Plant Physiology and Biochemistry 140 (2019) 55–67

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

# Arabidopsis thionin-like genes are involved in resistance against the beet-cyst nematode (*Heterodera schachtii*)

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*Keywords:*
Thionin
Antimicrobial peptide
Arabidopsis
*Heterodera schachtii*
Plant defense

A B S T R A C T

Plants express various antimicrobial peptides including thionins to protect themselves against pathogens. It was recently found that, in addition to four thionin genes, Arabidopsis contains 67 thionin-like (ThiL) genes including six pseudogenes. It is known that thionins have antimicrobial activity and are part of the plant defense system, however, nothing is known about ThiL genes.

In this study, we present a bioinformatic analysis of the (ThiL) gene family in Arabidopsis. We identified 15 different motifs which positioned the ThiL peptides in four groups. A comparison of amino acid sequences showed that the ThiL peptides are actually more similar to the acidic domain of thionin proproteins than to the thionin domain.

We selected 10 ThiL genes to study the expression and possible function in the Arabidopsis plant. RT-PCR and promoter:GUS fusions showed that most genes were expressed at a very low level but in several organs and at different developmental stages. Some genes were also expressed in syncytia induced by the beet cyst nematode *Heterodera schachti* in roots while others were downregulated in syncytia. Some overexpression lines supported lower number of nematodes that developed on the roots after inoculation. Two of the genes resulted in a strong hypersensitive response when infiltrated into leaves of *Nicotiana benthamiana*. These results indicate that ThiL genes might be involved in the response to biotic stress. ThiL genes have been expanded in the *Brassicales* and specifically the *Brassicaceae*. The most extreme example is the CRP2460 subfamily that contains 28 very closely related genes from Arabidopsis which are mostly the result of tandem duplications.

## 1. Introduction

Plants use different mechanisms to defend themselves against pathogens. One of the important defensive strategies employed by plants to restrict the invasion and establishment of pathogens is the use of antimicrobial peptides (AMPs) such as plant-defensins, thionins, cyclotides, lipid transfer-proteins and snakins (Broekaert et al., 1997). For various AMPs from plants and animals, an *in vitro* antimicrobial activity has been demonstrated (Bohlmann, 1994; Bohlmann et al., 1988; Broekaert et al., 1995, 1997; Epple et al., 1997; Garcia-Olmedo et al., 1998; Holtorf et al., 1998). Thionins from wheat endosperm with eight cysteine residues, and viscotoxins from mistletoes with six cysteine residues, were among the first antimicrobial peptides that were characterized in detail and shown to have antimicrobial and toxic activities (Bohlmann, 1994; Bohlmann and Apel, 1991). All thionins are characterized by two consecutive cysteines at position three and four. They have been isolated as 5 kD peptides from different plant species but are produced as preproproteins. These consist of a signal peptide, the mature thionin and a so-called acidic domain (Bohlmann and Apel, 1991).

To date, there is no experimental information available about possible functions of the acidic domain. But it is clearly not dispensable as shown by the high conservation of the cysteine residues, even in the

*Abbreviations:* AMP, antimicrobial peptide; CRP, cysteine rich peptide; ThiL, thionin-like; HR, hypersensitive response; dpi, days post inoculation

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https://doi.org/10.1016/j.plaphy.2019.05.005
Received 25 January 2019; Received in revised form 2 May 2019; Accepted 2 May 2019
Available online 03 May 2019

case of viscotoxin precursors which have several deletions in the acidic domain (Schrader and Apel, 1991). Furthermore, Florack et al. (1994) found that expression of α-hordothionin in transgenic tobacco plants without the acidic domain resulted in significantly lower levels of the mature thionin. One possible function might be that the acidic domain contains information to guide the thionin through the secretory pathway to its final destination, i.e. vacuoles, cell walls, and protein bodies. Such a function has been demonstrated for the pro region of human defensins (Liu and Ganz, 1995). Furthermore, the acidic domain might function as an intramolecular chaperone to assist in the folding of the thionin. We recently found that the proprotein is processed to produce the mature thionin by a subtilase in barley which degraded the acidic domain (Plattner et al., 2014, 2015). This explains why a protein corresponding to the acidic domain has never been isolated from plants.

Thionins are found in various plant species, for instance cereals, mistletoes and plants of the family *Brassicaceae*, including *Arabidopsis thaliana*. Inspection of the Arabidopsis genome sequence revealed that in addition to four thionin genes, it contains a large number of so-called thionin-like (ThiL) genes. Related genes are also found in other plant species. They are included in the CRP2200–CRP2610 groups of cysteine rich peptides as defined by Silverstein et al. (2007). Most of these genes had not been annotated before in the Arabidopsis genome due to their small size. Similarly, most of them are also not included on the Affymetric GeneChip. Therefore, not much is known about the expression of these genes. Their function has also not been explored earlier.

Here we have done a bioinformatic analysis of the Arabidopsis ThiL genes. Our analysis showed that the ThiL peptides are more similar to the acidic-domain of the thionin precursor than to the thionin domain. We selected 10 genes from different CRP groups to study their expression in Arabidopsis and a possible biological function. We have concentrated on the interaction of these ThiL genes with *Heterodera schachtii*, the beet cyst nematode.

## 2. Materials and methods

### 2.1. Sequence retrieval and in silico characterization of ThiL genes and proteins

Arabidopsis ThiL genes were originally identified by Silverstein et al. (2007). Accordingly, genomic DNA, coding (CDS) and amino acid sequences of all Arabidopsis thionin and ThiL genes were retrieved in FASTA format from TAIR (https://www.arabidopsis.org/). ThiL genes and proteins were characterized on the basis of different properties such as amino acid length, CDs length, genomic DNA length, number of exons and start as well as end position of the gene on the corresponding chromosome. To predict the signal peptide cleavage site present in the polypeptide sequences of ThiL proteins, the online SignalP 4.1 Server (Petersen et al., 2011) (http://www.cbs.dtu.dk/services/SignalP/) was used. Similarly, isoelectric points of ThiL proproteins (with signal peptides) and mature peptides (without signal peptides) were calculated through the online tool from ExPASy (Gasteiger et al., 2003) available at https://web.expasy.org/compute_pi/. Likewise, to compute the grand average of hydropathy (GRAVY), the Sequence Manipulation Suite (SMS) (Stothard, 2000), was accessed at http://www.bioinformatics.org/sms2/proteingravy.html.

To position the thionin family genes on their respective chromosomes, MapChart (Voorrips, 2002) was used under default parameters. The assessment of sequence similarity patterns among mature ThiL peptides was accomplished with the help of Uni-Pro U-Gene by using ClustalW algorithm with default parameters. After that, different parts of sequences were labeled to show the resemblance among the proteins under study. Phylogenetic analysis of thionin and thionin-like protein sequences was performed by MEGA v. 7.0 (Kumar et al., 2016). For this purpose, amino acid sequences from ThiL and Thionin proproteins were aligned using ClustalW followed by the development of phylogenetic tree based on neighbor-joining method under default parameters. The tree was further validated with different models of Maximum Likelihood Methods as well (data not shown). Conserved motifs among the proproteins were discovered through the online MEME SUITE tool (Bailey et al., 2009). ThiL proteins were assigned different CRP families defined by Silverstein et al. (2007) and labeled with different colors. Gene structure analysis on the basis of position, numbers and length of untranslated regions (UTRs), exons and introns was performed with the help of Gene Structure Display Server 2.0 (Hu et al., 2015) available at http://gsds.cbi.pku.edu.cn/.

### 2.2. Cloning of binary vectors

We used the vector pPZP3425 (Szakasits et al., 2007) for promoter analysis and overexpression studies. The plasmid contains a double enhanced 35S promoter and TMV omega element driving an intron containing GUS gene. For cloning of the overexpression vectors, coding sequences were amplified from genomic DNA by PCR using primers (Table S3a) containing the restrictions sites NcoI and BamHI. The amplicons were digested with NcoI and BamHI and ligated into the vector pPZP3425 digested with the same enzymes. The final constructs were confirmed by sequencing. The promoter regions were amplified by PCR using Arabidopsis genomic DNA as template. PCR primers (Table S3b) included restriction sites for KpnI and NcoI. After digestion the PCR fragment was ligated to the large vector fragment of pPZP3425 digested with the same enzymes, thus replacing the 35S promoter by the different ThiL promoters. All constructs were verified by sequencing.

For transient expression ThiL coding sequences were cloned into the pPZPTRBO vector (Shah et al., 2013). Therefore, the coding sequences were first cloned in an intermediate cloning vector (pUC-HB3) using NcoI and BamHI sites contained in the forward and reverse primer, respectively. They were then isolated as NotI-PacI fragments and ligated to the vector pPZPTRBO cut with the same restriction enzymes.

### 2.3. Plant material and growth conditions

Arabidopsis seeds (ecotype Columbia) were surface sterilized for 20 min in 6% (w/v) sodium hypochlorite and subsequently washed three times with sterile water and grown on Murashige and Skoog (MS) medium or Knop medium (Sijmons et al., 1991). Plants were grown on soil in a growth chamber at 25 °C in a 16 h light and 8 h dark cycle for seed production.

### 2.4. Arabidopsis transformation

Binary vectors were introduced into *Agrobacterium tumefaciens* GV3101 by a freeze-thaw method (Holsters et al., 1978) for transformation of *Arabidopsis* plants by a modified floral dip method (Logemann et al., 2006). Transformed seedlings were selected on MS medium with 50 μg/ml kanamycin and 250 μg/ml timentin at 22 °C with a photoperiod of 16 h light and 8 h dark until kanamycin-resistant seedlings could be clearly identified. Seedlings that were resistant to kanamycin were transferred to soil for seed production.

