Functions of Systemin and Systemin-Related Peptides in Tomato Plants

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M. Sc. Nga-Thanh Pham
aus Hanoi, Vietnam

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Dekan:	Prof. Dr. Thilo Stehle
1. Berichterstatter/-in:	Prof. Dr. Georg Felix
2. Berichterstatter/-in:	Prof. Dr. Thorsten Nürnberger

Table of Contents

Summary

Systemin was the first plant hormone to be discovered in plants (Pearce *et al.*, 1991; McGurl *et al.*, 1992). This opened a whole field of research centered around the understanding of peptide signals and their perception in plants. However, the specific mechanisms by which systemin regulates plant physiology was not deeply studied and the functional receptor of systemin peptide, SYR1, was only recently identified (Wang *et al.*, 2018). The role of systemin at the molecular level is still unclear and the process by which the systemin gets released in an active form into the cytoplasm is still under debate (Beloshistov *el al.*, 2017).

Recently, several systemin-like peptides were discovered which are also perceived by the systemin receptor SYR1. For one of them we provide evidence that it acts as a potent antagonistic of systemin and discuss its possible function in balancing the action of agonistic systemin peptides. As observed with a knockout mutant of the anti-systemin gene, this balancing function seems crucial for different steps in development of tomato plants, including seed germination, plant growth, flowering, fruit setting, and production of viable seeds.

The three other agonistic systemin-like peptides are agonists like systemin. We show that the agonistic systemin-like peptides elicit different response patterns than the authentic systemin in tomato leaf assays monitoring the production of the stress hormone ethylene. This finding will provide leads to further mechanistic and physiological studies, with the aim to understand this sophisticated regulatory pathway present in tomato.

Zusammenfassung

Systemin war das erste Pflanzenhormon, das in Pflanzen entdeckt wurde (Pearce *et al.*, 1991; McGurl *et al.*, 1992). Dies eröffnete ein ganzes Forschungsgebiet, das sich mit dem Verständnis von Peptidsignalen und ihrer Wahrnehmung in Pflanzen befasst. Die spezifischen Mechanismen, durch die Systemin die Pflanzenphysiologie reguliert, wurden jedoch nicht eingehend untersucht, und der funktionelle Rezeptor für Systemin-Peptid, SYR1, wurde erst kürzlich identifiziert (Wang *et al.*, 2018). Die Rolle von Systemin auf molekularer Ebene ist immer noch unklar, und der Prozess, durch den das Systemin in aktiver Form in das Zytoplasma freigesetzt wird, wird immer noch diskutiert (Beloshistov *et al.*, 2017).

Kürzlich wurden mehrere Systemin-ähnliche Peptide entdeckt, die auch vom Systemin-Rezeptor SYR1 wahrgenommen werden. Für eines von ihnen weisen wir nach, dass es als potenter Antagonist von Systemin wirkt und diskutieren seine mögliche Funktion beim Ausgleich der Wirkung von agonistischen Systemin-Peptiden. Wie bei einer Knockout-Mutante des Anti-Systemin-Gens beobachtet wurde, scheint diese ausgleichende Funktion für verschiedene Schritte in der Entwicklung von Tomatenpflanzen entscheidend zu sein, einschließlich der Samenkeimung, des Pflanzenwachstums, der Blüte, des Fruchtansatzes und der Produktion von lebensfähigen Samen.

Die drei anderen agonistischen Systemin-ähnlichen Peptide sind Agonisten wie Systemin. Wir zeigen, dass die agonistischen Systemin-ähnlichen Peptide in Tests an Tomatenblättern, die die Produktion des Stresshormons Ethylen überwachen, andere Reaktionsmuster hervorrufen als das authentische Systemin. Diese Erkenntnis wird zu weiteren mechanistischen und physiologischen Studien führen, die darauf abzielen, diesen hochentwickelten Regulationsweg in der Tomate zu verstehen.

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1. Introduction

1.1. An introduction to plant hormones and the identification of the systemin peptide

Plants, like all other living organisms, must be able to perceive changes in their surrounding and make appropriate responses in order to ensure their survival. Such changes could be the availability of environmental resources which influence the physiological traits (Chapin III *et al.*, 1987; Meinzer, 2002), or mechanical and chemical stimuli that require diverse and impressively elaborate network of perception, signal transduction, and response regulation (Walling, 2000; Braam, 2004; Jones & Dangl, 2006; Katagiri & Tsuda, 2010; Han, 2018). The wound response of plants is one such complex mechanism, and much of current knowledge stem from the experimental studies conducted in tomato (*Solanum lycopersium*) by Clarence Ryan and co-workers (Bowles, 1991). The initial works focused on the production of proteinase inhibitor in response of insect or mechanical wounding of single leaflets of young tomato plants (Green & Ryan, 1972; Graham *et al.*, 1986). Later, as it was discovered that the induced production of proteinase inhibitors occurred not only in the wounded leaflet, but throughout the entire plants (Green & Ryan, 1972), further studies has been dedicated to the identification of the proteinase inhibitor inducing factor (PIIF) which would be critical in mediate the systemic events (Doherty et al., 1988; Malone & Alarcon, 1995). Suggestion for the putative, hormone-like signal included abcisic acid (McGurl *et al.*, 1994), oligosaccharides, chitin and chitosan oligomers (Bergey *et al.*, 1996), and even electrical activity (Rhodes *et al.*, 1996). One such candidate, the 18-amino-acid-long, later-named systemin peptide, which was purified from tomato leaf extract (Pearce *et al.*, 1991), and was shown to induce systemic production of proteinase inhibitors when applied to fresh wounded tomato leaves (Pearce *et al.*, 1991; McGurl *et al.*, 1992; Constable *et al.*, 1995) with evidence of the translocation of the peptides from the wounded to distant, healthy leaves (Narváez-Vásquez *et al.*, 1995; Bergey *et al.*, 1996), is the subject of this doctoral thesis.

Following the discovery of systemin, a steady stream of plant peptides with signalling activity was identified (Franssen & Bisseling, 2001). Among these dozen peptide ligands, many have their corresponding receptors also identified and their signalling mechanism as well as their biological function elucidated. In contrast, studies on systemin lagged, possibly due to the early report on its identification. Another explanation for the lack of research interest might be that systemin and systemin perception only occur in some Solanaceae species, but not in plant species more amenable to experimental and genetic approaches like the model species *Arabidopsis thaliana*. Furthermore, there was the erroneous identification of a receptor kinase as the systemin receptor (Scheer & Ryan, 2002; Scheer *et al.*, 2003), which later turned out to be a tomato ortholog of the receptor BRI1 that detects the plant steroid hormone brassinolide (Holton *et al.*, 2007). Recently, two closely related receptor kinases have been identified that act as the cognate receptors for systemin (Wang *et al.*, 2018). This report also showed that systemin perception, while not essential for systemic activation of wound responses, was important for the protection against insect attack.

In further work from Dr. Felix, it was discovered that extract of tomato cells and leaves contain an antagonistic activity that specifically inhibits the responses to systemin (Felix *et al.*, unpublished data, University of Tübingen). This antagonistic compound was characterized as a peptide similar to systemin, only 2 amino acids shorter, and later named anti-systemin (aSys). With the advance of the Sol Genomic Project for sequencing the genome of species in the Solanaceous family, we have discovered three more genes models which are structurally related to the gene encoding aSys; however, the encoded peptides have agonistic systemin activity (Voigtländer & Wang, unpublished data, University of Tübingen). The three additional genes are clustered together on a different chromosome (Chromosome 4) than the one having the original systemin gene (Chromosome 5). Our colleagues from the research group led by Dr. Schaller have examined these systemin-like peptides and noticed difference in the scope of regulated genes downstream of peptide perception (Schaller *et al.*, unpublished data, University of Hohenheim).

1.2. *Functions of anti-systemin (aSys)*

In previous studies, the *Lycopersicum peruvianum* cells served as sensitive experimental system that responds to sub-nanomolar concentrations of systemin with responses like extracellular alklinization (Felix & Boller, 1995). The application of the extracted fraction containing aSys to a fresh cell suspension aliquot prevented an alkalinization response to subsequent treatment with systemin (**Figure 1.2.1**). Column purification and mass spectrometry analysis led to the identification of the peptide sequence, which is structurally similar to that of the systemin peptide (**Figure 1.2.2**). This antagonistic systemin peptide, which is named anti-systemin (aSys), was demonstrated to affect the EC_{50} of systemin in the *L. peruvianum* cell suspension culture by 100-fold at a low concentration of 10 nM (**Figure 1.2.3.A**), while having no effect upon co-treatment with flg22 even with a concentration of 100 nM (**Figure 1.2.3.B**). The K_i of aSys was determined to be 0.2 nM (**Figure 1.2.3.C**).

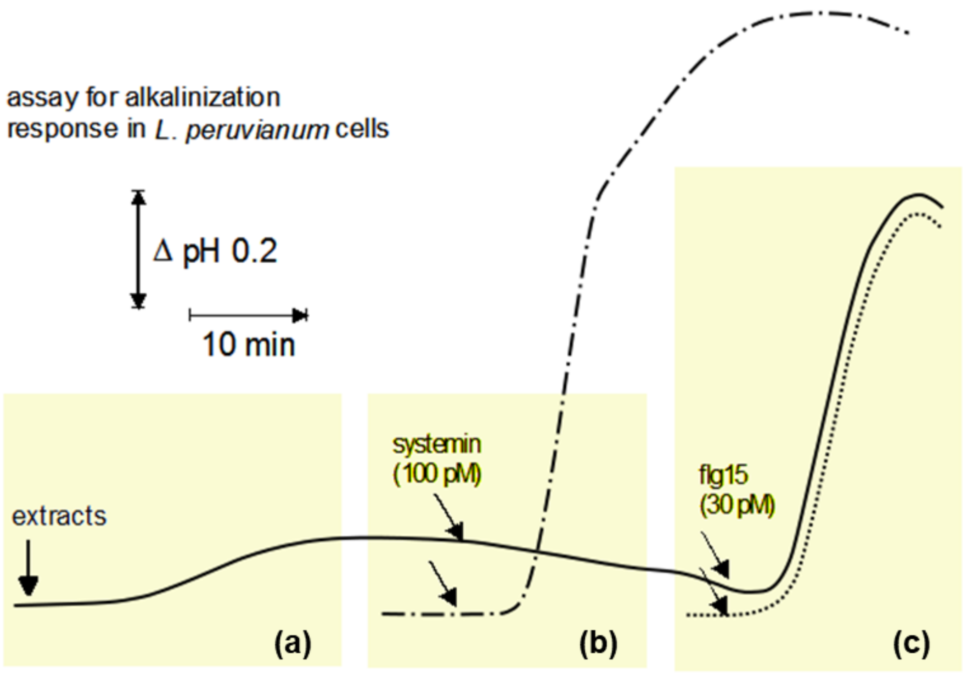


Figure 1.2.1. The alkalinization response of an aliquot of *L. peruvianum* cell suspension culture normally elicited by treatment of systemin was inhibited by pre-treatment with extract from a different batch of *L. peruvianum* cell suspension culture. The inhibition was exclusively systemin-type, as third treatment using flg15 elicited normal response as with fresh aliquot.

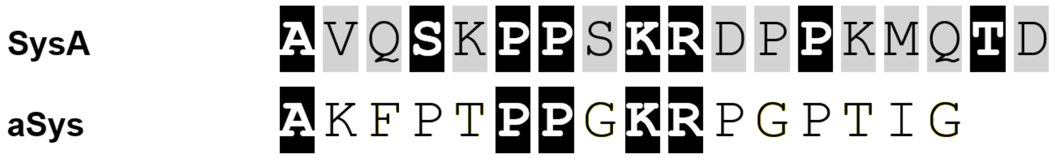


Figure 1.2.2. Comparison of the amino acid sequences of SysA and aSys.

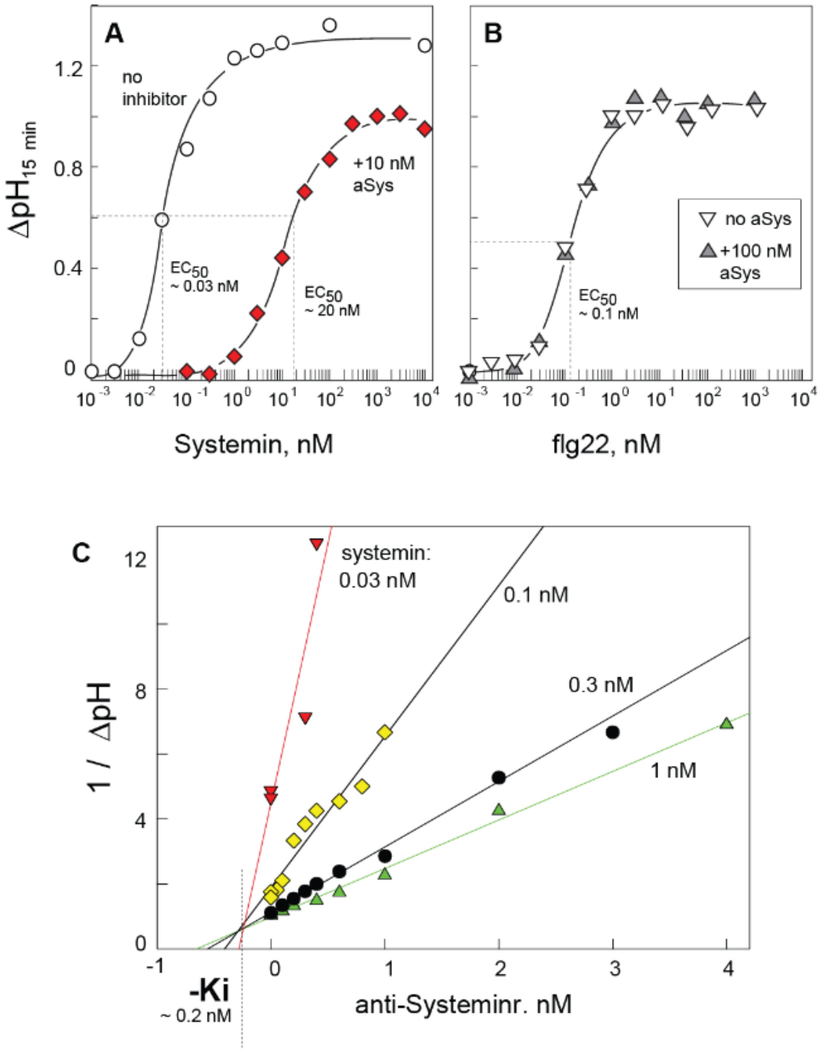


Figure 1.2.3. Anti-systemin (aSys) effectively inhibited the activity of **(A)** systemin but not **(B)** flg22 in the *L. peruvianum* cell suspension culture, with **(C)** estimation of the K_i value of aSys against systemin to be approximately 0.2 nM.

1.3. Functions of systemin-like peptides

The genes encoding the systemin-like peptides were discovered through alignment of the sequence of the aSys gene to the sequenced tomato genome (Voigtländer & Wang, unpublished data). The encoded peptides were then synthesized and tested for activity, which included medium alkalinization and production of oxidative species in the *L. peruvianum* cell suspension culture, as well as production of ethylene by *S. lycopersicum* leaf pieces.

