrium (Fig. 7). The mechanism of the 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 (Lin et al., 2009). OsD27 also isomerizes other carotenoids, such as the mono-hydroxylated β -carotene derivative β,β -cryptoxanthin (leading to 9-*cis*- β,β -cryptoxanthin), and α -carotene (leading to 9-*cis*- α -carotene and 9'-*cis*- α -carotene), that have at least one unmodified β -ionone ring (Bruno and Al-Babili, 2016). While 9-*cis*- β,β -cryptoxanthin, 9-*cis*- α -carotene and 9-*cis*- β -carotene can be converted by CCD7 into the same SL biosynthesis intermediate 9-*cis*- β -apo-10'-carotenal, the question about the biological significance of 9'-*cis*- α -carotene produced by CCD7 remains open.

In both rice and Arabidopsis, *d27* mutants display less severe tillering/branching phenotype, compared to *ccd7* or *ccd8* mutants (Lin et al., 2009; Waters et al., 2012a). This difference might

be explained by spontaneous isomerization of all-*trans*- β -carotene that can partially compensate lack of D27 activity. However, it is also possible that the D27 homologs (D27-LIKE 1 and D27-LIKE 2) present in Arabidopsis, rice, and other plant species also contribute to the formation of 9-*cis*- β -carotene. It should also be mentioned that 9-*cis*- β -carotene is not only a precursor of SLs but is also a structural component of the photosynthetic cytochrome *b6f* complex (Cramer et al., 2006).

1.2.5 From Carlactone to Canonical and Non-canonical SLs.

Cytochrome P450 (CYP) enzymes are heme-containing monooxygenases that form a large enzyme superfamily in plants, animals, fungi and bacteria. CYPs catalyze different types of reactions, such as C-hydroxylation, dealkylation, and epoxide formation (Isin and Guengerich, 2007; Werck-Reichhart and Feyereisen, 2000) and are involved in a wide range of biochemical pathways including lipids, alkaloids, terpenoids, and phenylpropanoids, as well as plant hormones (Chapple, 1998; Werck-Reichhart and Feyereisen, 2000).

The Arabidopsis *max1* mutant is disrupted in a gene encoding a CYP of the 711 clade (CYP711A1; MAX1) and shows a high branching phenotype similar to that of *max2*, *max3*, and *max4* mutants (Booker et al., 2005; Stirnberg et al., 2002). Genetic analysis and reciprocal grafting experiments suggested that MAX1 catalyzes a reaction downstream of MAX3 (CCD7) and MAX4 (CCD8) to produce the shoot branching inhibitory signal (SLs, Booker et al., 2005). This order of reactions is supported by the plastid localization of D27, CCD7 and CCD8 that act on carotenoids/apocarotenoids precursor (s) present in plastids and by the cytosolic localization of MAX1. The similarity of carlactone to SLs suggested that only a few steps would be required to convert carlactone into real SLs (Alder et al., 2012). In the absence of further candidate enzymes, it was assumed that carlactone may be a direct substrate of MAX1. Supporting this assumption, it was reported that the content of carlactone in the Arabidopsis *max1* mutant is approximately 700-fold higher than in wild type plants (Seto et al., 2014). In addition, feeding experiments of *max4* and *max1max4* mutants with ^{13}C -labeled carlactone confirmed the role of MAX1 in carlactone conversion. The direct evidence for the role of MAX1 in this process was provided by *in vitro* studies using yeast as expression system. Incubation of yeast microsomes expressing

MAX1 with carlactone showed the consecutive oxidations of the C19-atom, leading to a molecule designated by Abe et al. (2014) as carlactonoic acid (CLA) (Fig. 9). The authors confirmed the *in vitro* results by feeding *max4* and *max1max4* mutants with ^{13}C -labeled carlactone, which showed the formation of ^{13}C -labeled CLA in *max4* but not in *max1max4* double mutant. CLA is an intermediate in the Arabidopsis SL biosynthesis pathway and is methylated by an unidentified methyltransferase into methyl carlactonoic acid (MeCLA) (Fig. 9, Abe et al., 2014).

Recently, transcriptomic studies led to the identification of a further Arabidopsis SL biosynthesis enzyme. The enzyme *LATERAL BRANCHING OXIDOREDUCTASE (LBO)*, a member of the 2-oxoglutarate and Fe^{II} -dependent dioxygenase family, is co-expressed with MAX3 (Brewer et al., 2016), and an *lbo* loss-of-function mutant showed a more branching phenotype, which is, however, less pronounced than in *max3* and *max4* mutants. Further grafting and genetic analysis suggested that LBO acts downstream of MAX1 in the SL biosynthetic pathway (Brewer et al., 2016). Moreover, investigation of the activity of heterologously produced LBO *in vitro* showed the conversion of MeCLA into an unidentified product with a molecular weight equal to MeCLA+16 Da, suggesting the introduction of an oxygen atom in this substrate (Fig. 9, Brewer et al., 2016).

The rice genome encodes five *MAX1* homologs including a truncated, non-functional one (Challis et al., 2013). Zhang et al., (2014) investigated the activity of the four functional rice *MAX1* homologs, aiming at the identification of carlactone-metabolizing enzymes. For this purpose, the authors expressed the four *MAX1* enzymes in yeast and isolated the corresponding microsomes for *in vitro* studies utilizing carlactone as substrate (Zhang et al., 2014). In addition, they used the *Nicotiana benthamiana* transient expression system, by simultaneous introduction of genes encoding the carlactone biosynthetic genes alone, as a control, and together with each of the functional rice *MAX1*s. Leaves of transformed *N. benthamiana* plants were then analyzed by LC-MS to determine carlactone conversion and identify any product(s) formed. The two approaches led to the identification of the rice *MAX1* homolog Os900 (carlactone oxygenase, CO) as the enzyme that converts carlactone into 4-deoxyorobanchol (4DO; or *ent*-2'-*epi*-5-deoxystrigol), the parent molecule of the orobanchol-type, canonical SLs (Zhang et al., 2014). The conversion of carlactone into 4DO possibly involves a series of reactions resulting in oxidation, ring closure, and

B/C lactone moiety formation, all catalyzed by Os900 (Fig. 9 and Fig. 10). Intriguingly, simultaneous transient expression of *Os900*, *Os1400*, *Os5100* and *Os1900* with the carlactone biosynthetic genes caused a significant decline in 4DO and an increase in orobanchol, compared with co-expression of *Os900* (Zhang et al., 2014). This suggested that one of the other MAX1s (*Os1400*, *Os5100* or *Os1900*) oxidizes 4DO to form orobanchol. Further analysis in *N. benthamiana* and *in vitro* assays using yeast microsomes showed that Os1400 is an orobanchol synthase that hydroxylates 4DO to form orobanchol (Fig. 9, Zhang et al., 2014).

