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

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Chapter 6

General discussion

The strigolactones (SLs) are a fascinating, highly diverse, class of molecules with multiple biological functions in and outside the plant. In the past decades, more and more new SLs were structurally identified in the root exudates of a range of different plant species (Kim et al., 2014; Ueno et al., 2014; Charnikhova et al., 2017; Xie et al., 2017; Charnikhova et al., 2018; Xie et al., 2019; Yoneyama et al., 2020). Among the identified SLs there are also several so-called non-canonical SLs such as zealactone, zeapyranolactone, heliolactone, lotuslactone, 1'-OH-MeCLA and avenaol that do have the conserved D-ring, but greatly differ from the canonical SLs with regard to the ABC part. These non-canonical SLs are present in maize (*Zea mays*), sunflower (*Helianthus annuus*), *Lotus japonicus* and *Avena strigosa*, among others. Whether there are principal differences in the activities and functional roles of canonical and non-canonical SLs distributed in the plant kingdom has been discussed (Yoneyama et al., 2018; Yoneyama et al., 2018). However, a lack of knowledge about the biosynthetic pathway genes (enzymes) of these different SLs limits the possibilities to investigate the specific roles of these SLs and also prevents practical applications, for example to tackle ecological and agricultural problems such as yield loss in cereals due to *Striga* infection.

In my thesis, I studied this mysterious and important class of compounds from several different angles. In **Chapter 2**, the importance of the SLs as germination stimulants of parasitic plants is reviewed. In **Chapters 3 and 4**, using a combination of approaches, including co-expression analysis based on RNA-seq data, heterologous expression in *Nicotiana benthamiana* and *Saccharomyces cerevisiae*, and metabolite analysis, I characterized the functions of several new SL biosynthetic genes from maize and rice, identified several new non-canonical SLs, and hence elucidated the intricate biosynthetic pathways of SLs in both maize and rice (Fig. 1). The discovered new SL biosynthetic genes are located together in the same region of the chromosome, forming a gene cluster. Differences in the expression of these genes are responsible for differences in the SL composition of the root exudate, and this affects the communication of these plant species with other organisms (i.e *Striga* and AM fungi). In **Chapter 5**, I optimized/increased the heterologous production of SLs using transient upregulation of their precursor pathway in *N. benthamiana*. Finally, in **Chapter 6**, I present the discussion on the results of my thesis from a broader perspective. I discuss approaches used to discover new candidates in SL biosynthesis and their functional characterization, the novel functions of SL biosynthetic genes in maize and rice, how selection pressure drives SL structural diversification, and how to apply our knowledge on SL diversification and biosynthesis to practical applications in the field, for example to combat parasitic plants.

