Solanoeclepin A

Characterization of a rhizosphere communication molecule in tomato and potato

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Chapter 6

General discussion

When I started my PhD, not much was known about Solanoeclepin A (SolA). Even though it was discovered as an active hatching factor (HF) for Potato cyst nematode (PCN) already in 1996 (Mulder et al. 1996), very little literature was available and only on the relationship of SolA with PCN. I therefore started my work with very little information, focusing on giving answers to questions such as: is SolA commonly produced by solanaceous species or is it species-specific? How is it produced and regulated? Does SolA have other roles in the rhizosphere?

In my PhD, I uncovered that there is substantial natural variation in SolA production in solanaceous species. I discovered that SolA is present not only in potato and tomato root exudates (REs), but also in related species such as *Solanum pimpinellifolium*, *Solanum pennellii*, *Solanum habrochaites*, *Solanum sisymbriifolium* as well as many relatives of potato from the *Petota* section (e.g. *Solanum kurtzianum*, *Solanum morelliforme*, *Solanum pinnasectum*) (Chapter 2 and 3). This discovery allows me to trace the SolA production back to its center of origin in South America where parasitic PCN also originated (Jenkins 1948) and lays the foundation for future studies on co-evolution of SolA biosynthesis and the interaction of Solanum species with parasitic PCN.

In addition, I improved our understanding of SolA biosynthesis and its regulation by environmental factors (Chapter 5). I discovered that the production of SolA is stimulated by nitrogen deficiency and the presence of microorganisms. Taking advantage of these findings, through RNAseq I subsequently identified a number of putative biosynthetic genes that are upregulated by nitrogen deficiency. As a proof-of-concept, in Chapter 5, using virus induced gene silencing in combination with root microbiome phenotyping, I developed an approach to transiently engineer the production of rhizosphere signals to allow studying their role in signaling to nematodes and the rhizosphere microbiome.

Importance of SolA for Potato Cyst Nematode

The Solanaceae originate from the Andes in South America, where potato and tomato speciation occurred and the interaction with plant-parasitic PCN evolved (Jenkins 1948). According to Grenier et al. (2010), the different populations of the genus *Globodera* all derive from a small region in southern Peru, where plant biodiversity strongly shaped the speciation of these parasitic nematodes. Intriguingly, according to the results presented in Chapter 3, the majority of potato relatives that originated from South America show the ability to induce hatching in PCN. It is believed that PCN was imported from South America into Europe with potato tubers that were going to be used to breed new varieties resistant to late blight, a disease caused by the oomycete *Phytophthora infestans*, responsible for the infamous Irish potato famine in the 19th century (Grenier et al. 2010). SolA was identified as a hatching agent for PCN in 1996 (Mulder et al. 1996) and is referred to as the major hatching factor (HF), since it induces PCN hatching in extremely low concentrations (Tanino et al. 2011; Ochola et al. 2020). Despite the perceived importance of SolA as PCN HF, intriguingly, as shown in Chapter 2, some of the tomato relatives that do not produce high amounts of SolA, such as *S. pennelii*, still induce

