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RESEARCH ARTICLE

Are the cyst nematode hatching factor eclepins rhizosphere signalling molecules? Solanoeclepin A regulates gene expression in plants

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Abstract

Background: The eclepins are a group of root-exuded compounds that induce the hatching of cyst nematodes, economically relevant pests for crops such as soybean and potato. From an evolutionary standpoint a beneficial effect of eclepins would be expected for the plant.
Methods: To identify this beneficial effect, we analysed the transcriptome of two plant species in response to treatment with an eclepin. One of the eclepins, solanoeclepin A (solA), produced by members of the *Solanaceae*, was used in this study to treat tomato (*Solanum lycopersicum*) and Arabidopsis seedlings grown *in vitro*. Samples were used to call differentially expressed genes (DEGs) using RNAseq and followed by GO term enrichment analysis.
Results: RNAseq analysis showed that solA downregulates the immune and hypoxia response, as well as ethylene biosynthesis and signalling, and promotes root growth. This response was particularly strong under N and P deficiency in Arabidopsis.
Conclusions: Hence, solA affects gene expression in species it is not produced by. Furthermore, these results suggest that solA prepares the plant for colonization by microorganisms, possibly playing a role in nutrient uptake. Moreover, the change in hypoxia response and ethylene signalling and growth stimulation point toward the induction of an altered root architecture, possibly further improving the plant's ability to recruit beneficial microbes and absorb nutrients.

Keywords

Rhizosphere, solanoeclepin A, kairomone, tomato, Arabidopsis, hatching factor

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Introduction

Secondary metabolites are organic low-molecular mass compounds produced by bacteria, fungi and plants. They are often species-specific, important in intraspecies communication and thereby increasing the survival of the producer (Böttger *et al.*, 2018). Plants produce a large variety of metabolites with defensive roles against herbivores and pathogens or providing protection against abiotic stress (Harborne, 1999). For a small fraction of the large chemical diversity produced by plants there is evidence that they attract beneficial organisms such as predators of herbivores and symbiotic micro-organisms. An example of the latter is a group of secondary metabolites called strigolactones (SLs): they attract arbuscular mycorrhizal (AM) fungi, which help the plant cope with phosphate (Pi) stress (Akiyama *et al.*, 2005; López-Ráez *et al.*, 2008; Ruyter-Spira *et al.*, 2011). This is beneficial for the plant, because AM fungi supply the plant with extra P, thus overcoming suboptimal growth conditions (Akiyama *et al.*, 2005; López-Ráez *et al.*, 2008). Moreover, SL regulates, among others, shoot branching and root architecture (Akiyama *et al.*, 2005; Gomez-Roldan *et al.*, 2008; Ruyter-Spira *et al.*, 2011; Umehara *et al.*, 2008), further improving P uptake and efficient P use. However, contrastingly, SLs were initially discovered for their detrimental effect on the plant host that secretes them, since they induce germination of parasitic plants (Cook *et al.*, 1966). Hence, parasitic plants have hijacked the communication system between plant host and AM fungi.

The eclepins are another group of secondary metabolites that have been discovered for their role as kairomone (chemical emitted by one organism that is perceived by another species which gains advantage from this). Glycinoeclepin A, B and C and solanoeclepin A (solA) induce hatching of the parasitic cyst nematodes of the genera *Heterodera* and *Globodera*, respectively (Fukuzawa *et al.*, 1985; Masamune *et al.*, 1982; Schenk *et al.*, 1999). These triterpenoid secondary metabolites are exuded into the rhizosphere by kidney bean (glycinoeclepins) and *Solanaceae* (solA) where they are perceived by dormant cyst nematode eggs, which subsequently hatch (Masamune *et al.*, 1982; Tanino *et al.*, 2011). The hatched larvae migrate towards the host root where they establish a syncytium (Hunt *et al.*, 2005). Through this specialised feeding structure, cyst nematodes extract nutrients from the plant and thereby cause large yield losses. Potato cyst nematode (PCN) species *Globodera pallida* and *Globodera rostochiensis* are economically relevant pests of crops of the *Solanaceae* family, such as tomato (*Solanum lycopersicum)* and potato (*Solanum tuberosum*) (Jones *et al.*, 2013). Sub-nanomolar concentrations of solA are enough to induce high hatching rates in PCN (Guerrieri *et al.*, 2021; Sakata *et al.*, 2021; Tanino *et al.*, 2011).

Hence, SL and solA have in common that they are plant-derived cues/signalling molecules that betray the plant's presence to its enemies. For the SLs there is compelling evidence that its signalling function for symbiotic micro-organisms was hijacked as a reliable host presence cue by parasitic plants (Bouwmeester *et al.*, 2021). Here, we use early transcriptional event monitoring in tomato and Arabidopsis in response to exogenous solA treatment to show that solA also has a signalling role. SolA application inhibits the immune and hypoxia response, as well as ethylene biosynthesis and signalling, and promotes growth. This response is particularly strong under simultaneous N and P deficiency.

Methods

Plant material and growing conditions

Tomato Moneymaker and Arabidopsis Col-0 lines were used for all experiments. In this study, we used synthetic solA, kindly provided by K. Tanino (Tanino *et al.*, 2011). Tomato seeds were sterilized by immersion in 70% EtOH (2 min), 25% bleach (20 min) and 6x 6 min wash in sterilized milliQ. Subsequently, they were germinated in between wetted filter paper at 24°C. Seedlings were grown for 20 days in glass vials, containing 1 mL of ½ MS medium (20x diluted MS basal salt micronutrient solution (Merck, Darmstadt, Germany), 0.825 g/L NH_4NO_3, 0.22 g/L $CaCl_2{\cdot}2H_2O$, 0.185 g/L $MgSO_4{\cdot}7H_2O$, 0.95 g/L KNO_3, 0.085 g/L KH_2PO_4 and 0.04% agar). in a climate room at 24°C with alternating 8h dark, 16h light. On day 20, 20 µL of 250 µM solA in 50% ethanol (v/v) or 50% ethanol (v/v) carrier was pipetted on the agar close to the stem to reach an end concentration of 5 µM; control seedlings were treated with the same volume of ethanol/water, etc. Each treatment consisted of 6 replicates. Seedlings were harvested after 2h (root and shoot separately), which is the typical timeframe for transcriptomic experiments with externally applied hormones, such as for example SL (Zhan *et al.*, 2018; Zha *et al.*, 2019). Samples were collected into Eppendorf vials, snap-frozen in liquid nitrogen and stored at -80°C until further use. The 5 replicates with the best RNA quality after extraction were used for sequencing.

Arabidopsis seeds were sterilized by submersion in 1% bleach with 0.1% Tween for 5 min and were sown, 3 seeds per well, 6 replicates per treatment, in a 96-well plate containing ½ MS medium as described above but supplemented with 1g/L sucrose (control medium). After 10 days of growth, seedlings were transferred to a new plate with ½ MS with or without N and/or P (Table S1, (Vlaar, 2022)). For control conditions, we transferred seedlings into the same medium. For P deficiency, we used 20x diluted MS basal salt micronutrient solution (Merck, Darmstadt, Germany), 0.825 g/L NH_4NO_3, 0.22 g/L $CaCl_2{\cdot}2H_2O$, 0.185 g/L $MgSO_4{\cdot}7H_2O$, 0.95 g/L KNO_3, 0.932 g/L KCl and 0.04% agar), for N deficiency we used 20x diluted MS basal salt micronutrient, 0.22 g/L $CaCl_2{\cdot}2H_2O$, 0.185 g/L $MgSO_4{\cdot}7H_2O$, 0.085 g/L KH_2PO_4, 0.35 g/L KCl, and 0.04% agar), for combined N and P deficiency we used 20x diluted MS basal salt micronutrient solution, 0.22 g/L $CaCl_2{\cdot}2H_2O$, 0.185 g/L $MgSO_4{\cdot}7H_2O$, 0.397 g/L KCl and 0.04% agar. Seedlings were grown in a climate room at 22°C with alternating 8h dark, 16h light. After 24h in the new plate, 5 µL of 200 µM solA standard in 40% ethanol (v/v) or 40% ethanol carrier (v/v) was applied on the agar to reach an end concentration of 5 µM. After 2 h, the three seedlings of one well were combined into one

sample (Table S1). Samples were collected into Eppendorf vials, snap-frozen in liquid nitrogen and stored at -80°C until further use.

cDNA library preparation and high throughput sequencing

For RNA extraction, frozen samples were ground to a fine powder in a cooled mortar and pestle, and total RNA was extracted using the RNeasy Micro Kit (Qiagen, cat nr 74004) and stored at -80°C. RNA-Seq libraries were generated from 500 ng RNA according to the manufacturers' protocols using the QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, cat nr 015.96). The size distribution of the libraries with indexed adapters was assessed using a 2200 TapeStation System with Agilent D1000 ScreenTapes (Agilent Technologies). The libraries were quantified on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) using the NEBNext Library Quant Kit for Illumina (New England BioLabs, cat nr E7630S) according to the instructions of the manufacturer. The libraries were clustered and sequenced (1 x 75 bp) on a NextSeq 550 Sequencing System (Illumina) using a NextSeq 500/550 High Output Kit v2.5 (75 Cycles) (Illumina, cat nr 20024906).