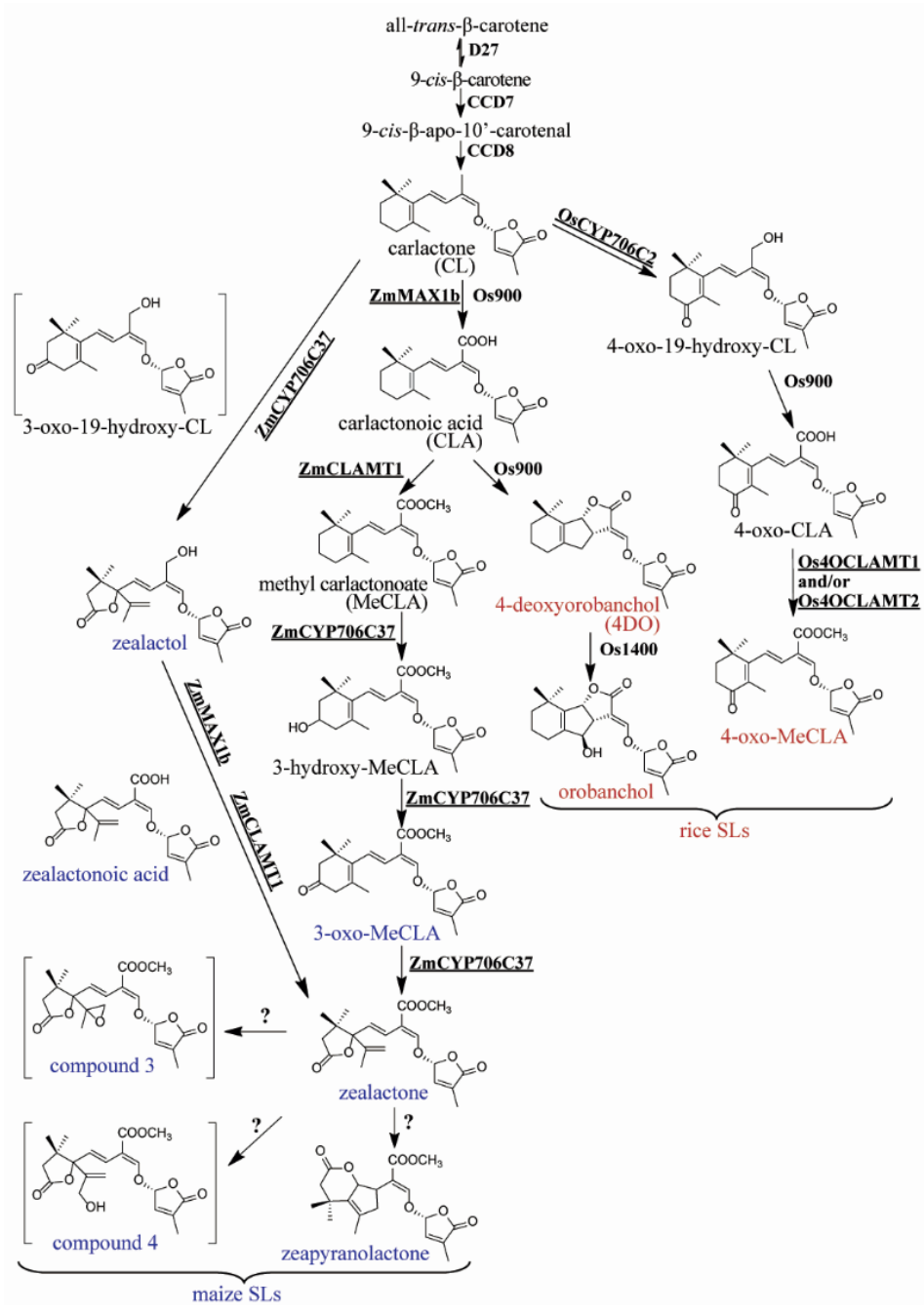

Figure 1. SL biosynthetic pathway of SLs in maize (indicated in blue) and rice (indicated in red). The enzymes characterized in this thesis are underlined. Question marks indicate as yet unidentified biosynthetic steps.

SL biosynthetic gene discovery and their functional characterization

So far, many SL biosynthetic and signaling genes were discovered using forward genetic screening and mapping mutations in increased tillering/branching mutants from several plant species. These mutants include *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). However, the forward genetics tool is less effective if the mutants have a too weak or invisible phenotype (Brewer et al., 2016). Therefore, other tools have been employed in recent years, such as the use of RNA sequencing data and bioinformatics tools have been used for the discovery of new SL biosynthetic genes, such as *CYP722Cs*, *LBO*, *LLD* and *CYP712G1* (Brewer et al., 2016; Wakabayashi et al., 2019; Mori et al., 2020; Wang et al., 2022). With all these approaches to discover new SL candidate genes and identify their functions in mind, in **Chapter 3 and 4**, I combined and optimized several methods, which resulted in the elucidation of the SL biosynthesis pathways in two major crops, maize and rice. In **Chapter 3**, bioinformatics analysis of maize transcriptome data helped me find and narrow down new candidate genes for maize SL biosynthesis. Specifically, these transcriptome analyses include mutual rank (MR) co-expression, graphical Gaussian model (GGM) gene network and differentially expressed gene analysis. Outputs of genes of interests were first obtained from the individual analyses, and then the candidates overlapping between these methods were selected for further characterization. In **Chapter 4**, similar approaches were used to find rice SL biosynthetic candidate genes from published transcriptome data of rice roots (Secco et al., 2013).