For each promoter:GUS construct, 10–12 independent transgenic plants were generated and tested for GUS activity to choose a representative line, which were grown further to produce homozygous seeds. For overexpression lines, 12 independent transgenic T2 lines were generated and applied to RT-PCR using the primers described in Table S3d to select the best expressing lines. These were then made homozygous to be used in the nematode resistance tests.

### 2.5. Resistance tests

*Heterodera schachtii* was cultured *in vitro* on mustard (*Sinapis alba* cv. Albatros) roots growing on 0.2 concentrated Knop medium supplemented with 2% sucrose (Sijmons et al., 1991). Hatching of J2 larvae from the cysts, collected from the mustard stock cultures, was

stimulated by soaking in 3 mM $ZnCl_2$. Larvae were washed three times in sterile water and the J2 were resuspended in 0.5% (w/v) gelrite (Duchefa, Haarlem, The Netherlands) for infection of Arabidopsis roots. Roots of 12 days old Arabidopsis seedlings on Knop medium were inoculated with approximately 50–60 J2 larvae per plant. Three independent experiments were performed, each had five Petri dishes and one Petri dish contained approximately 10 seedlings. At 14 dpi female and male nematodes were counted and the number of males and females per cm of root length was calculated. The data regarding the number of nematodes were analysed using single factor ANOVA ($P < 0.05$, $P < 0.01$) while the means were compared using least significant difference (LSD) test at 95% level of confidence.

### 2.6. GUS reporter analysis

Histochemical detection of GUS activity was performed according to Jefferson (1989) as described by Ali et al. (2013).

### 2.7. Semi-quantitative RT-PCR

Total RNA was purified from all independent plant lines using the "NucleoSpin® RNA Plant" from Macherey-Nagel. 2 µL of eluted RNA was used for photometric measurement of RNA concentration (NanoDrop, 2000; Thermo Scientific). Semi-quantitative RT-PCR was done with a "One-step Master Mix RT-PCR Kit" (Affymetrix) according to the manufacturer's instructions. Primers are listed in Table S3c. Three primer pairs could also amplify other closely related genes as indicated.

### 2.8. Transient expression

Agrobacteria (GV3101) were grown overnight in YEB liquid medium with appropriate antibiotics (25 µg/ml gentamycin and 35 µg/ml rifampicin for Agrobacteria and 200 µg/ml spectinomycin for pPZPTRBO (Shah et al., 2013) containing ThiL genes to $OD_{600}$ 0.8 in an incubator/shaker at 28 °C. The bacteria were harvested by centrifugation at 5000 rpm for 6 min in a Heraeus Megafuge 16 R refrigerated table top centrifuge (Thermo Scientific) at room temperature (RT). The pellet was resuspended in infiltration buffer (10 mM MES pH 5.6, 10 mM MgCl2, 100 µM acetosyringone). The Agrobacteria were diluted in the infiltration buffer to $OD_{600}$ of 0.4. After incubation for 2–4 h at RT, the Agrobacteria were infiltrated in the abaxial side of the leaves of five weeks old *N. benthamiana* plants by using a 1 ml syringe without needle. Infiltrated plants were kept in a plant growth chamber at 25 ± 1 °C temperature with 65 ± 2% humidity.

## 3. Results

### 3.1. Positioning of different ThiL genes on arabidopsis chromosomes

Arabidopsis has 67 ThiL genes (including six pseudogenes) in addition to the four thionin genes. We used MapChart to locate the four thionin genes and 67 ThiL genes on their corresponding chromosomes. Different chromosomes contain a different number of ThiL genes (Fig. 1). Out of total 71 genes, 40 ThiL genes as well as *Thi2.1* and *Thi2.4* were positioned on chromosome 1. Members of the CRP2460 family are mostly (i.e. 23 out of 28 genes) located on chromosome 1. Chromosomes 2, 3, 4 and 5 contain eight, five, one and 14 ThiL genes, respectively, including two thionin genes (*Thi2.3* on chromosome 2 and *Thi2.2* on chromosome 5).

### 3.2. Phylogenetic analysis and conserved motifs analysis of the ThiL family

The results of protein based phylogenetic and conserved motif analysis were described simultaneously for comparison of similarities and differences among ThiL genes. Phylogenetic analysis divided the ThiL peptides into four main groups (Fig. 2). Using maximum likelihood tools (Schwarz and Dayhoff, 1979; Zuckerkandl and Pauling, 1965) resulted in very similar trees which are included in the supplement (Figs. 1A and B). The largest group of these was group 1, which contained 28 proteins including all members of CRP2460. The first 13 members of group 1 from At1g21835 to At1g34830 showed maximum similarity in terms of motif type and their position in the polypeptide sequences. All of these members exhibited seven different motifs (motif 1 to 7) with nearly the same place of occurrence in the sequences (Fig. 2). Furthermore, in this group, motifs 1 and 3 are repeated twice (except At1g21835). The rest of the CRP2460 family includes several pairs of closely related peptides. Many of them have the new motif 10 in addition to a number of other motifs. Group 1 also includes one peptide (At1g12665) from CRP2310 with a new motif 8, repeated twice.

Group 2 includes nine members from four different cysteine-rich peptide families (CRP2550, CRP2310, CRP2360 and CRP2480). In all of these nine peptides, motif 8 was conserved in all members except At1g11572. Moreover, motif 8 was displayed four and three times in the amino acid sequences of At5g63063 and At3g4820, respectively. Motif 1, 2, 5, 11 and 14 were also presented by some members of this family but not in a persistent way.

Group 3 was the second largest group with 18 members belonging to five different CRP families, i.e. CRP2465, CRP2490, CRP2530, CRP2610 and CRP2200 including four thionin proproteins. Two members (At1g30972 and At1g30974) of the CRP2610 family were found in the form of a pair, exhibiting motifs 1, 8 and 11 at the same position. The four thionin members appeared in the form of a group with unique motifs 11 and 13 in addition to motif 8. Motif 2 was additionally present in At1g72260. At1g25275, the only member of CRP2490, appeared with motifs 2, 5 and 8 and was followed in the phylogenetic tree by At1g77682 (only member of CRP2465) that showed only motif 1. Group 3 also included nine of the 11 members of CRP2530. The first three members (At5g26717, At5g26692 and At5g26673) are characterized by motif 7 and a duplicated motif 8 while At3g44718 exhibited motifs 2 and 14 and duplicated motif 1. The remaining members presented several conserved motifs (1, 8, 11 and 14), but were characterized by motif 12 which was only found in this family. Motif 8 was one of the most prevalent motifs in this group.

Group 4 was the smallest group with only seven members from CRP2480, CRP2420, CRP2530 and CRP2310. Both, At1g53285 and At1g53282 (CRP2480) displayed motifs 2 and 8, whereas At5g64816 (CRP2420) showed only motif 1 and At5g58412 (CRP2530) exhibited motifs 1 and 8. At1g12660 and At1g12663 (CRP2310) appeared with a three times repetition of motif 8. Among all 65 members, there were three ThiL proteins (At1g12665, At3g48208 and At5g35698) which represented outgroups. Of them, At1g12665 and At3g48208 had only motif 8, whereas At5g35698 showed motifs 1, 8 and 11. Among all 15 discovered motifs, the most abundant one was motif number 8 with three cysteine residues.

### 3.3. Gene structure analysis of ThiL genes

Gene structure analysis of 61 ThiL genes and four thionin genes was carried out to compare the patterns of introns and exons in the genomic DNA. Exon-intron patterns were also conserved in different clusters of the phylogenetic tree and various CRP families (Fig. 3). Members of group 1 showed only one exon of similar size and at the same position in their DNA sequence; however, some members exhibited untranslated regions in addition to an exon. Within group 3, genes belonging to the thionin family (CRP2200), appeared with a precise gene structure of three exons and two introns followed by the UTRs. A similar gene structure with three exons and two introns was only found in two other genes. These were the only gene from CRP2490 (*At1g25275*) which clustered together with the thionin genes and gene *At1g12672* from CRP2310 which was found in group 4. All other members of group 3 did not show a considerable degree of similarity in terms of gene structure.