Tomato, like Arabidopsis, contains only one CYP711A1 (SIMAX1, Zhang et al., 2018). A *Slmax1* mutant showed several SL deficiency phenotypes in plant architecture and development and displayed significantly reduced production of SLs. Transient expression of *SIMAX1* together with *SID27*, *SICCD7* and *SICCD8* in *N. benthamiana* leaves demonstrated that SIMAX1 catalyzes the conversion of carlactone to CLA, just as Arabidopsis MAX1. Intriguingly, in contrast to the situation in rice, orobanchol (one of the major SLs in tomato) and its direct precursor 4DO were not directly produced from carlactone by SIMAX1. Plant feeding assays indicated that CLA, but not 4DO, is a precursor of orobanchol, which in turn is the precursor of the other known SLs in tomato (Zhang et al., 2018).

The investigation of the activity of AtMAX1 and homologs from rice and tomato unraveled differences in their substrates and formed products. However, feeding of fluridone-treated plants from different species, including sorghum, cotton, moonseed, cowpea and sunflower, with ¹³C-labeled carlactone resulted in the formation of ¹³C-CLA, indicating that the synthesis of CLA is a common step in SL biosynthesis (Fig. 9, Iseki et al., 2018). A recent, comparative study on AtMAX1 and homologs from rice, maize, tomato, poplar and the lycophyte *Selaginella moellendorffii*, which used different heterologous systems to determine the substrate specificities, revealed the presence of three groups. The first one, called A1 type MAX1s, includes AtMAX1 and homologs from Arabidopsis, tomato and poplar, which convert carlactone into CLA. A2 type MAX1s, represented by the rice Os900 and the *Selaginella* SmMAX1a/b, produce 4DO from carlactone. The A3 type of MAX1s, which includes the maize the maize ZmMAX1b and the rice Os1400, forms both CLA and 4-deoxyorobanchol from carlactone *in vitro* (Yoneyama et al., 2018). However, it can be speculated that A3 type MAX1s may have different catalytic activities

in vivo. For instance, there is some evidence for the formation of orobanchol from 4-deoxyorobanchol by Os1400 in planta.

1.2.6 The Biological Significance of Diversification in SLs

So far, around 25 different SLs have been characterized in different plant species, and even a single plant species can usually produce many different SLs (Abe et al., 2014; Awad et al., 2006; Charnikhova et al., 2017; Kohlen et al., 2013; Xie, 2016; Yoneyama et al., 2015). This is likely a consequence of the different biological functions exerted by SLs and indicates that structurally diversified SLs might be active in some biological functions but not in others (Wang and Bouwmeester, 2018). Moreover, there is likely a selection pressure for plants producing SLs with minor alterations in the structure, which could maintain the interactions with AM fungi but avoid the infestations of parasitic weeds. Indeed, different parasitic species show divergent responses to the root exudates from different host species. For instance, sorgomol, a SL found in root exudates of sorghum, is much more active in inducing seed germination in *Striga hermonthica*, compared to *Orobanche minor* (Xie et al., 2008). *Ent-2'-epi-orobanchol* and its acetylated derivative are two SLs found in both cowpea and red clover. It has been shown that acetylated *ent-2'-epi-orobanchol* has higher germination inducing activity towards *Orobanche minor* and *Striga hermonthica* than its non-acetylated form (Ueno et al., 2011), suggesting that a small change in SL structure can influence its biological activity in inducing seed germination in roots parasitic plant.

Recently, *LGS1* (*LOW GERMINATION STIMULANT 1*), which encodes an enzyme annotated as a sulfotransferase, was identified as a gene that determines the stereochemistry of canonical SLs released by sorghum roots (Gobena et al., 2017). Functional loss of *LGS1* altered the pattern of released SLs in sorghum, 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). Most intriguingly, this alternation in SL composition did not affect the other essential SL biological functions, such as the capability to promote AM fungi colonization and the inhibition of tillering (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 new possibilities for generating resistance to *Striga*.

1.3 REGULATORY CONTROL OF SL PRODUCTION

1.3.1 Regulation by Nutrient Availability

During evolution, plants have acquired the ability to sense the availability of soil nutrients and to respond accordingly. Nitrogen and phosphorus are two major nutrients required for plant growth and development, however, both are quite limiting nutrients in soil due to their low availability or low mobility. Therefore, plants modulate their root architecture and recruit symbiotic partners, such as AM fungi to increase the soil volume available for nitrogen and especially for phosphate uptake (Gutjahr, 2014). SLs have a critical role in both processes. Accordingly, SL biosynthesis and production are promoted when nitrogen and particularly when phosphate supply are insufficient, as shown for rice (Jamil et al., 2011; Umehara et al., 2010), Arabidopsis (Kohlen et al., 2011), sorghum (Jamil et al., 2013), maize (Jamil et al., 2012) red clover (Yoneyama et al., 2007). Similarly, root exudates of tomato plants grown under low phosphate conditions showed increased activity in inducing seed germination in *Orobancha ramosa*, compared to control plants (Lopez-Raez et al., 2018). LC-MS/MS analysis confirmed the increased production of several SLs, including orobanchol, solanacol and didehydro-orobanchol isomers under phosphate starvation. Similarly, the release of heliolactone by sunflower roots is induced upon phosphate starvation (Ueno et al., 2014). Recently, the effect of phosphate availability on germination stimulating activity of *Physcomitrella patens* exudate was examined also using *O. ramosa* seeds (Decker et al., 2017). It was demonstrated that SLs are released by *Physcomitrella* as confirmed by seed germination bioassay and that this release is increased by phosphate deficiency indicating the evolutionarily conserved role of SLs as well as their regulation by phosphate availability in plants (Decker et al., 2017).