considerable hatching in PCN. This trend is further supported in Chapter 3, where some of the wild potato relatives that do not produce SolA at all, such as for example *S. pinnatisectum* and *S. pampasense*, do induce high hatching of PCN, suggesting that SolA is not the only HF in the Solanaceae. Moreover, different cyst nematode species seem to differ in their affinity for different HFs. Indeed, Sakata et al. (2020) showed that not all plant parasitic nematodes belonging to the genus *Globodera* are equally sensitive to SolA or tomato RE. For examples when treated with SolA, the hatching activity in *Globodera. pallida* and *Globodera. ellingtonae* was lower than in *Globodera. rostochiensis* and *Globodera. tabacum*, while *Globodera. artemisiae* the only cyst nematode they tested that does not infect solanaceous species, did not respond to any concentration of SolA. In general, all the species, except for *G. artemisiae* responded better to tomato RE than to pure SolA, suggesting that SolA is not the only HF. I also found substantial differences between *G. rostochensis* and *G. pallida* response to different REs (Chapter 3). This indicates that despite their shared origin, the two PCN species respond differently to the different compounds present in the host REs, as also pointed out by Sakata et al. (2020).
As PCN completely depends on the presence of a suitable host for the completion of its life cycle, they likely also use other cues from their host, for example chemo-attractants which are often referred to as hatching stimulants (HSs). HSs are compounds that stimulate hatching, but with much lower activity than the HFs and have been demonstrated to be present in the root exudate of potato and tomato (Shimizu et al. 2020a). For examples, Shimizu et al. (2020) showed that steroidal glycoalkaloids (SGAs) are HSs, and solasonine, produced by potato, can induce up to 80% hatching in *G. rostochiensis* at a concentration of 5 μM. For reference, SolA is more active, inducing around 70-90% hatching at a concentration of 2 to 500 pM (Guerrieri et al. 2021; Chapter 2; Shimizu et al. 2020). In Chapter 4 I silenced the SGA biosynthetic gene *STEROID SIDE CHAIN 2* (*SSR2*) and this resulted in a decrease in tomato SGAs in the root exudate and, possibly due to reduced competition for substrate, an increase in the SolA concentration. Hatching assays with the root exudate of these VIGSed plants showed an increase in PCN hatching, confirming the superior role of SolA as HF compared with the HS SGAs.

Chemical diversification in the eclepins

In Chapter 3 I showed that there are Petota species that do not produce SolA but induce the hatching of PCN. This is a clear indication that SolA is not the only HF. Also, sometimes not all accessions of the same species produce SolA although they induce hatching. This may suggest that subtle genetic variation in SolA biosynthetic genes causes mutations or results in the formation of other compounds similar to SolA that have not yet been identified.
This raised the question of whether a SolA-like compound is present in potato RE. This would be supported by the fact that Fukuzawa et al. (1985), identified two noritriterpenes from dried and powdered roots of kidney bean that closely resembled the natural hatching factor of soybean cyst nematode (SCN), glycinoeclepin A. Due to their structural

similarity, they were named glycinoeclepin B and C. Glycinoeclepin A was isolated by Masamune et al. in 1982, from powdered roots of kidney bean and subsequent bioassay-guided fractionation. Glycinoeclepin A stimulates hatching of SCN in a concentration of as little as 10^{-12} g/mL. On the other hand, glycinoeclepin B stimulates hatching at 10^{-9} g/mL, while glycinoeclepin C shows no activity even at concentrations as high as 10^{-7} g/ml (Fukuzawa et al. 1985c). This structural diversification could be the consequence of a selection pressure to produce molecules that can still perform their (unknown!) beneficial role in the rhizosphere while being different enough to prevent SCN hatching. This could also apply to SolA and the putative SolA-like compound, with Solanaceae evolving the production of different eclepins with similar structure but lower hatching activity to PCN.

The isolation and identification of such compounds from potato and tomato REs would be an important next step in unraveling this structural diversity and its biological significance. As shown in Chapter 3, where I used fractionation with the help of Liquid-chromatography mass-spectrometry (LC-MS), root exudate samples can be divided into different compounds according to their polarity. Hatching assays subsequently revealed that some fractions that did not contain SolA still induced high hatching. This should be followed up to identify possible new hatching factors. Moreover, using both *G. pallida* and *G. rostochiensis* in the hatching assays on such fractions could result in the identification of several new HFs and give more insight into the differences in affinity to HFs between these species.

