Elucidation of the biosynthetic pathway and biological roles of strigolactones in maize and rice

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Chapter 1

General introduction

This Introduction is partially based on:
Jia, Kun-Peng, Changsheng Li, Harro J. Bouwmeester, and Salim Al-Babili.
Overview of strigolactones

Discovery of strigolactones and their biological functions

Strigolactones (SLs) have dual activities as plant hormone with various developmental and stress-related roles and as communication signal in the rhizosphere (Cook et al., 1966; Siame et al., 1993; Akiyama et al., 2005; Gomez-Roldan et al., 2008; Umehara et al., 2008). Interestingly, it was the latter activity in the rhizosphere that led to the discovery of SLs. The first identified SL, strigol, was isolated in 1966 from root exudates of cotton, as seed germination stimulant of the root parasitic weed, *Striga lutea* (Cook et al., 1966) (Fig. 1 gives an overview of the discovery of SLs and their biological functions). Since then, a series of structurally related compounds have been isolated from root exudates of different plant species, based on their capacity to trigger seed germination in root parasitic plants, i.e. *Striga*, *Orobanche*, *Alectra*, and *Phelipanche* spp. (Xie et al., 2010; Yoneyama et al., 2013; Al-Babili and Bouwmeester, 2015; a, 2021). The collective name “strigolactones” was coined to designate this group of intriguing compounds (Butler, 1995).

![Figure 1. Chronological overview of the discovery of SLs and their biological functions.](image)

These root parasitic plant species are obligate parasites, which produce enormous amounts of tiny seeds (Bouwmeester et al., 2003; Bouwmeester et al., 2021). The seed germination of these root parasitic plant species is tightly regulated, and only occurs when a host root is nearby. This synchronization is brought about by the strict germination dependency on host released chemical signals, in most cases SLs. The infestation of crops by root parasitic plants is a severe agriculture problem which causes tremendous yield losses in crops, such as cereals, legumes, rapeseed, tomato and sunflower (Parker, 2009). A more detailed analysis of the role of the SLs as germination stimulants of parasitic plants is described in **Chapter 2**.

The question why plants exude SLs into the rhizosphere, which triggers the germination of harmful parasitic plants, was answered in 2005 (Akiyama et al., 2005). It was shown that SLs are essential factors in establishing the symbiosis between plants and arbuscular mycorrhizal (AM) fungi (Akiyama et al., 2005). SLs induce hyphal branching in AM fungi,
allowing them to grow towards and colonize the host root. AM symbiosis contributes to the growth and survival of plants, as evidenced by its presence in around 80% of all land plants (Gutjahr and Parniske, 2013; Bonfante and Genre, 2015). 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. In return, the heterotrophic partner obtains reduced carbon, formed by photosynthesis, from the host (Gutjahr and Parniske, 2013; Bonfante and Genre, 2015). This benefit of AM symbiosis provides an explanation for the increased exudation of SLs into the soil under phosphorus deficiency (Gutjahr, 2014; Khosla and Nelson, 2016). It can be assumed that root parasitic weeds, which evolved much later than AM symbiosis, have hijacked the SL signal as a reliable indicator for host presence, by evolving a highly sensitive SL detection system coupled with the induction of seed germination.

The plant hormonal function of SLs was deciphered three years after uncovering their role in AM symbiosis (Gomez-Roldan et al., 2008; Umehara et al., 2008). This was discovered due to the research on increased shoot branching/tillering mutants from several plant species. Genetic analysis and grafting studies enabled the classification of these mutants into two groups. One that is deficient in the synthesis of a, mobile, shoot branching-inhibitory signal; the second mutated in the perception of this signal. The deficiency of SLs in mutants and the successful rescue of their high-branching/-tillering phenotype by the SL analog GR24 suggested that SLs are the 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). Indeed, today SLs are recognized as a plant hormone that mediates the adaptation to nutrient deficiency and is involved in the regulation of plant growth and development, such as root development, stem secondary growth and senescence (Brewer et al., 2013; Al-Babili and Bouwmeester, 2015; Waters et al., 2017; Bouwmeester et al., 2021; Mashiguchi et al., 2021; Xu et al., 2021). In addition, several studies show that SLs are involved in the response to biotic and abiotic stress such as pathogens, drought and salt stress, and osmotic stress (Torres-Vera et al., 2014; Van Ha et al., 2014; Liu et al., 2015; Decker et al., 2017) (Fig. 2 illustrates the major biological functions of SLs).