The up-regulation of SL content and release upon nutrient deficiency and during the AM symbiosis is mainly a result of increased transcript levels of SL biosynthesis genes, as shown for rice, *Medicago truncatula* and tomato (Bonneau et al., 2013; Sun et al., 2014; Wen et al., 2016; Stauder et al., 2018). Accordingly, sufficient phosphate availability reduces transcript levels of SL

biosynthesis and transporter genes, as shown for petunia *DAD1/CCD8* and the SL transporter gene *PDR1* (Breuillin et al., 2010; Kretzschmar et al., 2012). Recently, it was shown that even the supply of carotenoid precursors (β -carotene) for SL production is upregulated by phosphate starvation and during root colonization by AM fungi. This supply is mediated by a distinct symbiosis-inducible isoform of phytoene synthase and precursor supply can be limiting for SL production in dicot roots (Stauder et al., 2018).

1.3.2 SL Homeostasis and Regulation by Other Hormones

Like other plant hormones, SL biosynthesis is regulated within complex networks built by other hormones. In addition, SL biosynthesis is governed by a negative feedback mechanism maintaining SL homeostasis. For instance, application of the SL analog GR24 to Arabidopsis plants led to a decrease of *CCD7* and *CCD8* transcript levels (Mashiguchi et al., 2009). Consistently, SL biosynthesis and perception mutants in Arabidopsis, pea, rice and petunia all showed elevated transcript levels of SL biosynthetic enzymes. This increase was observed with *CCD8* transcript 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 a gain of function *d53* mutant that displayed increased transcript levels of SL biosynthesis genes and elevated SL contents (Jiang et al., 2013; Yao et al., 2016).

The plant hormone auxin is a major regulator of plant growth and development. The feedback inhibition of SL biosynthesis is dependent on auxin levels and signaling. On the other hand, auxin is a major regulator of SL biosynthesis at the transcriptional level, as shown for pea, rice, Arabidopsis, Chrysanthemum, and tomato. In pea, removing the auxin source by decapitation or reducing polar auxin transport (PAT) by 1-N-naphthylphthalamic acid (NPA) treatment, 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 transcript levels of *DgD27* in Chrysanthemum and *MAX3* in Arabidopsis (Hayward et al., 2009). Auxin also positively regulates the expression of *MAX4* and *D10* in Arabidopsis and rice, respectively (Arite et al., 2007; Wen et al., 2016). In

Arabidopsis, the induction of *MAX3* and *MAX4* transcripts by auxin is dependent on *AUXIN RESISTANT 1 (AXR1)*, a subunit of the RUB1 activating enzyme that regulates auxin receptor complex (Hayward et al., 2009). In tomato, silencing of the gene encoding the auxin signaling component AUX/IAA protein SI-IAA27 resulted in down-regulation of SL biosynthetic genes, *D27* and *MAX1* (Guillot et al., 2017).

ABA is an important plant hormone regulating various processes in plants and is mainly known for its role in the response to abiotic stresses (Sah et al., 2016; Vishwakarma et al., 2017). Several studies have shown that SLs are involved in stress response. In Lotus, osmotic stress decreased the SL content in tissues and root exudates by reducing transcript levels of SL biosynthetic and transporter genes (Liu et al., 2015). In tomato, it was shown that inhibition of NCED, a key enzyme in ABA biosynthesis, by applying AbaminSG decreased SLs levels, indicating that ABA maybe a positive regulator of SL biosynthesis (Lopez-Raez et al., 2010). Accordingly, ABA-deficient mutants, such as *notabilis*, showed reduced transcript levels of the SL biosynthetic genes *LeCCD7* and *LeCCD8*, and released lower SL amounts (Lopez-Raez et al., 2010). A positive role of ABA in regulating SL biosynthesis in maize is indicated by decreased SL content in root exudates of the ABA-deficient mutant *viviparous14* (Lopez-Raez et al., 2010). In addition, a recent study indicated interference between ABA and SL biosynthesis in rice, which is caused by an involvement of the SL biosynthesis enzyme OsD27 in determining ABA level. Analysis of *Osd27* mutant unraveled lower ABA content shoot and higher susceptibility to drought, compared to wild-type (Haider et al., 2018).

Gibberellins (GAs) are plant hormones that regulate a series of developmental processes, such as germination, stem elongation, dormancy and flowering. A recent investigation of the effect of GAs on SL biosynthesis in rice suggested a role as a negative regulator of SL biosynthesis. Treatment with GA1, GA3 or GA4 resulted in decreased 4-deoxyorobanchol levels in root tissues and exudates. The decrease in SL biosynthesis is caused by down-regulation of *D27*, *D10*, *D17*, *Os900* and *Os1400* transcript levels (Ito et al., 2017).

1.4 SL TRANSPORT AND EXUDATION

Grafting is a commonly used technique for investigating metabolite and hormone fluxes between shoots and roots. SL-deficient mutants display an obvious “more branching/high-tillering” phenotype. However, the “bushy” phenotype could be fully rescued when mutant scions were grafted onto WT rootstocks, indicating that SLs are transported from roots to shoots (Kohlen et al., 2010). On the other hand, grafting wild-type scions onto rootstocks of SL-deficient mutants did not lead to increased branching, which suggests that SLs can be synthesized in aerial tissues as well (Kohlen et al., 2010). Grafting studies also indicated that SL biosynthesis intermediates can be transported from roots to shoots. Grafting of *max3* or *max4* scions onto *max1* rootstocks restored wild-type branching to *max3* and *max4* shoots, which suggests that carlactone or its derivative(s) accumulated in *max1* rootstocks is transported into *max3* or *max4* carlactone-deficient shoots where it can be further metabolized into SLs that inhibit the shoot branching (Booker et al., 2005).