Read quality control, mapping and counting was done with a Snakemake pipeline (Mölder *et al.*, 2021) containing fastp v0.19.5 (Chen *et al.*, 2018), STAR v2.7.6a (Dobin *et al.*, 2013) and featureCounts v1.6.0 (Liao *et al.*, 2014) (Bliek *et al.*, 2021). The ITAG4.0 and the TAIR9 releases were used as a reference genome for tomato and Arabidopsis, respectively. For detailed settings we refer to the /input/ folders in the Supplemental materials. Shortly, reads were checked for quality with default settings of fastp, then mapped with STAR. Multimappers were allowed to a maximum of 10 and mismatches to a maximum of 4 for both species. A raw read count table was produced with featureCounts and this was used for all downstream analyses.

Clustering, differential gene expression analysis and GO term enrichment analysis

PCA analysis, heatmap plotting and Differentially Expressed Gene (DEG) analysis and visualization were done using packages ggplot2 (v3.3.3), pheatmap (v1.0.12), DESeq2 (v1.28.1), ComplexUpset (v1.3.0) and EnhancedVolcano (v1.6.0) in R (v4.0.2) (Blighe *et al.*, 2018; Kolde, 2015; Krassowski & Lagger, 2021; Love *et al.*, 2014; Wickham, 2016). A gene was considered differentially expressed if log2FC $\neq$ 0 and adjusted p value < 0.05, as determined by the Wald test in the DESeq2 package. The log2FC values were shrunk using the package apeglm (v1.10.0) (Zhu *et al.*, 2019). Detailed scripts can be found in R markdown files in the Supplemental materials. GO enrichment analysis was done in Panther (go.pantherdb.org) using the statistical overrepresentation test GO biological process with FDR correction < 0.05.

Results

SolA affects gene expression in tomato seedlings

Tomato seedlings were treated with 5 µM solA or carrier, and roots and shoots were harvested separately, after which RNA was extracted and sequenced. On average, 18.8M reads per sample were obtained. Fastq files were trimmed using fastp (Chen *et al.*, 2018), and over 93% of the sequences passed the quality filters (average 96.9%). The mapping percentage was 65.8% on average (Table 1), which is within the expected range for the library preparation method used (Moll *et al.*, 2014).

Principal component analysis (PCA, unsupervised) plots revealed a strong difference in transcription between shoot and root, but not of solA treatment (Figure 1A). When root and shoot samples were analysed separately, root samples clustered mostly according to solA treatment especially on the second component that explains roughly 20% of the total variance (Figure 1B). Shoot samples also clustered according to solA treatment but to a lesser extent (PC5, 9% of the total variation, Figure 1C). Differentially expressed genes were called using the DESeq2 package in R, using the Wald test (Love *et al.*, 2014). This resulted in the identification of 46 and 22 unique DEGs, respectively (Table S2).

SolA affects transcription of genes involved in biosynthesis of secondary metabolites, ethylene signalling, immunity, and cell wall modification in tomato

In the root, solA treatment resulted in the upregulation of genes annotated as being involved in biosynthesis of specialised metabolites, such as *STRICTOSIDINE SYNTHASE 2* (*STR-2*) and *UDP-GLYCOSYL TRANSFERASE* (*UGT-73C3*) (Solyc07g055740.1.1, log2 fold change (log2FC) 6.1, and Solyc10g085870.1.1, log2FC 2.3) (Table S2). In *Catharanthus roseus, STR-2* catalyses the production of strictosidine, the precursor for terpenoid indole alkaloids, compounds with pathogen repellent effects and anti-cancer drugs (Stöckigt & Zenk, 1977). Tomato does not contain indole alkaloids, but *STR-2* and *UGT-73C3* are involved in the biosynthesis

Table 1. Mapping percentages for experiments in this study (averages).

	Average # reads (xM)	Quality filter pass %	Unique mapping %	Total mapping %
S. lycopersicum n = 20	18.8	96.9%	54.0%	65.8%
Arabidopsis control n = 10	19.6	97.4%	59.7%	70.8%
Arabidopsis starvation n = 25	19.2	97.6%	63.9%	73.2%

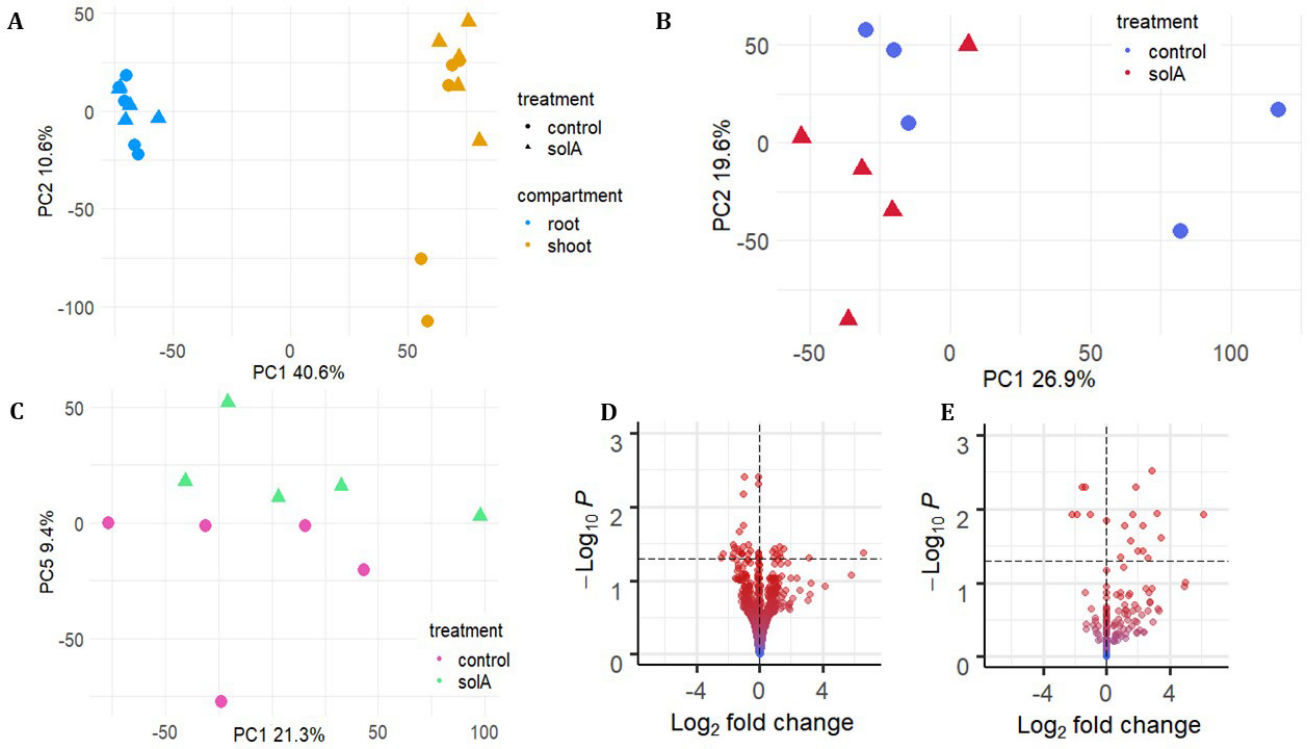

Figure 1. Two-week-old *S. lycopersicum* seedlings were treated with 5 uM solanoeclepin A and shoot and root were harvested separately after 2h of incubation and gene expression analysed using RNAseq. A: PCA plots of tomato root and shoot samples treated with/without solanoeclepin A showing the clustering based on organ. **B**: PCA plots of tomato root samples treated with/without solanoeclepin A. **C**. PCA plots of tomato shoot samples treated with/without solanoeclepin A. **D**. volcano plot showing DEGs distribution in shoot **E**. volcano plot showing DEGs distribution in root. The particular shape of the plot in **E** is caused by many root genes having a low base mean of counts, resulting in a log2 fold change of 0 after shrinkage with apeglm.

of α-tomatine and anthocyanins (Chen *et al.*, 2019). Furthermore, the expression of tomato flavonoid-3-O-rutinoside-4‴-phenylacyl transferase (Solyc12g088170.2.1) and *CHALCONE SYNTHASE 1* (*CHS1,* Solyc09g091510.3.1) – both also involved in flavonoid biosynthesis (O'Neill *et al.*, 1990; Tohge *et al.*, 2015) – were induced by SolA with a log2FC of 4.3 and 2.0, respectively (Table S2).

The transcription of genes involved in ethylene biosynthesis and signalling were affected by solA treatment, but exclusively in the shoot. Expression of the gene encoding rate-limiting biosynthetic enzyme *ACC OXIDASE1* (*ACO1*), was down-regulated (Solyc07g049530.3.1, log2foldchange -1.3), just as its close homologue, but not functionally characterized, *ACC OXIDASE 4* (*ACO4*) (Solyc02g081190.5.1, log2fold-change -1.0) (Houben & Van de Poel, 2019). Furthermore, the transcription of *ETHYLENE RESPONSE FACTOR 10-2* (*ERF10-2,* Solyc08g007820.1.1, log2foldchange -0.1), a small *HEAT SHOCK PROTEIN* regulated by ethylene (Upadhyay *et al.*, 2020) (*HSP17.7B*, Solyc09g015020.1.1, log2foldchange -0.1) and *ETHYLENE RESPONSE HEAT SHOCK PROTEIN COGNATE 70* (Solyc04g011440.4.1, log2foldchange -1.0) were all down-regulated by solA. Interestingly, previous studies showed that the latter gene is upregulated during the immune response against nematode invasion in tomato (Kulshrestha *et al.*, 2020).