The importance of analytical methods

When I started my PhD work, there were no existing analytical methods for the detection of SolA, let alone for the analysis of SolA in the RE of a single plant, a prerequisite to progress with experimental work on the biosynthesis and biological importance of SolA. Due to the very low abundance of the molecule in the rhizosphere, in previous research hundreds to thousands of plants were needed to collect enough exudate for the isolation of a few µg of eclepins. For example, Mulder et al. in 1996 collected root exudate from 700 potato plants to get 245 µg of SolA. In Chapter 2 the highest amount of SolA that I found, in *S. habrochaites* PI127826, was 0.135 µg in the RE of a single plant, which is roughly 95 µg of SolA in 700 plants so roughly the same order of magnitude. In 2011, Tanino et al. managed to chemically synthesize SolA, and the hatching activity of a solution of 10^{-8} g/ml was comparable with that of 500 L of RE collected from 12.000 tomato plants grown in hydroponics. I wanted to be able to analyse SolA routinely, preferably in the exudate of single plants and therefore it was necessary to develop a standard protocol for sample prep and analytical detection of SolA in the background of a large amount of other metabolites present in RE. In Chapter 2, I describe the combination of solid phase extraction and targeted LC-MS/MS coupled with multiple reaction monitoring (MRM) analysis which allowed me to detect SolA in 5 ml of RE collected from a single plant. This method will be instrumental for the discovery of a

potential beneficial role of SolA in the rhizosphere and the elucidation of its biosynthesis in the plant. This approach has been used in other studies, for example, the ability to chemically analyse strigolactones (SLs) in the RE of single plants, allowed to analyse mutants that then led to the (partial) elucidation of its biosynthetic pathway (López-Ráez et al. 2008; Floková et al. 2020).
With the analytical method that I developed we can now also determine which solanaceous species produce SolA. This is crucial in the development of new methods to prevent or reduce PCN infection, for example using a trap crop strategy. Trap crops are plant species of which the root exudate contains high amounts of compounds that induce PCN hatching while they are not a suitable host for the PCN or are destroyed before the nematode has completed its life cycle (Scholte 2000c). Through this strategy, the infestation level of fields can be decreased (Scholte 2000). With my analytical method we can now select trap crops with a high amount of SolA in their RE, which would ensure high hatching of PCN eggs present in the field, thus inducing strong hatching, before the cultivation of the desired crop. The analytical method can also be used to select low SolA producing genotypes that can be used as a source of genetic material for breeding programs. In Chapter 3, I identify several accessions of wild potato relatives that do not produce SolA and can perhaps be used for this purpose.

Biotic and abiotic factors that stimulate SolA production

In Chapter 5, I discovered that SolA production in tomato is strongly activated when grown under nitrogen starvation. Intriguingly, this is analogous to what has been reported for the plant hormone and rhizosphere signaling molecule, the strigolactones, under phosphate deficiency (Yoneyama et al. 2007; López-Ráez et al. 2008). Also for other aspects there are similarities between the histories of strigolactones and SolA. The discovery of both compounds started with a negative impact on the plant: something in the RE was stimulating a parasitic interaction. In the case of SolA this was the stimulation of PCN hatching (Mulder et al. 1996), for the strigolactones it was the germination of root-parasitic plants belonging to the *Orobanchaceae* (Cook et al. 1966). At the time of its discovery in 1966, the biological role of strigolactones in the plant was also unknown, as it is for SolA today, but thanks to stable knockouts and mutants we now know that the strigolactones are a plant hormone (Gomez-Roldan et al. 2008). For SolA there are no mutants known yet, so generation of knock-out lines through transformation could be an interesting option to study its role in the plant.
Moreover, through a fractionation approach, for the strigolactones it was discovered that their increased production under phosphate deficiency serves to attract and stimulate the symbiotic interaction with arbuscular mycorrhizal fungi (Akiyama et al. 2005b; Bouwmeester et al. 2007). Intriguingly, in my work, I discovered that SolA presence in tomato RE under nitrogen deficiency requires the presence of micro-organisms and correlates with the presence of certain bacteria. Perhaps SolA is a rhizosphere signaling molecule evolved in the *Solanaceae* to attract microbes that can help mitigate nitrogen