The question on how SLs are transported from roots to shoots is still largely elusive. It has been assumed that SLs are transported through the xylem (Kohlen et al., 2010). However, upon feeding rice roots with labeled orobanchol and 4-deoxyorobanchol, both compounds were detected in the shoot, but not in the xylem sap (Xie et al., 2015). This experiment provided further evidence in favor of a different transport mechanism. Time kinetic studies showed that the transport of labeled SLs was much slower than expected for xylem-mediated movement (Xie et al., 2015). Thus, further studies are still required to understand the transport of SLs between organs.

In addition to root/shoot transport, SLs are released into the rhizosphere to facilitate the symbiosis with AM fungi, which is supported by the presence of specific transporters. In petunia, *P. hybrida* *PLEIOTROPIC DRUG RESISTANCE 1* (*PhPDR1*), a member of the ATP-binding cassette (ABC) transporter superfamily, was shown to mediate the release of SLs from the roots into the soil (Kretschmar et al., 2012). Localization studies indicated that *PhPDR1* is predominantly present in the plasma membrane of individual sub-epidermal cells in lateral roots which largely correspond to hypodermal passage cells that act as entry points for AM fungi hyphae. This may suggest that the *PhPDR1* activity leads to a local SL maximum guiding the hyphae of AM fungi hyphae to the hypodermal passage cells. *PaPDR1*, a *PhPDR1* ortholog from *P. axillaris*, exhibits a cell-type-specific asymmetric localization in different root tissues (Sasse et al., 2015). This

transporter is localized at the apical membrane of root hypodermal cells in root tips, which may explain the shoot-wards transport of SLs. Above the root tips, in the hypodermal passage cells, PaPDR1 is localized in the outer-lateral membrane, consistent with its postulated function to transport SLs from the roots into the rhizosphere.

1.5 SL PERCEPTION AND SIGNALING

1.5.1 Perception of SLs

Perception and signal transduction of various plant hormones including SLs share a number of common features, of which the regulation via the ubiquitin 26S proteasome system (UPS) of the receptor protein complex including transcriptional repressors is the most-intensely studied part (Hershko and Ciechanover, 1998). This main proteolytic pathway in eukaryotic cells is involved in ABA, auxin, brassinosteroid, cytokinin, ethylene, GAs, jasmonic acid and also in SL perception (Dharmasiri et al., 2005; Jiang et al., 2013; Katsir et al., 2008; Kepinski and Leyser, 2005; Mockaitis and Estelle, 2008; Santner and Estelle, 2009; Yao et al., 2016). UPS sequentially utilizes three type of ligases, E1, E2 and E3 to attach multiple units of ubiquitin to protein substrates, marking them for degradation by the 26S proteasome. There are five types of E3 ligases, including the SCF (SKP1, CULLIN and F-BOX) type that play an essential role in the signal transduction of plant hormones. SCF E3 ligase is a complex consisting of a conserved SKP1, a CULLIN protein, and a specific F-BOX protein that determines the substrates recognized by the E3 ligase (Shu and Yang, 2017). In Arabidopsis, the F-box protein MAX2 (rice ortholog is DWARF3, D3) is a signal transduction component for both SLs and karrikins. Accordingly, *max2* mutants show all phenotypes related to SL deficiency, but also reduced photo-morphogenesis and decreased seed germination, two karrikin-regulated processes (Shen et al., 2012; Waters et al., 2014).

However, a special feature of SL perception is the involvement of DWARF14 (D14), a α/β -fold hydrolase that binds and catalyzes the hydrolysis of the hormone signal, in this case of SLs (Fig. 11) (Hamiaux et al., 2012; Yao et al., 2016). The capability to hydrolyze the ligand is an intriguing feature of D14, which distinguishes it from other hormone receptors, such as the gibberellin receptor GID1 (Ueguchi-Tanaka and Matsuoka, 2010), and which has been demonstrated to be required for transducing the SL signal (Yao et al., 2016). SL hydrolysis is mediated by a conserved

Ser/His/Asp catalytic triad and results in the formation of 5-hydroxy-3-methylbutenolide (D-ring) and a tricyclic lactone. However, when applied to plants, none of the two products showed any SL activity, suggesting that the purpose of SL hydrolysis is not to release an active final SL product. Hence, it was assumed that a conformational change in D14, which accompanies hydrolysis, is critical for SL signal transduction. Indeed, the SL analog GR24 promotes the physical interaction between D14 and MAX2 and triggers the consequent degradation of D14 by MAX2, which require the active Ser/His/Asp catalytic triad in D14 protein (Hamiaux et al., 2012).

The crystal structure of the SL-induced rice AtD14-D3-ASK1 complex explained how the conformational change of AtD14 mediates SL signal transduction (Yao et al., 2016). Following binding and hydrolysis, the D-ring of SLs forms a covalently linked intermediate molecule (CLIM) with the receptor D14, while D14 undergoes an open-to-closed state conformational change to facilitate the interaction with D3 (Fig. 11) (Yao et al., 2016). Furthermore, it was demonstrated that the Ser in the Ser/His/Asp catalytic triad is responsible for docking of SLs into D14 (Zhao et al., 2013). The four helices near the active site pocket of D14 are also important for its activity, as corresponding mutations resulted in SL insensitivity due to a reduction in the aperture size of the active site pocket (Kagiyama et al., 2013; Nakamura et al., 2013). The formation of CLIM by D14 was also shown for pea D14 (de Saint Germain et al., 2016) and the *Striga* SL receptor ShHTL7 (Yao et al., 2017).

Karrikin-Insensitive2 (KAI2) and *DWARF14-LIKE2 (DLK2)*, are two close homologs of *AtD14* in *Arabidopsis* (Waters et al., 2012b). Despite its high similarity to D14, KAI2 shows different ligand specificity, binding karrikin and mediating the plant response to this compound that regulates photomorphogenesis and leaf development (Nelson et al., 2010; Soundappan et al., 2015; Waters et al., 2013). KAI2 has the conserved Ser/His/Asp triad that is required for karrikin response and possesses a smaller ligand-binding pocket than D14, indicating that this receptor recognizes ligands smaller than SLs (Zhao et al., 2013). However, the crystal structure of KAI2-KAR1 (KAR1 is a karrikin) complex indicates that KAI2-KAR1 creates a contiguous interface that allows the binding of signaling partners in a ligand dependent manner and likely does not transduce the ligand-signal by hydrolysis and CLIM formation, in contrast to D14 (Guo et al., 2013). There is not much information about the biological functions of DLK2, because *Arabidopsis dlk2*

mutants do not show any obvious phenotype (Waters et al., 2012b). Root parasitic plants contain D14 paralogs termed *Striga hermonthica* HYPO-SENSITIVE TO LIGHT/KARRIKIN INSENSITIVE2 proteins (ShHTLs/ShKAI2s) that perceive SLs to induce seed germination (Conn et al., 2015; Tsuchiya et al., 2015). By experimental evidences, it was demonstrated that ShHTL7, similar to D14, is a non-canonical receptor that hydrolyzes SLs and generates a CLIM, which enhances the interaction between ShHTL7 and ShMAX2 to trigger *Striga* seed germination (Yao et al., 2017).