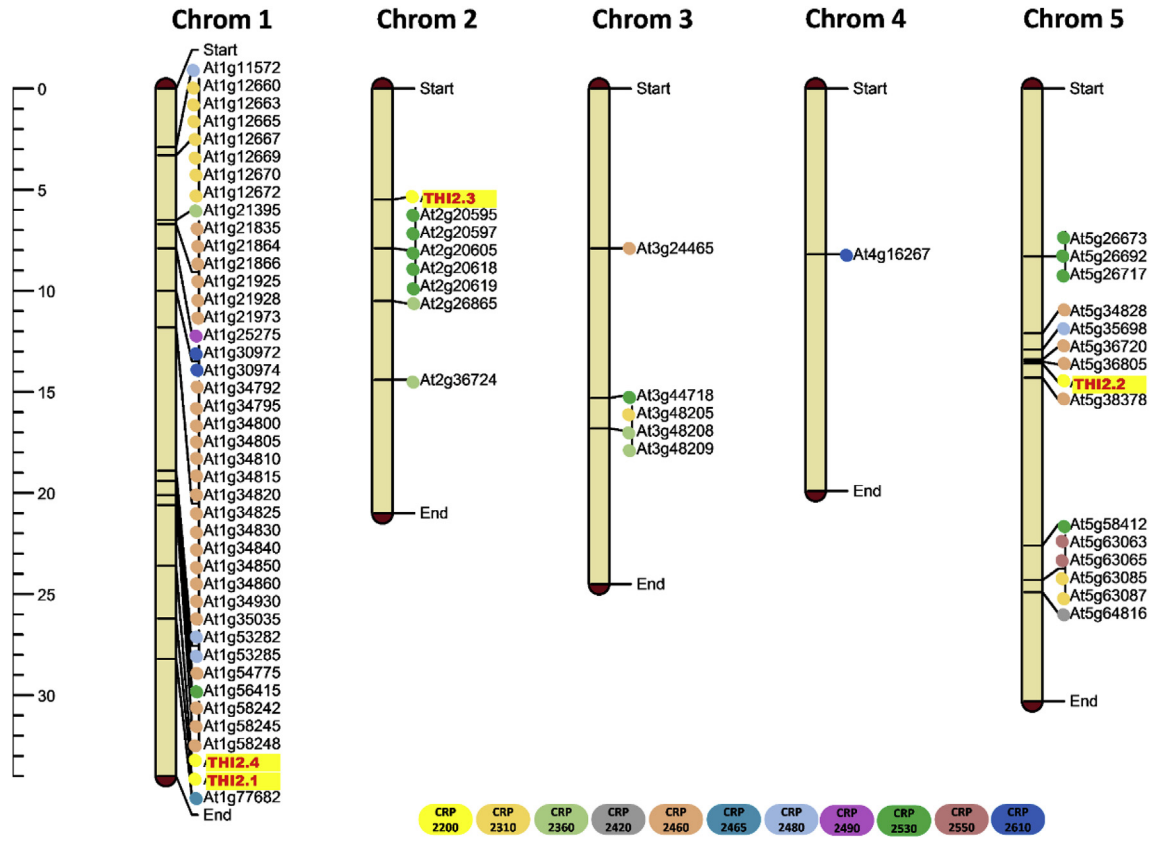


**Fig. 1. Chromosomal positioning of 67 ThiL genes, including six pseudogenes, and four thionins**. Scale on the left side shows chromosomal length in mega base pairs (Mbp). Four yellow shaded genes belong to the thionin family, whereas all other ThiL genes are labeled by colored dots. These colors are according to their CRP families (according to Silverstein et al. (2007)). Colors provided at the bottom of the Figure. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### 3.4. Evolution of the ThiL genes

The availability of genome sequences from different plant species allows to identify the occurrence of homologs of the Arabidopsis ThiL genes in other plants. We used PLAZA (Van Bel et al., 2018) to identify gene families corresponding to the 10 ThiL CRP families defined by Silverstein et al. (2007). The single Arabidopsis genes in CRP2420 (*At5g64816*) and CRP2490 (*At1g25275*) correspond to PLAZA gene families HOM04D004102 and HOM04D008388, respectively. These families include 84 genes in *Viridiplantae* and 26 genes in *Eudicotyledons*, respectively (Fig. S2). For most CRP groups ThiL genes are restricted to *Brassicales* or even *Brassicaceae*. The single Arabidopsis gene from CRP2465 (*At1g77682*) has only one homologous gene in *A. lyrata* (Fig. S2). CRP2460, corresponding to the PLAZA gene family HOM04D006842 with 59 genes, includes 28 genes from Arabidopsis and 11 from *A. lyrata*, indicating a strong expansion in Arabidopsis. Many of the CRP2460 genes are tandem duplicates, which can also be seen in Fig. 2. Tandem duplicates are also found in other CRP groups, for instance *At1g53282* and *At1g53285* from CRP2480. The majority of the CRP2460 genes are located on chromosome 1 with a cluster of 13 genes (from *At1g34792* to *At1g35035*) within a DNA sequence of 45 kb. They are interspersed with eight copies of pseudogenes of *At1g34790* (*Transparent Testa 1*). This cluster is flanked by several transposable elements (Fig. S9). If these elements were involved in duplication of these ThiL genes and Transparent Testa 1 pseudogenes is not known.

### 3.5. ThiL genes encode peptides with similarity to the thionin acidic domain

All ThiL genes encode peptides that are preceded by a signal peptide except *At5g64816* as seen in Table S1. The exception is the only gene of CRP2420 (*At5g64816*) where SignalP does not predict a signal peptide. The majority of the peptides are basic and they have a conserved cysteine pattern. Alignment of the sequences of thionin proproteins and ThiL peptides showed that a small part of the ThiL peptides aligned with the last part of the thionin domain whereas the larger part aligned with the acidic domain (Fig. 4). The ThiL peptides also miss the characteristic double cysteine motif at position three and four that is characteristic for thionins. In a strict sense we would rather have to call them “acidic-domain-like peptides”. While the 3D structures of a large number of thionins have been determined experimentally (Mendez et al., 1990; Ohtani et al., 1977; Stec, 2006) nothing is known about the 3D structure of the acidic domain. We run the sequences of the 10 peptides that we selected for further study on Phyre 2 (Kelley et al., 2015). However, the program could not predict a structure with strong confidence (Table S2 and Fig. S3).

Thionin proproteins are further processed to produce the mature thionin which has repeatedly been isolated from different plant species. If the ThiL peptides are also processed is not known but maybe unlikely as there is not the typical thionin proprotein structure.

### 3.6. Expression analysis

RT-PCR was used to determine the expression of 10 ThiL genes in different organs and growth stages of Arabidopsis plants (Fig. 5). In general, expression was low and expression was mostly found in siliques and stems. In case of *At1g12665,* a clear signal was only obtained for siliques. The strongest expression was found for *At1g25275*. *At5g64816* showed a similar expression as *At1g25275* but at a lower level. Some of the genes are represented on the Arabidopsis GeneChip. These are *At1g25275*, *At5g36720*, and *At5g64816* (Fig. S4) which

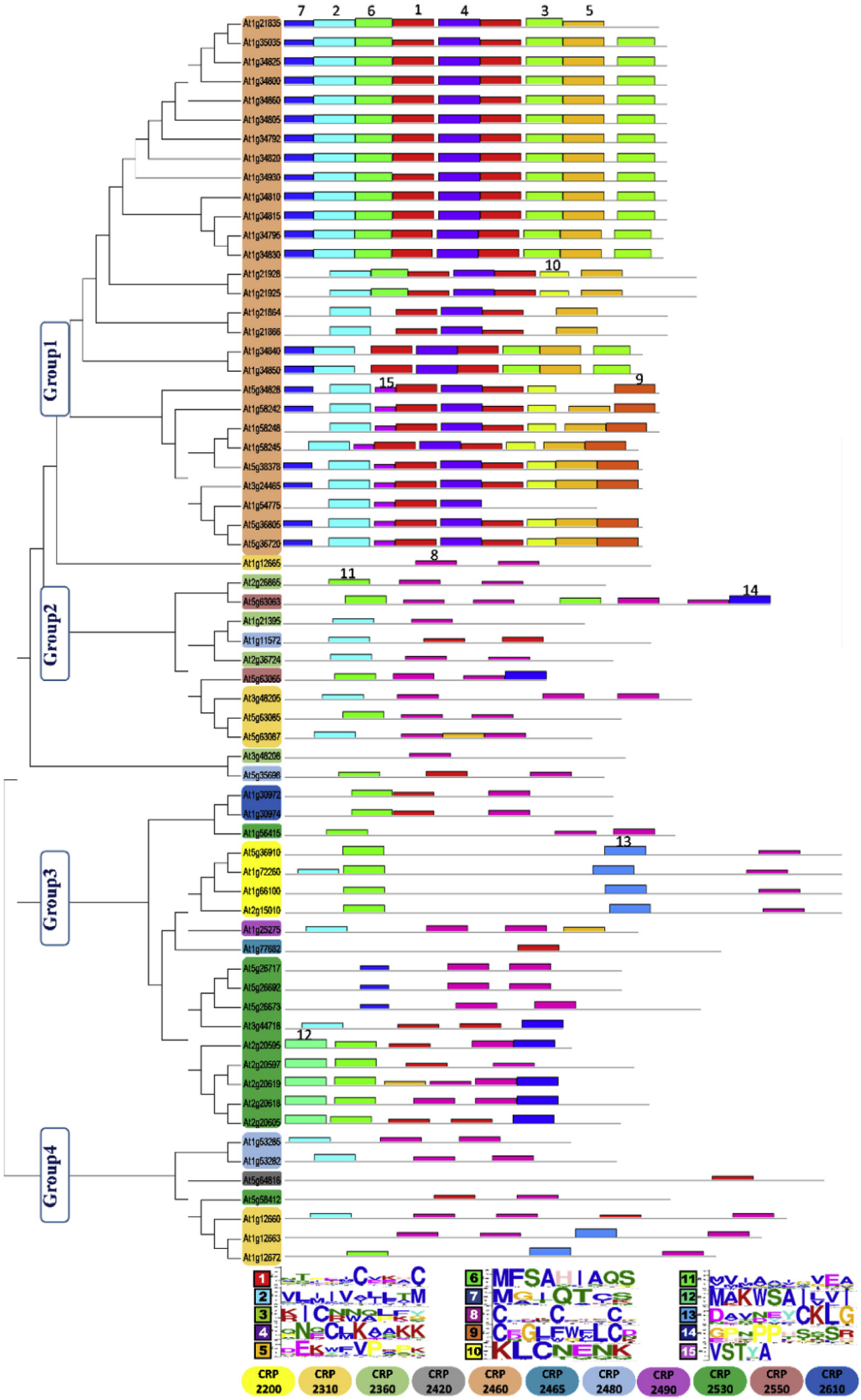


**Fig. 2. Phylogenetic and conserved motif analyses of 61 ThiL genes and four thionins**. Motif numbers assigned by MEME suite tool are mentioned on each motif. Motifs heights depict the significance of the match as taller are more significantly conserved than lower motifs. Sequence logos of all motifs are provided at the bottom. Members of different groups clustered by phylogenetic analysis are shaded by different colors based on their CRP family according to Silverstein et al. (2007). Colored scheme of all 11 CRP families is given at the bottom. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

confirm our results. In addition to the GeneChip data, RNAseq data are available (Fig. S5) for these genes. They confirm the expression of *At1g25275* at a high level and of *At5g64816* at a lower level while expression of the other genes was found at a very low level. For *At1g12665* the RNAseq data show no expression in all tissues except very low expression in receptacles.