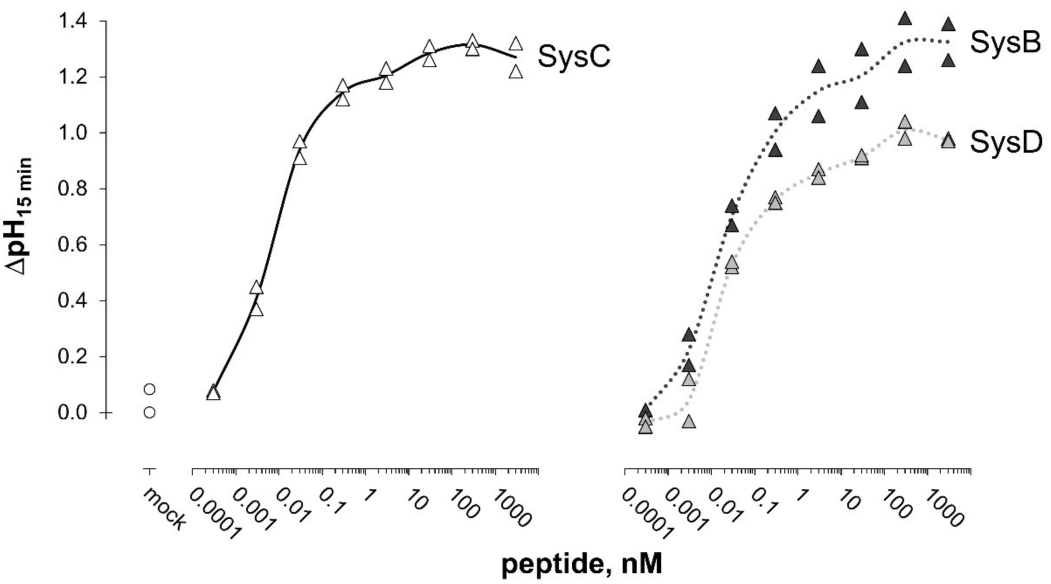


Figure 1.3.1. The alkalinization response of an aliquot of *L. peruvianum* cell suspension culture to increasing amount of systemin (SysA) and systemin-like (SysB/C/D). The mock samples were measurement of medium culture to addition of same volume of peptide solvent.

In the assay with *L. peruvianum* cell suspension culture, the systemin-like peptides (SysB/C/D) elicited the alkalinization response in a dose-dependent manner like the original systemin peptide (from here on referred to as SysA) (**Figure 1.3.1**). The cell suspension culture responded to the systemin-like peptides with the same amplitude showed for SysA (**Figure 1.2.3B**), indicating that the SysB/C/D as agonistic systemin are as good as the authentic SysA. Interestingly, in the assay measuring production of oxidative species, the cell suspension culture showed response to smaller doses of systemin-like peptide (SysC) compared to SysA (**Figure 1.3.2**). Furthermore, the SysC appeared to induce production of ethylene in tomato leaf pieces to a higher threshold, especially at higher peptide concentrations (**Figure 1.3.3**).

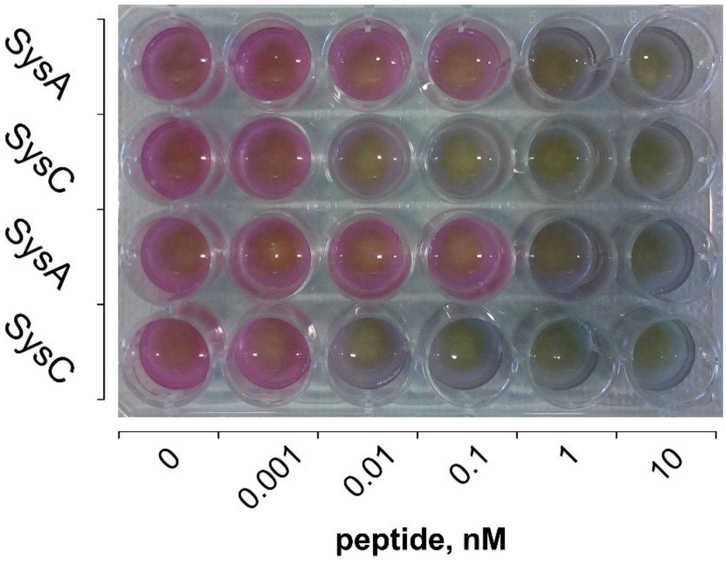


Figure 1.3.2. *L. peruvianum* cell suspension aliquots (3 ml, with chlorphenol-red) were treated with different concentration of SysA and SysC and incubated for 15 min (room temperature, with agitation), followed by the addition of the same amount of 1M NaOH. The production of reactive oxygen species results in the oxidation and discoloration of chlorphenol-red in the medium.

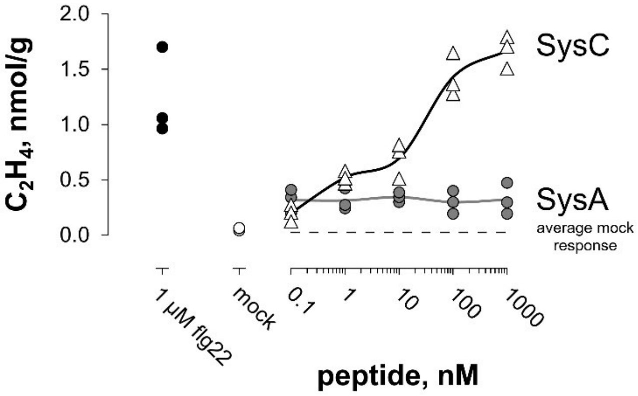


Figure 1.3.3. Ethylene production by tomato leaf pieces (cv. MoneyMaker) after 4 hours of incubation following peptides treatment. The level of ethylene produced in response to SysA treatment was significantly higher than the average mock response.

1.4. *Research objectives*

It is interesting that the plant would produce both systemin and aSys concurrently. According to McGurl *et al.* (1994), overexpression of prosystemin gene led to constitutive production of proteinase inhibitors throughout the tomato plants, which understandably affected the plant growth and development, as limited resources were used up by costly chemical defence proposition (Chapin III *et al.*, 1987). However, there are evidences showing the prosystemin mRNA is constantly presented at low levels in the cytosol and the nucleus of vascular parenchyma cells (Narváez-Vásquez & Ryan, 2004). The limited proteolysis principle has been proposed to explain this contradicting facts: at the site of wounding, the biologically competent systemin peptide is excised from its precursor and triggers the wound response (Schaller & Ryan, 1996). With the identification of the anti-systemin peptide, we propose a new hypothesis where aSys plays important roles in the regulation of systemin level in unwound plants. In order to verify this hypothesis, we set out to first quantifying the amount of endogenous sytemin and anti-systemin peptides in tomato leaves. In the next step, we generate transgenic tomato plants in which the anti-systemin gene is mutated resulting in aSys peptide not being produced. The transgenic plants are analysed to determine what are the effects from the lack of aSys upon the physiological state of the plants.

The newly discovered systemin-related genes and the respective systemin-like peptides were initially not very exciting for further study, as they behave similar to the original systemin in all our common bioassays. However, after reviewing the proteomics results from Dr. Schaller and co-workers (University of Hohenheim) which suggested systemin-like peptides trigger broader scope of genes regulation, we adjust the setup of our ethylene biosysthesis assay to have a more dynamic observation of the response. The results we obtain are surprisingly fascinated, and are further discussed in detailed in the next chapter.

2. Results

2.1. Systemin-related activities in tomato leaves

The original purification protocol developed by Pearce *et al.* (1991) (also summarized in **Section 3.5.1**) consumed a total of 30 kg of leaf material and resulted in 1 µg of a purified peptide—later named systemin—that proved highly active in inducing the production of proteinase inhibitor I and II in tomato leaves. Although the multistep purification procedure likely diminished the final yield considerably, the approx. 20 pmol of purified systemin obtained per kg of leaves suggested that the concentration of systemin present in non-wounded tissue might be indeed very low, presumably in the picomolar range.

Since the time of the original study, analytical techniques have improved considerably. Moreover, measurement of systemin-induced alkalinization in *L. peruvianum* cells as a bioassay offered a way to quantify the presence of systemin-type of activity in plant extracts. The alkalinization assay offered a convenient, robust and equally sensitive alternative to the measurement of proteinase inhibitors produced and accumulated in tomato leaves. Therefore, we wanted to use this assay to try to detect and quantify the activities of SysA, the aSys peptide, and the newly discovered systemin-like peptides in extracts of tomato plants.

In order to evaluate the efficiency of extraction, we first practiced on *N. benthamiana*, which has no endogenous systemin detectable by the cell culture bioassay. We added 1000 pmol of synthetic systemin to 2 g of fresh leaves of *N. benthamiana* prior to freezing the leaves in liquid nitrogen, grinding to powder, and applying the extraction steps from the original purification protocol, with the many enrichment steps replaced by the use of a solid phase C18 pre-column, eluting with 0.1% TFA in water and step gradient of methanol. By testing different amounts of the eluted fractions in the alkalinization assay we quantified systemin activity but could only observe a recovery of 9.3% of the added systemin standard (**Figure 2.3.1**). Next, we concentrated on improving the recovery of systemin as well as further simplifying the extraction steps.

Our first attempt for improving the recovery of systemin peptides was to avoid exposing the peptides to endogenous enzymes (i.e. proteinases) by reducing the time the leaf mixtures are handled at conditions suitable for enzymatic activities.

Therefore, we substituted water with 50 mM MES (pH 6.0) solution for the initial homogenizing step and performed the extraction at 4°C. This protocol worked with our practice plant model *N. benthamiana*, resulting in an increased recovery rate of approx. 50% of the initially added standard systemin (**Figure 2.3.2**). However, when we applied the protocol to tomato leaves, the filtered leaf solution still appeared to contain significant number of components which could interfere with our bioassay. Thus, we added a heating step before enrichment, where the filtered leaf solution was incubated at 95°C for 20 min to further remove unrelated sediments.

The response elicited by the fractions eluted from C18 pre-column was inhibited by pretreatment of the *L. peruvianum* cells with aSys only very weakly, suggesting the presence of one or more stimuli different from systemin in this fraction (**Figure 2.3.3**). In order to further search for the presence of systemin, the fraction eluted from the pre-column was fractionated by HPLC on C8 column using a gradient of Acetonitrile as eluent. The responses of the 1-ml fractions are discussed in **Section 2.2**.

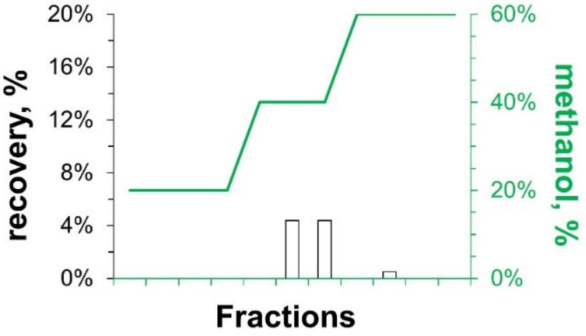


Figure 2.1.1. Solid phase C18 column elution of extract from *N. benthamiana* leaves. The bars represent the amount of spiked systemin peptide recovered using this extraction protocol.

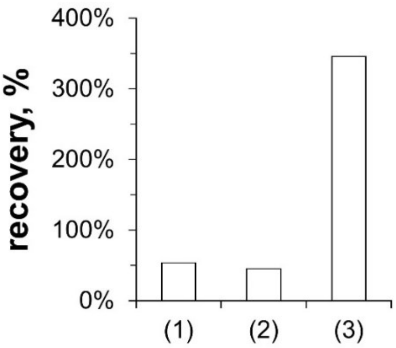
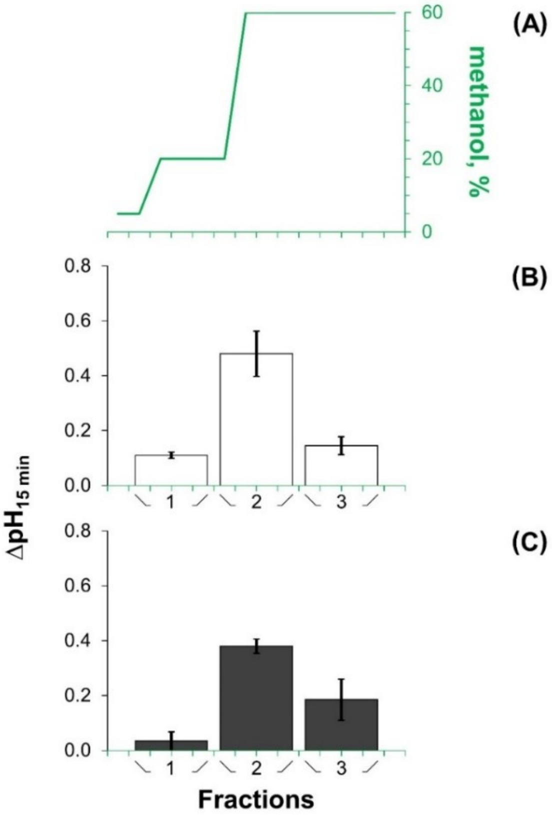


Figure 2.1.2. Solid phase C18 column elution of extract from leaves of (1,2) *N. benthamiana* & (3) *S. lycopersicum* (MoneyMaker cultivar) spiked with same amount of synthetic systemin.

Figure 2.1.3. Solid phase C18 column elutions of extract from tomato leaves (*S. lycopersicum*, MoneyMaker cultivar) **(A)** elicited response from *L. peruvianum* cells **(B)**, which could only be partially inhibited by pretreatment with aSys **(C)**.



2.2. *Determining the presence of systemin agonist and antagonist in tomato leaves*

Tomato leaf crude extract was enriched using a C8 pre-column, then fractionated on an HPLC C8 column, eluted with a gradient from 0% to 50% of Acetonitrile (in TFA 0.1%) (**Figure 2.2.1.A**). The fractions were reduced by vacuum to less than 100 µL in volume and the pH was adjusted to the pH of the cell suspension medium before being tested in the bioassay. Results from testing of 3 µL of each fraction to a 1-mL aliquot of cell suspension (comprising of approx. 0.2 g of cells) are summarized in **Figure 2.2.1.B**. The fractions which caused alkalinization of the cell suspension aliquots (#3, #7, #10, #14-17, #20) were tested again after pretreatment with 100 nM of aSys. As shown in **Figure 2.2.1.C**, the response was not reduced by the pre-treatment. Hence, most if not all of the activity observed in these fractions was likely due to elicitors different from systemin-type signals that cannot be inhibited by the antagonist. Candidates for such non-systemin signals known from tomato include peptides of the RALF (rapid alkalinization factor) (Scheer *et al.*, 2005) and Pep (plant elicitor peptides) categories (Trivilin *et al.*, 2014; *Zelman* & Berkowitz, 2023). In comparison, 30 pM of standard systemin, added as a control, was completely inhibited in the presence of 100 nM aSys (**Figure 2.2.1.C**, right panel). The amplitude of the response to standard systemin also indicated that activity extractable from 1 g of leaf material contained less than 30 fmol equivalents of standard systemin. In a further test we looked for the presence of aSys activity in the factions. For this, the cell suspension was first treated with aliquots of the eluted fractions and then with 100 pM systemin standard. This assay showed a clear reduction of the systemin response with fractions #13 and #14 (**Figure 2.2.1.D**).