1.5.2 Transduction of the SL Signal

As described above, SCF E3 ligase-regulated UPS plays a central role in the signal transduction of plant hormones. The transduction of the SL signal uses the SCF^{MAX2} complex that contains the F-box protein MAX2 and the SCF subunits ASK1 and AtCUL1. A study of a rice high-tillering gain-of-function mutant led to the identification of SMXLs/D53 proteins as the SCF^{MAX2/D3} substrates in the SL signaling pathway (Fig. 11) (Jiang et al., 2013; Yao et al., 2016). In rice, D14 directly interacts with D53 and D3 in a SL-dependent manner, leading to poly-ubiquitination and degradation of D53. A small deletion in the C-terminal of D53 stabilizes this protein, preventing its degradation, which results in phenotype similar to those of SL-deficient mutants (Jiang et al., 2013; Yao et al., 2016). This mutation and the resulting phenotype suggest that D53 is a negative regulator of SL signaling. In Arabidopsis, the D53 orthologues, SMXL6, SMXL7 and SMXL8, also interact with AtD14 and MAX2 and are negatively regulating shoot branching (Wang et al., 2015). There are eight members in the SMXLs/D53 family in rice and Arabidopsis, respectively (Stanga et al., 2013). It is assumed that SCF^{MAX2/D3} may have different substrate preferences and hence regulate different SL responses. In Arabidopsis, it was shown that SMAX1 and SMXL2, two members of the SMXLs/D53 protein family, are involved in SL-independent processes, i.e. karrikin-dependent photomorphogenesis and seed dormancy (Soundappan et al., 2015; Stanga et al., 2013). Moreover, SMXL3, SMXL4 and SMXL5 were demonstrated to regulate phloem development, independent of both SLs and karrikins (Wallner et al., 2017). Sequence analysis showed that all SMXLs/D53 proteins contain a conserved D2 ATPase domain (Zhou et al., 2013). This D2 domain includes a conserved ethylene-responsive element binding factor-associated amphiphilic repression motif of Phe-Asp-Leu-Asn-Leu, which has been assumed to interact with TOPLESS/TOPLESS RELATED PROTEIN2 (TPL/TPR2) (Fig. 11) (Ke et al., 2015; Pauwels et al., 2010;

Soundappan et al., 2015; Zhou et al., 2013). Members of the TPL/TPR2 family usually act as transcription co-repressors that inhibit the expression of genes related to plant hormones by repressing the transcriptional activity of interacting transcriptional factors (Ke et al., 2015). Indeed, it was also shown that D53, SMXL6, and SMXL8, all directly interact with TPL or TPR2 *in vivo* (Wang et al., 2015), suggesting that SMXLs/D53 proteins likely act as transcriptional repressors in SL signal pathway.

1.5.3 Downstream Transcription Factors in SL Signaling

Although the D3-D14-SMXLs/D53 regulatory module has been well characterized, the direct downstream transcriptional factors that regulate SL responsive gene expression are still largely unknown. One class of transcription factors (TFs), termed the *TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1* (TCP) family, has been shown to act downstream of *SMXLs/D53* in SL signaling, such as *FC1/OsTB1* in rice (Takeda et al., 2003), *BRC1* and *BRC2* in Arabidopsis (Aguilar-Martinez et al., 2007), *TB1* in maize (Doebley et al., 1997; Guan et al., 2012), and *PsBRC1* in pea (Braun et al., 2012). These genes are mainly expressed in axillary buds, and corresponding loss-of-function mutants showed an increased branching/tillering phenotype that could not be recovered by exogenous SL application. In Arabidopsis, the expression level of *BRC1* was shown to be decreased in *max3* and *max2* but enhanced in *smxl6/7/8* (Soundappan et al., 2015; Wang et al., 2015). Similarly, expression of *HB53*, one of the known target genes activated by *BRC1*, was also increased in *smxl6/7/8* triple mutants (Wang et al., 2015). Moreover, the expression of *BRC1* and *PsBRC1* was induced by SL treatment in Arabidopsis and pea, respectively (Braun et al., 2012). However, in monocots, such as rice and maize, SL signaling and the expression of *TB1* seem to be uncoupled; the expression of *FC1/OsTB1* and *TB1* is not induced by SLs (Guan et al., 2012; Minakuchi et al., 2010). In addition, even though *BRC1* works downstream of *SMXLs/D53* in Arabidopsis SL signaling, there is no direct interaction between *BRC1* and members of the *SMXLs/D53* family. Hence, the connection between *SMXLs/D53* and *BRC1* is still missing.

Ideal Plant Architecture 1 (*IPA1*) (Jiao et al., 2010; Miura et al., 2010), a *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) transcription factor (Wang and Wang, 2015), is a key regulator of

the plant architecture in rice that triggers the expression of *D53* (Song et al., 2017). Interestingly, IPA1 also interacts physically with D53, as shown by *in vivo* and *in vitro* studies. This interaction suppresses the transcriptional activity of IPA1, generating a negative feedback loop that controls *D53* expression, which is regulated by SLs (Song et al., 2017). IPA1 is the first example that shows how the SMXLs/D53 transcriptional repressors directly regulate downstream transcriptional factors (Fig. 11).

1.6 CONCLUSIONS

Strigolactones (SLs) are carotenoid-derived plant hormones and rhizosphere signaling molecules mediating the communication with symbiotic mycorrhizal fungi, which have been coopted by root parasitic weeds as seed germination signal.