Indeed, also transcription of other genes involved in the plant immune response were affected by solA treatment. In both root and shoot, expression of *SERINE PROTEIN INHIBITOR3* (*Serpin 3*) (Solyc04g079450.4.1) was strongly downregulated (log2foldchange -7.1). Serpins play a role in neutralizing pathogen effector proteins (Clemente *et al.*, 2019). A *MULTIDRUG AND TOXIC COMPOUND EXTRUSION (MATE) DETOXIFICATION TRANSPORTER PROTEIN* (Solyc02g032660.3.1), which extrudes xenobiotics, was downregulated in the root as well, with a log2FC of -1.0. Other plant-pathogen interaction-related transcripts, too, were repressed under solA treatment: in the shoot, *DISEASE RESISTANCE PROTEIN* (Solyc07g052790.3.1) (log2FC -1.4); in the root *GERMIN-LIKE PROTEIN 4* (*GLP4,* Solyc01g102895.1.1) (log2FC of -1.4). Remarkably, in the shoot, another *GLP* homologue, Solyc03g123410.1.1, was upregulated with a log2FC of 1.3. *GLP*s play a role in defence reactions, such as the catalysis of dismutation of reactive oxygen species (Dunwell *et al.*, 2008). Hence, we see mostly an inhibiting effect of solA on immunity-related transcripts in the root, while in the shoot there is a combination of up- and downregulation.

Among the genes of which expression is upregulated by SolA, there are four that are related to photosynthesis. Curiously, three of these are specifically upregulated in the root, some involved in light-independent reactions (a ribulose bisphosphate carboxylase/oxygenase activase (Solyc09g011080.3.1) and *TRANSKETOLASE* (*TKL1*, Solyc10g018300.3.1)), but also some involved in light-dependent reactions (*RUBP CARBOXYLASE SMALL SUBUNIT* (Solyc03g034220.3.1)), with a log2FC of 1.6, 1.5 and 1.2, respectively. In the shoot, a photosynthetic *NDH subcomplex B2* (*NDF2*, Solyc03g062720.3.1, log2FC 0.9) was upregulated.

Lastly, two transcripts encoding cell wall modifying proteins, *CELL WALL PROTEIN* (*CWP*) Solyc09g097770.3.1 and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE-HYDROLASE 7* (*XTH7*) Solyc02g091920.3.1 were strongly upregulated in the roots (log2FC of 2.6 and 2.9, respectively). These proteins contribute to modifications to the cell wall in response to abiotic and biotic stresses (Iberkleid *et al.*, 2015) and cell wall expansion (Rose *et al.*, 2002), respectively. In the shoot, a cell-wall degrading enzyme was downregulated with a log2FC of -1.6 (Solyc07g064870.3.1).

SolA induces a similar transcriptional response in Arabidopsis

To evaluate the conservation of the transcriptional response to solA between plant species, we set out to analyse the transcriptional response to solA in Arabidopsis. In the Arabiodpsis RNAseq analysis, the average number of reads per sample was 19.6M and the total mapping percentage 70.8% (unique mapping percentage 59.7%, Table 1), within the range expected for the library preparation method used (Moll *et al.*, 2014). PCA showed partial clustering according to treatment in PC 2 and 3 (Figure 2A), and DEG analysis resulted in the identification of 110 significant DEGs, of which 21 were up- and 89 were downregulated (Figure 2B, Table S3).

GO term/pathway analysis showed that solA treatment upregulates specialised metabolite biosynthesis and cell wall expansion, and downregulates ethylene biosynthesis and signalling, and the immune response, just as in tomato (Figure 2C), although no direct homologues were similarly differentially expressed. The only aspect of solA-induced gene expression of tomato that could not be confirmed in Arabidopsis was the upregulation of photosynthesis-related genes; the only photosynthesis-related gene in the Arabidopsis DEG list (Table S3) is *PHOTOSYSTEM II BY* (*PSBY*, AT1G67740.1) which was slightly downregulated (log2FC -0.4). The confirmation of the upregulation of secondary metabolite biosynthesis was not ubiquitous, but significant: the downregulation of two F-box/kelch repeat proteins (AT1G80440.1 and AT1G23390.1, log2FC -0.4 and -0.3, respectively) which inhibit phenylpropanoid (precursor of, among others, flavonoids) biosynthesis. Ethylene biosynthesis was inhibited

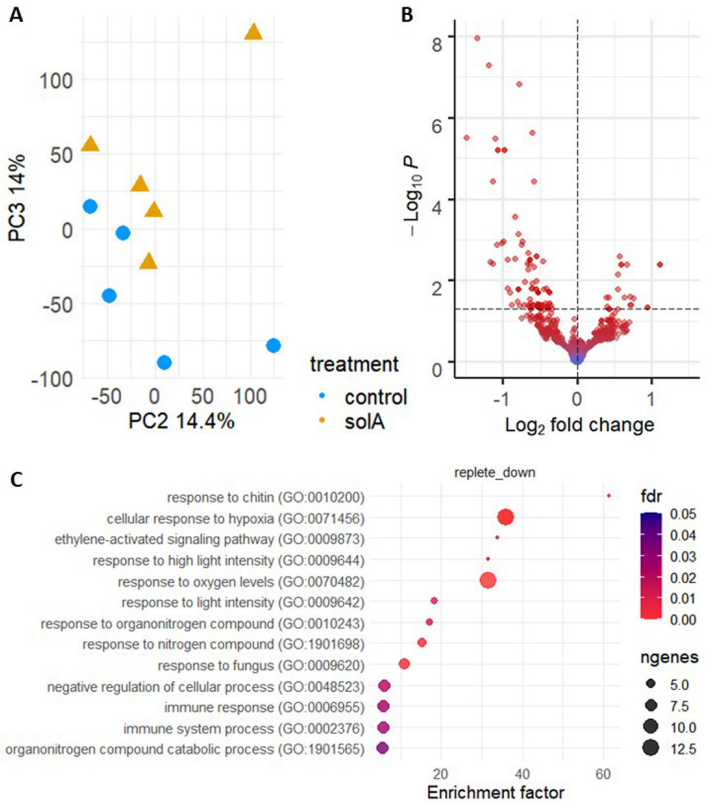

Figure 2. SolA effect on gene expression in Arabidopsis in **A**) PCA plot and **B**) volcano plot, and **C**) the significantly enriched GO terms for downregulated DEGs, summarized using the ReviGO tool (removal of redundant GO terms).

through the downregulation of *ACC SYNTHASE 7* (*ACS7*, AT4G26200.1, log2FC -0.9) and *MITOGEN-ACTIVATED PROTEIN KINASE KINASE 9* (*MKK9*, AT1G73500.1, log2FC -0.6); *MKK9* induces, and *ACS7* is involved in ethylene biosynthesis. Furthermore, ethylene signalling was inhibited as well, visible in the GO term analysis where "ethylene-activated signalling pathway" was significantly enriched in the down-regulated DEGs (Figure 2C). Although the direct homologue of the tomato ERF that was downregulated, *Sl-ERF10-2,* was not affected, several other ERFs were downregulated: *ERF060* (AT4G39780.1, log2 foldchange -1.0), *ERF6* (AT4G17490, log2FC -0.7), *ERF1A* (AT4G17500.1, log2 foldchange -0.6), *ERF104* (AT5G61600.1, log2FC -0.5), *RELATED TO AP2.4* (*RAP2.4*, AT1G22190.1, log2FC -0.3), *TEMPERANILLO 1* (*TEM1/RAV1*, AT1G25560, log2FC -0.6) and *TEMPERANILLO 2* (*TEM2/RAV2*, AT1G68840, log 2 FC -0.6). Interestingly, one of the ERFs, *RAP2.4*, belongs to the ERFVII transcription factors that mediate the elongation of adventitious root growth under hypoxic conditions (Eysholdt-Derzsó & Sauter, 2019). Indeed, the expression of several hypoxia-induced genes is downregulated by solA and multiple GO terms related to hypoxia are enriched for the downregulated genes (Figure 2C). Moreover, under solA treatment, a gene involved in N-end rule degradation is downregulated. This pathway degrades ERFVII proteins in an oxygen-dependent manner. In short, solA results in downregulation of the response to hypoxia and ethylene.

The downregulation of immunity observed in tomato was reflected by the downregulation in Arabidopsis of *NEMATODE-RESISTANCE PROTEIN-LIKE 2* (*HSPRO2,* AT2G40000.1, log2 foldchange -0.8), *AVIRULENCE PEPTIDE AVR9* (AT1G32928.1, log2 foldchange -1.1), *RESPONSE TO WOUNDING PROTEIN* (AT1G32920.1, log2FC -1.4) and *BOTRYTIS-INDUCED KINASE 1* (*BIK1*, AT2G39660.1, log2FC -0.4). Additionally, RING-type ubiquitin ligases *ATL6* and *ATL31* (AT3G05200.1 and AT5G27420.1) were both downregulated (log2FC -0.5 and -0.9, respectively). These are upregulated in response to microbe-associated molecular patterns elicitors such as chitin or flg22 and overexpression of both ATL's results in higher resistance against *Pseudomonas syringae* (Maekawa *et al.*, 2012). The downregulation of immunity was confirmed by the GO term "Response to chitin", "Response to fungus", "Immune system response" and "Immune response" that were enriched for the downregulated DEGs. Lastly, the upregulation of cell wall expansion was confirmed through the upregulation of *UDP-D-GLUCURONATE 4-EPIMERASE 6* (*GAE6*, AT3G23820.1, log2FC 0.4), which is involved in pectin biosynthesis in the cell wall. In conclusion, the results of solA-induced/repressed gene expression in tomato were largely confirmed by the results in Arabidopsis, except for the upregulation of photosynthesis-related genes.