The characterization of the biochemical functions of these SL related genes is achieved through heterologous expression and/or mutant analysis. For the functional characterization of the rice SL biosynthetic genes *Os900* and *Os1400*, for example, transient expression in *N. benthamiana* and *S. cerevisiae* were used (Zhang et al., 2014) (Fig. 1). More recently, an *E. coli*–yeast consortium was established as a new platform for producing SLs and testing the function of candidate genes (Wu et al., 2021). In this consortium, carlactone is produced by *E. coli*, which is then further converted by P450s expressed in yeast. For the other method, mutant analysis, a gene of interest is knocked down or out (reverse genetics), after which phenotypes, such as plant architecture, SL production & composition, and the interaction with other organisms are analyzed. With the great progress in gene editing techniques, generating gene knockout lines in more plant species is feasible, which will accelerate the exploration of gene functions in SL

biosynthesis. In **Chapter 3 and 4**, the functions of the top candidate genes were tested in two expression systems. Agroinfiltration in *N. benthamiana* with known SL precursor pathways and in combination with candidate gene(s), together with (semi-)targeted metabolomics analysis showed us if the enzyme(s) catalyzed conversions of SL precursors to the anticipated intermediates or SL products. The fact that many SLs and pathway intermediates have not been elucidated greatly hampers the elucidation of the reactions catalyzed by enzymes, even if we have candidates in hand. In **Chapter 3 and 4**, using educated guessing and retrosynthesis, we got access to synthetic standards for several maize and rice SLs (and intermediates), which facilitated the elucidation of the SL biosynthetic pathways.

In **Chapter 5**, we aimed at promoting SL production in *N. benthamiana*. There, we tried two different strategies: overexpression of heterologous isoprenoid pathway genes and RNAi silencing of competing pathway genes to boost the production of the common SL precursor, carlactone. Among the candidate genes and constructs tested, two constructs (*Arabidopsis PSY-GGPS11* and *Zea mays ZmPSY1*) driving the overexpression of upstream precursor pathway genes indeed increased carlactone production (around 3-fold higher). However, the competing pathway silencing approach did not show a positive effect on carlactone production. Other candidates or strategies might need to be tested in the future. To show that carlactone boosting also results in a larger flux towards real strigolactone end products, I also transiently co-expressed the maize SL biosynthetic genes I discovered in **Chapter 3** as well as two rice SL biosynthetic genes published previously (Zhang et al., 2014). The production of the maize and rice SLs were also enhanced by the coexpression of the pathway boosting constructs, *Arabidopsis PSY-GGPS11* and *Zea mays ZmPSY1*.

To supply additional support for the biochemical function of the two P450 genes that I discovered, *ZmCYP706C37* and *OsCYP706C2*, I used *in vitro* yeast microsome assays using SL precursors and intermediates as substrate. The advantage of this yeast -based assay is that we get a cleaner product and have less side effects because it is a cleaner, simpler system. When I used this in my work, I could confirm that these two P450s catalyze the steps in SL biosynthesis that we also found in *N. benthamiana*. The disadvantage of this yeast-based system is that pure/synthetic compounds are needed as substrate, which sometimes is not easily obtained. In *N. benthamiana* we make those substrates enzymatically. In **Chapter 3**, I also used the application of fluridone and synthetic maize SLs and precursors to maize seedlings to further proof the postulated SL pathway. Hereto, with the carotenoid inhibition effect of fluridone and detection of compounds (the added ones and the accumulated SLs in root exudates), the intermediates in maize SL biosynthesis and the mechanism regulating maize SL profiles were determined. However, prerequisites for this approach are a rational postulated mechanism

and availability of compounds and detection methods. Moreover, in **Chapter 3 and 4**, mutants from maize and rice, generated by transposon or EMS, and CRISPR-Cas9 knock outs, provided us with more clear evidence of the intricate biosynthetic pathways of SLs from both species.