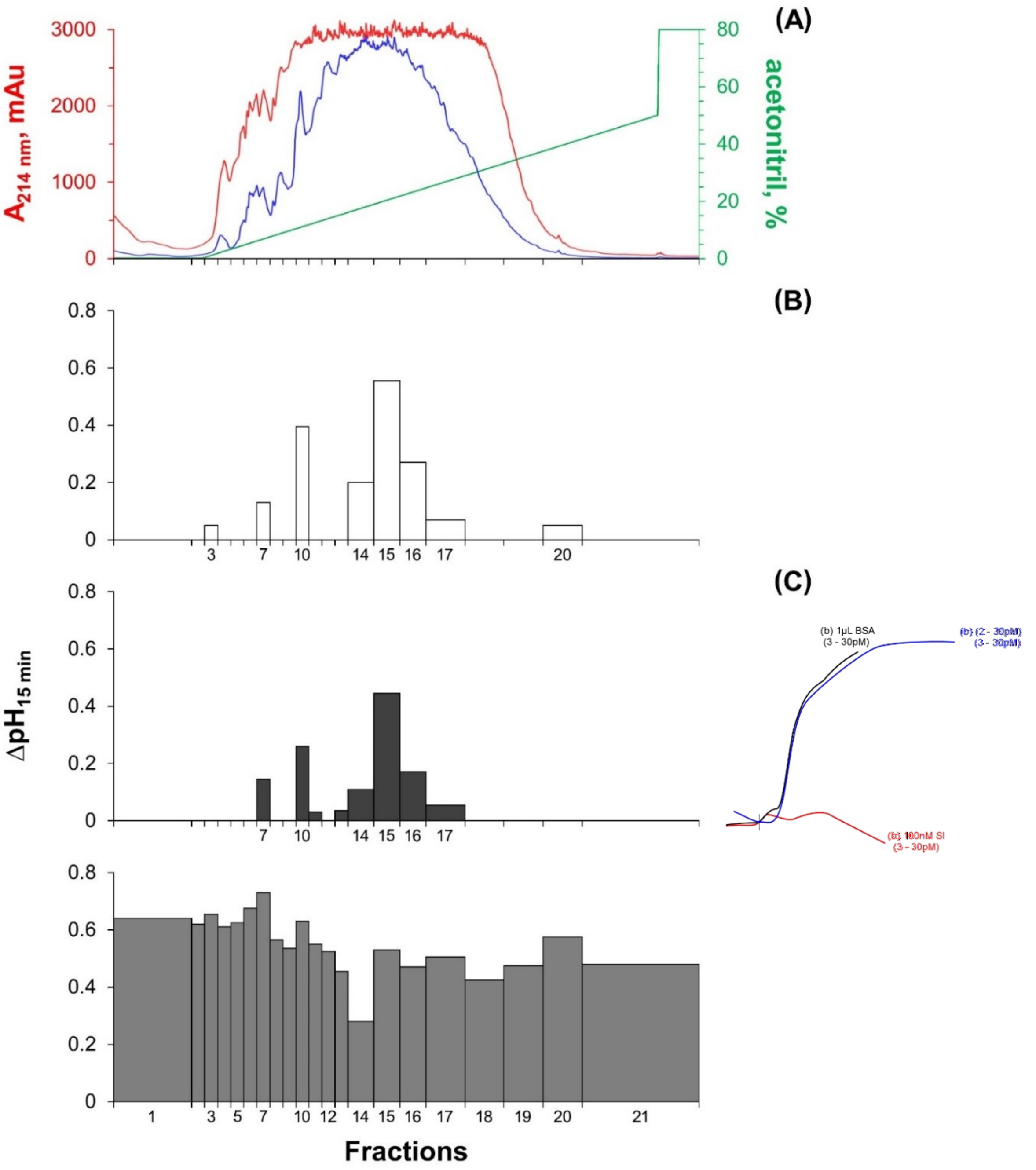


Figure 2.2.1. Fractionation of systemin-type activities on a reversed phase C8 column. An extract from 30 g tomato leaves, pre-purified on C8 pre-columns, was fractionated on a C8 HPLC column eluted with a gradient from 0% to 50% of Acetonitrile (in TFA 0.1%). Fractions were reduced by speed-vac to approx. 100 µl.

(A) elution profile: green = eluent gradient, red = UV detection 214nm, blue = UV detection 280nm; **(B)** alkalinization response to 3 µl of the fractions; **(C)** alkalinization response in cell cultures pre-treated with 10 nM aSys for a few sec before treatment with 3 µl of the fractions (scheme at the right demonstrates the response of cell cultures to 30 pM of SysA, and the inhibition effect of 100nM aSys); **(D)** alkalinization response to an excess amount of standard SysA (3 nM) after pre-treatment of the cell cultures with 3 µl of the fractions.

In summary, this experiment with extracts from healthy, non-wounded leaves of young tomato plants resulted in evidence for the presence of systemin inhibitor, but not for agonistic systemin activity. Agonistic systemin activity might be too low, or it might be masked by the presence of other signals like RALFs and PEPs, or by the concomitant presence of aSys. However, systemin has been discovered in tomato leaves (Pearce *et al.*, 1991; McGurl *et al.*, 1992), albeit at very low concentration. In our experiment we started out with 30 g rather than 30 kg of leaf tissue used in Pearce *et al.* (1991). Thus, the 3 µL sample tested contained the equivalent of extracts from 1 g leaf tissue. With none of these samples we could observed a response such as the one observed with 30 pM of standard systemin (**Figure 2.2.1.C**, right panel). This indicates less than 30 pM of systemin extractable from tomato leaf tissue. Starting from more plant tissue and more extensive chromatographic separation of interfering activities present in leaf extracts may be required to detect and quantify systemin activity in non-injured tissue.

Our results suggested presence of antagonistic aSys seems higher than agonistic systemin activity in extracts of non-wounded leaves. There are studies demonstrated that systemin is constitutively produced as inactive prohormone which is localized to the cytosol and nucleus of vascular parenchyma cells (Narváez-Vásquez & Ryan, 2004), requiring enzymatic cleavage into active peptide (Beloshistov *et al.*, 2018). This raises the question regarding the relationship and physiological roles of agonistic and antagonistic systemin peptides in tomato plants. Could it be possible that the presence of the antagonistic aSys might contribute to preventing the processing of prosystemin into systemin peptide in non-triggered plants? Could they also be involved in some mechanisms other than immune signaling and defense?

2.3. Transgenic tomato lines with modification in the aSys gene displayed phenotypical effects in development and reproduction

In order to elucidate the potential biological role of the antagonistic systemin peptide aSys in tomato plants, we generated transgenic tomato lines with mutation in the aSys gene so that there would be no functional aSys produced. We decided against interfering with the expression of systemin and systemin-like peptides, as there had been reports on transgenic tomato plants expressing antisense mRNA for systemin (Pearce *et al.*, 1991) with little phenotypical difference in the plants, which we suspected might be due to compensation by systemin-like peptides. Using the CRISPR/Cas genome editing tool to target multiple sites near the coding regions on the aSYS gene (**Figure 2.3.1**), we transformed 160 tomato cotyledons (cv. MoneyMaker), of which 33 produced callus tissue with shoots (T0 generation) selected on media containing basta (**Figure 2.3.2**). The shoots were separated for root formation (Whitman *et al.*, 2016), resulting in 22 plantlets with sufficient amount of roots and were transferred to soil for further growth in the greenhouse. Genotyping by PCR was performed at this point to determine whether the plantlets carried mutations in the aSYS gene.

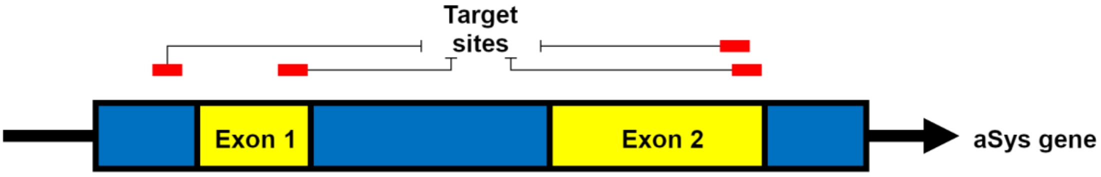


Figure 2.3.1. The aSys gene with target sites. The size of components in the scheme are not to scale.

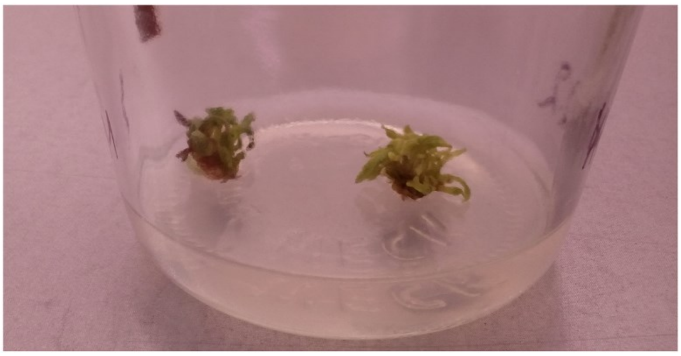


Figure 2.3.2. Tomato cotyledons (cv MoneyMaker), which were inoculated with *Agrobacteria* carrying the CRISPR construct, growing and developing shoots on basta selective media.

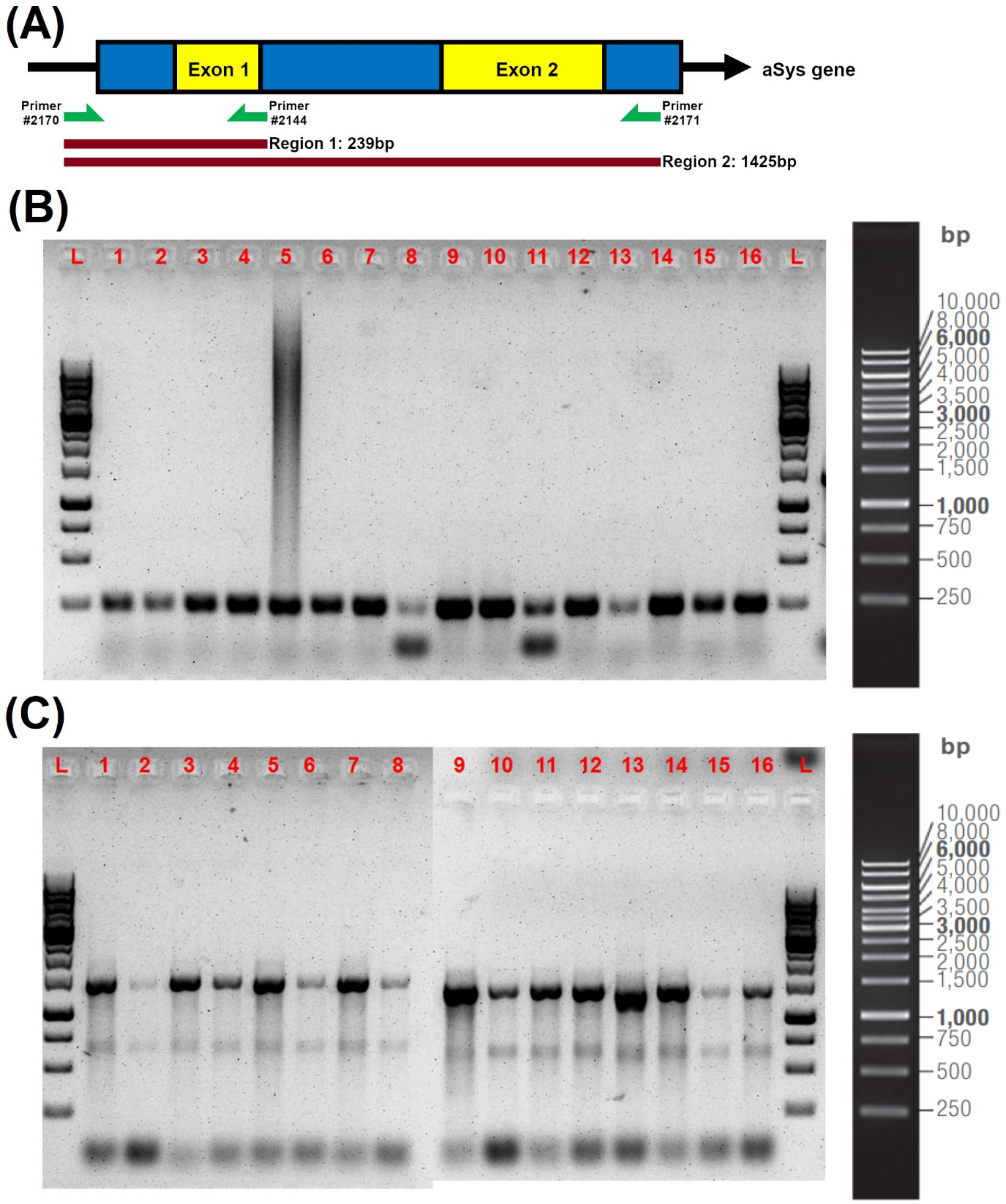


Figure 2.3.3. Genotyping by PCR of 16 tomato transformants.

(A) The two regions of aSys gene amplified for genotyping using the indicated primers.

(B) Amplification of region 1 using primers #2170 and #2144 (start of gene to end of exon 1; 239bp).

(C) Amplification of region 2 using primers #2170 and #2171 (the complete aSys gene; 1425bp).

Reference for PCR fragment size used the GeneRuler 1 kb DNA ladder from ThermoFischer Scientific.

We amplified two regions of the aSys gene: (1) from start of gene to end of the first exon (size 239 bp), and (2) the entire gene, encompassing both exons (size 1425 bp) (**Figure 2.3.3.A**). The genotyping results of the first 16 plantlets are shown in **Figure 2.3.3.B** (amplification of region 1) and **Figure 2.3.3.C** (amplification of region 2), which suggested mutations in the first half of the aSys gene in plantlets #8, #11, and #13; and mutations in the second half of the gene in plantlets #2, #6, #8, #10, #13, and #15. We further analyzed the mentioned transformants by sequencing the amplified region 2, and in the end, only two actually contained mutations: plantlets #13 and #15, which were named C8.1 and C8.4 (the corresponding names of the other transformants can be found in **Table 2.3.1**). The last 6 plantlets of the total 22 T0-generation plantlets did not contain any aSys mutation (data not presented).

Table 2.3.1. Tomato transformants.

Plantlet	Name	Plantlet	Name
#1	C2.1	#9	C6.1
#2	C2.2	#10	C6.5
#3	C2.3	#11	C6.6
#4	C2.4	#12	C6.8
#5	C5.1	#13	C8.1
#6	C5.3	#14	C8.3
#7	C5.5	#15	C8.4
#8	C5.6	#16	C10.1

The plantlets suspected to have mutation after genotyping are underscored.