SolA treatment in Arabidopsis did also result in a number of interesting DEGs that we did not see in tomato. For example, the expression of *RAV1/TEM1* was downregulated by solA treatment (log2FC of -0.6). Interestingly, *RAV1/TEM1* expression is also inhibited by epi-brassinolide (Hu *et al.*, 2004), which is a hormone that is structurally related to solA. *RAV1/TEM1* inhibits lateral root growth and hence its downregulation by solA might promote lateral root outgrowth. Furthermore, *ZAT10* and *ZAT12* (AT1G27730.1 and AT5G59820.1), two zinc-finger proteins that are involved in the activation of ROS-related antioxidant genes upon cold stress, were both downregulated with log2FCs of -0.7 and -0.6, respectively. Together with the downregulation in the response to hypoxia, this indicates not only a downregulation of the response to biotic, but also abiotic stress.

N deficiency affects the Arabidopsis transcriptome more than P

Since SL biosynthesis is upregulated under N and particularly P deficiency and has an important role in helping the plant cope with that stress (Akiyama *et al.*, 2005; López-Ráez *et al.*, 2008; Ruyter-Spira *et al.*, 2011), we decided to assess if the effect of solA on gene expression is affected by nutrient availability. Since the transcriptional starvation response is highly context-dependent (Gutiérrez *et al.*, 2007), we first tested the effect of several starvation conditions - P starvation, N starvation and N+P starvation - on transcription under our experimental conditions. For this experiment, the average number of reads per sample was 19.2M and total mapping percentage was 73.2% (Table 1), within the range anticipated for the used library preparation method (Moll *et al.*, 2014). Seedlings were grown for 10 days on complete medium and subsequently transferred to starvation or complete medium (control). PCA revealed a clear effect for N starvation on transcription, while the effect of P starvation was not clearly visible on PC1 and PC2 (Figure 3A, Figure S1C), nor in the other first 10 PCs (results not shown).

DEG analysis resulted in the identification of 45 DEGs for P starvation, of which only 15 with a $|\log2FC| > 1.0$ (Figure 3B). The P stress response was clear from the upregulation of *PHOSPHATE STARVATION INDUCED PROTEIN 3* (*PS3*, AT3G47420, log2FC 2.7), *SPX1* (AT5G20150.1, log2FC 2.5), *INDUCED BY PI STARVATION 2* (*IPS2*, AT5G03545.1, log2FC 2.4), *PEPC1* (AT1G17710, log2FC 1.8) and *PHOSPHATE TRANSPORTER 1* (*PHT1*, log2FC 1.7). *PS3* and *PHT1* were upregulated in tomato, rice and Arabidopsis in earlier studies, confirming their ubiquitous P starvation responsiveness (Cai *et al.*, 2012; Wang *et al.*, 2021b) and *SPX1* is regarded as a P starvation marker (Puga *et al.*, 2014). Strongly downregulated under P starvation were genes involved in iron uptake, such as Fe-uptake inducing peptide 3 (*FEP3*, AT1G47400.1, log2FC -3.3) and *FEP2* (AT1G47395.1, log2FC -3.0). Additionally, plants showed a response to oxidative stress by the upregulation of *FE-SUPEROXIDE DISMUTASE* (*FSD1*, AT4G25100, log2FC 0.7), *FERRITIN-1* (*FER1*, AT5G01600, log2FC 1.0), *ARGININE DECARBOXYLASE 2* (*ADC2*, AT4G34710.1, log2FC 0.5) and the downregulation of *COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE* (AT1G12520, log2FC -0.7) and *ISOFLAVONE REDUCTASE P3* (AT1G75280, log2FC -0.7). The metal, and specifically iron, status of the plant has been linked to phosphate availability

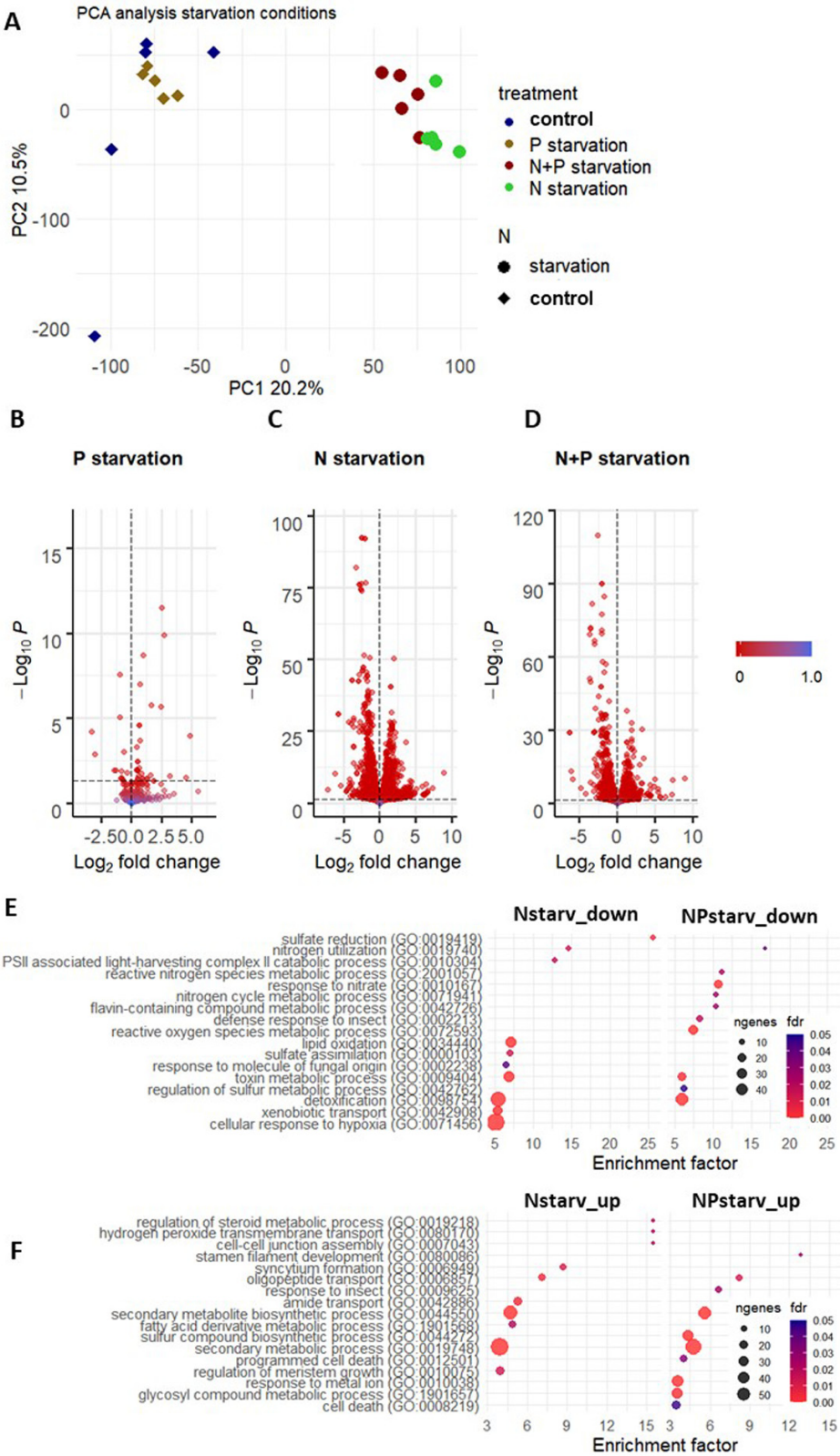

Figure 3. **A**, **B**) PCA plots, **C**, **D**) volcano plots showing the effect of P and N+P starvation vs. control conditions on gene expression in Arabidopsis and **E**–**F**) dot plots showing the top 10 enriched GO terms (redundancy removed using ReviGO) in down- (**E**) and upregulated (**F**) genes of N and N+P starvation vs. control conditions. A full list of all enriched GO terms is shown in Table S5. The comparison P starvation vs. control did not yield any significantly enriched GO terms.

before (Hirsch *et al.*, 2006). The upregulation of *PURPLE ACID PHOSPHATASE 17* (*PAP17*, AT3G17790, log2FC 0.5) signifies the strategy from the plant to solubilize inorganic P via the excretion of phosphatases (Zhang *et al.*, 2014). Finally, differential expression of multiple transporter genes was observed, for example *CATION/H+ ANTIPORTER 17* (*CHX17*, AT4G23700, log2FC 1.3) and *CATION/CALCIUM EXCHANGER 1* (*CCX1*, AT5G17860.2, log2FC 0.5).

N starvation treatment of Arabidopsis seedlings in our system resulted in 5249 DEGs, of which 1459 had a |log2FC| > 1.0 (Table S4). Hence, the plant's transcriptional response to N starvation was much stronger than to P starvation. The N starvation gene expression response shows considerable overlap with N starvation studies in Arabidopsis reported earlier, such as upregulated auxin transport, affected cell wall biogenesis and nitrogen-containing compound transport (Krapp *et al.*, 2011). GO term analysis on downregulated DEGs showed highest enrichment for sulphate reduction and assimilation, nitrogen utilization, PSII associated light-harvesting complex II catabolic process, lipid oxidation, response to molecule of fungal origin, toxin metabolic process and detoxification, xenobiotic transport and cellular response to hypoxia (Figure 3E, full list of enriched GO terms in Table S5). For upregulated DEGs, enrichment was highest for regulation of steroid metabolic process, hydrogen peroxide transmembrane transport, cell-cell junction assembly, syncytium formation, oligopeptide transport, amide transport, secondary metabolite biosynthetic process, fatty acid derivative metabolic process, sulphur compound biosynthetic process and regulation of meristem growth.