In recent years, it has been shown that SLs also play a role in nodulation. For instance, in soybean, SL biosynthetic gene mutants exhibited decreased nodulation while overexpression of SL biosynthetic genes resulted in an increased nodule number (Haq et al., 2017; Rehman et al., 2018). The signaling roles of SLs in both AM symbiosis and nodulation suggest they may possibly also function in other relations with the rhizosphere microbial community/microbiome. Indeed, in Arabidopsis, the SL deficient max4 mutant, displayed an altered composition of the fungal community while no clear effects were found on the bacterial community (Carvalhais et al., 2019). In contrast, overexpression of SL related genes in soybean significantly affected the bacterial community but had no
influence on the fungal community (Liu et al., 2020). However, in rice, differences in both the bacterial and fungal community were observed in rice SL biosynthesis and signaling mutants compared with wild type (Nasir et al., 2019). And a recent analysis of 16 rice genotypes with different SL production confirmed the latter study and, moreover, showed that structurally different rice SLs correlate with different microbes in the rhizosphere and roots (Kim et al., 2022).

**Figure 2. The 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). This figure is from a book chapter (Jia et al., 2019).

**Regulation of SL production 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, phosphate supply are insufficient, as shown for rice (Umehara et al., 2010; Jamil et al., 2011), Arabidopsis (Kohlen et al., 2011), sorghum (Jamil et al., 2013), tomato (Kohlen et al., 2012; Wang et al., 2022), maize (Jamil et al., 2012; Charnikhova et al., 2017, 2018), red clover (Yoneyama et al., 2007) and sunflower (Ueno et al., 2014). In addition, the effect of phosphate availability on germination stimulating activity of Physcomitrella patens exudate was examined using O. ramosa seeds (Decker et al., 2017). It was demonstrated that SLs are released by Physcomitrella, as confirmed by the 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 is mainly a result of increased transcript levels of SL biosynthesis genes, as shown for rice, tomato, maize, Dendranthema grandiflorum and Medicago truncatula (Bonneau et al., 2013; Sun et al., 2014; Wen et al., 2016; Ravazzolo et al., 2019; Wang et al., 2022). Accordingly, sufficient phosphate supply reduces transcription of SL biosynthesis and transporter genes, as shown for petunia DAD1 (Decreased Apical Dominance 1) / CCD8 (Carotenoid Cleavage Dioxygenase 8) and the SL transporter gene PDR1 (Pleiotropic Drug Resistance 1) (Breuillin et al., 2010; Kretzschmar et al., 2012).

**SL perception and signaling**

SL perception and signal transduction proceed via the ubiquitin 26S proteasome-based pathway similar as for other plant hormones (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Katsir et al., 2008; Mockaitis and Estelle, 2008; Santner and Estelle, 2009; Jiang et al., 2013; Yao et al., 2016; Seto et al., 2019) (Fig. 3). An SCF complex composed of SKP1, CULLIN and F-BOX protein (MAX2, D3, or RMS4) 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 (Hamiaux et al., 2012; Jiang et al., 2013; Lei et al., 2015; Yao et al., 2016; Shu and Yang, 2017; Seto et al., 2019). This results in the degradation of these repressors by the 26S proteasome, and subsequent activation of the transcription of SL responsive genes (e.g OsTB1, BRC1, BRC2, TB1, PsBRC1, IP41) (Doebley et al., 1997; Aguilar-Martínez et al., 2007; Jiao et al., 2010; Miura et al., 2010; Takeda et al., 2010; Braun et al., 2012; Guan et al., 2012; Song et al., 2017). Different models for SL perception and signaling have been postulated especially with regard to the binding and (need for) hydrolysis of the SL ligand (Yao et al., 2016; Seto et al., 2019; Bürger and Chory, 2020; Mashiguchi et al., 2021). In my work I focused on the structural diversity and intricate biological significance in the SLs and therefore in the next sections and chapters, I mainly emphasize SL biosynthesis.
Figure 3. SL 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. This figure is from a book chapter (Jia et al., 2019).