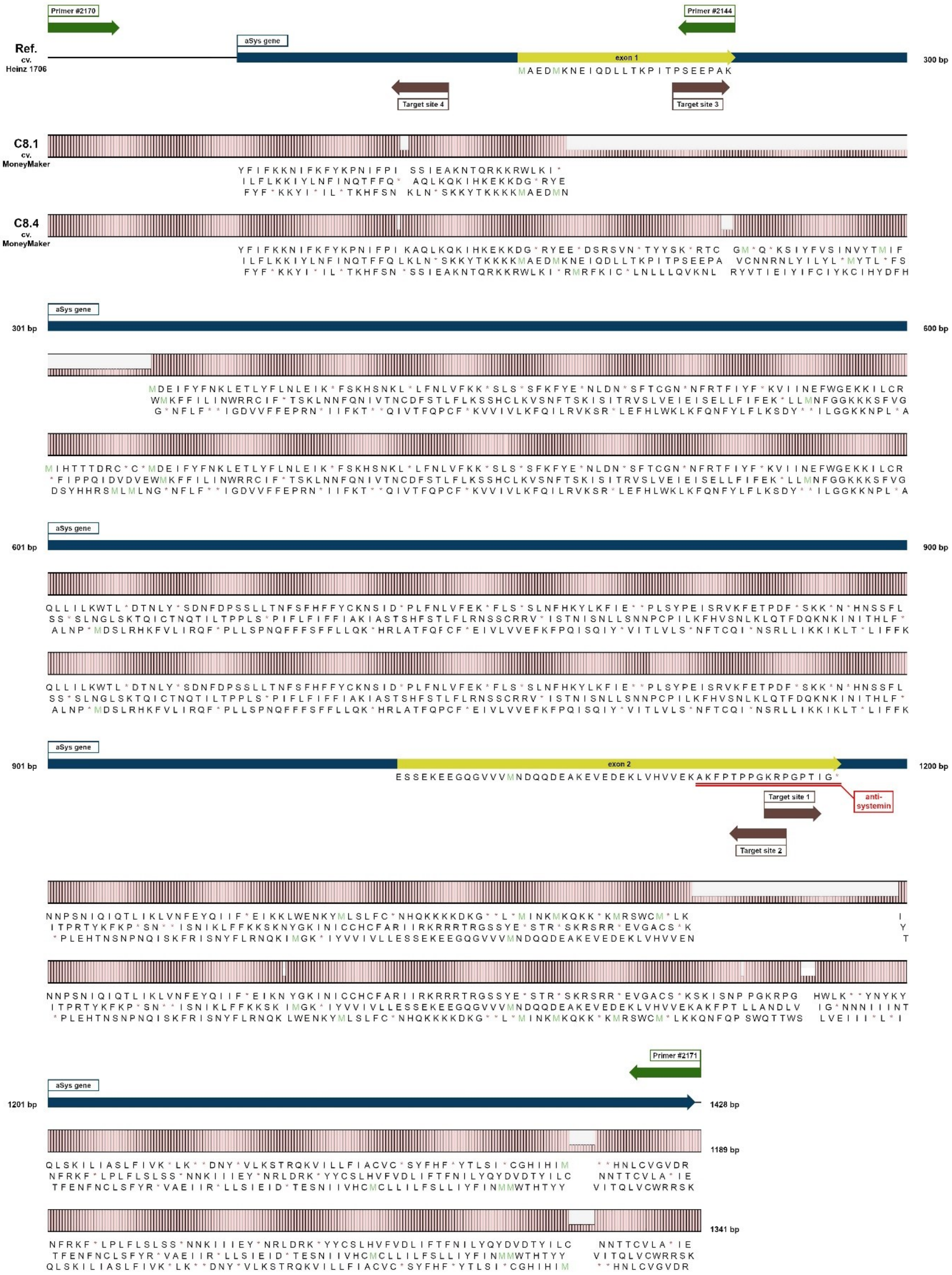


Figure 2.3.4. The genomic sequences of the transgenic lines (C8-1 and C8-4) were compared to the reference gene (tomato cultivar Heinz 1702, annotation version SL2.0; the data is courtesy of the Sol Genomic Project). The bar graphs are for visualization of the difference between the mutated genes and the reference gene. The transcripts of the mutated genes do not encode for the amino acid sequence of aSys (red, double underlined.)

The two transformants have two different mutations: one with a large deletion (C8.1), and one with a small, few base-pairs deletion (C8.4) within the coding region of the aSYS gene, respectively (**Figure 2.3.4**). Each transformant produced less than 10 seeds, and all these seeds were sown. For both mutant lines, only one single plant of each (T1 generation) grew to flowering stage. The aSys transgenic lines are phenotypically different from the wild type, having difficulties producing flowers, fruits, and seeds. For increasing the chances of seed production, the T1 generation plants were also propagated into several plants by shoot cutting. Nevertheless, compared to the representative transgenic line without mutations in the aSys gene (C6.5), the aSys mutants developed poorly and produced fruits with very low number of seeds: we obtained 42 seeds from 94 ripened tomato fruits of C8.1, and 118 seeds from 92 fruits of C8.4, while C6.5 produced more than 200 seeds from less than 90 fruits. This might be the first indication of effects due to disruption in aSys production.

To further understand the possible effect of the absence of aSys on reproduction, we conducted a germination assay on the seeds collected from the T1 plants (aSys mutants C8.1 and C8.4, and another aSys non-mutant C6.1). These T2 seeds were sown concurrently with seeds of MoneyMaker cultivar (transformation background). While seeds from MoneyMaker cultivar and C6.1 line germinated at the same rate (80-90% of the sown seeds germinated after 4-5 days), seeds of C8.1 and C8.4 had very low germination rate: only 1-2 seeds germinated for every 50 seeds sown (**Figure 2.3.5**). The non-germinated seeds were recollected from the soil. Some of these non-germinated mutant seeds showed visible swelling and embryonic development when dissected and analyzed under the microscope (**Figure 2.3.6**), indicating that the ability to break the seed coat was impaired. Further efforts by Dr. Lei Wang (University of Bern) to propagate tomato lines lacking aSys, either C8.1 and C8.4 in the MoneyMaker background (presented in this work) or a mutant line in the M82 background generated by Dr. Yan Wang (unpublished data, University of Tübingen), continuously resulted in plants with severe developmental problems and phenotypical differences compared to wild-type plants (**Figure 2.3.7**). Compared to non-mutated plants, the aSys-mutated plants were shorter (as much as half the length), with visibly thicker stem. While 6 weeks after sowing, the non-mutated plants already started producing flowers, with at least one branch with fully blown flowers and new branch sprouting from the apical meristem showing flower buds; the mutated plants had no flower buds at all.

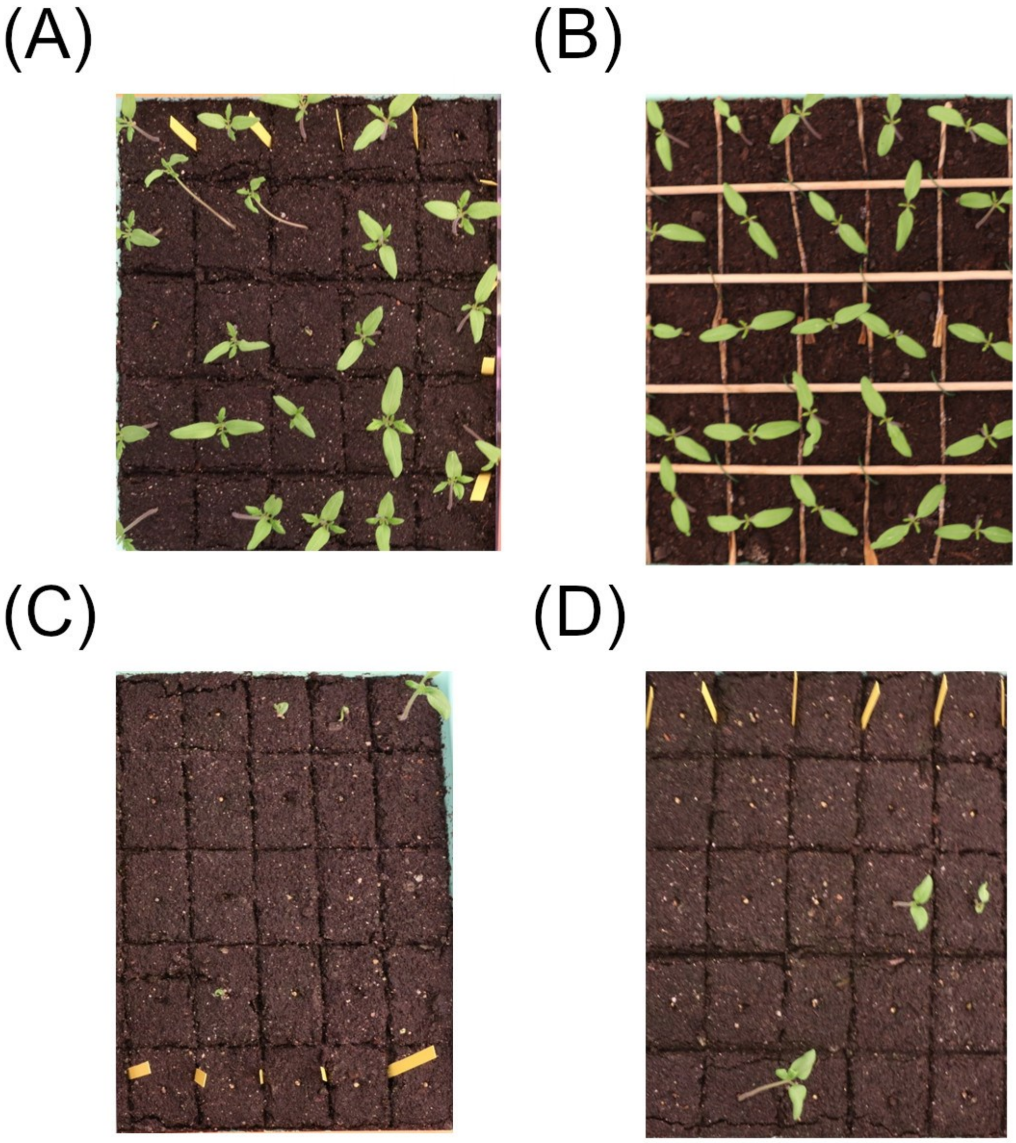


Figure 2.3.5. Germination assay of the aSys mutated tomato lines. **(A)** Wild-type Money Maker and **(B)** non-aSys-mutated C6.1 line showed a germination rate of more than 90%, while the mutation lines **(C)** C8.1 and **(D)** C8.4 had less than 10% germinating seeds.
Images taken 10 days after the seeds were sown.

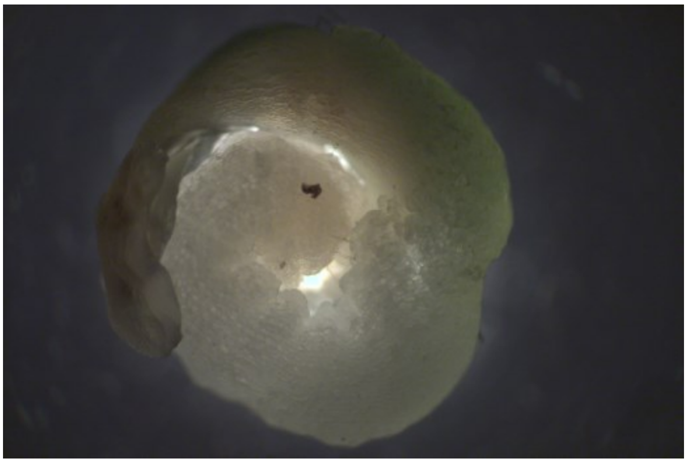


Figure 2.3.6. Microscopic image of a non-germinated seed from the aSys-mutated tomato line (C8.1/C8.4), showing normal development of the plant embryo.
(Image taken by David Molina, University of Tübingen)

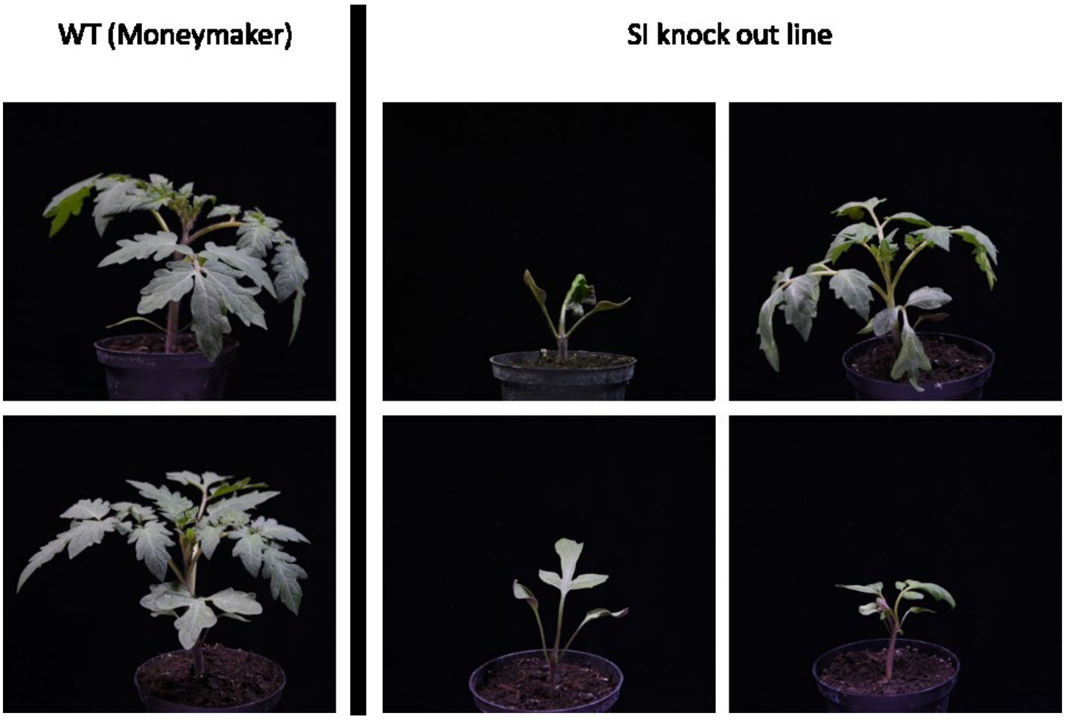


Figure 2.3.7. The tomato plants lacking aSys (Sl knock out line) displayed phenotype difference from wild-type plants.
(Image courtesy of Dr. Lei Wang, University of Bern)

2.4. Difference in perception patterns of systemin and systemin-like peptides

In parallel with studying the effects of lacking aSys on development and reproduction of tomato plants, we compared the activity of the systemin-like peptides (SysB, SysC, and SysD), with the activity of SysA. Leaf pieces from MoneyMaker plants which were 4- to 6-week-old were treated first with 1 µM aSys, and after 15 minutes followed by either mock or 10 nM the classic systemin SysA, or the systemin-like peptides SysB, SysC, and SysD. The amount of ethylene accumulating in the air space after 4 hours of incubation was measured with gas chromatography and the results are presented in **Figure 2.4.1**. The results illustrate the ability of aSys to inhibit the activity of all the systemin peptides. Curiously, there seemed to be a stronger stimulation by the three systemin-like peptides compared to the classic SysA, which led to the question whether this enhanced response might be due to a more potent or a more prolonged activity of the systemin-like peptides. For this we conducted time-course assays with measurement of ethylene production in hourly intervals over 6 hours. The result of the time-course assay (**Figure 2.4.2**) revealed that SysA elicited a transient ethylene production which tapered off 2 hours after peptide treatment. In contrast, response to SysB, SysC, and SysD continued to increase for at least 4 hours after peptide treatment.