For some of the genes the primers could also amplify closely related genes as indicated in Table S3D. Therefore, and in order to study the

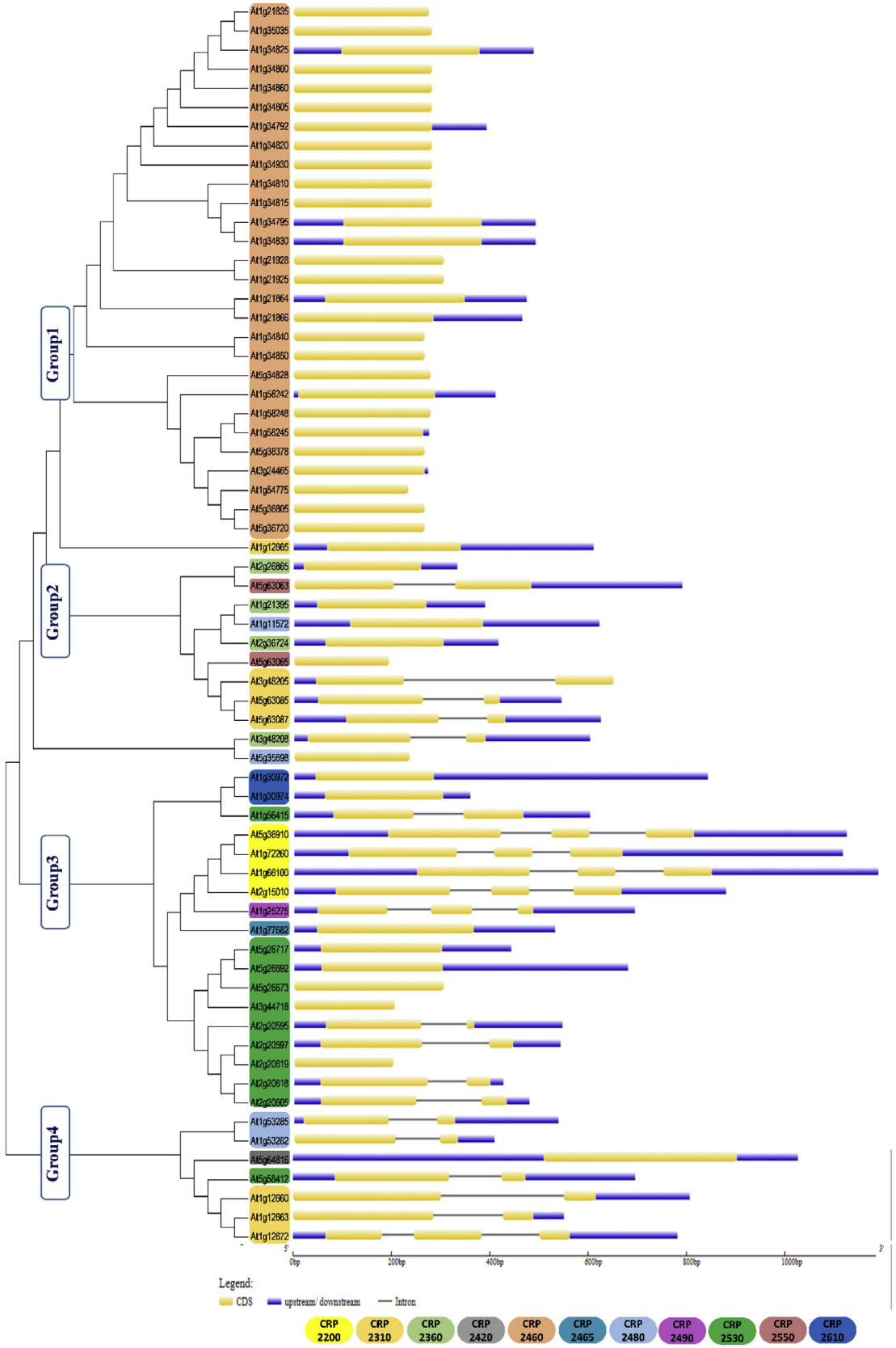


**Fig. 3. Phylogenetic analysis in line with exon-intron structure analysis of 61 ThiL genes and four thionins.** Members of the same CRP family are shaded with the same color. Scale below the Figure compares the length of gene in base pairs (bp). Golden, blue and thin grey colored lines show positions of exons, UTRs and introns, respectively. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

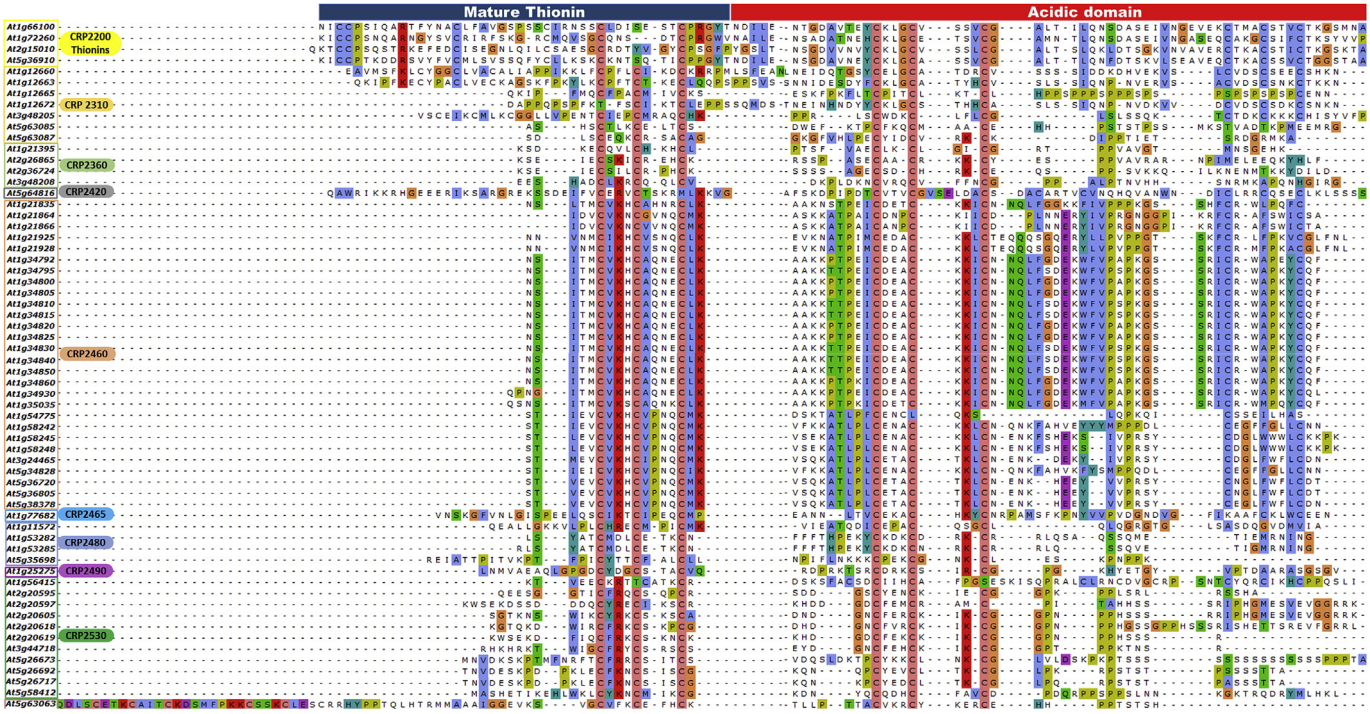


**Fig. 4. Multiple sequence alignment of 61 ThiL genes with four thionins**. First four members are thionins which belong to the CRP2200 family. The position and length of mature peptide and acidic domain of thionins have been labeled with blue and red colored boxes, respectively. Members of different CRP families are highlighted along with the name of their CRP family. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

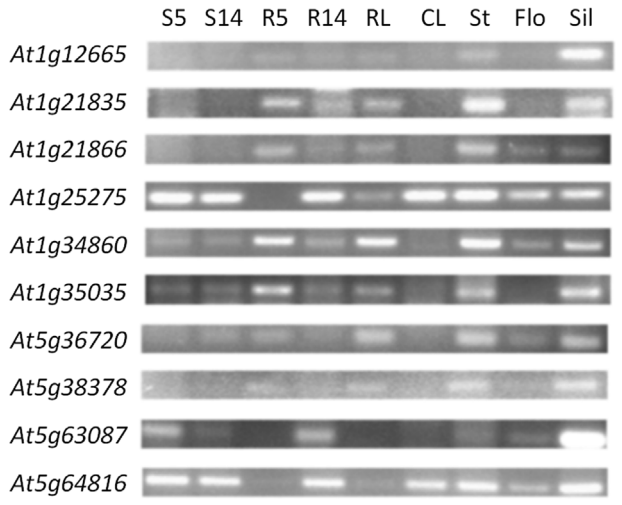


**Fig. 5. RT-PCR expression analysis in different Arabidopsis tissues and developmental stages**. Total RNA was transcribed using oligo-dT and amplified using primers described in Table S3c. Expression was analysed in different organs and developmental stages of wild-type Columbia: five days old seedlings (S5), 14 days old seedlings (S14), five days old roots (R5), 14 days old roots (R14), five weeks old rosette leaves (RL), cauline leaves (CL), stems (St), flowers (Flo) and silliques (Sil).

tissue-specific expression in more detail, we produced promoter:GUS lines for all genes that were tested with RT-PCR using the vector pPZP3425 (Szakasits et al., 2007). For each gene approximately 10 independent lines were obtained and a representative homozygous line was used for a detailed analysis using X-gluc staining (Fig. 6). None of the GUS lines that we obtained for *At1g12665* showed any GUS expression. The GUS expression was also not observed in siliques although we had obtained a signal in RT-PCR (Fig. 5). *At1g34860*:GUS was expressed only in some spots at the edge of older leaves and cauline leaves. In case of the other genes, GUS expression was found in most organs.