Biosynthesis of carlactone

SLs are carotenoid derivatives

For almost 40 years after the discovery of the first SL, there was no progress in elucidating SL biosynthesis because of uncertainty regarding their biosynthetic origin. SLs were supposed to be sesquiterpene lactones (Butler, 1995; Yokota et al., 1998), a class of secondary metabolites with medical importance, which originate from the cytosolic C_{15} 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) (Parry and
Horgan, 1992; Tan et al., 1997; Bouwmeester et al., 2003). Both carotenoids and sesquiterpenes are isoprenoids, i.e. they derive from the universal C₅ building block isopentenyl diphosphate (IPP). However, plants utilize two different pathways for IPP biosynthesis: the mevalonate pathway that leads to sesquiterpenoids (C₁₅), triterpenoids (C₃₀), steroids and other cytosolic isoprenoids, and the plastidic methylerythritol 4-phosphate (MEP) pathway that provides the IPP precursor for the biosynthesis of monoterpenoids (C₁₀) and diterpenoids (C₂₀), chlorophylls, carotenoids and tocopherols.

To answer the question on the SL origin, Matusova et al. applied mevastatin and fosmidomycin to maize seedlings, isoprenoid biosynthesis inhibitors specific for the mevalonate and MEP pathway, respectively, and fluridone, an inhibitor of carotenoid biosynthesis (Matusova et al., 2005). After treatment, Matusova et al. 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 seed germination stimulating activity was greatly decreased in root exudates of fluridone treated maize (Matusova et al., 2005). Similar results were also examined for fluridone-treated cowpea and sorghum, which also induced much lower germination of seeds of Orobanche 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 indicate that SLs derive from carotenoids and should be called apocarotenoids (carotenoid cleavage products) rather than sesquiterpene lactones.

Conversion of β-carotene to carlactone: the core pathway

At about the same time, plant scientists were trying to identify a putative plant shoot branching regulator that is transported from the root to the shoot. The presence of this inhibitory signal, which we now know is SL, was proposed upon the discovery of mutants with increased shoot branching/tillering. These mutants were called more axillary growth (max) in Arabidopsis (Stirnberg et al., 2002; Sorefan et al., 2003; Booker et al., 2004; Booker et al., 2005; Stirnberg et al., 2007), ramosus (rms) in pea (Beveridge et al., 1996; Morris et al., 2001; Sorefan et al., 2003; Foo et al., 2005), dwarf (d)/high tillering dwarf (htd) in rice (Ishikawa et al., 2005; Arite et al., 2007; Arite et al., 2009; Lin et al., 2009; Zhou et al., 2013), and decreased apical dominance (dad) in petunia (Snowden et al., 2005; Simons et al., 2007; Drummond et al., 2009; Drummond et al., 2012; Hamiaux et al., 2012) (Fig. 4). Mapping experiments showed that max₃, rms₅, dad₃, and d₁₇/htd₁ is one gene, and max₄, rms₁, dad₁ and d₁₀ is another, identified as CCD7 (Carotenoid Cleavage Dioxygenase 7) and CCD8 (Carotenoid Cleavage Dioxygenase 8) respectively.
Additionally, rice D27 (DWARF27) and Arabidopsis AtD27 were discovered as iron-binding enzymes involved in SL biosynthesis (Lin et al., 2009; Waters et al., 2012). Later on, the sequential steps catalyzed by these three enzymes (D27, CCD7, and CCD8) were characterized by in vitro assays (Alder et al., 2012) (Fig. 4). This revealed the SL biosynthesis core pathway (carlactone pathway), which starts from all-trans-β-carotene. After the formation of 9-cis-β-carotene by D27, CCD7 and CCD8 catalyze sequential conversions, to produce 9-cis-β-apo-10’-carotenal and eventually carlactone. Currently, it is generally assumed that carlactone is the common precursor for all SLs (Fig. 4).

Figure 4. An overview of SL biosynthesis pathways in plants. Three types of SLs are indicated. The names of characterized enzymes are shown along the arrows with solid line. The arrows with dashed line present unknown steps.
**Diversification of the SL biosynthesis pathway, from carlactone to other SLs**

All natural SLs share the C-2’ R-configured D-ring and they can be divided into two groups, canonical and non-canonical SLs (Zwanenburg and Pospíšil, 2013; Wang and Bouwmeester, 2018; Yoneyama et al., 2018) (Fig. 4). SLs in the former group have an intact tricyclic lactone (ABC-rings) and can be divided into strigol- and orobanchol-like SLs based on the stereochemistry of the C-ring. In the past years, also non-canonical SLs have been identified, such as zealactone, zeapyranolactone, heliolactone, lotuslactone and avenaol (Kim et al., 2014; Ueno et al., 2014; Charnikhova et al., 2017; Xie et al., 2017; Charnikhova et al., 2018; Xie et al., 2019) (Fig. 4).