We initially suspected the cause of the response pattern divergence was due to a lower stability of the SysA peptide, presumably due to a faster proteolytic degradation in the assays with the leaf pieces. To test this hypothesis, we applied a second dose of SysA at time t=4 hours after the first peptide treatment (**Figure 2.4.3**). However, there was no response to the second dose of SysA. Interestingly, in contrast, there was a second response when these leaves were treated with either one of the systemin-like peptides SysB, SysC, or SysD, respectively (**Figure 2.4.4**). These results demonstrated that the cause for the transient response to SysA was not because of inactivation of the SysA peptide but rather due to a process that rendered the leaves refractory to further respond to this stimulus. More intriguingly, these results hinted at differential perception mechanisms for SysA and the systemin-like SysB, SysC, and SysD, respectively.

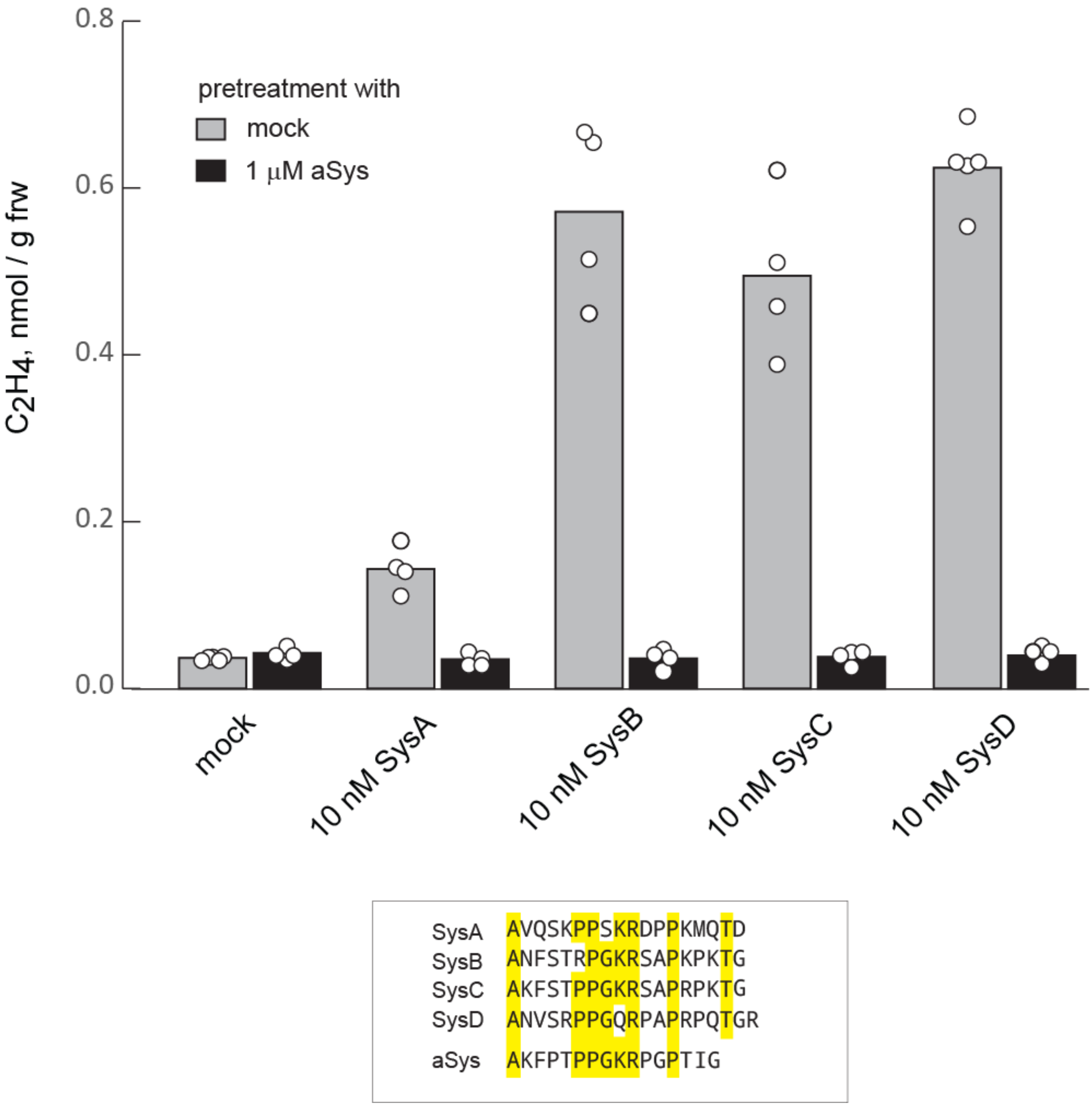


Figure 2.4.1. Systemin (SysA) and the systemin-like peptides SysB, SysC and SysD induce ethylene biosynthesis in tomato leaves.

Leaf pieces were pretreated for 15 min with the solution used to dilute the peptides (mock) or 1 µM of the peptide aSys, followed by the addition of mock or 10 nM peptide agonists, as indicated. Ethylene accumulating over 4 h is shown for n=4 replicates as symbols and bars (averages). Results shown are representative for n > 4 independent repetitions.

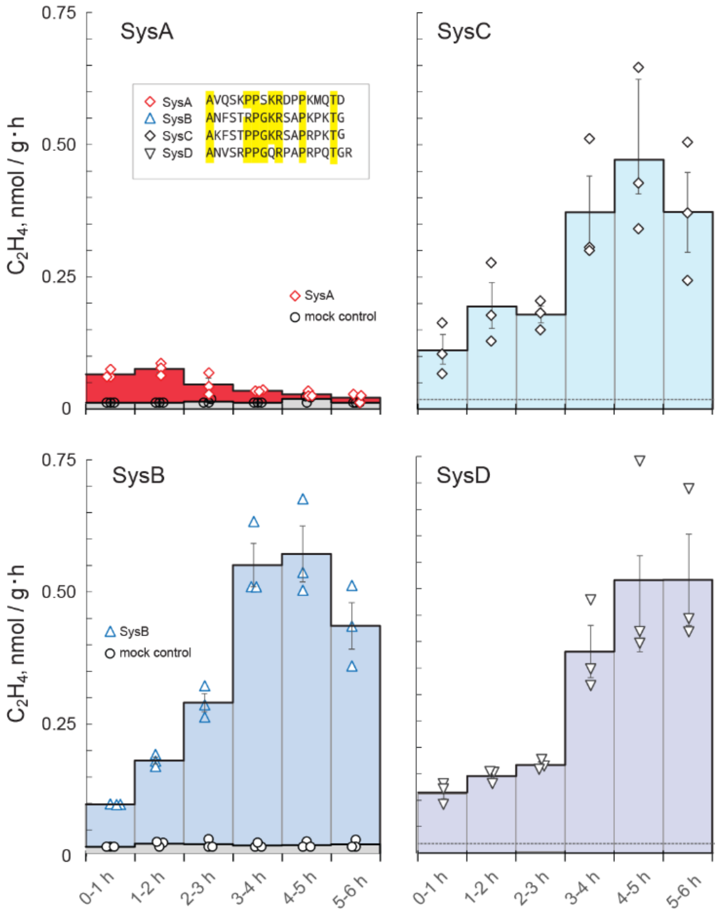


Figure 2.4.2. Systemin (SysA) induces ethylene production in tomato leaves less strongly and more transiently than SysB, SysC and SysD.

Leaf pieces were treated at t=0h with 1 µM of the peptides, ethylene accumulating within 1 hour was measured and air was exchanged for the next interval. Data are shown for n=3 replicates (symbols) with averages (bars) and standard errors (error bars). The results, shown for leaves of the variety MoneyMaker, are representative for n>4 independent repetitions (including M82 as a second tomato variety). The difference in kinetics of C_2H_4 production between sysA and the other systemins was observed in all of n>10 experiments.

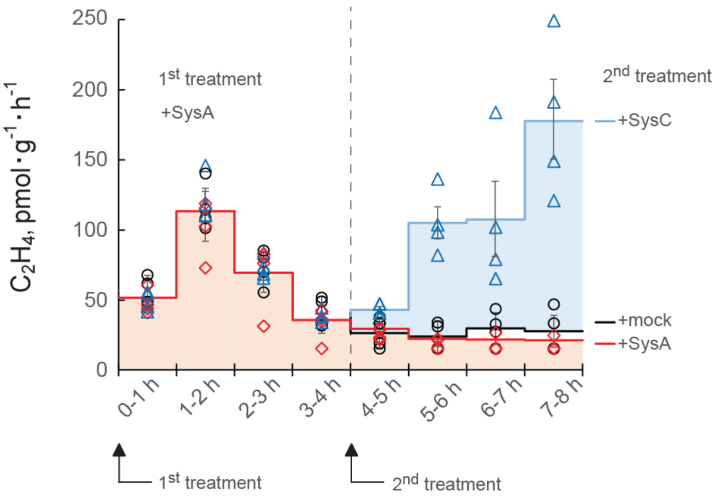


Figure 2.4.3. The transient response to SysA is not due to disappearance of the inducing peptide.

Leaf pieces (variety MM) were treated at t=0h with 1 µM of SysA and received a second treatment at t=4h with 1 µM of SysA, or SysC, or mock solution (the buffer used to dissolve the peptides).

Data are shown for n=3 replicates (symbols) with averages (bars) and standard errors (error bars).

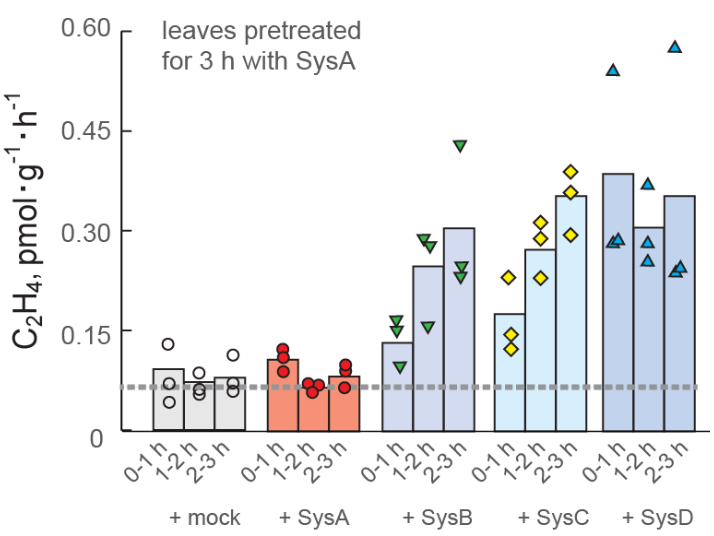


Figure 2.4.4. Leaves pretreated with SysA are refractory to a second treatment with SysA but still respond to treatment with SysB, SysC and SysD.

Leaf pieces (variety M82) were pretreated with 1 µM of the peptide SysA for 3 hours and received a second treatment with 1 µM of SysA, SysB, SysC and SysD as indicated. Data are shown for n=3 replicates (symbols) with averages (bars) and standard errors (error bars).

In order to identify characteristic(s) or feature(s) of the systemin peptides that lead to the differential perception pattern, we used synthetic peptides with chimeric sequences derived from SysA and SysC, and narrowed down the specificity factor to the C-terminal part: transient response if the SysA sequence is present and persistent response if the SysC sequence is present (**Figure 2.4.5.A**). Next, we tested single amino acid replacements of the SysA sequence at position 15 (Met → Pro), position 16 (Asn → Lys) and position 18 (Asp → Gly). The first two exchange did not affect the response pattern, while the last one converted the response pattern from transient to persistent, similar to that of SysC (**Figure 2.4.5.B**). Glycine at position 18 is also presented in SysB and SysD (**Figure 2.4.1**, lower panel).

The C-terminal part of SysA has previously been found to be required for activation of response output (Meindl *et al.*, 1998). This might suggest differences in the recruitment of co-receptors to the SysA/SYR1 or SysC/SYR1 complexes, either with/without co-receptor or each complex matching up with different co-receptors. Nevertheless, as it has been shown that in tomato plants, SysA is recognized by two receptor proteins SYR1 and SYR2, with SYR2 being the weaker receptor to SysA (Wang *et al.*, 2008), we first considered that SYR2 might be the genuine receptor for the systemin-like peptides, and when perceiving these peptides, might cause the prolonged response pattern. However, when we performed the time course assay with the *pCa*MV35S::SYR1_GFP reconstituted IL3-3 tomato plants, the response patterns to SysA and SysC were similar to those displayed by the standard wild-type tomato plants: SysA caused a transient ethylene production, while SysC elicited a clearly more prolonged response (**Figure 2.4.6**). We have also checked the response patterns in *syr1* and *syr2* single mutants (mutant lines are courtesy of Schaller *et al.*, University of Hohenheim), in which only one functional systemin receptor protein under its native promoter was expressed (SYR2 and SYR1, respectively). Leaf pieces from *syr1* mutant expressing only SYR2 did not show any responses significantly different from background level even to 1 µM of systemin. In contrast, the *syr2* mutant expressing only SYR1 showed the same response patterns as in wild-type and SYR1-overexpression plants (**Figure 2.4.7**). These results led us to believe that the difference in perception might be due to some other factor that gets in contact with the systemin ligands and could differentiate between SysA and SysB/C/D. The plant LRR receptor kinase studied so far all seem to require a co-receptor that also interacts with the respective ligands bound to their receptor sites for activation (Böhm *et al.*, 2014; Wan *et al.*, 2019). Thus, such co-receptors might contribute to ligand specificity of perception systems.

The systemin receptor protein is a member of the large protein family of leucine-rich repeat receptor-like kinases (LRR-RLKs) (Wang *et al.*, 2018; Wan *et al.*, 2019), and many of these receptor proteins have been found to associate with another receptor protein from the LRR-RLKs group called SERK (somatic embryogenesis receptor kinase), after binding to their respective molecular patterns (Schmidt *et al.*, 1997; Chinchilla *et al.*, 2009; Li, 2010; Ma *et al.*, 2016). As the SERKs, as coreceptors, have such diverse roles in both plant development and immunity, we tested the possibility that SysA and systemin-like (SysB, SysC, and SysD) would recruit different SERKs by treating leaves of IL3-3 plants expressing SYR1 with either SysA or SysC, then immuno-precipitating SYR1 via its GFP-tag, and analyzing the precipitate for the presence of the potential coreceptors by MS/MS (**Figure 2.4.8**). The result of a first such analysis indicated that treatment with SysA and SysC both led to co-precipitation of SERK3B but only treatment with SysC also led to an association between SYR1 and SERK3A.