N and P starvation responses have been shown to be linked. For example, P starvation reduces the uptake, transport and assimilation of N (Krouk & Kiba, 2020). However, N starvation strongly represses the P starvation response, suggesting that N deficiency responses are prioritised over the P deficiency response (Krouk & Kiba, 2020). In our experiment, combined N+P starvation resulted in the identification of 3403 DEGs, of which 948 had a |log2FC| > 1.0. There was indeed considerable overlap between enriched GO terms of N and N+P starvation for the induced DEGs (Figure 3E, F, Table S5), such as for the upregulation of nitrogen-containing compound transport and photosynthesis, and the downregulation of amino acid metabolic processes and sulfate reduction and assimilation. Unique GO terms for N+P starvation are marked (Table S5) and were mostly related to response to reactive oxygen species, response to light, development and biosynthetic/metabolic processes for downregulated DEGs, and to response to metal ions (iron and zinc), immune response, response to hypoxia and nutrient starvation response for upregulated DEGs.

An upset plot (Figure S3) shows the overlap of DEGs between the different treatments. There are only 20 genes that are differentially expressed in all three treatments. Most overlap is indeed visible between the N and N+P starvation treatments, although N starvation alone leads to much more unique DEGs, which make up more than half of the total DEGs for that treatment. In conclusion, nutritional starvation treatments of Arabidopsis seedlings in our growth system resulted in considerable differential gene expression, especially in the N and N+P starvation treatments. The DEGs show considerable overlap with other studies on the transcriptional response in plants to N and P deficiency.

SolA-induced transcriptional changes in P starved Arabidopsis are similar as in non-starved plants

In a next step we decided to analyse the effect of SolA on gene expression under nutrient deficiency conditions. Because of the overlap in the transcriptional response between N and N+P starvation we did this analysis on the P and N+P starvation treatments. Hereto, plants were grown for 24h under nutrient deficiency and then treated with solA for 2 h.

SolA treated and untreated P-starved plants separated in a PCA plot in the third component (Figure 4A) and DEG analysis resulted in the identification of 36 DEGs (Table S3). From the strongly downregulated genes, 3 are heat shock proteins (AT5G59720.1, AT3G46230.1 and AT1G16030.1, log2FCs -2.5, -1.4 and -1.2 respectively), of which the two former were also downregulated by solA treatment in the shoot of tomato, but not in Arabidopsis (grown under normal nutrient conditions). Furthermore, three of the ERFs that were downregulated by solA under control conditions were downregulated by solA under P starvation as well (*ERF071*, *TEM1*, *TEM2*). Enriched GO terms were only detected for downregulated DEGs and included response to hypoxia, response to organonitrogen compound, response to chitin and protein folding.

In conclusion, the effect of solA under P starvation is roughly comparable to the effect of solA under control conditions (Figure 2C, Figure 4E). Under both conditions, solA results in downregulated genes involved in chitin response, hypoxia response, ethylene signalling, and organonitrogen compound response. Furthermore, solA treatment of Arabidopsis seedlings under P starvation shows overlap with solA treatment of tomato seedlings under control conditions, since in both cases several heat shock proteins, some of which are homologues, are downregulated, and XTH genes and genes involved in photosynthesis, such as *LIGHT HARVESTING COMPLEX PHOTOSYSTEM II* (*LHCB4.2*, AT3G08940, log2FC 0.4), are upregulated.

SolA treatment of N+P starved plants results in strong downregulation of ethylene, immune and hypoxia response, and upregulation of photosynthesis and carbon fixation

In the N+P starvation treatment, one outlier (sample S46) was detected after visual inspection and removal was justified by the fact that it lies more than six standard deviations from the other points in the group in PC1 (Figure S1A, B). Analysis of the solA effect showed that the solA-treatment clustered separately from the control samples along PC1 (Figure 4B), showing that solA application represents the main source of variation.

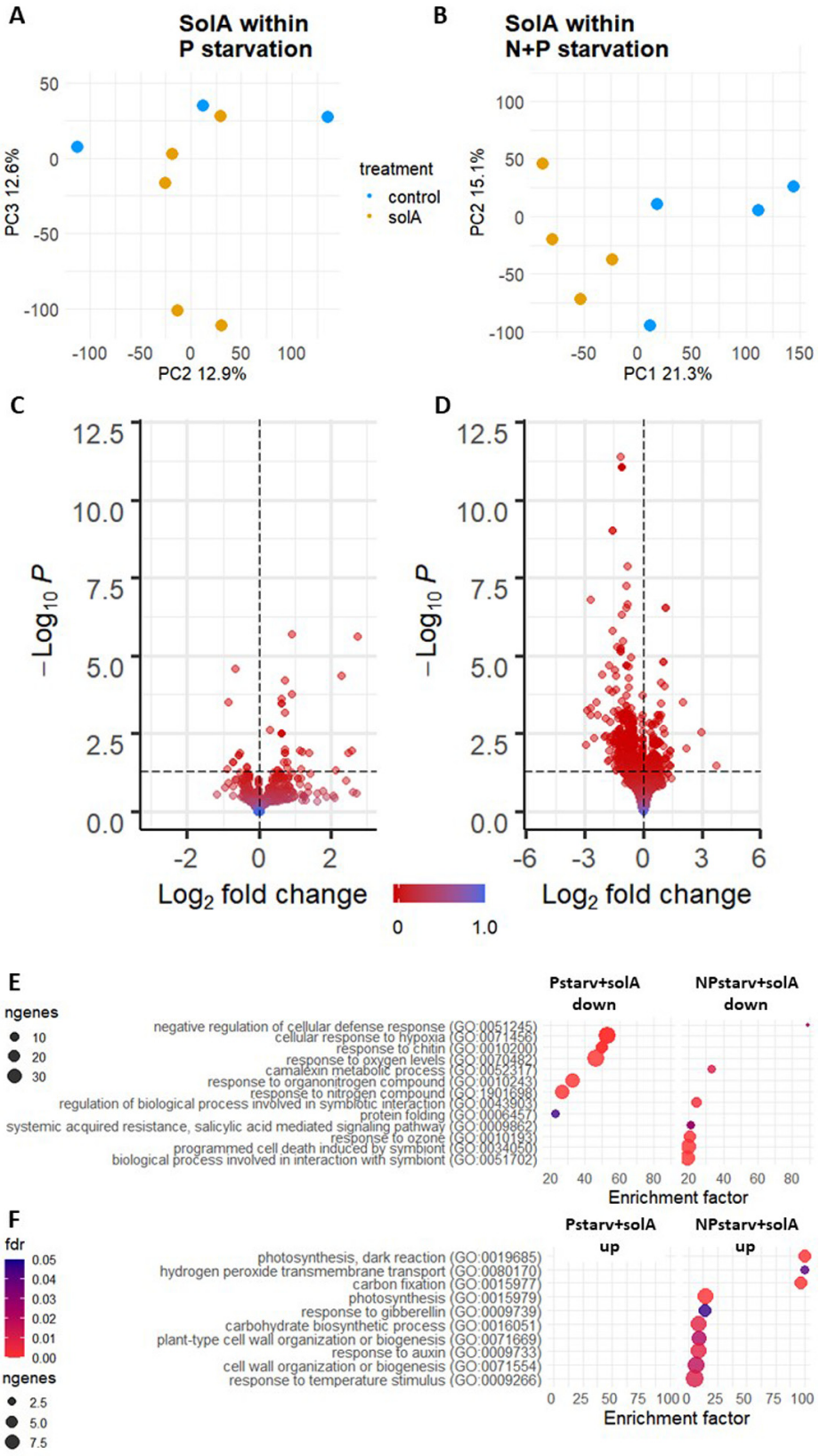

Figure 4. **A**, **B**) PCA plots, **C**, **D**) volcano plots and **E**–**H**) dotplots with top enriched GO terms (redundancy removed using ReviGO) showing the effect of solA within the P and N+P starvation treatments on gene expression in Arabidopsis. The full list of enriched GO terms is shown in Figure S4.

The treatment with solA under N+P starvation resulted in many more DEGs than under control (Figure 2B) or P starvation conditions (Figure 4C) that were also more strongly differentially expressed (Figure 6, Figure S2). Interestingly, the overlap between solA-induced DEGs under the different nutritional conditions was low (Figure 5). Only 6 genes were differentially expressed in all three conditions, and 382 out of 412 DEGs (93%) under N+P starvation were unique for that condition.

GO term analysis of N+P starved solA treatment retrieved mostly enriched terms in the downregulated DEGs. The top 10 terms were related to immune response, response to hypoxia, and camalexin biosynthesis (Figure 6, Figure 4E), but digging deeper shows also enrichment for phytoalexin biosynthesis and a wider array of terms linked to immune response and symbiont relationships (Figure S4A). Upon further inspection, these terms are enriched because of a downregulation in defense-related genes, which could point towards the facilitation of (a) symbiotic relationship(s). There is overlap with the solA induced response in tomato with *JACALIN-LIKE LECTIN 3* (AT5G18130, log2FC -0.5, in tomato -1.1) and two heat shock proteins that share a homologue in tomato (AT5G59720.1 and AT3G46230.1, log2FC -0.1 and -1.5, in tomato -0.1).