Expression of all genes was also tested after infection of the promoter:GUS lines with *H. schachtii* at 5 and 15 dpi (Fig. 7). No expression was found for *At1g12665*, *At1g21835*, *At1g21866*, *At1g34860,* and *At5g63087,* which showed also a very low expression in roots. Expression of the lines containing promoter:GUS constructs for *At1g35035* and *At5g38378* was downregulated in syncytia. *At5g36720* showed expression in syncytia but not in roots. A more detailed analysis was done for *At1g25275,* which indicated that the expression was downregulated in syncytia from 7 dpi on and for *At5g64816* which showed a weak expression in syncytia up to 10 dpi but not in 15 dpi syncytia (Fig. 8).

### 3.7. Functional analysis

We selected 10 genes for transient expression in *Nicotiana benthamiana.* The coding regions (including intron) of these genes were cloned in the vector pPZPTRBO (Shah et al., 2013). These constructs were transformed into Agrobacteria for infiltration into leaves of six weeks old *N. benthamiana* plants. Expression was confirmed by RT-PCR (Fig. S8). Infiltration of the empty vector resulted in chlorotic spots at the point of infiltration. In case of *At1g21835* and *At1g35035,* we observed a strong hypersensitive response (HR) which was clearly visible at 3 dpi and onward and which eventually covered almost the whole leaf (Fig. 9). For the *At1g34860* expression construct we found some small necrotic spots at 5 and 7 dpi. For all other genes we did not observe any visible reaction (data not shown).

Overexpression constructs containing the coding regions (including intron) for transformation in Arabidopsis were produced using the vector pPZP3425 (Szakasits et al., 2007) which includes the 35S promoter with doubled enhancer and an omega translational enhancer. We selected eight homozygous lines for each construct and tested the expression of the transgene with RT-PCR (Fig. S7). For each gene we selected one line with a good expression level. None of these showed any

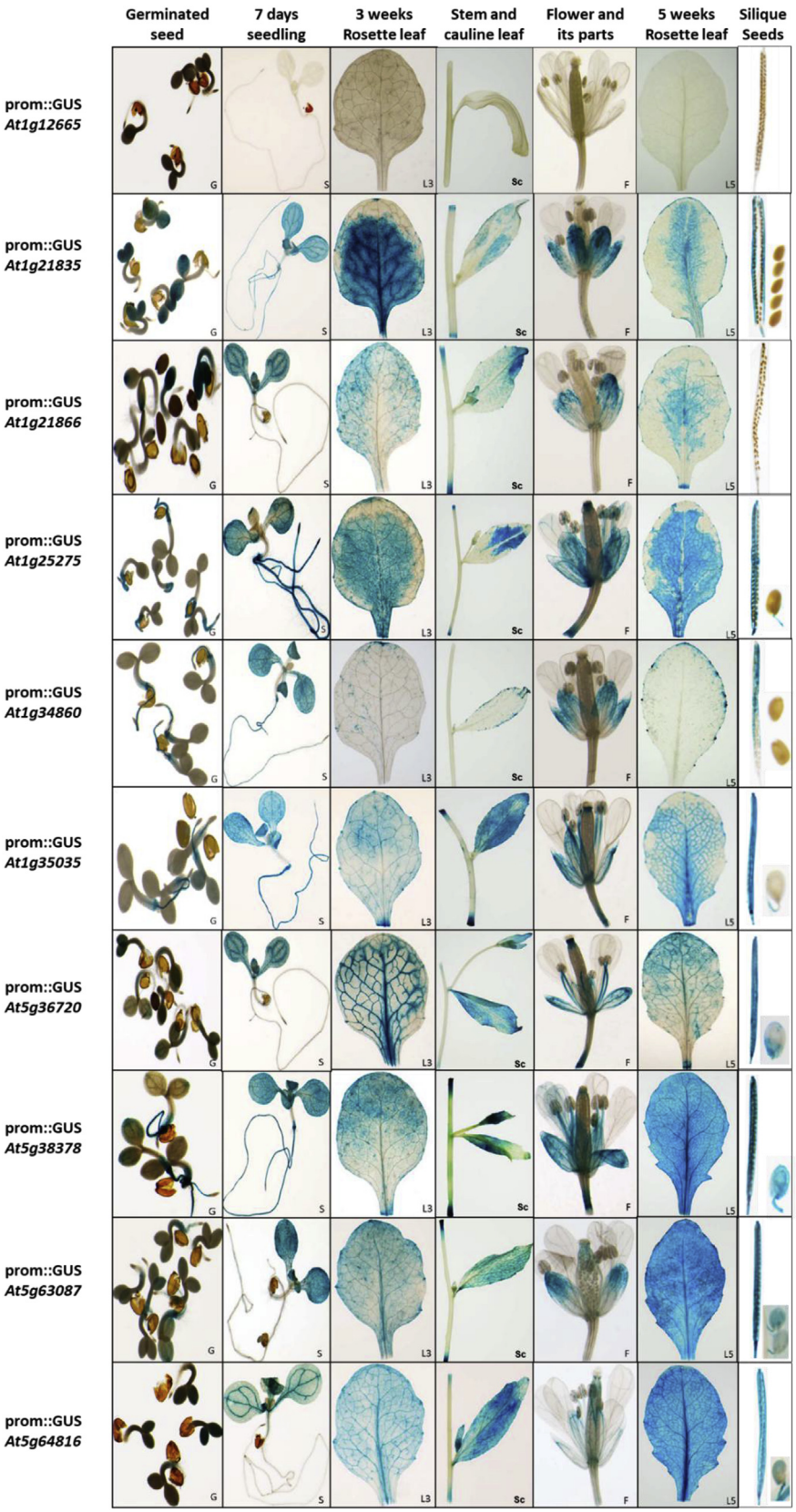


**Fig. 6. GUS expression of thionin like genes in Arabidopsis organs.** Shown is the expression in germinated seeds, seven days old sedlings, three weeks old rosette leaves, stem sections with cauline leaves, flowers, five weeks old rosette leaves and siliques. For some lines enlarged pictures of seeds are shown together with the siliques.

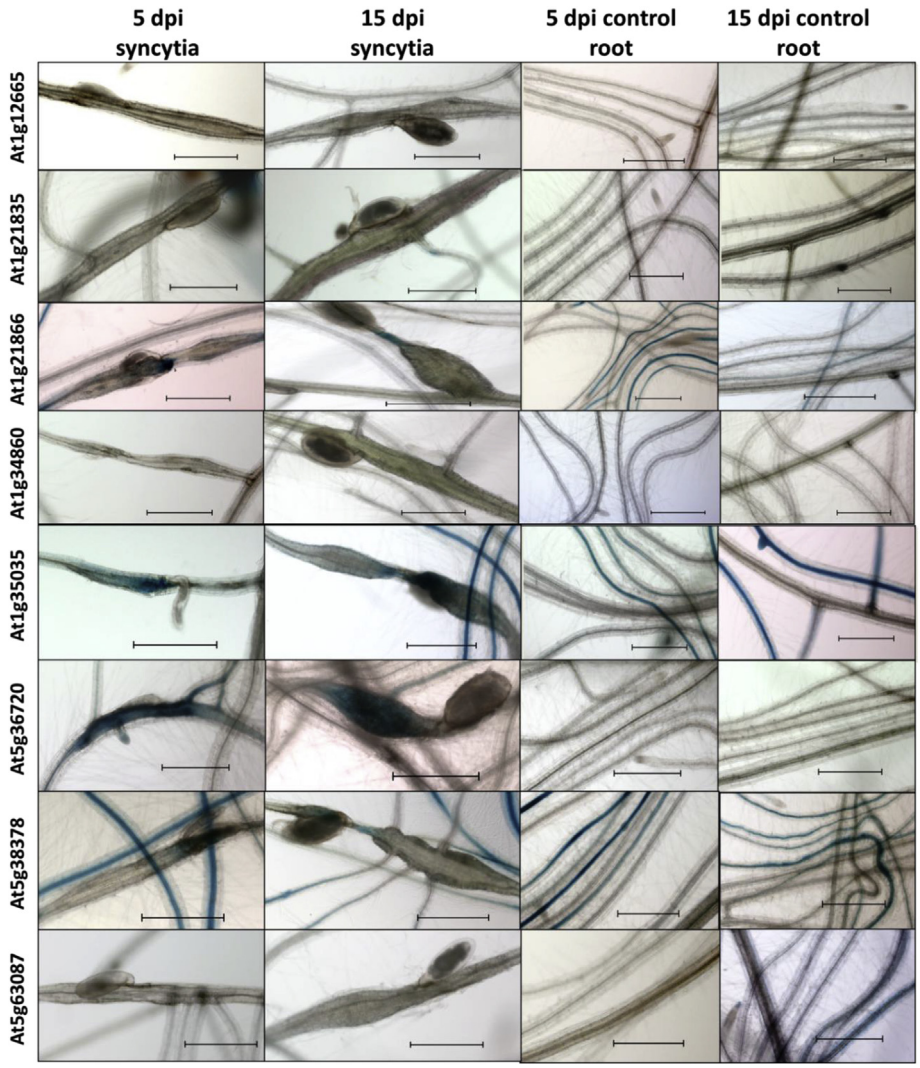


**Fig. 7. GUS expression of ThiL genes in 5 and 15 dpi syncytia and corresponding uninfected control roots**. Syncytia of promoter:GUS lines are shown at 5 and 15 dpi together with roots from uninfected plants of the same age. Bar = 500 µm.

obvious phenotype (Fig. S8). These overexpression lines were infected with 2nd stage juveniles of the beet cyst nematode *H. schachtii*. For *At1g12665* and *At1g25275* the number of females and males was significantly reduced on the overexpression lines as compared to wildtype plants. For *At1g35035* and *At5g64816* we found a reduction of the number of females but not of males. In all other cases there was no significant reduction of females or males as compared to the wildtype (Fig. 10).