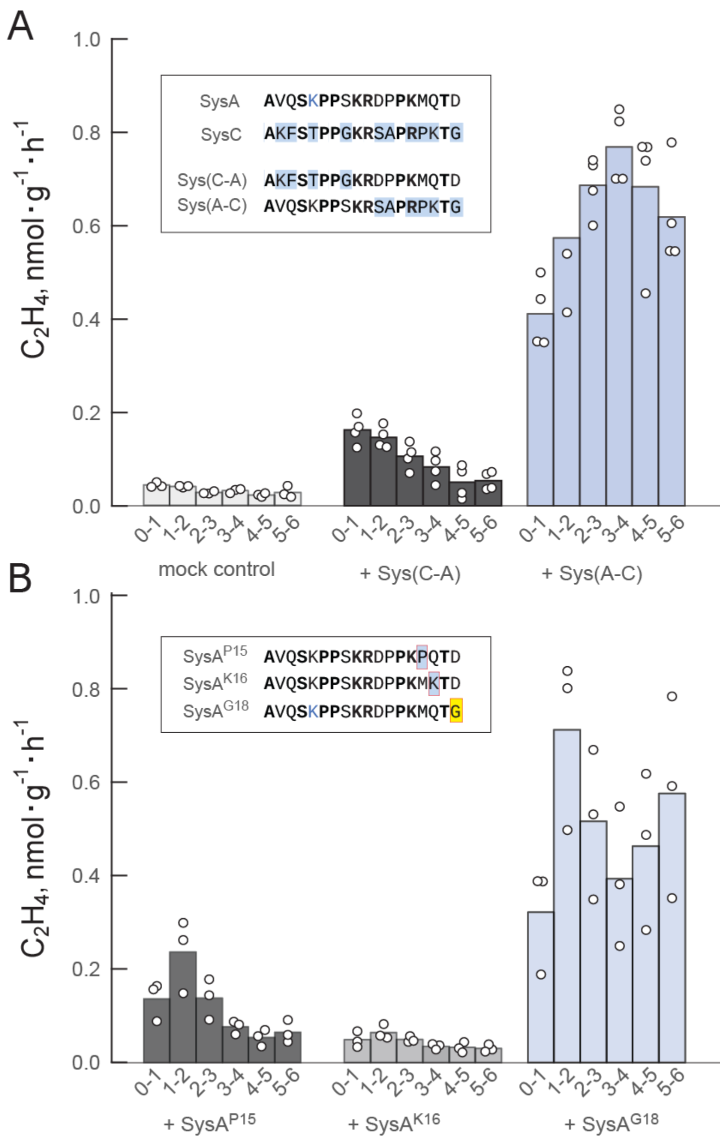


Figure 2.4.5. The amino acid at position 18 of the systemin peptides is decisive for the different response patterns in ethylene assay. **(A)** Half of the peptide sequence (8 aa) was swapped, then **(B)** single residues at position 15, 16, and 18 were exchanged.

Data are shown for n=3 replicates (symbols) and averages (bars).

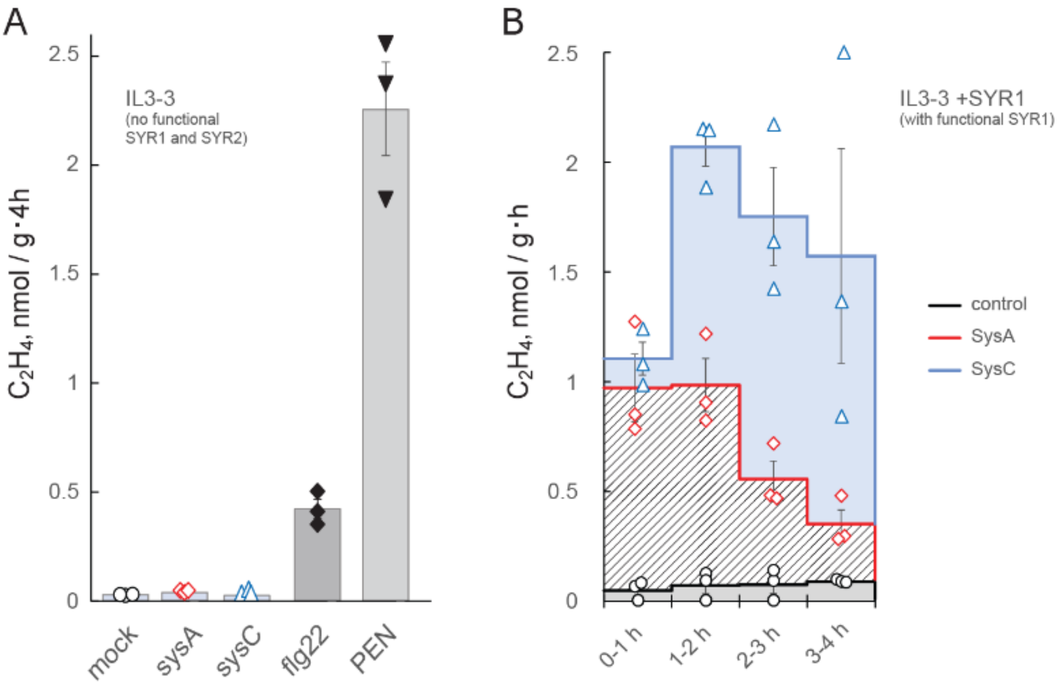


Figure 2.4.6. The differential pattern caused by the different systemin peptides are not due to different systemin receptors. **(A)** IL3-3 has no functional SYR1 and SYR2 receptors and does not respond to SysA and SysC. **(B)** IL3-3 reconstituted with SYR1 responds transiently to SysA, and in a prolonged way to SysC, respectively. Data are shown for n=3 replicates (symbols) with averages (bars) and standard errors (error bars).

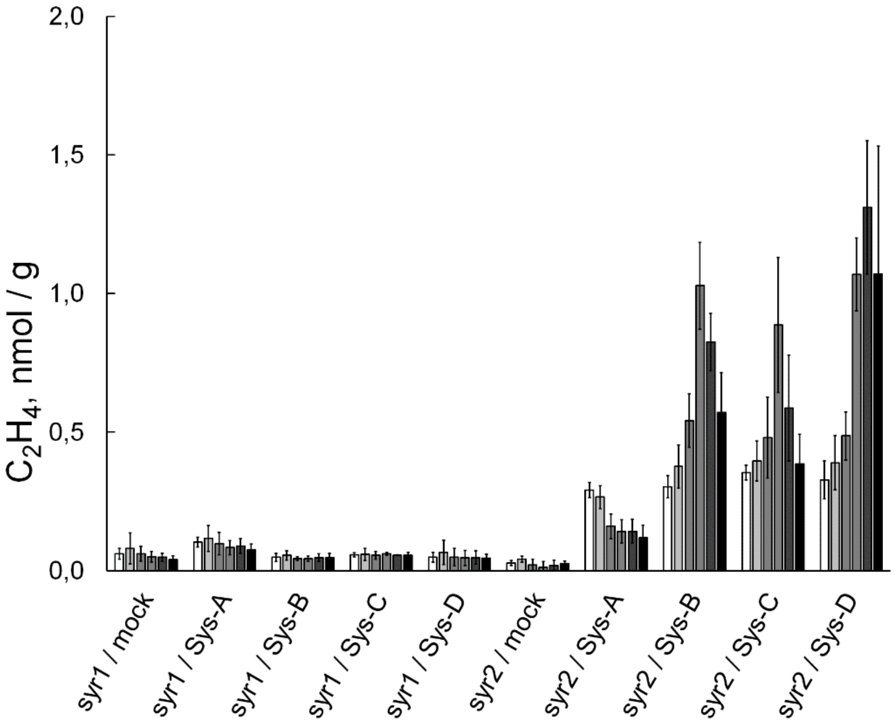
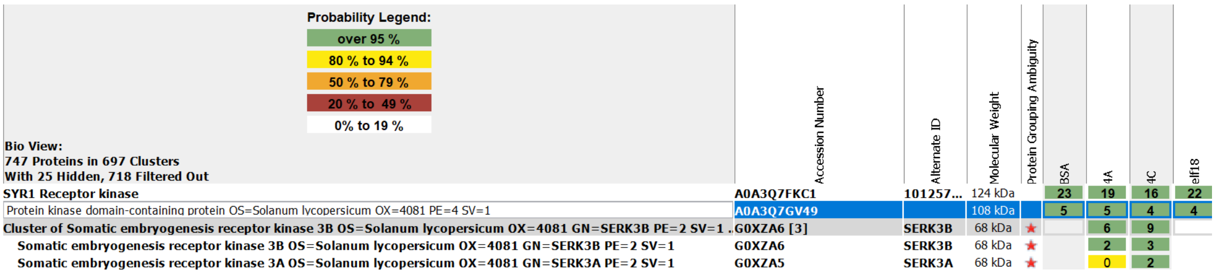


Figure 2.4.7. The response of plants lacking either SYR1 (*syr1* plants) or SYR2 (*syr2* plants) to 1 µM SysA and systemin-like peptides (SysB/C/D). The *syr1* and *syr2* mutant plants (in MoneyMaker background) were 5 weeks old. Ethylene production measured in hourly intervals for 6 hours are shown for n=3 replicates with averages (bars; from white=0-1h to black=5-6h) and standard errors (error bars).



Probability Legend:
🟩 over 95 %
🟨 80 % to 94 %
🟧 50 % to 79 %
🟥 20 % to 49 %
⬜ 0% to 19 %

Bio View:
747 Proteins in 697 Clusters
With 25 Hidden, 718 Filtered Out

	Accession Number	Alternate ID	Molecular Weight	Protein Grouping Ambiguity	BSA	4A	4C	elf18
SYR1 Receptor kinase	**A0A3Q7FKC1**	**101257...**	124 kDa		23	19	16	22
Protein kinase domain-containing protein OS=Solanum lycopersicum OX=4081 PE=4 SV=1	**A0A3Q7GV49**		108 kDa		5	5	4	4
Cluster of Somatic embryogenesis receptor kinase 3B OS=Solanum lycopersicum OX=4081 GN=SERK3B PE=2 SV=1 ..	**G0XZA6 [3]**	**SERK3B**	68 kDa	★		6	9	
Somatic embryogenesis receptor kinase 3B OS=Solanum lycopersicum OX=4081 GN=SERK3B PE=2 SV=1	**G0XZA6**	**SERK3B**	68 kDa	★		2	3	
Somatic embryogenesis receptor kinase 3A OS=Solanum lycopersicum OX=4081 GN=SERK3A PE=2 SV=1	**G0XZA5**	**SERK3A**	68 kDa	★		0	2	

Figure 2.4.8. MS/MS analysis of the pull-down sample revealed potential co-receptor.

The MS/MS data was generated in collaboration with the research group of Dr. Andreas Schaller from University of Hohenheim. This was only the first preliminary result, and required confirmation from subsequent repetitions.

3. General Discussion

Systemin elicits an alkalinization response in *L. peruvianum* cell suspension cultures at a very low concentration of 0.01 nM (Felix & Boller, 1995). We had initially hoped that this sensitive bioassay could aid in the quantification of endogenous systemin levels in tomato leaves. Our attempts to quantify systemin-related components in the tomato leaf extract, while not excluding the presence of agonistic systemin peptides, could not observe such activity, likely due to the use of insufficient amount of starting material and the presence of interfering activities not related to systemin (**Figure 2.2.1.C**). In contrast, the presence of the antagonistic systemin peptide, the anti-systemin (aSys) was easily detectable. The presence of aSys suggests that this factor might have a balancing effect contributing to the dampening of inadvertent systemin activity.

Evidence for such a balancing effect comes from observations with the two tomato transformants C8.1 & and C8.4 that have disruption in the pro-aSys peptide. These mutants displayed stunted growth phenotype, abnormal plant development, reduced seed formation, as well as low seed germination rate. Interestingly, the overexpression of SysA also resulted in a developmental phenotype in which the transformed plants looked less healthy than the wild-type plants (McGurl *et al.*, 1994). In both cases, it is possible that the equilibrium between agonistic and antagonistic systemin was disturbed. The phenotypical traits displayed by the transgenic lines C8.1 and C8.4 might have been due to the complete lack of aSys to counter the continuous presence of systemin and systemin-like peptides in tomato tissues. Further analysis in term of quantifying the amount of agonistic and antagonistic systemin peptides, or by measuring the response of leaf pieces to standard systemin peptide treatment would give us more understanding of how the absence of indigenous functional aSys affects the plant biochemical system. In addition, analysis of the expression of all the systemin genes (both agonistic and antagonistic) in the mutated lines as well as the two wild-type tomato lines (MoneyMaker and M82) would provide further evidence of the balancing of these peptides in the plants.

The systemin-like are also under study by Dr. Andreas Schaller and his research group at the University of Hohenheim. They invested in an RNA-seq approach after treatment of tomato seedlings with SysA, SysB, SysC and SysD.

Their data suggested that the systemin-like peptides SysB/C/D induced the expression of many genes in addition to the ones induced by SysA (personal communication, unpublished data). This was surprising with respect to our initial findings that all four systemin peptides exhibited similar activity and triggered responses with similar efficiency when assayed for rapid responses like medium alkalinization in cell suspensions (**Figure 1.3.1**) and production of oxidative species in leaves (**Figure 3.1**). We thus checked whether the different peptides would also show similar activity in the somewhat slower induction of ethylene biosynthesis. Thereby we noticed two different response patterns: (1) the original systemin peptide (SysA) causes a rapid and transient ethylene production with peak production within 2 hours after peptide treatment, while (2) the systemin-like peptides (SysB/C/D) initiate a somewhat slower but steadily increasing production of ethylene (for at least 4 hours after treatment). Our initial hypothesis was that the difference in response might be due to the two distinct receptors SYR1 and SYR2. However, the assay with single-receptor mutants indicated that the two types of systemin response patterns depend on SYR1 alone. In turn, at least for the induction of the ethylene response in leaves, SYR2 seems to play no role in perception of any of the systemin peptides (**Figure 3.2**). According to the research of Wang *et al.* (2018), despite the protein sequence of SYR2 being 89% identical to that of SYR1, SYR2 is significantly less sensitive to systemin than SYR1. Observable perception of systemin by SYR2 in bioassay such as ethylene biosynthesis or ROS production was only possible when the protein was transiently expressed in *N. benthamiana*, or was stably overexpressed in the introgression line IL3-3. Even then, SYR2 required a larger amount of systemin peptide to elicit a comparable response to SYR1.

Co-immunoprecipitation data suggested that the complex with the co-receptor SERK3A might mediate the prolonged response pattern. On the peptide side, we were able to determine the single amino acid which is responsible for the different perception patterns, locating at the C-terminus of the 18-aa peptide sequence. This expands on the results presented by Meindl *et al.* (1998) which states that the N-terminal part of systemin is responsible for binding of peptide to receptor protein while the C-terminal part activates response from the receptor. We now learn that the response activation of the systemin receptor is further regulated by the amino acid at position 18 of the peptide sequence, mediating formation of complex with SERK co-receptors and prolonging the production of response signal.

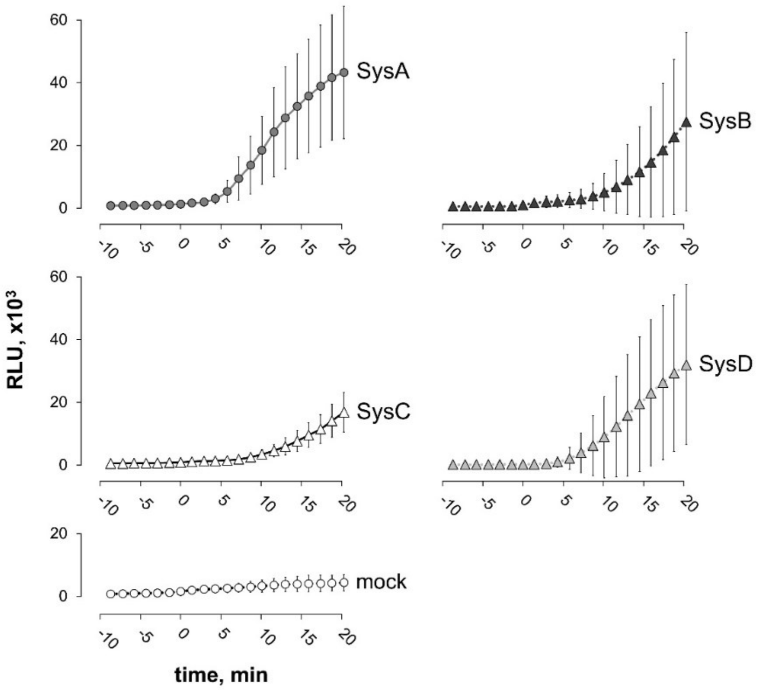


Figure 3.1. ROS production by leaf pieces from 5-week-old tomato plants (MoneyMaker) to 1 nM SysA and systemin-like peptides (SysB/C/D). Values are shown for n=4 replicates with averages (symbols) and standard errors (error bars).