The core pathway of SL biosynthesis is strictly stereospecific and leads from all-*trans*- β -carotene to the central intermediate carlactone, catalyzed by the sequential action of three enzymes, the all-*trans*/9-*cis*- β -carotene isomerase (D27), the 9-*cis*-specific CCD (CCD7) and the unusual CCD, CCD8, which catalyzes a combination of reactions on the 9-*cis*-product of CCD7.

CYP enzymes of the clade 711 (the Arabidopsis MAX1 and homologs) catalyze the conversion of carlactone to various canonical and non-canonical SLs, including carlactonoic acid, a supposed intermediate in the synthesis of both SL classes.

Plants produce different SLs, as observed in exudates of single plants. In addition, different plant species produce diverse SL compositions.

A set of largely unknown enzymes, including the recently identified oxoglutarate-dependent oxidoreductase, LBO, is responsible for the structural diversity of SLs. The elucidation of the biosynthesis routes behind this diversity will require combining multiple omic approaches, i.e. genomics, transcriptomics, and targeted metabolomics.

SL biosynthesis and release are regulated by the availability of nutrients, mainly phosphate, and governed by other plant hormones.

SL perception and signal transduction proceed via the ubiquitin 26S proteasome-based pathway that is used by other plant hormones. An SCF complex composed of Skp, Cullin and the F-box protein (MAX2 or D3) in combination with the α/β -hydrolase fold receptor (D14 or DAD2) catalyzes the ubiquitination of transcriptional repressors, such as D53 and SMXL6/7/8, upon binding of the SL ligand to the receptor; this results in the degradation of these repressors by the 26S proteasome, and subsequent activation of the transcription of SL responsive genes. The SL receptor differs from receptors of other plant hormones, as it acts as an enzyme that hydrolyses the SL ligand and binds covalently to the released D-ring moiety.

It can be assumed that the structural diversity of SLs is a result of an evolution towards particular functions in the communication with other organisms and in regulating plant development and stress response. Hence, the identification of this functional specificity and the elucidation of the biosynthesis routes leading to the different SLs are expected to open up new possibilities in developing crops with optimized architecture, increased resistance to root parasitic weeds and/or higher mycorrhization rates. Knowledge about particular functions of different SLs may also pave the way for designing SL analogs with specific applications.

1.7 APPLICATION POTENTIAL FOR SLs

The diversity of the functions predestines SLs, SL analogs/mimics and antagonists for application in agriculture and horticulture and makes the biosynthesis, release and perception of this hormone an excellent target for genetic engineering and breeding towards crops with improved performance.

Optimal plant architecture is essential for exploiting the production and yield potential of crops. SLs are a key regulator of shoot and root architecture, as suggested by the effect of SL deficiency on shoot branching and plant height. Therefore, modulation of SL biosynthesis and perception holds promise for generating crops and ornamental plants with the architecture, e.g. tiller/branch number, pattern of roots types, etc., desired for different agriculture and horticulture systems. Moreover, the recently discovered role of SLs in plant response to biotic and abiotic stress opens up a further possibility of enhancing crop productivity by manipulating SL biosynthesis and response towards increasing drought-, salt- and/or pathogen-resistance. In

this context, application of SL analogs and antagonists might be a promising, more straightforward approach to improve the resilience and architecture of plants in agriculture and horticulture.

The root parasitic weed *S. hermonthica* is a major threat to global food security, affecting many regions in the world, particularly sub-Saharan Africa. SLs are a major factor in the Striga/host plant interplay and a key component in dealing with this threat. Recent data indicate that pattern and amount of released SLs determine the susceptibility to Striga. Strigol-type SLs seem to be much better germination stimulants, compared to the orobanchol-like ones. Related *Phelipanche* and *Orobanche* spp. have also adapted to certain type of SLs. Hence, alteration of SLs pattern is an attractive possibility to alleviate the infestation by root parasitic weeds. This alteration can be approached either by breeding or by genetic engineering. Better knowledge on late steps of SL biosynthesis as well as on release mechanisms, which determine type and of released SLs, is required to achieve this target.

Considering the essential role of SLs in establishing the mycorrhizal symbiosis and the different efficiency of various SLs in this process, understanding the biological reason for SL diversity and the mechanism of their release will also help in increasing the mycorrhization, which improves the growth of host plants and reduce the demand for fertilizers.

Root parasitic plants produce very large amounts of seeds that can remain viable for more than a decade in the soil. Thus, seed banks of Striga and other root parasitic plants, which have accumulated over decades in infested soils, are major constraints in combating these weeds. The application of SL analogs to induce seed germination of these obligate parasites in the absence of a host is a promising approach that can significantly reduce or even eliminate accumulated seed bank. This method, suicidal germination, can be combined with other approaches, such as planting varieties with increased resistance, in an integrated strategy to successfully control root parasitic weeds.

Table 1: Genes involved in strigolactone (SL) biosynthesis and signaling (modified from Jia et al., 2018)

Abbreviations: CL: carlactone; CCD7: Carotenoid Cleavage Dioxygenase 7; CCD8: Carotenoid Cleavage Dioxygenase 8; MAX: MORE AXILLARY GROWTH; RMS: RAMOUSUS; DAD: DECREASED APICAL DOMINANCE; HTD: HIGH TILLERING DWARF; D: DWARF; LBO: LATERAL BRANCHING OXIDOREDUCTASE; SMXL: SMAX1-LIKE; TPL/TPR: TOPLESS/ TOPLESS-RELATED PROTEIN; BRC: BRANCHING; IPA: Ideal Plant Architecture.