## 4. Discussion

### 4.1. In silico characterization of the ThiL gene family in arabidopsis

Thionin genes are known for quite some time in different plant families and four genes are found in the Arabidopsis genome. In their search for CRPs in the Arabidopsis genome, Silverstein et al. (2007) defined a group of so-called ThiL peptides. They found that Arabidopsis contains 67 ThiL genes of which 6 are pseudogenes.

About 94% of all CRP genes from Arabidopsis either lack introns or contain a single intron which is usually located between the exons encoding the signal peptide and mature peptide (Silverstein et al., 2007). Gene structure analysis of ThiL genes revealed that introns were present in 17 out of 61 genes studied here. Thionin genes from different plant families were found to have two small introns within the acidic domain (Bohlmann et al., 1988). This pattern is also found in the Arabidopsis thionin genes. In addition to these, there are only two ThiL genes with two introns. Similarly, the pattern of intron-exon structure was partially conserved in different groups and subgroups of the ThiL genes.

We performed different *in silico* analyses to characterize the ThiL peptides. The results obtained by these analyses indicated various analogies to the previously reported ThiL peptides from other plant species. Our results predicted ThiL peptides with a length between 66 and 135 amino acid residues. Thionin-like peptides 1 and 2 from *Capsicum annuum* have 66 and 59 amino acids residues, respectively (Taveira et al., 2014). Similarly, another peptide that is described as thionin-like from *Triticum aestivum* (UniprotKB ID: Q5BQ30) exhibits 89 amino acids length. Isoelectric point of most ThiL peptides was in the basic range, but some had an acidic pI, down to approximately five. The known AMPs are usually basic peptides but it is currently not known if the pI of the ThiL peptides is related to a possible antimicrobial activity.

A comparison of the ThiL peptides with the four thionins through multiple sequence alignment showed that ThiL peptides are actually more similar to the acidic domain of the thionin proproteins but have little similarity with the thionin domain. The acidic domains of thionin proproteins have a characteristic three + three cysteine pattern which is also found in many of the ThiL peptides. If these cysteines form a cystine knot as in knottins (Postic et al., 2018) is not known. The

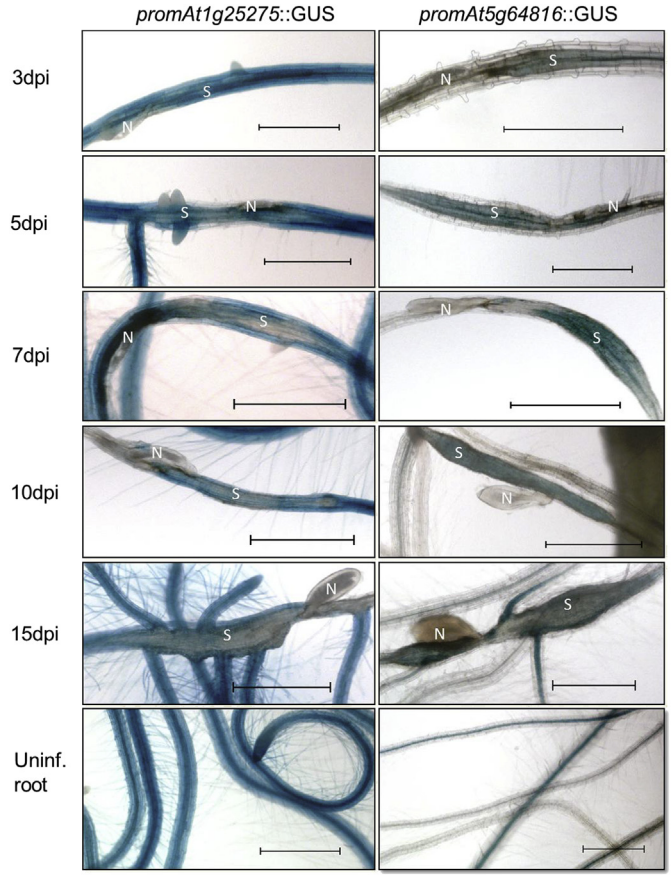


**Fig. 8. GUS expression of two ThiL genes in syncytia.** GUS staining was performed for 3, 5, 10, 12, and 15 dpi syncytia. N = nematode, S = syncytia and bar = 100 μm.

characteristic two introns found in the acidic domain of thionin genes are, however, found in only two ThiL genes (Fig. 3). The alignment with the acidic domain also explains why the Phyre program could not predict a 3D structure for the ThiL peptides with high level of confidence. While 3D structures have been obtained for many thionin peptides with different methods, no structure is known for an acidic domain.

### 4.2. Expression analyses of ThiL genes

Expression analysis from various developmental stages demonstrated that most of the genes were ubiquitously expressed in most plant parts through various developmental stages. However, the expression level as shown by RT-PCR and supported by publicly available RNAseq data is mostly very low, including all six of the CRP2460 genes that we have studied. An exception is *At1g25275* and with lower levels *At5g64816*, both of which are expressed in most of the plant organs at higher levels. The very low expression levels raise the question if these genes have a function. Of course, it cannot be excluded that these genes are expressed after encounter of specific biotic or abiotic stresses. Indeed, a transcriptome analysis of Arabidopsis pistils showed that several ThiL genes were upregulated in interspecific pollination with pollen from other Arabidopsis species (Mondragon-Palomino et al., 2017). Among the genes studied here, they include *At1g21866*, *At5g36720*, and *At5g38378*. Moreover, *At5g36720* is reported to be specifically expressed in pollen tubes but not in pollen grains and it requires MYB transcription factors i.e., *MYB97*, *MYB101*, and *MYB120* for its expression in the pollen tube (Leydon et al., 2013). This further supports the evidence provided by Mondragon-Palomino and colleagues that this gene could be involved in pollen tube burst (Leydon et al., 2013; Mondragon-Palomino et al., 2017). On the other hand, Karunanandaa et al. (1994) characterized a pistil expressed plant defensin from *Petunia inflata* and suggested that this peptide might be involved in defending the pistil against pathogen infection. Thus, expression of ThiL genes in the pistil might also be part of the defense against microorganisms. Our study revealed that *At5g36720* was not expressed in roots but expressed in syncytia induced by *H. schachtii* in roots which suggests its involvement in plant-nematode interactions. However, expression of most of the ThiL genes studied here was suppressed in response to nematode infection. This confirms our previous hypothesis that *H. schachtii* is able to suppress the expression of defense related genes (Ali, 2012; Ali et al., 2017). Our results exhibited downregulation of *At1g35035* and *At5g38378* from early time points post inoculation while *At1g25275* was downregulated in syncytia from 7 dpi on. Conversely, gene *At1g25275* is induced in response to karrikin (Nelson et al., 2010) but it is not known if it has any function in that interaction.

### 4.3. Function of ThiL peptides

Thionins are an important part of the plant defense to cope with invading pathogens and have *in vitro* antimicrobial activity against various plant pathogens (Epple et al., 1995, 1998, 1997). Thionin preproproteins comprise a signal peptide, mature thionin and an acidic domain (Bohlmann and Apel, 1991). The mature thionin is the part that has antimicrobial activity while the proper function of the acidic domain is not yet known (Plattner et al., 2015).

With one exception, all ThiL genes encode preproteins containing a signal peptide according to SignalP (Petersen et al., 2011). If the mature peptides end up in the vacuole or in the apoplast is not known. For a plant defensin from *A. halleri,* it was shown that, although there was a signal peptide, the mature peptide was found in intracellular compartments (Oomen et al., 2011).

Our results of the nematode infection tests showed that two genes, *At1g12665* and *At1g25275*, supported significantly less numbers of female and male nematodes in the overexpression lines as compared to the wild type. Overexpression lines for *At1g35035* and *At5g64816,* on the other hand, only showed a significant reduction of the number of females but not males. The motif analysis revealed that motif 8 rich in cysteine residues was present twice in At1g12665 and At1g25275 peptides but not in At1g35035 and At5g64816 peptides. One might therefore speculate that this motif, which contained two lysine residues in addition to three cysteines, may be important for reducing nematode numbers in addition to rendering the stability to the peptide through disulfide bridges. The only member from CRP2420 family, *At5g64816* was expressed in syncytia up to 10 dpi. The At5g64816 peptide contains 10 cysteine residues and a typical serine rich C-terminal motif (SSSSSSSRYS). However, it is not known if this motif is involved in the resistance against *H. schachtii*.

While we found that overexpression of *At1g25275* resulted in decreased susceptibility against the beet cyst nematode, another publication has recently reported that overexpression of *At1g25275* increased susceptibility (Dobón et al., 2015). The authors identified genes that were upregulated in four transcription factor mutants, resulting in increased susceptibility to the necrotrophic plant pathogenic fungi *Botrytis cinerea* and *Plectosphaerella cucumerina*. *PROVIR2* (*At1g25275*) was induced by inoculation with *P. cucumerina* but downregulated by *Pseudomonas syringae* DC3000 (*AvrRpm1*). The authors claimed that overexpression of *PROVIR2* (*At1g25275*) conferred enhanced susceptibility to *P. cucumerina*. However, this result has to be treated with caution because all PROVIR proteins were expressed as fusion with GFP which might render the proteins inoperable. The expression of the At1g25275:GFP fusion might then lead to co-suppression of the endogenous *At1g25275* gene which could explain the observed susceptibility.