For the upregulated genes, the top-10 enriched GO terms were related to photosynthesis and carbon fixation, response to hormones, hydrogen peroxide transmembrane transport and cell wall biogenesis and organization (Figure 4F, Figure 6, Figure S4B). The hormone response to gibberellin is enriched because of the upregulated expression of the aquaporin *TIP1-1* (AT2G36830.1, log2FC 0.4), the expansin *EXPA1* (AT1G69530, log2FC 0.6), the transcription factor *GLABROUS INFLORESCENCE STEMS* (*GIS*, AT3G58070.1, log2FC 2.0) and *GATA TRANSCRIPTION FACTOR 21* (AT5G56860.1, log2FC 0.5) (Table S3). The latter gene is also responsible for the "response to auxin" enriched term, in addition to 4 small auxin upregulated RNA (*SAUR*) transcripts: *SAUR78* (AT1G72430.1, log2FC 0.6), *SAUR16* (AT4G38860.1, log2FC 0.8), *SAUR14* (AT4G38840.1, log2FC 0.9) and *SAUR6* (AT2G21210.1, log2FC 1.0) (Table S3). *SAUR*s play a role in cell elongation and growth, which they might achieve by regulating expansins (Stortenbeker & Bemer, 2019). SAURs induce cell elongation by inhibiting PP2C.D phosphatases, which leads to cell wall acidification and thereby elongation according to the acid growth theory (Spartz *et al.*, 2014). This matches with the results in tomato, in which *CWP* (*CELL WALL-MODIFYING* PROTEIN) and *XTH-7*, which affect cell wall expansion, were upregulated. Further overlap with tomato is the upregulation of *CHS* (AT5G13930.1, log2FC 0.5, in tomato 2.0) which is involved in flavonoid biosynthesis. There was also overlap in carbon fixation-related genes between tomato and Arabidopsis under N+P starvation with the upregulation of *NDH-DEPENDENT CYCLIC ELECTRON FLOW*, *NDF2* (AT1G64770, log2FC 0.5, in tomato 0.9), a component of photosystem I, and *TRANSKETOLASE 1* (*TKL1*, AT3G60750.1, log2FC 0.3, in tomato 1.5), which participates in the pentose phosphate and the Calvin cycle.

Discussion

Plants have been shown to exude compounds into the rhizosphere that not only reflect the plant's nutritional status but are also involved in the adaptation to the corresponding nutrient deficiency stress (Canarini *et al.*, 2019). For example, SLs play a role in alleviation of P starvation stress by altering root architecture and attracting beneficial micro-organisms (Akiyama *et al.*, 2005; López-Ráez *et al.*, 2008). Here, we hypothesized that solanoeclepin A (solA), hitherto only known as potent hatching factor for Potato Cyst Nematode, might have similar specific effects under

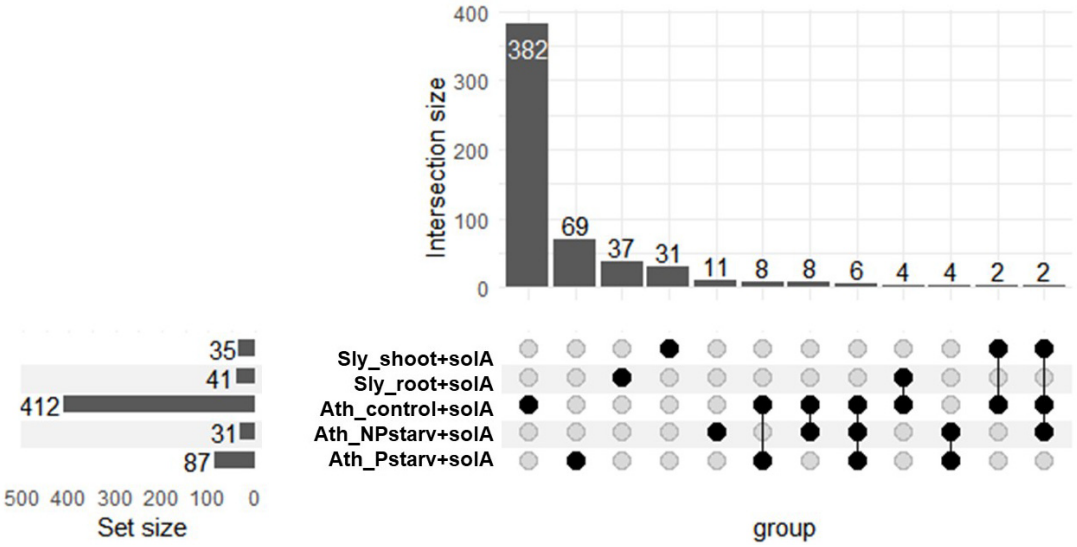

Figure 5. Upset plot showing the overlap between all solA-treatments and the total number of DEGs per treatment.

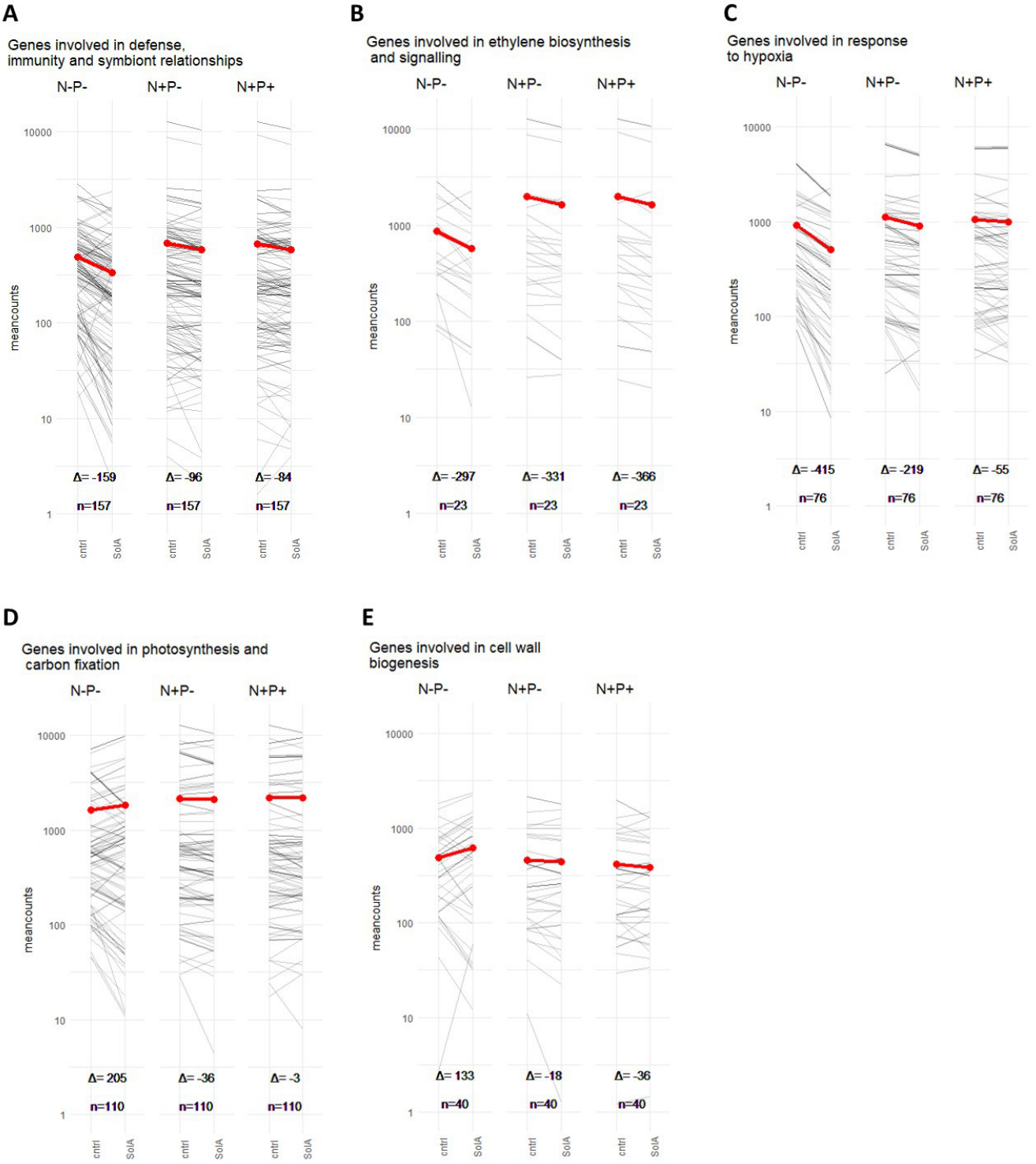

Figure 6. Plots of normalized gene counts of all genes labelled with specific GO terms in gray, with in red the average normalized gene count, under control or solA treatment, respectively. A) genes involved in defense, immunity and symbiont relationships. **B**) genes involved in ethylene biosynthesis and signalling **C**) genes involved in response to hypoxia **D**) genes involved in photosynthesis and carbon fixation **E**) genes involved in cell wall biogenesis. Δ indicates the difference between average normalized gene count with solA treatment and average normalized gene count control, and hence the slope of the red line. n is the number of genes in the group on which the averages were based.

nutrient deficiency which we tested for the two most crucial elements for plant growth, P and N. We show, through transcriptomics analysis, that solA affects gene expression in tomato and Arabidopsis particularly under nutrient starvation (Figure 1, Figure 2, Figure 4). SolA treatment of N+P starved Arabidopsis induces an approximately 10-fold stronger response in gene expression (412 DEGs) than in tomato (shoot and root, 35 and 41 DEGs, respectively) and Arabidopsis grown under control nutrient conditions or P starvation (87 and 31 DEGs, respectively; Figure 5). Downregulated DEGs are ubiquitously involved in immune response and symbiont relationship signalling (Figure 6A), hypoxia and ethylene signalling (Figure 4E, Figure 6B, C) and upregulated DEGs are mostly involved in photosynthesis and carbon fixation (Figure 6D), and growth and development through hormone signalling of auxin and gibberellin and cell wall expansion (Figure 4F, Figure 6E). Additionally, some genes involved in secondary metabolism are up- (for example, chalcone synthase involved in flavonoid biosynthesis) or downregulated (for example, camalexin biosynthesis). For most of these processes, the differential expression encompassed more genes and a higher absolute log2FC on average if the plants were grown under N+P deficiency (Figure 6).