Novel functions SL biosynthetic genes in rice and maize

As described above, several approaches were combined and used to discover and characterize new genes in SL biosynthesis in maize and rice. This resulted in the identification of genes from two families, the cytochrome P450s and methyltransferases.

The cytochrome P450 (CYP) enzymes form a superfamily that plays a very important role in plant metabolism (Mizutani and Ohta, 2010; Nelson and Werck-Reichhart, 2011; Dimaano and Iwakami, 2021). Among its subfamilies, MAX1 or CYP711A, was the first P450 that was shown to be involved in SL biosynthesis (Booker et al., 2005). A few years later, the function of Arabidopsis MAX1 was revealed, and shown to be responsible for the conversion of carlactone to carlactonoic acid (CLA) (Abe et al., 2014). Intriguingly, in rice five homologs of MAX1 are present, in contrast to the one copy in Arabidopsis, two of which (Os900, Os1400) catalyze the conversion of carlactone not to CLA, but to 4-deoxyorobanchol (4DO) and from 4DO to orobanchol, respectively (Zhang et al., 2014) (Fig. 1). In a comprehensive study, the function of MAX1 homologs from multiple plant species was tested and this showed that maize homolog, ZmMAX1b, *in vitro* converted carlactone to CLA, and 4DO to orobanchol (Yoneyama et al., 2018). The latter is surprising as there is no clear evidence indicating that maize produces canonical SLs such as 4DO and orobanchol (Charnikhova et al., 2017, 2018), suggesting that this is an *in vitro* artefact when 4DO is used as substrate. Also other cytochrome P450s have been identified to participate in SL biosynthesis, such as CYP722Cs from tomato and cowpea that catalyze the conversion of CLA to orobanchol, without the formation of 4DO as an intermediate (Wakabayashi et al., 2019; Mori et al., 2020; Wakabayashi et al., 2020). Interestingly, the CYP722C homologs from lotus and cotton catalyze the biosynthesis of 5-deoxystrigol (5DS), a strigol-type SL, from CLA. Recently, a new P450 member from tomato, SlCYP712G1, was identified to be responsible for the biosynthesis from orobanchol of the so-called DDH isomers, the precursor of solanacol, an important tomato SL (Wang et al., 2022).

Finally, although the involvement of a methyltransferase is very obvious considering the many methoxylated SLs, only very recently the first methyltransferase, CLAMT, was identified in Arabidopsis and shown to convert CLA to MeCLA (Mashiguchi et al., 2022).

In **Chapter 3 and 4**, I describe the discovery of two genes from a new P450 subfamily, CYP706, and methyltransferases as SL biosynthesis enzymes in maize and rice (Fig. 1). In **Chapter 3**, I demonstrated that ZmCYP706C37 is essential for the biosynthesis of all

maize SLs. Surprisingly, there are two parallel pathways towards zealactone biosynthesis (Fig. 1). In one of these, carlactone is converted by ZmCYP706C37 to form zealactol after which ZmMAX1b and ZmCLAMT1 catalyze the conversion from zealactol to zealactone (Fig. 1). In the other branch, the order for these three downstream SL biosynthetic enzymes is different and MeCLA is produced by ZmMAX1b and ZmCLAMT1 from carlactone via CLA as an intermediate (Fig. 1). Then the conversion from MeCLA to zealactone is catalyzed by ZmCYP706C37 (Fig. 1). I managed to detect some of the intermediates in these pathways (3-oxo-MeCLA, zealactol, zealactonoic acid) also in maize root exudate (Fig. 1). In **Chapter 4**, the homolog of ZmCYP706C37 in rice also showed up as one top candidate in our bioinformatics screening. It is mainly expressed in root tissues and is induced by phosphate starvation, which might be a indicative it is SL related (López-Ráez et al., 2008; Umehara et al., 2010). In addition to 4DO and orobanchol, rice also produces other SLs, among which one was previously named "methoxy-5DS-isomer" but with unknown structure. With the mutant analysis and chemical synthesis, I showed that OsCYP706C2 is required in the biosynthesis of this new SL, we coined 4-oxo-MeCLA. Unlike the activity of ZmCYP706C37 in using both carlactone and MeCLA as substrate, to my surprise OsCYP706C2 was virtually inactive with MeCLA as substrate. However, it consumed carlactone when transiently expressed in *Nicotiana benthamiana*, which resulted in the formation of 4-oxo-carlactone that was then further converted by rice MAX1 and methyltransferase to 4-oxo-MeCLA (Fig. 1). Taken together, the knowledge from maize and rice biosynthetic genes/enzyme functions indicates that the biosynthetic pathways downstream of carlactone can be complicated, and the involved enzymes can even play dual or even multiple roles.