Role		Protein Identity/Function	Gene name			
			Arabidopsis	Pea	Petunia	Rice
SL Biosynthesis	From β -carotene to CL	9- <i>cis</i> /all- <i>trans</i> - β -carotene isomerase	<i>AtD27</i>	-	-	<i>D27</i>
		CCD7	<i>MAX3</i>	<i>RMS5</i>	<i>DAD3</i>	<i>HTD1/D17</i>
		CCD8	<i>MAX4</i>	<i>RMS1</i>	<i>DAD1</i>	<i>D10</i>
	From CL to various SLs	Cytochrome P450, 711 (CYP711)	<i>MAX1</i>		<i>PhMAX1</i>	<i>CO</i> (<i>Os01g0700900</i>), <i>OS</i> (<i>Os01g0701400</i>), <i>Os02g0221900</i> , <i>Os06g0565100</i>
		Other downstream enzymes	<i>LBO</i>			
SL Signaling	Perception	α/β hydrolase	<i>AtD14</i>	<i>RMS3</i>	<i>DAD2</i>	<i>D14/ D88/ HTD2</i>
		F-Box protein	<i>MAX2</i>	<i>RMS4</i>	<i>PhMAX2A</i> , <i>PhMAX2B</i>	<i>D3</i>
	Transcription regulation	Repressor of SL signaling	<i>SMXLs</i>			<i>D53</i>
		Corepressors	<i>TPR2</i>			<i>TPL/TPR2</i>
		Transcriptional factors	<i>BRC1</i> , <i>BRC2</i>			<i>IPA1</i>

Figure legends:

Figure 1: Timeline of the discovery of SLs and their biological functions. AMF, arbuscular mycorrhizal fungi.

Figure 2: Biological functions of SLs. SLs inhibit the outgrowth of axillary buds, which prevents the formation of tillers/branches. SLs also accelerate leaf senescence, promote the growth of internode elongation and stem secondary growth, and determine root growth and architecture.

Released into the rhizosphere, SLs induce seed germination of root parasitic weeds, such as *Striga* spp. and induce hyphal branching in symbiotic arbuscular mycorrhizal fungi (AMF).

Figure 3: Structures and classification of SLs and analogs. Canonical SLs are characterized by the presence of a tricyclic lactone (ABC-ring) connected to a second lactone (D-ring) in *R*-configuration (2' *R*). They are divided into strigol- and orobanchol-like SLs based on the stereochemistry of the B/C-ring junction, with the C-ring in β orientation (up) and α orientation (down) in strigol-like and orobanchol-like SLs, respectively. Non-canonical SLs contain different structural elements instead of the tricyclic lactone. *rac*-GR24 is the most commonly used SL analog; CN-debranone and methyl phenlactonoate 3 (MP3) are two SL-analogs with a simple structure; KAR1, KAR2 and KAR3 are examples of karrikins.

Figure 4. Plant isoprenoid biosynthesis pathways (adapted from Matusova et al., 2005). Plants utilize two different IPP and DMAPP biosynthesis routes, the mevalonate pathway and the MEP pathway (also called as non-mevalonate pathway). The two pathways are present in different compartments and are used to produce the IPP building block for diverse classes of isoprenoids, such as carotenoids and sesquiterpene lactones. Structures of examples of these two classes are shown in the inset next to the structure of strigol.

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; MVA, mevalonic acid; MVP, mevalonate-5-phosphate; MVPP, mevalonate-5-pyrophosphate; FPP, farnesyl pyrophosphate; GA-3P, D-glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP-MEP, CDP-ME 2-phosphate; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate; HMBPP, 1-hydroxy-2-methyl-2-butenyl 4-pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate.

Figure 5: Representative examples of the two classes of carotenoids: the oxygen-free carotenes and the xanthophylls that carry oxygen-containing functional groups.

Fig. 6: Carotenoids are susceptible to oxidative cleavage that leads to a wide range of different compounds generally called apocarotenoids. The cleavage reaction is mediated by carotenoids cleavage dioxygenases (CCDs) but can also occur spontaneously triggered by reactive oxygen species. The structure of all-*trans*- β -carotene depicts the numbering of the C-atoms, which is used to designate the cleavage site and the resulting apocarotenoid.

Figure 7: SL biosynthesis. The 9-*cis*/all-*trans*- β -carotene isomerase D27 catalyzes the reversible conversion of all-*trans*- β -carotene (C_{40}) into 9-*cis*- β -carotene that is cleaved by the stereospecific carotenoid cleavage enzyme CCD7 at the C9'-C10' double bond into 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 (C₁₉) and the C₈ compound ω -OH-(4-CH₃)heptanal. The C- skeleton of carlactone corresponds to the shaded part of 9-*cis*- β -apo-10'-carotenal. D27, CCD7 and CCD8 are all localized in the plastids. Carlactone is the central metabolite of SL core pathway, which are catalyzed by cytochrome P450 enzymes, such as the Arabidopsis MAX1 and the rice Carlactone oxidase (CO) and Orbachol synthase (OS), into carlactonoic acid and canonical SLs. Other enzymes, such as the lateral branching oxidoreductase are involved in later steps of SL biosynthesis, which contribute to SL diversity.

Figure 8. A proposal for the mechanism of CCD8-catalyzed reactions (modified from Jia et al., 2018 and Bruno et al., 2017). (A) A proposal for the formation of β -apo-13-carotenone by CCD8 from all-*trans*- β -apo-10'-carotenal. R1 and R2 correspond to the structures present in the same color, respectively. The reactive Fe^{III}-O-O· species attacks the C14 of all-*trans*-substrate, forming an instable dioxetane intermediate at the C13-C14 double bond. The dioxetane breaks into two carbonyl products, β -apo-13-carotenone and a yet unconfirmed dialdehyde. (B) A simplified proposal for the mechanism of the formation of carlactone (**c**) from 9-*cis*- β -apo-10'-carotenal (**a**). The C numbering in **b-d** corresponds the C numbering in **a**. R3 and R4 correspond to the β -ionone moiety and the C17-C21 chain of the substrate (**a**), respectively. Oxygen atoms are depicted in red. It is proposed that the formation of carlactone (**c**) is initiated (step I) by converting the *transoid* configuration of the bonds depicted in magenta into a *cisoid* one, by rotating at C12-C13 in (**a**). The attack of Fe^{III}-O-O· species, formed by the activation of atmospheric O₂ by the ferrous iron in CCD8 active center, at C14 in (**b**), followed by repeated oxygenation and intramolecular rearrangements, leads to the production of carlactone (**c**) and an extensively conjugated ω -OH-aldehyde group (**d**). C11 in carlactone (**c**) corresponds to C11 in 9-*cis*- β -apo-10'-carotenal (**a**), as confirmed by using accordingly ¹³C-labeled 9-*cis*- β -apo-10'-carotenal (**a**).