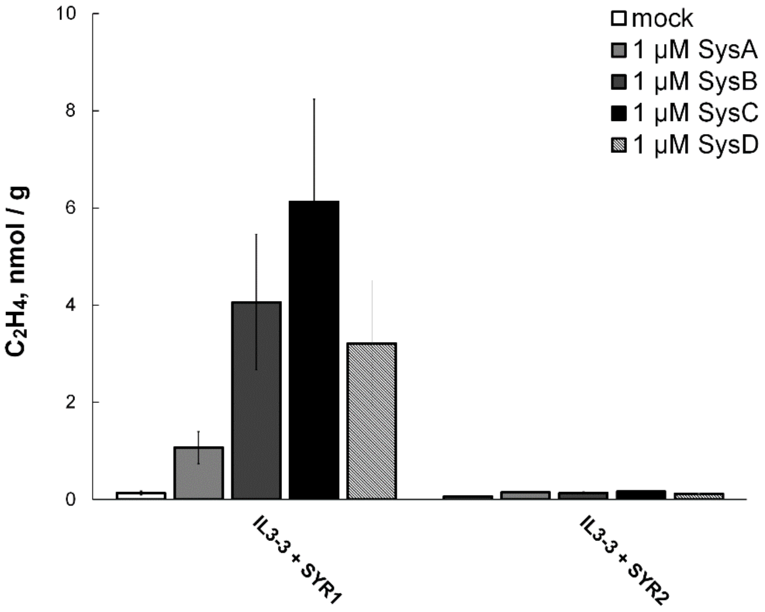


Figure 3.2. Ethylene biosynthesis by leaf pieces from 5-week-old tomato plants (IL3-3 complemented with either SYR1 or SYR2) to 1 µM SysA and systemin-like peptides (SysB/C/D). Values are shown for n=3 replicates with averages (bars) and standard errors (error bars).

3. General Discussion

The diverse role of SERKs as co-receptors has been intensely studied in many plants, especially the model plant *Arabidopsis thaliana* (Fan *et al.*, 2016; Ma *et al.*, 2016; Kumar & Van Staden, 2019; Zhuo *et al.*, 2019); however, there are only few studies which focus on the functions of SERKs in tomato (Mantelin *et al.*, 2011; Peng & Kaloshian, 2014). The results of our experiment provide new evidence for the role of SERKs as co-receptors in tomato. On the other hand, it is possible that the prolonged ethylene production response might be artefactual due to the saturating amount of systemin signals used in the experiment. It is very unlikely that tomato plants under will encounter such high concentration of peptides under physiological conditions. Nevertheless, the fact that SERK3A interacts with SYR1 to form complex that mediates response to peptide signals provides potential leads for further studying on the mechanism in which SERKs aid in the signaling pathway in tomato.

What was only one single peptide systemin and its two receptor proteins SYR1 and SYR2, has expanded into a collection of one antagonist, the anti-systemin, and four agonists, the original systemin (SysA) and the systemin-like (SysB/C/D), and the more sensitive receptor kinase SYR1 might form complex with different co-receptors (e.g. SERKs) to perceive different qualities of the systemin ligands. Such complex perception systemin tomato is surprising and fascinating, which we only touch on the surface. There are still many open questions: which mechanism for the differentiation of perceived systemin ligand; what possible role of SYR2 in this complex system, a back-up receptor for when SYR1 is exhausted, or maybe SYR2 act as a negative control; is the SYR1/SERK3A complex stabilizing the signal which leads to the prolonged production of ethylene; since tomato mutants lacking aSys show that this factor is relevant to plant reproduction, hinting at the importance of the balance of antagonist and agonist systemin regulating plant development. All these questions are potential for further research and study.

4. Materials and Methods

4.1. Plant material

The plant materials that were used in the experiments of this project included: tomato *Solanum lycopersicum* cultivar Moneymaker (MM), cultivar M82, M82/*Solanum pennellii* introgression lines IL3-2 & IL3-3 (Eshed *et al.*, 1995), IL3-3 complemented with either of the systemin receptors SYR1 or SYR2 (Wang *et al.*, 2018), and *Nicotiana benthamiana* wild-type. Tomato and *N. benthamiana* seeds germinated in soil and were cultured in the greenhouse under long day condition with daily watering and constant temperature of 20°C.

4.2. Cell suspension culture

The cell suspension of *Lycopersicon peruvianum* (Mill.) was originally provided by L. Nover (University of Frankfurt) and were grown in Murashige/Skoog-type medium containing 5.0 mg/l 1-naphthylacetic acid under constant light on a rotary shaker at 26°C, with weekly sub-culturing of 1-3 ml of the suspension in 70 ml fresh medium (Nover *et al.*, 1982). The conditions of the cell suspension and equipment setup for the measurement of medium alkalinisation of the cell suspension *L. peruvianum* (Mill.) in response of different peptide treatments were as described by Felix *et al.* (1995 & 1998).

4.3. Bacterial strains

The bacterial strains employed for the relevant cloning and transformation processes, *Escherichia coli* (strain TOP10 from Thermo Scientific) and *Agrobacterium tumefaciens* (strain GV3101), were grown at 37°C for *E. coli* or 28°C/30°C for *A. tumefaciens*, either on LB agarose plates (in stationary incubator) or in liquid LB medium (with rotary shaking at 200 rpm). The LB contained appropriate antibiotics (gentamycin, carbenicillin, kanamycin, streptomycin, hygromycin) and selection compound (sucrose 50%).

4.4. Peptides, oligonucleotides, and plasmids

The 18-amino-acid peptide systemin (Pearce *et al.*, 1991) and the 16-amino-acid systemin antagonist were synthesized by F. Fischer (Friedrich Miescher-Institute). All other peptides were synthesized by GenScript Biotech (Netherlands). Stock peptide solutions of 10 mM were first prepared in deionized water and were stored at –20°C. Subsequent dilutions were prepared in a solution of 10 mg/ml bovine serum albumin (BSA) and 100 mM NaCl to avoid adsorption to plastic tubes. Dilutions were stored at –20°C, and kept on ice while in use. All peptides are listed in **Table 4.4.1**.

The oligonucleotides used for CRISPR-Cas modification of the aSys gene in tomato were designed using CLC Work Bench 8.1 software based on annotated gene sequence (*S. lycopersicum* str. Heinz 1706, SL2.50sc05650) obtained from the NCBI database, with target sites selection based on the analysis results generated from the CRISPRdirect website (https://crispr.dbcls.jp/). Other oligonucleotides included the primers for genotyping (by PCR) and sequencing of the aSys gene. All oligonucleotides (see **Table 4.4.2**) were produced by Merck KGaA, and were delivered in dry powder form in tubes. Stock solutions (100 pmol/µL) and working dilutions (10 pmol/µL) were prepared in nuclease-free water and stored at –20°C.

The CRISPR-Cas components, e.g. the *Cas9* and *Csy4* genes, Csy4, the guide RNA scaffold and the binding site for Csy4 protein, were cloned from the plasmid pDIRECT-22c (Cermark *et al.*, 2017; supplied by Addgene) and assembled with GoldenGate cloning (Binder *et al.*, 2014). All subsequent plasmid constructs are listed in **Table 4.4.3**.

Table 4.4.1. Peptide sequences.

Peptide	Symbol	Sequence
systemin *	SysA	AVQSKPPSKRDPPKMQTD
anti-systemin †	aSys	AKFPTPPGKRPGPTIG
systemin B ⁺	SysB	ANFSTRPGKRSAPKPKTG
systemin C ⁺	SysC	AKFSTPPGKRSAPRPKTG
systemin D ⁺	SysD	ANVSRPPGQRPAPRPQTGR
	Sys(A-C) †	AVQSKPPSKRDPPKPKTG
	Sys(C-A) †	AKFSTPPGKRDPPKMQTD
	$SysA^{P15}$ †	AVQSKPPSKRDPPKPQTD
	$SysA^{K16}$ †	AVQSKPPSKRDPPKMKTD
	$SysA^{G18}$ †	AVQSKPPSKRDPPKMQTG
	flg22 ‡	QRLSTGSRINSAKDDAAGLQIA

* Pearce *et al.*, 1991
⁺ Voigtländer & Wang (University of Tübingen, unpublished data)
† This thesis
‡ Felix *et al.*, 1999

Table 4.4.2. Oligonucleotides.

ID	Sequence	Note
#2056	TCT GCAG GTTCACTGCCGTATAGGCAG **aaacgacc**	SI_oligo-a [+]
#2057	**accaggtcgttt** CTGCCTATACGGCAGTGAAC CTGC	SI_oligo-b [+]
#2058	TCGTCTCA **tggtcctaccat** GTTTTAGAGCTAGAAATAGC	SI_primer-c1 [+]
#2059	TCGTCTCA **tggcaaacgacc** CTGCCTATACGGCAGTGAAC	SI_primer-d2 [+]
#2060	TCGTCTCA **gccaggaggagt** GTTTTAGAGCTAGAAATAGC	SI_primer-c2 [+]
#2061	TCGTCTCA **ttcttcacttgg** CTGCCTATACGGCAGTGAAC	SI_primer-d3 [+]
#2062	TCGTCTCA **agaacctgctaa** GTTTTAGAGCTAGAAATAGC	SI_primer-c3 [+]
#2063	TCGTCTCA **ctcaattgaagc** CTGCCTATACGGCAGTGAAC	SI_primer-d4 [+]
#2064	TCGTCTCA **tgagctttatat** GTTTTAGAGCTAGAAATAGC	SI_primer-c4 [+]
#2065	AGCTCTTCGATT CTGCCTATACGGCAGTGAAC	SI_primer-e [+]
#2144	CTTTAGCAGGTTCTTCACTT	SI promoter Rv [*]
#2170	GTATAATCATGTGCACGCCATCTTA	SI-CRISPR Fw [†]
#2171	TCTTTCGATCTACGCCAACACACAA	SI-CRISPR Rv [†]
#2277	CCTGGTCCTCTACTGTATTT	CRI_SI sequenc Fw [‡]
#2278	CTTTCTGTCTAGTCGATTTC	CRI_SI sequenc Rv [‡]

[+] Oligonucleotides used for CRISPR-Cas modification of aSys gene: sections with single underline are binding site of Csy4 protein; the ones with double underline are the type-IIS restriction site; and those in lower case letters are fragments of the target sites.

[*],[†] Primers used for checking presence of modified aSys gene in transgenic tomato plants.

[‡] Primer pair used for sequencing of the aSys gene.

Table 4.4.3. Plasmids.

Name	Construct
(LIIc_3-4)_CRI1a ⁺	p*Cm*YLCV::*sac*B::35S-T
(LIIc_3-4)_CRI1b	p*Cm*YLCV::guide-RNA-array::35S-T
(LIIc_1-2)_CRI2 ⁺	2x35S-Ω::*Csy4*-P2A_*Cas9*-NLS::nos-T::BastaR
(LIIIa)_CRI-E	2x35S-Ω::*Csy4*-P2A_*Cas9*-NLS::nos-T::BastaR__ p*Cm*YLCV:: guide-RNA-array::35S-T

⁺ These constructs were designed and assembled by M. Lammers and L.-P. Maier (University of Tübingen).
The relevant GoldenGate modules and vectors were provided by Dr. R. Morbitzer (University of Tübingen; Binder *et al.*, 2014).

4.5. Tomato leaf extracts

4.5.1. The original purification protocol by Pearce *et al.* (1991)

Tomato leaves *S. lycopersicum* (formerly *Lycopersicon esculentum*) harvested from 20-day-old plants were homogenized (using mortar and pestle) for 5 min with distilled water. The mixture was filtered and the liquid was adjusted to pH 4.5 with HCl, then centrifuged at 1,000 rcf for 10 min. The supernatant was adjusted to pH 6.1 with NaOH, centrifuged at 10,000 rcf for 10 min, and decanted through filter paper. The filtrate was chromatographed on DEAE cellulose column, followed by reversed-phase C18 flash chromatography, Sephadex G25 gel filtration, and CM Sephadex chromatography. The crude sample from the above preliminary extraction step was further processed through several rounds of chromatographic fractionation of reversed phase and strong cation exchange columns, with diverse types of solvent and eluting conditions (e.g. eluent gradient, flow rate, and duration of elution).

4.5.2. The modified extraction protocol

Mature and fully expanded leaves without the petioles of 4-week-old MM or M82 plants were collected and wiped with paper towels to remove surface water and quickly frozen in liquid nitrogen. Leaf material was either processed directly or kept at –80°C before grinding to a fine powder with pre-cooled mortar and pestle. Aliquots of leaf powder (8 to 10 g in 50-ml falcon tubes) were mixed with two volumes (w/v, 16-20 ml) of the extraction buffer (MES 50 mM, pH 6.0), and the mixture was left to thaw briefly at room temperature before being moved to constant mixing at 4°C for 10 min. The plant material was removed by spinning the mixture at 10,000 rcf for 15 min. The supernatant was then heated to 95°C for 20 min and precipitated material was removed by spinning the mixture again at same speed and time.

The supernatants of 3 aliquots were combined (volume of 50-60 ml) and applied to small solid-phase C8 pre-columns (CHROMABOND®), which had been conditioned with 6 ml of methanol, then with 6 ml of 0.1% TFA in water. After sample application and washing with 3 ml of 5% methanol, columns were eluted with 2 ml 20% methanol, and 4 ml of 60% methanol (all methanol solutions were prepared with 0.1% TFA in water). The combined eluates were concentrated to approx. 500 µl by Speed-Vac vacuum centrifugation and fractionated on C8 HPLC column (Agilent), using a gradient of 0% to 50% acetonitrile in 0.1% TFA. Fractions of 1 ml were collected, volume reduced to 50-100 µl (Speed-Vac) and stored at –20°C. Each fraction was adjusted to a pH of 5.5-5.8 (corresponding to the pH of the cell suspension cultures) by adding NaOH.

4.5.3. Determination of systemin-related activities

Aliquots of the *L. peruvianum* cell suspension culture were treated with 3-5 µl of the extracted fractions, and pH of the medium was measured after 13-17 min. To identify whether SysA or aSys was present in the extracted fractions, standard aSys or SysA would be added before or after fraction treatment (time between standard and fraction treatments was appx. 15 sec). Final concentration of anti-SYS in cell suspension aliquot was 30 nM, and for SYS was 3 nM.

4.6. Transgenic tomato lines with modification of systemin antagonist gene

4.6.1. CRISPR construct assembly

The anti-SYS gene was modified using the genome editing tool of CRISPR-Cas9 (Doudna & Charpentier, 2014). The CRISPR construct was assembled using the GoldenGate method (Binder *et al.*, 2014), which included the genes encoding the Cas9 and Csy4 proteins, as well as the polycistronic gRNA array under the *Cs*YMLV promoter (Cermark *et al.*, 2017) (**Figure 4.6.1**), and transformed into *A. tumefaciens*.