deficiency. Indeed, as I have shown in Chapters 2 and 3, SolA is quite conserved among different solanaceous species, suggesting that it evolved with a positive function. There are quite a few other examples of molecules secreted into the rhizosphere with positive functions (see my General Introduction). In the interaction between legumes and rhizobia, flavonoids are secreted as chemo-attractants to induce the first step of the symbiosis (Phillips and Tsai 1992). Once the first contact is established and the colonization takes place, the bacteria induce the formation of specific structures: the root nodules where the nitrogen fixation takes place (Masson-Boivin and Sachs 2018).
Solanaceae do not establish a symbiotic relationship with rhizobia. However, other types of symbiotic relationships occur in the rhizosphere, such as with non-endosymbiotic bacteria that supply nitrogen. As pointed out by Franche et al. (2009), there is an increasing number of studies reporting nitrogen-fixing bacteria associated to plant roots, without the intimate symbiotic relationship typical for the legumes. For example, the free-living bacteria *Azospirillum* spp. associated with grasses have been studied for their nitrogen-fixing properties, and are known as plant-growth-promoting rhizobacteria (PGPB) (Steenhoudt and Vanderleyden 2000). It is believed that their function in the rhizosphere of grasses is not only limited to nitrogen-fixation, but also to enhance mineral uptake and the production of plant growth-promoting substances. More recently, Yu et al. (2021) found that under nitrogen starvation, maize plants produce flavonoids to attract bacteria belonging to the Oxalobacteriacea that improve lateral root growth in maize and thus nitrogen acquisition. Also other root exuded triterpenoids have been shown to shape the microbial community in the rhizosphere. Huang et al. (2019), for example, showed that *Arabidopsis thaliana* triterpenoids, thalianol, arabidin and their derivatives, play a pivotal role in shaping the Arabidopsis microbiome partially by acting as promoting or inhibiting compounds. In the similar way, SolA could also act not only as a chemo-attractant for beneficial microbes, but also as a source for proliferation or anti-microbial agent.

How to select and prove candidate genes

Above I discussed that I believe SolA must have a beneficial role in the rhizosphere, and as it was for strigolactones, the elucidation of the SolA biosynthetic pathway would be of great help to further unravel this potential beneficial role. One of the hypotheses that we generated based on my results is that SolA is not produced by the plant alone, but that a precursor is exuded in the rhizosphere and then modified by microorganisms into SolA. The plant-based precursor is most likely a cycloartenol-derived molecule as its structure resembles SolA and it has been hypothesized to be the precursor also for glycinoeclepin A (Corey and Hong 1994).
In Chapter 5, I used RNA-seq to look for candidate genes for SolA biosynthesis. Using correlation analysis, I looked for genes with an expression profile that correlates with the SolA content in the root exudate. Even though the correlation between SolA and gene expression was quite low, I identified several interesting candidate genes that now need

to be further evaluated: a terpene cyclase and three cytochrome P450 monooxygenases. Judging from all the oxygen substituents in SolA, perhaps more cytochrome P450s must be involved, but on the other hand on multiple occasions these cytochrome P450s have been shown to oxidize multiple times and even on multiple positions for example in diterpenes (Bathe and Tissier 2019). Other enzymes involved in the biosynthesis might be one or more methyltransferases, alcohol dehydrogenase and a C-5 sterol desaturase to create the 7-carbon B ring, the main distinctive feature of SolA. However, I did not find candidate genes related to these classes of enzymes in the RNA-seq analysis I did, possibly because the massive changes in gene expression as a result of nitrogen starvation masked the genes involved in SolA production.

Nonetheless, RNA-seq is a powerful tool that has been previously used to elucidate biosynthetic pathways, such as strigolactones and capsaicin (Wang et al. 2022; Zhang et al. 2016). The approach only works, however, with a good experimental set up. The aeroponics system that I developed, provided exactly what we were looking for. I used it not only as a means to grow plants in such a way that we can easily access the root exudate, but also as a tool to explore the tomato response to nitrogen deficiency and different growing substrates such as soil and rockwool. Thanks to the versatility of the aeroponics system, I discovered how SolA is influenced by nitrogen starvation and microbes and I believe this is just the beginning. This system can be used not only to study SolA, but also to identify other compounds in the root exudate and elucidate their biosynthetic pathway, such as strigolactones.