Figure 9. Pathways from carlactone to SLs in different plant species. Names of the species are displayed in different colors, and names of enzymes from the same species are indicated in the corresponding color. *Intermediacy of 4-OH-CL in moonseed and MeCLA in sunflower has not been confirmed. Abbreviations: CL, carlactone; CLA, carlactonoic acid; MeCLA, methyl carlactonoic acid; 4-DO, 4-deoxyorobanchol; 4-OH-CL, 4-hydroxy carlactone; 5-DS, 5-deoxystigol.

Figure 10. Proposed mechanism for the formation of 4-deoxyorobanchol (4-DO) from carlactone oxidase (CO) (adapted from (Zhang et al., 2014)). The enzyme catalyzes three oxygenation reactions, with carboxylation of the C19, and hydroxylation of the C18 atom. Abstraction of a proton from the carboxyl group by an active center base (~B|) results in the formation of C ring, and synchronously the addition of a proton to the hydroxyl group at C18, triggers the stereospecific B ring closure and the release of water to form the canonical SL, 4-DO.

Figure 11. SL perception and signaling pathway. In the absence of SL, the transcriptional repressors SMXLs/D53 together with the co-repressor TPL/TPR interact with SPL transcription factors, inhibiting their transcriptional activity and repressing the expression of target genes. Binding of SL to the receptor D14 leads to the hydrolysis of the ligand, which releases the SL D-ring that covalently binds to the receptor. This process is accompanied by a simultaneous open-to-closed state conformational change that facilitates the interaction with the F-box protein D3 or MAX2. CLIM-D14-D3 further interacts with SMXLs/D53 to form a ternary complex, which will result in the polyubiquitination of D14 and SMXLs/D53 and, finally, in their degradation by the 26S proteasome. The degradation of D14 could desensitize SL signaling and the degradation of SMXLs/D53 will relieve the repression of SPL by SMXLs/D53-TPL/TPR, which activates SL-elicited responses.

Figure 1: Timeline of discovery of SLs and their biological functions. AMF, arbuscular mycorrhizal fungi.

Figure 2: Biological functions of SLs. SLs inhibits the outgrowth of axillary buds, which prevents the formation of tillers/branches. SLs also accelerate leaf senescence, promote the growth of internode elongation and stem secondary growth, and determine root growth and architecture. Released into the rhizosphere, SLs induce seed germination of root parasitic weeds, such as *Striga* spp. and induce hyphal branching in symbiotic arbuscular mycorrhizal fungi (AMF).

Figure 3: Structures and classification of SLs and analogs. Canonical SLs are characterized by the presence of a tricyclic lactone (ABC-ring) connected to a second lactone (D-ring) in *R*-configuration (2' *R*). They are divided into strigol- and orobanchol-like SLs based on the stereochemistry of the B/C-ring junction, with the C-ring in β orientation (up) and α orientation (down) in strigol-like and orobanchol-like SLs, respectively. Non-canonical SLs contain different structural elements instead of the tricyclic lactone. *rac*-GR24 is the most commonly used SL analog; CN-debranone and methyl phenlactonoate 3 (MP3) are two SL-analogs with a simple structure; KAR1, KAR2 and KAR3 are examples of karrikins.

Figure 4. Plant isoprenoid biosynthesis pathways (adapted from Matusova et al., 2005). Plants utilize two different IPP and DMAPP biosynthesis routes, the mevalonate pathway and the MEP pathway (also called as non-mevalonate pathway). The two pathways are present in different compartments and are used to produce the IPP building block for diverse classes of isoprenoids, such as carotenoids and sesquiterpene lactones. Structures of examples of these two classes are shown in the inset next to the structure of strigol.

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; MVA, mevalonic acid; MVP, mevalonate-5-phosphate; MVPP, mevalonate-5-pyrophosphate; FPP, farnesyl pyrophosphate; GA-3P, D-glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP-MEP, CDP-ME 2-phosphate; MEcPP, 2-C-methyl-D-erythritol 2,4-

cyclopyrophosphate; HMBPP, 1-hydroxy-2-methyl-2-butenyl 4-pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate.

Figure 5: Representative examples of the two classes of carotenoids: the oxygen-free carotenes and the xanthophylls that carry oxygen-containing functional groups.

Fig. 6: Carotenoids are susceptible to oxidative cleavage that leads to a wide range of different compounds generally called apocarotenoids. The cleavage reaction is mediated by carotenoids cleavage dioxygenases (CCDs) but can also occur spontaneously triggered by reactive oxygen species. The structure of *all-trans-β-carotene* depicts the numbering of the C-atoms, which is used to designate the cleavage site and the resulting apocarotenoid.

Figure 7: SL biosynthesis. The 9-*cis*/all-*trans*-β-carotene isomerase D27 catalyzes the reversible conversion of all-*trans*-β-carotene (C₄₀) into 9-*cis*-β-carotene that is cleaved by the stereospecific carotenoid cleavage enzyme CCD7 at the C9'-C10' double bond into 9-*cis*-β-apo-10'-carotenal (C₂₇) and β-ionone (C₁₃). In the third step, CCD8 converts 9-*cis*-β-apo-10'-carotenal into carlactone (C₁₉) and the C₈ compound ω-OH-(4-CH₃)heptanal. The C- skeleton of carlactone corresponds to the shaded part of 9-*cis*-β-apo-10'-carotenal. D27, CCD7 and CCD8 are all localized in the plastids. Carlactone is the central metabolite of SL core pathway, which are catalyzed by cytochrome P450 enzymes, such as the Arabidopsis MAX1 and the rice Carlactone oxidase (CO) and Orbachol synthase (OS), into carlactonoic acid and canonical SLs. Other enzymes, such as the lateral branching oxidoreductase are involved in later steps of SL biosynthesis, which contribute to SL diversity.

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Acknowledgement

We thank Justine Braguy and Jianing Mi for assisting drawing the illustrations and their critical reading to the manuscript.

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