Transient expression of two ThiL genes i.e. *At1g21835* and

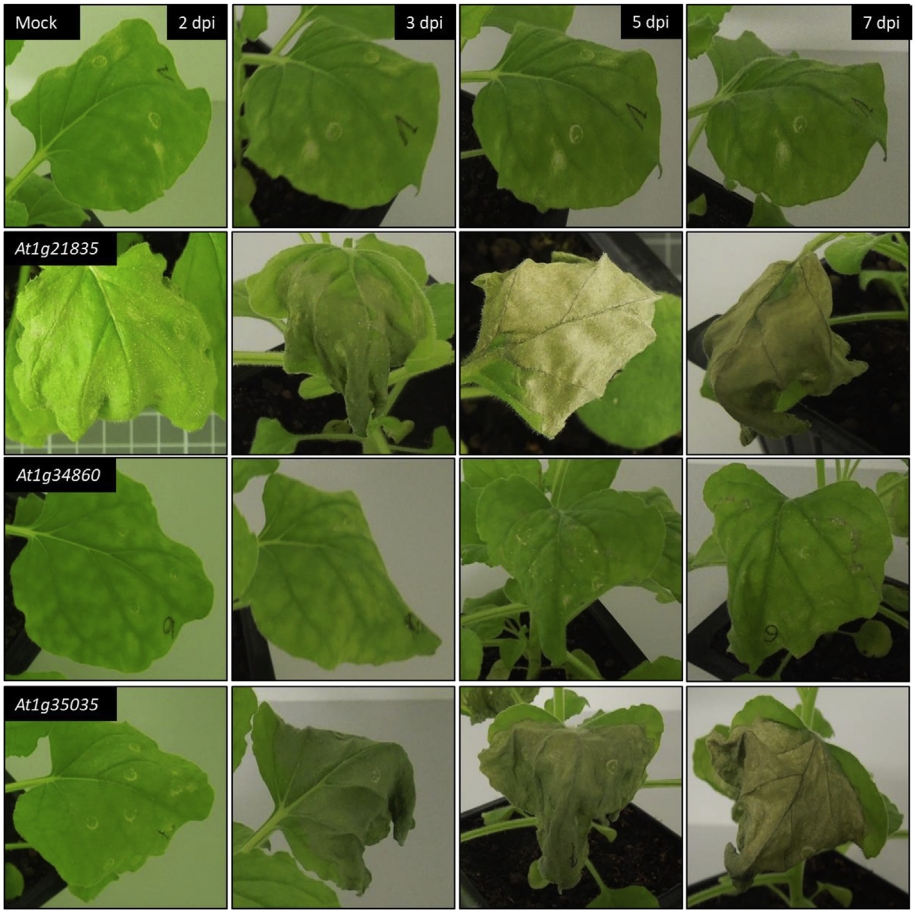


**Fig. 9. Transient expression in *Nicotiana benthamiana*.** Agrobacteria containing expression constructs for three different ThiL genes were infiltrated into leaves. Pictures were taken at 2, 3, 5 and 7 dpi. Mock indicates plants that were infiltrated with $MgCl_2$.

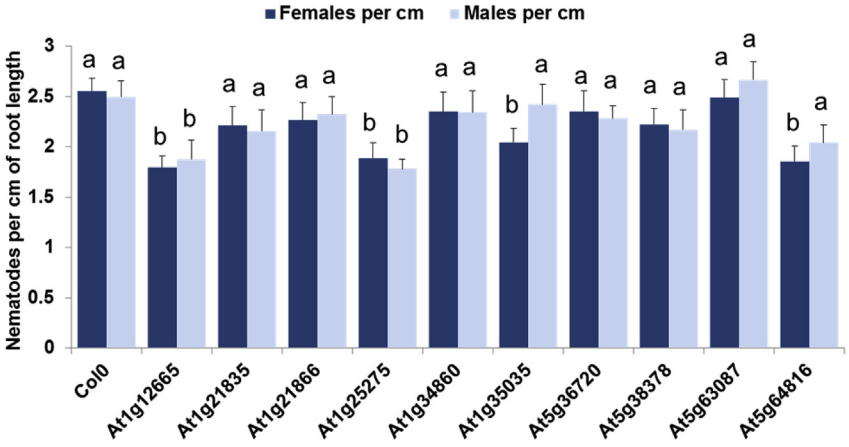


**Fig. 10. Nematode resistance test**. The resistance of overexpression lines of ThiL genes compared to wild type plants after infection with *H. schachtii*. Number of male and female nematodes per cm of root length calculated at 15 dpi setting the wild type as 100%. Infection rate is shown in column sets with different letters indicating significant differences ($P < 0.05$; ANOVA and LSD). The statistical significance was determined by three independent replicates. Values are means ± SE, n = 15. The bar shows standard error for the mean.

*At1g35035* gave a strong HR as early as 3 dpi in *N. benthamiana*. This suggests a possible role of these ThiL genes in HR-mediated defense responses, possibly through the induction of ROS and programmed cell death. Recently, a ThiL peptide, CaThi, from *C. annuum* has been reported to cause programmed cell death through activation of caspases and extracellular $H^+$ flux (Taveira et al., 2018). However, in the Arabidopsis overexpression lines of both these genes we did not see any sign of HR. There are two possible explanations for this. On one hand, it might be possible that these genes have a different effect in *N. benthamiana* and Arabidopsis. On the other hand, if strong expression of these genes would lead to an HR in Arabidopsis, we would not be able to isolate overexpression lines with a strong expression level but only ones with a low expression level which would not show an HR. While the overexpression line of *At1g21835* was not different from wild type in respect to nematode numbers, oxerexpression of *At1g35035* resulted in a lower number of female nematodes (but not male nematodes). *At1g21835* was not expressed in roots and in syncytia induced by *H. schachtii* while expression of *At1g35035* was downregulated in syncytia.

Up to date, very few thionin-like genes have been characterized. One of the best studied ThiL genes is *CaThi* from *C. annuum,* which showed a strong antimicrobial activity against different species of *Candida* genus which are opportunistic human parasites and cause gastrointestinal and mouth candidiasis (Taveira et al., 2016). This ThiL peptide also showed antimicrobial activity against *Fusarium solani*,

probably through apoptosis (Taveira et al., 2017). These reports and our results suggest that ThiL peptides might have anti-nematodal activity or they could be involved in HR and/or programmed cell death to enhance nematode resistance in Arabidopsis.

### 4.4. Evolution of ThiL genes

With a few exceptions, most of the Arabidopsis ThiL genes have no homologs outside the *Brassicaceae* or the *Brassicales* and have thus been expanded in the order *Brassicales*. The most extreme example is CRP2460 which contains 28 genes from Arabidopsis. Many of these genes are the result of tandem duplications, partly together with pseudogenes of *At1g34790* (*Transparent Testa 1*). This demonstrates that the ThiL family is specifically conserved in the *Brassicaceae* family of angiosperms. They do not include orphan genes in the strict sense, however, it has been shown that most orphan genes code for proteins that have the size of peptides (Arendsee et al., 2014). The Arabidopsis *QQS* orphan gene was for instance found to be a regulator of carbon and nitrogen allocation (Li et al., 2009). The function of most of the Arabidopsis ThiL genes is not known but the large number of recent tandem duplications indicates that many of these genes may still be in search of a function.

## 5. Conclusions

The Arabidopsis genome contains several gene families that code for thionin-like peptides. Many of these genes are tandem duplicates. The ThiL peptides are actually more related to the acidic domain of thionin proproteins than to the mature thionins. At least part of these could be involved in plant defense, although other functions cannot be excluded at the moment.

## Contributions

Bachar Almaghrabi did most of the experimental work. Muhammad Amjad Ali did the nematode experiments. Adil Zahoor and Muhammad Amjad Ali performed the bioinformatics analysis. Kausar Hussain Shah performed the Nicotiana benthamiana experiments. Holger Bohlmann supervised the study and wrote the manuscript together with Muhammad Amjad Ali. All authors approved the manuscript.

## Acknowledgements

This research was supported by grants P21896-B16 and P28984 of the Austrian Science Fund. The authors declare that they have no conflict of interest. Bachar Almaghrabi was supported by the Austrian agency for international mobility and cooperation in education, science and research (OeAD). Muhammad Amjad Ali and Kausar Shah were supported by Higher Education Commission (HEC) of Pakistan. We appreciate the technical assistance of Martina Niese. We thank Patricia Schekahn for taking some of the GUS pictures.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.05.005.