**Cytochrome P450s in SL biosynthesis**

Cytochrome P450 (CYP) enzymes are heme-containing monoxygenases that form a large enzyme superfamily in plants, animals, fungi and bacteria (Werck-Reichhart and Feyereisen, 2000). CYPs catalyze different types of reactions, such as C-hydroxylation, dealkylation, and epoxide formation (Werck-Reichhart and Feyereisen, 2000; Isin and Guengerich, 2007). They are involved in a wide range of biochemical pathways of for example 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 displays a branched phenotype similar to that of max2, max3, and max4 mutants (Stirnberg et al., 2002; Booker et al., 2005). 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). Indeed, 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 13C-labeled carlactone confirmed the role of MAX1 in carlactone conversion. 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 resulted in the consecutive oxidation of the C19-atom, leading to carlactonoic acid (CLA) (Abe et al., 2014).

Several MAX1 homologs have been characterized from different plant species. The rice MAX1 homolog Os900 (carlactone oxygenase, CO) 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). Further analysis in Nicotiana benthamiana and in vitro assays using yeast microsomes showed that Os1400 is an orobanchol synthase that hydroxylates 4DO to form orobanchol (Zhang et al., 2014). In tomato, an simax1 mutant showed several SL deficiency phenotypes in plant architecture and development and displayed significantly reduced production of SLs (Zhang et al.,
Transient expression in *N. benthamiana* leaves demonstrated that SlMAX1 catalyzes the conversion of carlactone to CLA. A comparative study on AtMAX1 and homologs from rice, maize, tomato, poplar and the lycophyte *Selaginella moellendorffii*, using different heterologous systems to determine the substrate specificities, revealed the presence of three MAX1 groups (Yoneyama et al., 2018). The A1 type includes AtMAX1 and homologs from tomato and poplar, and converts carlactone into CLA. A2 type MAX1s, represented by the rice Os900 and the *Selaginella* SmMAX1a/b, produce 4DO from carlactone. The A3 type, which includes the maize ZmMAX1b and the rice Os1400, forms CLA from carlactone and orobanchol from 4DO in vitro (Yoneyama et al., 2018). In Chapter 3 and 4 of my thesis I further describe the biochemical functions of MAX1s and other CYPs from maize and rice.

Another member of the CYP family participating in SL biosynthesis is CYP722C. It was shown that VuCYP722C from cowpea (*Vigna unguiculata*) and SlCYP722C from tomato catalyze the conversion of CLA to orobanchol via 18-hydroxy-CLA (Wakabayashi et al., 2019). This direct conversion without passing through 4DO is clearly different from the orobanchol biosynthesis in rice (described above). Moreover, two homologs from cotton (*Gossypium arboreum*) and *Lotus japonicus* GaCYP722C were shown to catalyze 5-deoxystrigol (5DS) biosynthesis from CLA (Mori et al., 2020; Mori et al., 2020; Wakabayashi et al., 2020).

**Other enzymes in SL biosynthesis**

Also, genes/enzymes other than CYPs have been identified to be involved in SL biosynthesis. CLA and methyl carlactonoate (MeCLA) were first identified in Arabidopsis, and feeding assays proved that CLA is the precursor of MeCLA (Abe et al., 2014; Seto et al., 2014). Very recently, an enzyme coined CLAMT from Arabidopsis was identified as the CLA methyltransferase, and shown to catalyse the formation of MeCLA from CLA (Mashiguchi et al., 2022).

In 2016, transcriptomic studies led to the identification of a further Arabidopsis SL biosynthesis enzyme, *LATERAL BRANCHING OXIDOREDUCTASE (LBO)* (Brewer et al., 2016). Functional analysis showed that LBO converts MeCLA into an unidentified product MeCLA+16 Da (Brewer et al., 2016). A few years later, the LBO product was characterized to be hydroxymethyl-carlactonoate (1'-OH-MeCLA) and similar activities as the Arabidopsis LBO were found from homologs in other plant species such as tomato, maize, and sorghum (Yoneyama et al., 2020).