The CYP706 family belongs to the CYP71 clan (Nelson and Werck-Reichhart, 2011). There are only a few reports about the functions of CYP706 members in plants. CYP706B1 from cotton (*Gossypium arboreum*), for example, was identified as a (+)-δ-cadinene-8-hydroxylase, in the biosynthesis pathway of gossypol (a sesquiterpene aldehyde, plant defense related compound) (Luo et al., 2001). CYP706M1 from Alaska cedar (*Callitropsis nootkatensis*) showed activity in catalyzing the oxidation of (+)-valencene to nootkatone (Cankar et al., 2014). CYP706C55 was shown to participate in cyanogenic glucoside biosynthesis in *Eucalyptus cladocalyx*, and catalyzes the dehydration of phenylacetaldoxime (Hansen et al., 2018). In Arabidopsis, a small gene cluster of two genes encoding a sesquiterpene synthase (*TPS11*) and *CYP706A3* was characterized and CYP706A3 efficiently oxidizes the TPS11 products and the products of other mono- and sesquiterpene synthases (Boachon et al., 2019). In **Chapter 3 and 4**, for the first time, we showed the functions of two CYP706s in SL biosynthesis of maize and rice. So far, studies on CYP706 enzymes report that they only catalyze one or two oxidization/dehydration steps. Surprisingly, maize ZmCYP706C37 is responsible for

several consecutive oxidative reactions in maize SL biosynthesis, involving hydroxylation, oxidation, epoxidation etc. These steps were further supported by modeling/docking and *in vitro* assays, from which some intermediates were characterized. Another intriguing discovery is the SL biosynthetic gene cluster (BGCs), which was found in both species. Extended analysis suggested that similar BGCs are also present in other plants, especially in Poaceae species. Intriguingly, there is just limited information about SLs in these other Poaceae, making it challenging to predict the functions of these other CYP706s in SL biosynthesis. With the characterization of new SLs in the plant kingdom, their role in SL biosynthesis will probably slowly be unraveled.

Importance of SL structural diversification and selection pressure

The two well-established roles for SLs outside the plant in its rhizosphere, are seed gemination stimulants for parasitic plant germination and hyphal branching inducers for AM fungi (Cook et al., 1966; Cook et al., 1972; Akiyama et al., 2005; Gomez-Roldan et al., 2008; Xie et al., 2010; Brewer et al., 2013; Al-Babili and Bouwmeester, 2015; Bouwmeester et al., 2021). It is intriguing and puzzling that plants produce compounds that contribute to both detrimental and beneficial processes.

As an ancient microorganism, AM fungi was estimated to exist from 600 million years ago (Redecker et al., 2000), older than the oldest land plants (Taylor et al., 1995). It was shown that the beneficial interaction (symbiosis) between AM fungi and plants started from about 460 million years ago when the bryophytes first colonized land (Simon et al., 1993). Even though there is no direct evidence to indicate that SLs played a role in the co-evolution of AM fungi and land plants, SLs have been reported as communication signals in algae and basal land plants, that also associate with AM fungi (liverworts and moss) (Parniske, 2008; Proust et al., 2011; Delaux et al., 2012). About the evolution of the role of SLs as plant hormone (Gomez-Roldan et al., 2008; Umehara et al., 2008; Ruyter-Spira et al., 2011; Guan et al., 2012; Zhang et al., 2018) we know even less. Higher plants produce a blend of structurally diversified SLs and this is induced by nutrient deficiency. Possibly this blend is the consequence of a selection pressure to keep the positive roles of the SLs (attraction of AM fungi and functioning as endogenous hormone), but not the negative such as the interaction with parasitic plants.