Before evaluating the effect of solA, we studied the effect of P, N and N+P starvation alone on Arabidopsis. In our system, P starvation only induced small transcriptional changes (45 DEGs) (Figure S3). The low amount of DEGs induced by P starvation was confirmed by other studies: for example, Hammond *et al.* (Hammond *et al.*, 2003) found only 4 DEGs after 28h of P starvation in Arabidopsis shoots in a microarray. Still, several P starvation marker genes were upregulated in our analysis (Table S4), proving that a P shortage was perceived by the plants. We found a downregulation of cation transporters, which is probably due to the fact that the absence of the phosphate anion results in higher availability of cations, for example Fe^{2+} (Li & Lan, 2015). This leads to oxidative stress, and indeed we saw altered expression levels of genes involved in the response to oxidative stress. N and N+P starvation resulted in a much stronger transcriptional response, with 5222 and 3384 DEGs, respectively, of which 2362 are overlapping (Figure S3). For both conditions, typical N starvation response genes were affected, such as the ubiquitous upregulation of 10 root nitrate transporters (NRT), with the exception of *NRT1.8* that was downregulated in both N and N+P starvation. N starvation in plants results in the recycling of vacuolarly stored N and hence an increase in transport (Krouk *et al.*, 2010), which is reflected in our data (Figure 3F, Table S5). Furthermore, N deficiency increases auxin transport towards the shoot and thereby represses lateral root formation, whereas mild N shortage results in accumulation of auxin in the root, which does result in increased lateral root growth (Asim *et al.*, 2020; Jia & von Wirén, 2020). Indeed, for both N and N+P starvation, this is reflected in our data (Table S4). However, N control of gene transcription is dependent on additional conditions, since from many microarray studies, only around 300 genes were found to be context-independently differentially expressed (Gutiérrez *et al.*, 2007). Overall, the transcriptional responses to the three starvation conditions in our study agree with the existing literature (Asim *et al.*, 2020; Cai *et al.*, 2012; Jia & von Wirén, 2020; Li & Lan, 2015; Puga *et al.*, 2014).

The most ubiquitously affected process in our analyses was immune response and defence (Figure 6A). These processes are downregulated under N and P starvation, and are even further repressed upon solA treatment. Indeed, the general idea is that under nutrient stress, plants favour nutrient acquisition over immune response. Hence, from our data, it can be speculated that solA promotes the inactivation of the immune system, possibly to preserve energy for coping with nutrient stress. Moreover, since solA is excreted into the rhizosphere, it could have a similar function to SLs, to recruit a beneficial rhizobiome or endophytes that enhance nutrient uptake. Lowering of the immune response can facilitate the colonisation of plant roots by beneficial micro-organisms and hence improve nutrient acquisition (Hacquard *et al.*, 2016). Rozpadek *et al.* suggested that SLs might mediate this immune suppression under P starvation for some endophytes (beneficial fungi) (Rozpadek *et al.*, 2018). Furthermore, solA induces the biosynthesis of alkaloids and flavonoids, in tomato, and there are indications that the same happens in Arabidopsis, where phenylpropanoid biosynthesis inhibitors are downregulated. These compounds are excreted in root exudates and function as repellents against pathogens (terpenoids/alkaloids) and attractants for AM fungi (flavonoids) (Tian *et al.*, 2021). On the other hand in Arabidopsis the biosynthesis of the antimicrobial camalexin (Umezawa & Shin, 1999) is downregulated. It is already known that P starvation induces metabolic changes (Pant *et al.*, 2015), and from our data it becomes apparent that solA further affects the plant's intricate cocktail of signalling molecules, on the one hand suppressing its immune system, possibly to welcome beneficial microbes that can help overcome nutrient deficiencies, on the other hand upregulating a selection of pathogen repelling metabolites.

Under both N and N+P starvation, GO terms related to response to hypoxia are enriched; under N starvation, only in downregulated genes, under N+P starvation, in both up- and downregulated genes. SolA induces a further downregulation of hypoxia response, especially under N+P starvation. Furthermore, solA downregulates ethylene biosynthesis and signalling (Figure 6). Hypoxia affects root architecture; it inhibits primary root growth, whereas it promotes adventitious root growth, which is achieved through ethylene response factor VII (ERFVII) signalling (Eysholdt-Derzsó & Sauter, 2017; Eysholdt-Derzsó & Sauter, 2019). ERFVII are unstable proteins, but are stabilized under hypoxia because of the inhibition by oxygen of plant cysteine oxidases. ERFVIIs then move to the nucleus to induce transcription of anaeroby related genes (Loreti & Perata, 2020). The resulting altered root architecture might aid the plant in nutrient acquisition and this would fit with the stronger downregulation by solA of genes involved in response to hypoxia under N+P stress compared to only P stress or control conditions (Figure 6D). However, there are also various other possible explanations for the reduction in hypoxia response under starvation conditions and under solA treatment. Some tissues, like the meristem,

have a permanently hypoxic state, and hence, it can be a non-stress state required for the production of new leaves (Weits *et al.*, 2019). The loss of the hypoxic state under nutrient starvation may be a means to inhibit growth. Alternatively, tissues can become hypoxic when metabolic activity is high, and removal of hypoxic state can imply a repression of anaerobic fermentation (Loreti & Perata, 2020). Thirdly, hypoxia is linked to biotic interactions, detrimental or beneficial. For example, nitrogen-fixing rhizobia need a hypoxic environment since the nitrogenase enzyme is inactivated by O_2. Hypoxia-induced genes are also activated at the interface between plant and pathogen, although it is unclear whether this is to help the plant fight the invader, or is caused by increased metabolic activity of both organisms (Loreti & Perata, 2020). In te present study, reduction of the hypoxia response can be linked to the suppression of the immune response that is detected mostly under N starvation in our dataset. Lastly, hypoxia signalling has been shown to use the same signal transduction pathway as P starvation response (Klecker *et al.*, 2014); hence, the GO term might be enriched because the DEGs involved are responding to the starvation, not a change in oxygen supply.

The downregulation of ethylene biosynthesis and signalling could, besides or instead of its putative role in altering root architecture described above, also be linked to suppression of defence. Ethylene Response Factors (ERFs) are involved in relay of signals that lead to the transcription of ethylene and jasmonate-induced defence genes (Huang *et al.*, 2016). For example, *ERF6*, which is downregulated in Arabidopsis both in control as well as in N+P starvation, is involved in resistance against *Botrytis cinerea* (Moffat *et al.*, 2012). Additionally, prevention of ethylene induced defense responses are essential for the symbiotic relationship with nitrogen-fixing rhizobia (Berrabah *et al.*, 2018). The resulting increased association with rhizobia might be a means for the plant to cope with and relieve N deficiency stress.

Lastly, genes related to growth are upregulated by solA in tomato and in Arabidopsis under N+P starvation. For example, cell wall expanding proteins and inducers thereof are upregulated in tomato and Arabidopsis, respectively. Furthermore, photosynthesis and carbon fixation-related genes are upregulated by solA, but in Arabidopsis only under N+P stress (Figure 6F). Upregulation of photosynthesis-related genes in root has been shown before, in *Brassica napus*, under SL treatment (Ma *et al.*, 2017). All this suggests that solA promotes growth. Indeed, plant immunity and growth are usually antagonistic, however, plants grown under nutrient stress usually exhibit reduced growth, rather than enhanced. Perhaps the increased growth is analogous to the stimulation of growth by SLs, simulating lateral root growth under P starvation, to improve uptake of phosphate from the soil (Ruyter-Spira *et al.*, 2011).

Conclusion and future perspective

In the present study, exogenous solA treatment followed by transcriptomics was used to explore a putative signalling function of solA in plants. Both solA and SLs were discovered because of their detrimental effect on the plant: solA for the hatching activity on parasitic cyst nematodes, and SLs for the germination of parasitic plants. Later, the SLs were discovered to also have an indispensable beneficial role for the plant, facilitating the symbiosis with AM fungi. In the present study, we show that solA represses the hypoxia response and ethylene biosynthesis and signalling, which might result in adapted root architecture. SLs affect root architecture as well (Ruyter-Spira *et al.*, 2011). Furthermore, the immune response was suppressed under exogenous solA treatment and specialised metabolite biosynthesis was affected. The relevance of these findings cannot be explained by only transcriptomics, but it is tempting to link this to the attraction of beneficial microbial symbionts, and facilitation of their colonization of the plant root. SLs, too, are signalling molecules in the rhizosphere (they induce hyphal branching of AM fungi (Akiyama *et al.*, 2005) and alter the rhizobiome (Schlemper *et al.*, 2017). Additionally, the biosynthesis of specialised metabolites was differentially expressed under exogenous SL treatment (Zhan *et al.*, 2018). Lastly, solA and SLs both enhance the expression of genes involved in photosynthesis and carbon fixation pathways (Zhan *et al.*, 2018), although solA does this mostly under N+P starvation.

SolA is only produced by members of the *Solanaceae* (Guerrieri *et al.*, 2021), but our experiments show that it elicits a similar response in tomato and a distantly related species that has not been reported to produce this metabolite, Arabidopsis. However, solA is not the only eclepin: glycinoeclepin A, B and C are produced by kidney bean and act as a hatching factor for soybean cyst nematode (*Heterodera glycines*) (Fukuzawa *et al.*, 1985). The cross-species activity of the eclepin used in our study may be qualified as interspecific facilitation (Wang *et al.*, 2021a). Furthermore, it raises the option that more eclepins exist in the plant kingdom, and that, although specific eclepins, such as solA, are only produced by a (group of) species, other plant species can detect a range of eclepins, which would qualify the eclepins as a new class of plant signalling molecules.