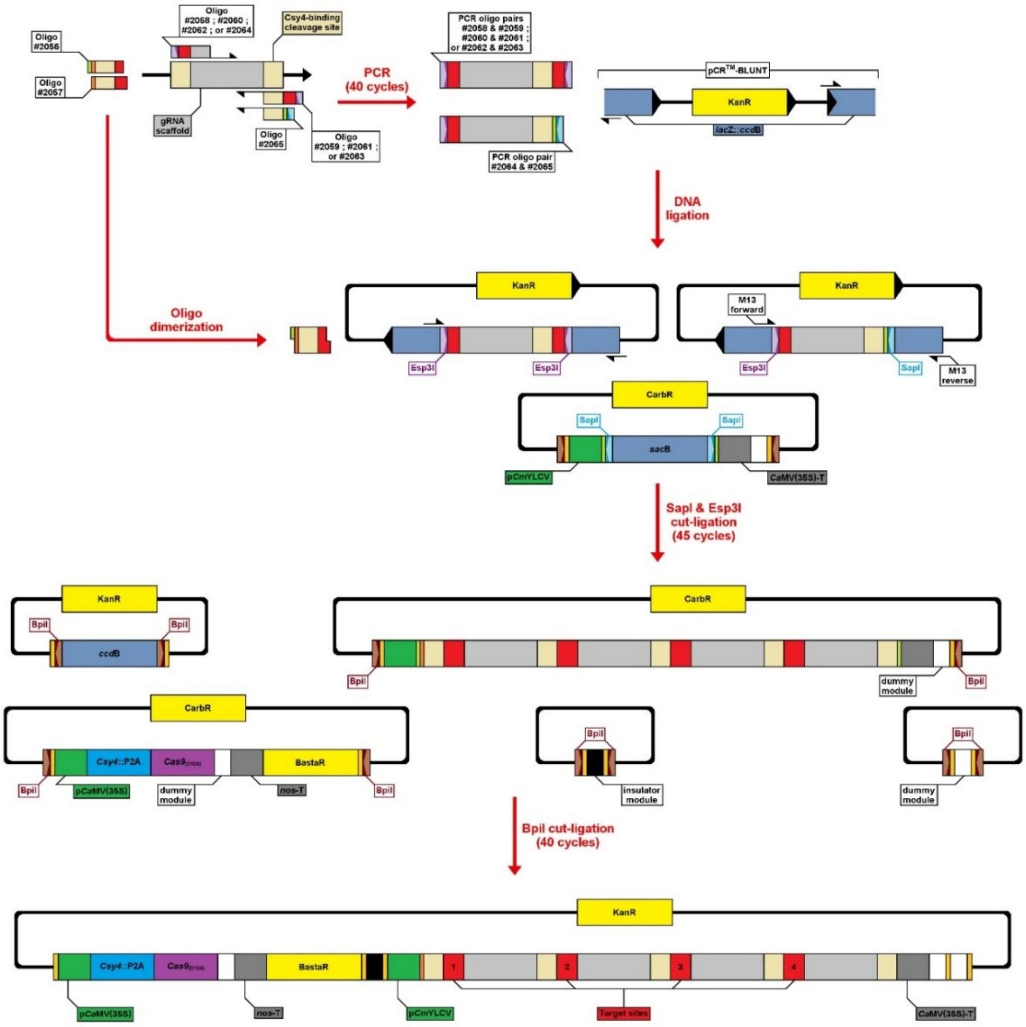


Figure 4.6.1. Cloning and GoldenGate assembling of CRISPR construct for the modification of the anti-SYS gene in *S. lycopersicum*. The components are not drawn to scale. Oligonucleotides and module-carrying plasmids are listed in **Table 4.4.2** and **Table 4.4.3**. Cycling programs for the polymerase chain reaction, and restriction enzyme cut-ligation reactions are listed in **Table 4.6.1**.

Table 4.6.1. PCR & restriction enzyme cut-ligation reaction programs.

Step	Temperature	Time	Cycle
Polymerase chain reaction			
Denaturation	95°C/98°C	15s	35/40x
Annealing	55°C/60°C	15s	
Extension	72°C	45s/1min	
Restriction enzyme cut-ligation reaction [+]			
Digestion	37°C	2 min	45x
Ligation	16°C	5 min	
Final digestion (for *Bpi*I)	37°C	5 min	
Final digestion (for *Bsa*I)	50°C	5 min	
Inactivation	80°C	5 min	

[+] Binder *et al.*, 2014.

4.6.2. Generation of the transgenic tomato lines

Tomato (*S. lycopersicum* v. Moneymaker) was transformed according to the protocol described by Wittmann *et al.* (2016). The transgenic shoots with sufficient development of adventitious roots were transferred to soil and grown in green house under long day condition at 20°C. Genomic analysis and seed propagation were repeated for at least two generations to obtain potential homozygous plants of the gene modification (**Figure 4.6.2**).

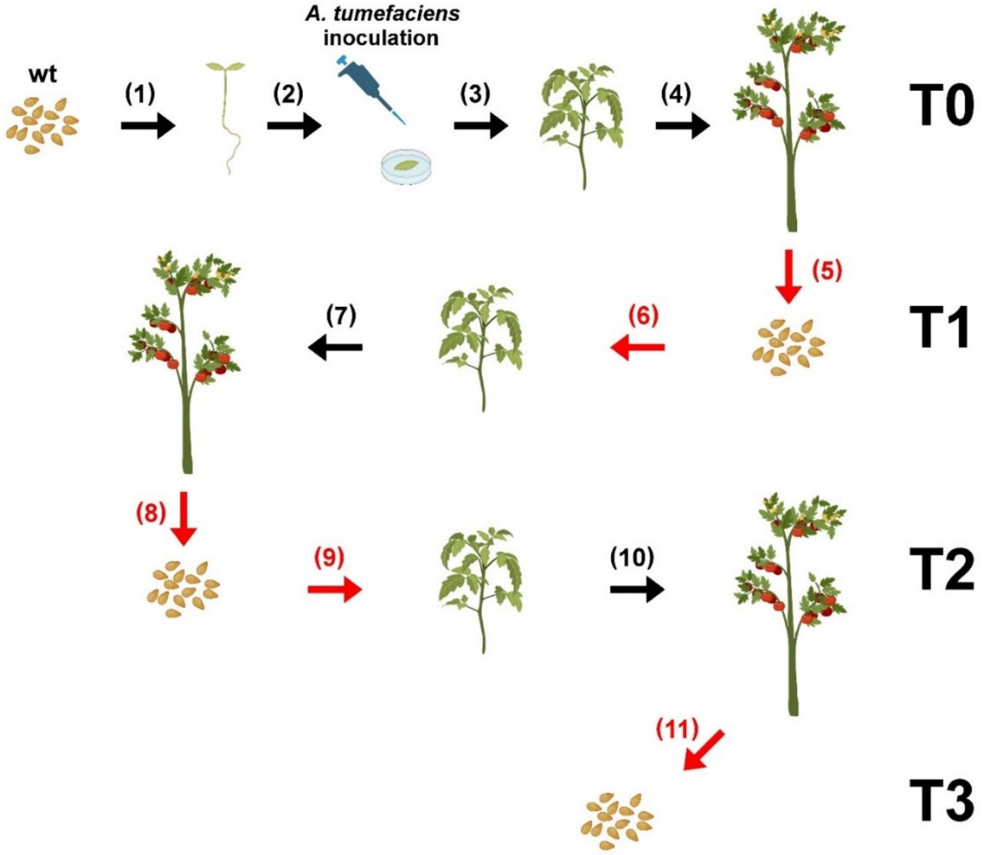


Figure 4.6.2. Transformation and propagation scheme for the stabled transgenic tomato lines.

(1) Tomato seeds (**wt**) of cultivar MoneyMaker were germinated for 12 days in the dark. **(2)** Cotyledons were harvested and placed on conditioning medium (0.1 µg/ml BAP and 1 µg/ml NAA). After 2 days of incubation in the dark, the cotyledons were inoculated with agrobacteria containing a T_i plasmid encoding the CRISPR construct. **(3)** Transformed cotyledon was cultured on medium containing 2 µg/ml BASTA (for T-DNA selection marker) and 250 µg/ml ticarcilin (for eliminating the agrobacteria), as well as 1 µg/ml of trans-zeatin for shoot-inducing. The emerging shoots were then separated into individual culture flasks until generation of sufficient roots for transferring to soil and further growth in the greenhouse. Genomic analysis was performed on the young plants and those with modification in the ant-SYS gene (and 1-2 negative lines for control) were let grown to maturation **(4)(7)(10)** for further propagation **(5,6)(8,9)(11)**.

(Tomato seeds: https://depositphotos.com; tomato plants & other tools: https://biorender.com)

4.6.3. Genotyping for presence of relevant mutations in transgenic plants

The genomic samples from the transgenic plants were obtained using the nexttec DNA Isolation Kit for Plants (Biozym), following the kit protocol for homogenised plant material, and were kept at 4°C. Genotyping by polymerase chain reaction (PCR) was performed by amplification of two regions of the aSys gene as shown in **Figure 4.6.3**. The reaction mixture included 5 ng of the DNA sample, 5 µl of highQu AllIn™ Red Taq mastermix 2x, 0.5 µl each of the primers (**Table 4.4.2**), and distilled water to a total volume of 10 µl (the reaction program was summarised in **Table 4.6.1**).

Figure 4.6.3. Amplified regions for genotyping of the aSys gene.

4.6.4. Measuring the germination rate of seeds from the confirmed transgenic lines

For uniform germination, we employed the bleach treatment recommended by C.M. Rick (TGRC; https://tgrc.ucdavis.edu/seedgermination) with some adjustment: analytical sodium hypochlorite was diluted to 2.7% solution; tomato seeds were soaked in the solution on a shaking platform for 30 minutes, before being sown in trays, which were divided into 50 sections for sowing individual seeds. The trays were kept covered to maintain humidity until seeds germinated or two weeks after date of sowing. The germinating status was recorded every 2 days. Alternatively, the seeds were sown on 1/2 MS with or without anti-SYS supplementation, and the germination dishes were kept in growing chamber with 16-hour light cycle, 28°C.

4.7. Bioassays with tomato leaf pieces

4.7.1. Cumulative ethylene production measurement

Leaf material from 4 weeks old tomato plants, with no visible damage or infection, was prepared as described and incubated on water over night (Albert *et al.*, 2015). Leaf pieces (3 per replicate) were transferred onto 500 µl water in 6-ml test tubes, after which peptide solution was added to the desired final concentration. The tube was capped and placed on shaker for constant agitation for 4 hours. The air space (1 ml) was collected and analysed with GC equipment.

4.7.2. Time-course and double-treatment ethylene assay

For the evaluation of the hourly synthesis of ethylene after elicitor treatment, leaf material from 4 weeks old tomato plants, with no visible damage or infection, was prepared as described and incubated on water over night (Albert et al, 2015). The air space (1 ml) was collected every hour after peptide treatment and analysed. After each collection, the reaction tubes were kept opened for 10-20 seconds, in order to correctly determine the hourly production, instead of the cumulative production. Dr. Felix contributed to the design of the bar figures illustrating results from this assay.

4.8. Oxidative burst assay with cell suspension culture

The *L. peruvianum* cell suspension culture (ready for alkalinisation assay and with added chloramphenicol-red) was aliquot (3 mL) into the wells of a 24-well cell culture plate (without lid). Each well was treated with known amount (0 to 10 nM) of either SysA or SysC. The plate was incubated on a rotating platform, at room temperature, for 15 min. The same excess amount of 1M NaOH was added to each well to estimate the amount of purple-coloured, non-oxidized chlorophenol-red left in the medium.

4.9. Western blot & Co-immunoprecipitation

Mature and fully expanded leaves of 4-week-old plants IL3-3 complemented with systemin receptor SYR1-GFP (Wang *et al.*, 2018) were collected, divided into portions of 2-3 g and placed in 100 mL flasks, filled with 80 ml of distilled water containing 1 µM of peptide (SysA, SysC, aSys or csp22) or an equivalent amount of the solution used to dilute the peptides (BSA/NaCl). The flasks were placed in sealed chamber and vacuum was applied for max. 3 minutes, and was then released slowly for uniform infiltration of the leaves. After removing from the infiltrating solution and excess liquid was blotted dry with paper towel, the leaves could then be processed immediately or frozen with liquid nitrogen and stored at –80^{o}C.

For immediate processing the leaves were quickly homogenized using cooled mortar and pestle, with 4-5 mL of solubilisation buffer. The homogenate was filtered through miracloth into a 15 mL falcon tube. Solubilisation buffer was added to the filtrate to a volume of 10 mL, which was then moved to overhead rotating at 5 rpm, 4°C for 1 hour, and afterward to centrifugation at 20.000 rcf, 4°C for 1 hour. The supernatant was transferred into new tube for incubation with immunoprecipitation beads.

Leaves stored at –80°C were ground to powder in cooled mortar and pestle, and the leaf powder was then transferred into 15 mL falcon tube to 2-mL mark (approx. 5 g), then solubilisation buffer was added to 10-mL mark. The mixture was moved to overhead rotating at 5 rpm, 4°C for 1 hour, then to centrifugation twice at 20.000 rcf, 4°C for 1 hour. The supernatant was then incubated with immunoprecipitation beads.

Commercial immunoprecipitation bead (GFP trap) mixture was prepared by first taking an aliquot of an appropriate volume (20 µL for each sample) of bead suspension. The beads were washed (1 time with 1 mL solubilisation buffer) and re-suspended in solubilisation buffer and kept on ice. The beads were added to the leaf supernatant, and the solution was incubated with overhead rotating at 5 rpm, 4°C for 2 hours, then centrifugated at 1.000 rcf, 4°C for 5 minutes. The supernatant was discarded, and the beads were transferred to a 1.5-mL tube for washing: 2x 1 mL solubilisation buffer, 2x 1 mL wash buffer. At the end, the beads were re-suspended in 50 µL wash buffer and could were used for Western blot analysis, or quickly frozen with liquid nitrogen and stored at –80°C.

For Western blot, the bead solution was first mixed with 2x SDS-PAGE sample buffer (62.5 mM Tris/HCl solution pH 6.8, 25% glycerol, 2% w/v of SDS, 0.001% w/v of bromophenol blue) and beta-mercapto-ethanol (to final concetration of 5%), then heated at 95°C for 10 minutes. The mixture was vortexed briefly then centrifuged at 13.000 rcf, at room temperature for 10 minutes. Appropriate amount of the supernatant was loaded onto protein gel. After separation, the proteins in the gel were transferred to blotting membrane, and the membrane was incubated with the antibody against GFP overnight for chemiluminescence detection. Final visualisation of the blot was recorded with a CCD camera.

4.10. Other chemical agents

Chemicals and reagents were generally of standard purity, purchased from Carl Roth (Karlsruhe), Sigma-Aldrich (Taufkirchen), Merck (Darmstadt), Duchefa

(Haarlem), Qiagen (Hilden), Invitrogen (Karlsruhe), or Fluka (Buchs), except for acetonitrile and methanol which were for HPLC and of respective grade. Restriction enzymes used for DNA studies and the kinase inhibitor K252a were obtained from Thermo Fisher Scientific (St. Leon-Rot).

Except for the AllIn^{TM} Red Taq mastermix 2x from highQu, all restriction enzymes (BsaI, BpiI, Esp3I, SalI) and the Phusion polymerase were products of Thermo Fischer.

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