The root parasitic plants evolved the use of the existence of SLs for host detection as an important survival strategy. Sorghum is a host plant of *Striga* and it produces several SLs in root exudate, including sorgolactone, strigol, 5DS, and sorgomol. It was shown that in sorghum, a mutation in *LOW GERMINATION STIMULANT1* (*LGS1*) changed the SL composition in root exudate (from 5DS to orobanchol), which gave rise to greatly lower inducing activity in *Striga* seed germination (Gobena et al., 2017). However, this mutation did not affect the root colonization by three representative AM fungi species (Gobena et

al., 2017). In **Chapter 3**, we showed that a maize commercial genotype NP2222 produces a unique SL profile. Two SLs, zealactol and zealactonoic acid were present in its root exudate while there was no zealactone (Fig. 1). Even though their structures are quite similar to zealactone, the activities of zealactol and zealactonoic acid in inducing *Striga* seed germination were much lower than that of zealactone, the major SL in our maize germplasm collection. Another two examples are the *zmcyp706c37* and *zmmax1b* mutants I characterized. Since ZmCYP706C37 is an essential enzyme in all maize SL biosynthesis pathways, most of maize SLs were almost at undetectable levels in *zmcyp706c37* mutant root exudate, giving rise to much lower *Striga* germination inducing activity. Lower amounts of zealactone and its derived SLs and no significant alteration of zealactol and zealactonoic acid were found in *zmmax1b* root exudate, compared with its wildtype. The exudate from the *zmmax1b* mutant also induced less *Striga* germination, in line with its SL profile.

In **Chapter 4**, we generated *oscyp706c2* CRISPR-Cas9 mutants in rice and found that the mutants were deficient in one SL with unknown structure. This SL was identified by comparing it with a synthetic standard of 4-oxo-MeCLA (Fig. 1). *Striga* germination rates showed no clear differences when incubated with root exudates of *oscyp706c2* mutant and control plants, although 4-oxo-MeCLA does have some activity in inducing *Striga* seed germination. This could be explained by the fact that the other two canonical rice SLs (4DO and orobanchol) were present in all the lines and already saturated the *Striga* germination response. However, AM fungi colonization in the mutant was reduced compared with that of wild type, especially in the early stages, indicating that this new rice SL contributes to the beneficial symbiosis process.

These results suggest that variations in SL composition might be consequences of selection (inducing less *Striga* germination while maintaining beneficial symbiosis) and could be one feasible target for us to develop *Striga* resistant lines of crops. In addition, it was also indicated that SLs are related to plant resistance/defense against biotic stress such as pathogenic fungi (Yoneyama, 2020). For instance, a tomato SL mutant *Slccd8* showed higher susceptibility to two fungal pathogens *Botrytis cinerea* and *Alternaria alternata* (Torres-Vera et al., 2014). But a pea *ccd8* mutant did not develop different disease symptoms in response to *Fusarium oxysporum* infection, compared with wildtype (Foo et al., 2016), suggesting that the influence of SLs might alter with different pathogens (Yoneyama, 2020). Thus, there could be also selection pressure on host/plant SL biosynthesis to avoid pathogenic fungi existing in the rhizosphere.

Control of parasitic plants through modification of SL biosynthesis

The parasitic witchweeds, such as *Striga hermonthica* and *Striga asiatica*, pose an enormous threat to the production of cereal crops and threaten the livelihood of millions