In *Lotus japonicus*, an enzyme belonging to the 2OGDs (2-oxoglutarate and Fe (II)-dependent dioxygenases), *lotuslactone defective (LLD)*, was identified to be playing a role in lotuslactone biosynthesis, although its function was not characterized in detail yet (Mori et al., 2020).
Approaches for SL biosynthetic gene discovery and their functional characterization

Omics technologies and their application in plant science have revolutionized the access to “plant data” (genome sequences, gene expression data, etc.). Using these data, bioinformatics tools such as RNA-seq and co-expression analysis have enabled the discovery of a number of SL biosynthetic gene, including the CYP722s, LBO, and LLD (Brewer et al., 2016; Wakabayashi et al., 2019; Mori et al., 2020). I have employed the use of these pre-existing data and creative combinations of analyses to find new maize and rice SL candidate genes in Chapter 3 and 4.

As mentioned above, heterologous expression systems have been successfully applied to characterize the functions of CYPs in SL biosynthesis. For instance, four rice MAX1 enzymes were expressed in yeast (Saccharomyces cerevisiae) and isolated microsomes tested in vitro for their capacity to use carlactone as substrate (Zhang et al., 2014). In addition, the authors used the N. benthamiana transient expression system, by simultaneous introduction of genes encoding the carlactone biosynthetic genes alone, as a control, as well as in combination with each of the rice MAX1s. Leaves of transformed N. benthamiana plants were then analyzed by LC-MS to determine carlactone conversion and identify any product(s) formed. These two approaches led to the identification of rice Os900 and Os1400, which are responsible for 4DO and orobanchol biosynthesis. Recently, an E. coli–yeast consortium was established as a new platform for producing SLs and to assess candidate gene function (Wu et al., 2021). In Chapter 3 and 4, I use the N. benthamiana transient expression system to reconstitute the maize and rice SL pathways and also employ in vitro yeast microsome assays to characterize the functions of P450 genes. In Chapter 5, I investigated the possibility of enhancing SL production in this transient N. benthamiana expression system.

Another method used to provide additional evidence on putative pathway intermediates is to supply (synthetic) intermediates to seedlings, preferably in combination with an inhibitor of SL biosynthesis such as fluridone. As an example, 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 SlMAX1. Assays in which putative intermediates were supplied to fluridone treated tomato seedlings showed that CLA, but not 4DO, is the precursor of orobanchol (Zhang et al., 2018). Indeed, later, tomato CYP722C was identified and shown to catalyze the reaction from CLA to orobanchol (Wakabayashi et al., 2019). In Chapter 3, I used a similar strategy to determine the intermediates in maize SL biosynthesis and the mechanism regulating the composition of the maize SL blend.

The function of genes can also be investigated using mutants generated in multiple different possible ways. Several examples (lbo, slced8, slmax1, slcyp722c) have been presented in SL biosynthesis research (Kohlen et al., 2012; Brewer et al., 2016; Zhang et
al., 2018; Wakabayashi et al., 2019). With the progress of genome editing, making targeted mutants in more plant species has become feasible. These mutants can then be analyzed for their phenotypes in several aspects (plant architecture, SL production & composition, and activities in interactions with other organisms). In Chapter 3 and 4, I use mutants of maize and rice to elucidate their intricate SL biosynthetic pathways.

Scope of this thesis
The objective of the research described in this thesis is to elucidate the biosynthetic pathway and biological roles of SLs in maize and rice. Chapter 1 provides a general introduction to this topic. In Chapter 2, I review the role of the SLs as germination stimulants of parasitic plants. In Chapter 3 and 4, I focus on the maize and rice SLs. By using a combination of approaches, including co-expression analysis and (transient) gene expression in N. benthamiana and yeast, I identify several new SL related genes as well as a number of new SLs, in both species. In these two chapters I show how intricate SL biosynthesis is and shed further light on their biological significance. In Chapter 5 I show how transient expression in N. benthamiana for functional characterization of candidate genes can be optimized and potentially used for the heterologous production of SL standards. Finally, in Chapter 6 I discuss several aspects of SL biosynthesis and the findings of my thesis, present an outlook on their societal relevance and discuss the remaining scientific challenges.

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