Pinpointing candidate genes is only the first step, they also need to be validated and characterized. As was shown in Chapter 4, silencing genes involved in the biosynthesis of cycloartenol-derived compounds such as glycoalkaloids, influences the SolA concentration in tomato RE, making Virus Induced Gene Silencing (VIGS) a suitable technique to validate genes. It was previously used by Cárdenas et al. (2016) to validate a C5-sterol desaturase involved in potato and tomato glycoalkaloid biosynthesis. Upon silencing this gene in tomato leaves and fruits, they found a significant reduction in α-tomatine concentration. What is special about my experiment is that I was able to systemically silence genes in the roots and observe a decrease in the concentration of compounds in tomato RE, which was never reported before. Thus, VIGS would be the perfect technique to validate the gene candidates I found with the RNA-seq experiment since SolA can be detected in tomato RE.

VIGS is not the only technique that can be used to validate gene candidates. Other techniques are for example transient expression in *Nicotiana benthamiana* and stable expression in *E. coli* and yeast. These techniques (including VIGS) allow to perform experiments in a short time and are quite easy to perform and scale-up, and since in the future there may be more candidate genes to validate for SolA biosynthesis, they are the best choice. Moreover, transient expression in *N. benthamiana* allows reconstituting longer biosynthetic pathways giving the chance to clone and express multiple genes at the same time, while stable expression in *E. coli* and yeast would help in studying in more

detail the enzyme-substrate interaction for individual enzymes in the SolA biosynthetic pathway.
On the other hand, stable transformation is also an option that allows to create mutants that can be propagated and maintained for further analysis. The most used techniques to produce stable transgenic lines are RNA interference (RNAi) and CRISPR/CAS9. Even though creating a stable mutant is convenient for future experiments, the process is time consuming, involves the creation and maintenance of tissue culture and is strictly regulated by national laws. Creating mutants of single genes involved in SolA biosynthesis would not only help in elucidating the rest of the pathway, but also in studying SolA function in both plant and rhizosphere.

Perspective

With my work, I have contributed to giving answers to some of the many questions on the presence of SolA in the RE of tomato and potato. For example, I discovered that SolA is produced by many different solanaceous species, including wild tomato and potato relatives from their center of origin in South America. Moreover, I found out that nitrogen plays a significant role in regulating SolA production in the rhizosphere and that microbes are involved in its biosynthesis.
However, also a number of unanswered questions remain, and my work has also generated new questions. Unanswered is the question of SolA biosynthesis and what the role is of SolA within the plant. New questions arose from the fact that I discovered other HFs in the RE that seem to contribute to PCN hatching. For some of these questions I already contributed pieces of the answer. For example, I managed to identify several candidate genes for SolA production. If these genes can be validated and shown to be involved in the SolA biosynthetic pathway, the creation of mutants becomes feasible and this would help to elucidate SolA function within the plant as well as its relationship with the rhizosphere microbial community.
As already mentioned in Chapter 3, the fractionation of REs is a promising method to discover new HFs. The REs of different varieties and wild relatives of tomato and potato could be fractionated to search for new hatching factors, possibly with a structure similar to SolA and to link the production of SolA to the *Solanaceae* phylogeny and discover how this trait evolved and was passed on.
The work presented in this thesis is the first step towards a better understanding of the SolA role in the rhizosphere of solanaceous species. It is also an important step towards options to deal with the threat of PCN and hence improve tomato and potato cultivation. With the accomplishment of my PhD study, we can now analyse SolA production in a single plant, which can help in the selection of varieties that do not produce SolA or produce less, and therefore induce no or less hatching in PCN. Moreover, if we can validate the SolA biosynthesis candidate genes, knock-down mutants can be generated that potentially display PCN resistance.

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