of people, especially in sub-Saharan Africa (De Groote et al., 2008; Scholes and Press, 2008; Badu-Apraku and Fakorede, 2017). Several different strategies to solve this problem have been proposed (Bouwmeester et al., 2021; Jamil et al., 2021). For instance, seed coating with herbicides was shown to be effective in reducing *Striga* emergence and thus improving yield of maize genotypes resistant to the herbicide (Menkir et al., 2010; Chikoye et al., 2011; Makumbi et al., 2015). Another applicable example is the use of *Fusarium oxysporum*, in the form of either inoculated rice culture or the coating of the crop seeds. This reduces *Striga* attachment to crops and eventually the seed amount in the infested field (Rebeka et al., 2013; Nzioki et al., 2016; Zimmermann et al., 2016). Additionally, this biocontrol agent did not show adverse effects on indigenous rhizosphere fungal communities (Zimmermann et al., 2016). Crop resistance breeding is also an attractive approach; however, progress has been slow. One successful example, is the breeding of low germination stimulant sorghum, which was introgressed through a mutation in *LOW GERMINATION STIMULANT* 1 (*LGS1*), which resulted in changes in the SL blend (from 5DS to orobanchol) and thus *Striga* resistance (Gobena et al., 2017). Indeed, sorghum lines with a high amount of 5DS in their root exudates are more susceptible to *Striga* than genotypes with orobanchol (Mohemed et al., 2018).

Maize is one of the most important crops for human beings and is also a favorable host for *Striga* species. There has been one report showing that *Striga* resistance in maize may be associated with a different SL composition (Yoneyama et al., 2015). The susceptible genotype mainly produced 5DS while sorgomol is the major SL in the resistant line. Later, the author claimed that the identities of the SLs in these maize cultivars need to be confirmed as these could be background noises in their old-LC–MS/MS system (Quattro LC, Micromass) (Yoneyama et al., 2018). Indeed, our group did not detect these canonical SLs in maize root exudate. Instead, we detected at least six non-canonical SLs, two of which were structurally identified previously as zealactone and zeapyranolactone (Charnikhova et al., 2017; Xie et al., 2017; Charnikhova et al., 2018). In **Chapter 3**, we demonstrated that there is natural variation in the composition of the blend of these SLs in the root exudate of maize varieties which exhibit different degrees of *Striga* resistance. Zealactone was the most abundant SL in most lines in our screening. However, one unique line NP2222 did not produce zealactone but only zealactol and zealactonoic acid. *Striga* germination bioassays indicated that zealactone is a *Striga* germination stimulant with high activity while zealactol and zealactonoic acid showed very low inducing activity. I subsequently identified and characterized all maize SL biosynthetic genes and the structures of three so far uncharacterized maize SLs. I also showed that changes in the expression of these genes can result in changes in the maize SL amount or blend, as shown in *zmccd8*, *zmmax1b*, and *zmcyp706c37* mutants, which are great potential targets for developing *Striga*-resistant maize varieties.

Conclusions and future perspective

In 2017, when I started my PhD, only two maize SLs (zealactone and zeapyranolactone) and two rice SLs (4DO and orobanchol) had been characterized (Fig. 1). The identity of the others has remained elusive as well as the role of individual SLs in *Striga* germination and AM fungi symbiosis, and their biosynthetic route. In this thesis, I elucidated the biosynthetic pathways of virtually all maize and rice SLs and characterized the functions of the biosynthetic genes involved, with the support of different tools. Moreover, I provided evidence on the roles of maize and rice SLs in the interactions with parasitic plant and beneficial AMF. This work lays the foundation for further investigation, for example using mutants, of the intricate functions of the multitude of different SLs in plants. For instance, microbiome analysis of bacterial and fungal communities could provide us with more information about the effects of different SLs in the rhizosphere.

Additionally, there are still unknown steps in the biosynthesis of three maize SLs, which are probably derivatives from zealactone. Screening more diverse lines to find material that accumulates or shows deficiency in these SLs might be helpful to address this enigma. Another interesting aspect that has not received much attention so far, would be to elucidate the transcriptional regulation of SL biosynthesis in maize and rice (and also other plant species). In terms of perception, there are still lots of questions about how plants and organisms in the plant rhizosphere (*Striga*, microbes) perceive different SLs and how this triggers the downstream signaling pathways. Taken together, additional research will still be needed but on the other hand I expect that in the near future, it will already be possible to transfer our knowledge on SL biology to applications in agriculture, and thus contribute to food security and an ecologically balanced agriculture.

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