## References

Ali, M.A., 2012. Induction of Resistance against Cyst Nematodes by Modifying Gene Expression in Syncytia. Department of Crop Sciences. University of Natural Resources and Life Sciences, Vienna, Austria.
Ali, M.A., Azeem, F., Li, H., Bohlmann, H., 2017. Smart parasitic nematodes use multifaceted strategies to parasitize plants. Front. Plant Sci. 8.
Ali, M.A., Plattner, S., Radakovic, Z., Wieczorek, K., Elashry, A., Grundler, F.M., Ammelburg, M., Siddique, S., Bohlmann, H., 2013. An Arabidopsis ATPase gene involved in nematode-induced syncytium development and abiotic stress responses. Plant J. 74, 852–866.
Arendsee, Z.W., Li, L., Wurtele, E.S., 2014. Coming of age: orphan genes in plants. Trends Plant Sci. 19, 698–708.
Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E., Clementi, L., Ren, J., Li, W.W., Noble, W.S., 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res. 37, W202–W208.
Bohlmann, H., 1994. The role of thionins in plant-protection. Crit. Rev. Plant Sci. 13, 1–16.
Bohlmann, H., Apel, K., 1991. Thionins. Annu. Rev. Plant Physiol. 42, 227–240.
Bohlmann, H., Clausen, S., Behnke, S., Giese, H., Hiller, C., Reimann-Philipp, U., Schrader, G., Barkholt, V., Apel, K., 1988. Leaf-specific thionins of barley-a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defence mechanism of plants. EMBO J. 7, 1559–1565.
Broekaert, W.F., Cammue, B.P.A., DeBolle, M.F.C., Thevissen, K., DeSamblanx, G.W., Osborn, R.W., 1997. Antimicrobial peptides from plants. Crit. Rev. Plant Sci. 16, 297–323.
Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., Osborn, R.W., 1995. Plant defensins - novel antimicrobial peptides as components of the host-defense system. Plant Physiol. 108, 1353–1358.
Dobón, A., Canet, J.V., García-Andrade, J., Angulo, C., Neumetzler, L., Persson, S., Vera, P., 2015. Novel disease susceptibility factors for fungal necrotrophic pathogens in Arabidopsis. PLoS Pathog. 11, e1004800.
Epple, P., Apel, K., Bohlmann, H., 1995. An arabidopsis-thaliana thionin gene is inducible via a signal-transduction pathway different from that for pathogenesis-related proteins. Plant Physiol. 109, 813–820.
Epple, P., Apel, K., Bohlmann, H., 1997. Overexpression of an endogenous thionin enhances resistance of Arabidopsis against Fusarium oxysporum. Plant Cell 9, 509–520.
Epple, P., Vignutelli, A., Apel, K., Bohlmann, H., 1998. Differential induction of the Arabidopsis thaliana Thi2.1 gene by Fusarium oxysporum f. sp. matthiolae. Mol. Plant Microbe Interact. 11, 523–529.
Florack, D.E., Dirkse, W.G., Visser, B., Heidekamp, F., Stiekema, W.J., 1994. Expression of biologically active hordothionins in tobacco. Effects of pre- and pro-sequences at the amino and carboxyl termini of the hordothionin precursor on mature protein expression and sorting. Plant Mol. Biol. 24, 83–96.
Garcia-Olmedo, F., Molina, A., Alamillo, J.M., Rodriguez-Palenzuela, P., 1998. Plant defense peptides. Biopolymers 47, 479–491.
Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D., Bairoch, A., 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 31, 3784–3788.
Holsters, M., Dewaele, D., Depicker, A., Messens, E., Vanmontagu, M., Schell, J., 1978. Transfection and transformation of agrobacterium-tumefaciens. Mol. Gen. Genet. 163, 181–187.
Holtorf, S., Ludwig-Muller, J., Apel, K., Bohlmann, H., 1998. High-level expression of a viscotoxin in Arabidopsis thaliana gives enhanced resistance against Plasmodiophora brassicae. Plant Mol. Biol. 36, 673–680.
Hu, B., Jin, J., Guo, A.-Y., Zhang, H., Luo, J., Gao, G., 2015. GSDS 2.0: an upgraded gene feature visualization server. Bioinformatics 31, 1296–1297.
Jefferson, R.A., 1989. The GUS reporter gene system. Nature 342, 837–838.
Karunanandaa, B., Singh, A., Kao, T.-h., 1994. Characterization of a predominantly pistil-expressed gene encoding a γ-thionin-like protein of Petunia inflata. Plant Mol. Biol. 26, 459–464.
Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., Sternberg, M.J., 2015. The Phyre2 web portal for protein modeling, prediction and analysis. Nat. Protoc. 10, 845–858.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Leydon, A.R., Beale, K.M., Woroniecka, K., Castner, E., Chen, J., Horgan, C., Palanivelu, R., Johnson, M.A., 2013. Three MYB transcription factors control pollen tube differentiation required for sperm release. Curr. Biol. 23, 1209–1214.
Li, L., Foster, C.M., Gan, Q., Nettleton, D., James, M.G., Myers, A.M., Wurtele, E.S., 2009. Identification of the novel protein QQS as a component of the starch metabolic network in Arabidopsis leaves. Plant J. 58, 485–498.
Liu, L., Ganz, T., 1995. The pro region of human neutrophil defensin contains a motif that is essential for normal subcellular sorting. Blood 85, 1095–1103.
Logemann, E., Birkenbihl, R.P., Ulker, B., Somssich, I.E., 2006. An improved method for preparing Agrobacterium cells that simplifies the Arabidopsis transformation protocol. Plant Methods 2.
Mendez, E., Moreno, A., Colilla, F., Pelaez, F., Limas, G.G., Mendez, R., Soriano, F., Salinas, M., de Haro, C., 1990. Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin, gamma-hordothionin, from barley endosperm. Eur. J. Biochem. 194, 533–539.
Mondragon-Palomino, M., John-Arputharaj, A., Pallmann, M., Dresselhaus, T., 2017. Similarities between reproductive and immune pistil transcriptomes of Arabidopsis species. Plant Physiol. 174, 1559–1575.
Nelson, D.C., Flematti, G.R., Riseborough, J.A., Ghisalberti, E.L., Dixon, K.W., Smith, S.M., 2010. Karrikins enhance light responses during germination and seedling development in Arabidopsis thaliana. Proc. Natl. Acad. Sci. U. S. A. 107, 7095–7100.
Ohtani, S., Okada, T., Yoshizumi, H., Kagamiyama, H., 1977. Complete primary structures of two subunits of purothionin A, a lethal protein for brewer's yeast from wheat flour. J. Biochem. 82, 753–767.
Oomen, R.J., Seveno-Carpentier, E., Ricodeau, N., Bournaud, C., Conejero, G., Paris, N., Berthomieu, P., Marques, L., 2011. Plant defensin AhPDF1.1 is not secreted in leaves but it accumulates in intracellular compartments. New Phytol. 192, 140–150.
Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.
Plattner, S., Gruber, C., Altmann, F., Bohlmann, H., 2014. Self-processing of a barley subtilase expressed in E. coli. Protein Expr. Purif. 101, 76–83.

Plattner, S., Gruber, C., Stadlmann, J., Widmann, S., Gruber, C.W., Altmann, F., Bohlmann, H., 2015. Isolation and characterization of a thionin proprotein-processing enzyme from barley. J. Biol. Chem. 290, 18056–18067.
Postic, G., Gracy, J., Perin, C., Chiche, L., Gelly, J.C., 2018. KNOTTIN: the database of inhibitor cystine knot scaffold after 10 years, toward a systematic structure modeling. Nucleic Acids Res. 46, D454–D458.
Schrader, G., Apel, K., 1991. Isolation and characterization of cDNAs encoding viscotoxins of mistletoe (Viscum album). Eur. J. Biochem. 198, 549–553.
Schwarz, R., Dayhoff, M., 1979. Matrices for detecting distant relationships. In: Dayhoff, M. (Ed.), Atlas of Protein Sequences. National Biomedical Research Foundation, pp. 353–358.
Shah, K.H., Almaghrabi, B., Bohlmann, H., 2013. Comparison of expression vectors for transient expression of recombinant proteins in plants. Plant Mol. Biol. Rep. 31, 1529–1538.
Sijmons, P.C., Grundler, F.M.W., Vonmende, N., Burrows, P.R., Wyss, U., 1991. Arabidopsis-Thaliana as a new model host for plant-parasitic nematodes. Plant J. 1, 245–254.
Silverstein, K.A., Moskal Jr., W.A., Wu, H.C., Underwood, B.A., Graham, M.A., Town, C.D., VandenBosch, K.A., 2007. Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants. Plant J. 51, 262–280.
Stec, B., 2006. Plant thionins–the structural perspective. Cell. Mol. Life Sci. 63, 1370–1385.
Stothard, P., 2000. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques 28, 1102–1104.
Szakasits, D., Siddique, S., Bohlmann, H., 2007. An improved pPZP vector for Agrobacterium-mediated plant transformation. Plant Mol. Biol. Rep. 25, 115–120.
Taveira, G.B., Carvalho, A.O., Rodrigues, R., Trindade, F.G., Da Cunha, M., Gomes, V.M., 2016. Thionin-like peptide from Capsicum annuum fruits: mechanism of action and synergism with fluconazole against Candida species. BMC Microbiol. 16.
Taveira, G.B., Mathias, L.S., da Motta, O.V., Machado, O.L., Rodrigues, R., Carvalho, A.O., Teixeira-Ferreira, A., Perales, J., Vasconcelos, I.M., Gomes, V.M., 2014. Thionin-like peptides from Capsicum annuum fruits with high activity against human pathogenic bacteria and yeasts. Biopolymers 102, 30–39.
Taveira, G.B., Mello, É.O., Carvalho, A.O., Regente, M., Pinedo, M., de La Canal, L., Rodrigues, R., Gomes, V.M., 2017. Antimicrobial activity and mechanism of action of a thionin-like peptide from Capsicum annuum fruits and combinatorial treatment with fluconazole against Fusarium solani. Biopolymers 108, e23008.
Taveira, Gabriel B., Mello, Érica O., Souza, Sávio B., Monteiro, Renan M., Ramos, Alessandro C., Carvalho, André O., Rodrigues, R., Okorokov, Lev A., Gomes, Valdirene M., 2018. Programmed cell death in yeast by thionin-like peptide from Capsicum annuum fruits involving activation of caspases and extracellular H + flux. Biosci. Rep. 38, BSR20180119.
Van Bel, M., Diels, T., Vancaester, E., Kreft, L., Botzki, A., Van de Peer, Y., Coppens, F., Vandepoele, K., 2018. PLAZA 4.0: an integrative resource for functional, evolutionary and comparative plant genomics. Nucleic Acids Res. 46, D1190–D1196.
Voorrips, R.E., 2002. MapChart: software for the graphical presentation of linkage maps and QTLs. J. Hered. 93, 77–78.
Zuckerkandl, E., Pauling, L., 1965. Molecules as documents of evolutionary history. J. Theor. Biol. 8, 357–366.