Phenotypic and molecular studies on the beneficial role of solA on the plant should shed more light on the effect of solA on plant development, which could potentially qualify it as a new plant hormone and for which we laid the foundation with this transcriptomics study. To this end, the molecule should become more readily available, possibly through purification from root exudates, since chemical synthesis is very complicated and time-consuming (Tanino *et al.*, 2011). SolA is produced in low sub-nanomolar concentrations by 4-6 week old *Solanaceae* species, but considerable variation exists between genotypes (Guerrieri *et al.*, 2021). Using high producers in combination with an optimized extraction and purification protocol should yield enough solA to test a variety of conditions, species and mutants.

In this study, for the first time, the beneficial role of a cyst nematode hatching factor, solA, was investigated. RNAseq experiments on both tomato and Arabidopsis showed overlapping results, namely, a suppression of the immune response, ethylene biosynthesis and signalling, and hypoxia response. Furthermore, solA induces growth through enhanced photosynthesis, carbon fixation and cell wall expansion. These effects were more severe under N+P starvation in Arabidopsis, and might aid in optimizing plant physiology towards uptake of nutrients and recruiting a beneficial rhizobiome. Therefore, this study improves our understanding of the plant response to an important, hitherto considered detrimental, rhizosphere signalling molecule, and the outcomes might aid in understanding the plant's adaptation strategy to cope with nutrient starvation.

Ethics and consent

Ethical approval and consent were not required.

Data availability

Underlying data

European Nucleotide Archive. Arabidopsis and tomato seedlings are treated with solanoeclepin. Accession number: PRJEB47417, https://identifiers.org/ena.embl:PRJEB47417 (Vlaar *et al.*, 2022).

Zenodo: Lieker/Sly_Ath_solA_RNAseq: v0.0.1 (the code used for data analysis), https://doi.org/10.5281/zenodo.7044590 (Vlaar & Galland, 2021).

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Extended data

Zenodo: Supplemental materials Vlaar *et al.*, 2022, Open Research Europe. https://doi.org/10.5281/zenodo.7044560 (Vlaar, 2022).

This project contains the following extended data:

- Supplementary material.docx
- Table S2.xlsx: all DEGs of *S. lycopersicum* root and shoot samples treated with solA and their annotation.
- Table S3.xlsx: DEGs under influence of solA treatment of Arabidopsis grown under control, P starvation and N+P starvation conditions
- Table S4.xlsx: Arabidopsis DEGs under influence of nutritional starvation treatments
- Table S5.xlsx: Enriched GO terms in down- and upregulated DEGs by N starvation and N+P starvation conditions.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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Reviewer Report 12 June 2023

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?

Minoru Ueda
Tohoku Daigaku, Sendai, Miyagi Prefecture, Japan

In this paper, the authors present intriguing findings demonstrating that SolA induces similar gene expression patterns in both tomatoes, a member of the Solanaceae family, and Arabidopsis, which belongs to the Brassicaceae family. While the primary focus of this paper is on gene expression analysis, the inclusion of the following additional experiments on biochemical analyses would greatly enhance the content:

- Plant hormones, such as those involved in immunity and secondary metabolite production, exert a strong influence on gene expression. The results of this paper strongly suggested the involvement of some plant hormones, on the mode of action of SolA. To confirm this, it would be valuable to investigate whether the addition of SolA affects the accumulation of plant hormones, particularly JA-Ile, SA, ET, and others.

- Notably, previous research has employed RNAseq analysis to study the effects of JA-Ile, SA, and ET in Arabidopsis. It would be beneficial to compare the results obtained from these studies with the findings presented in this paper.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Natural products chemistry and plant biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 01 June 2023

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?

Noel Blanco-Touriñan
DBMV, University of Lausanne, Lausanne, Vaud, Switzerland
Daniela Barro-Trastoy
Max Planck Institute of Molecular Plant Physiology, Potsdam, Germany

The manuscript by Vlaar *et. al* reports on the identification of differentially expressed genes (DEGs) in two plant species (Arabidopsis and tomato) in response to a secondary metabolite, namely solanoeclepin A (solA). Whether the effect of solA on gene expression was altered under nutrient deficiency conditions was also addressed. Briefly, overlapping DEGs were found in both plant species and solA induced stronger transcriptional changes under nutrient starvation. The manuscript is very well written and we appreciate all the details included in the methods section. The findings are interesting and the main conclusions are supported by the shown data.

Our main concerns are: (i) the concentration chosen for the transcriptomic analysis, (ii) the physiological relevance of the DEGs in response to solA, and (iii) the suggestion to consider solA as a new plant hormone. First, it was previously described that “sub-nanomolar concentrations of solA are enough to induce high hatching rates in PCN” (mentioned in the introduction). However, the authors analyzed solA-induced transcriptional changes at a final concentration of 5µM. Although we understand that the authors wanted to investigate the early transcriptional changes (2 h after the treatment), that concentration looks quite high and consequently the findings might be quite artificial (unless the activity of the solA stock used is low). We think that the manuscript might benefit from the analysis of transcriptional changes at lower solA concentrations (by qPCR for instance). The authors should explain clearly in the manuscript the criteria used for choosing the solA concentration. In line with this, the authors mentioned in the discussion that “which could potentially qualify it (solA) as a new plant hormone and for which we laid the foundation with this

transcriptomics study". We believe the data reported in this manuscript does not support this idea, especially considering the high concentration of solA used and the low amount of DEGs identified. Could the authors rephrase this and use a more general term to refer to solA (maybe plant-derived signaling molecule)? Please, find below some other comments that require the authors' input as well:

1. Page 4, methods section: "On day 20, 20 µL of 250 µM solA in 50% ethanol (v/v) or 50% ethanol (v/v) carrier was pipetted on the agar close to the stem to reach an end concentration of 5 µM; control seedlings were treated with the same volume of ethanol/ water, etc.". Could the authors provide a better explanation of how the treatment was performed? For instance, it is not clear for us why the authors indicated "water, etc.".

2. Page 4, methods section: "Seedlings were harvested after 2h (root and shoot separately), which is the typical timeframe for transcriptomic experiments with externally applied hormones, such as for example SL.". We are not convinced by this explanation since we should not assume that all the signaling compounds have the same effect at a specific time point.

3. Page 4, methods section: This might be irrelevant but why did the authors decide to use ITAG4.0 and TAIR9 releases instead of ITAG5.0 or TAIR10 for the tomato and Arabidopsis reference genomes, respectively?

4. Page 4, methods section + Page 6, results section: A gene was considered differentially expressed if log2FC ≠0. Although a log2FC of -0.1 (e.g., *HSP17.7B*) can be statistically significant, is this logFC relevant at the physiological level?

5. Page 8, results section: "The downregulation of immunity observed in tomato was reflected by downregulation in Arabidopsis of…". Could the authors tone down claims such as "the downregulation of immunity" throughout the manuscript? The authors only found some DEGs related to some particular responses.

6. Page 8, results section: "N deficiency affects the Arabidopsis transcriptome more than P" should be read as "N deficiency affects the Arabidopsis transcriptome more than P deficiency".

7. Typo on page 15. "In te present study" > "In the present study"

Although the manuscript is of interest to the scientific community, sometimes it seems repetitive in discussing the DEGs found and their role in plant development and in stress responses.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and does the work have academic merit?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant development, vascular development, hormone signaling and plasticity, gene expression regulation.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Reviewer Report 18 May 2023

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?

Danuse Tarkowska
Univerzita Palackeho v Olomouci, Olomouc, Olomouc Region, Czech Republic

The authors wrote the manuscript dealing with the role of solanoeclepin A (solA) which is a naturally-occurring compound produced by roots of plants from the Solanaceae family. Except for the already known effect of solA in the induction of the hatching of cyst nematodes, the authors found that this compound seems to be also involved in signaling leading to nutrient (N, P) uptake and promotion of root growth.

The science seems to be good but I have the following comments:

1. The article misses the paragraph about Chemicals in the Experimental section. This paragraph should contain a description of chemicals that were used in the study including their purity and supplier. I am, for example, missing the information what is exactly "ethanol carrier", what the authors mean by "bleach", what is the purity of solA standard used, what instrument produced "milliQ", supplier of sucrose, agar, etc.

2. The authors claim that "subnanomolar concentrations of solA are enough to induce high hatching rates in PCN" (Introduction part). If this is so, why do they use solA at a final concentration of 5 micromolar (section Methods)? Is it a result of any optimization procedure? I am asking because it is well known that the effect of studied compounds highly depends on the concentration, i.e. at low concentrations they can act as stimulants

while at higher concentrations they may often behave the opposite.

3. The authors use "Eppendorf vials". I guess it is more common to use "Eppendorf tubes" instead? The vials are the containers used in chromatography.

4. In the conclusion paragraph (page 15), the authors claim that "solA could be potentially qualified as a new plant hormone". I cannot agree with that. There is still a valid definition for plant hormone as a substance that is omnipresent in nature, i.e. it is produced by all plant species[1]. And this is not the case with solA. It is just signaling molecule produced by Solanaceae family only playing a certain role in allelopathy as many other compounds produced by plants to cope with various environmental influences.

   Although no clear boundaries exist between hormones and other types of signaling compounds, unsurpassable requirement for the hormone should be the presence of identified protein–receptor for its perception if we resign ourselves to the demand for omnipresence.

   I have discussed this issue also: Tarkowská (2019)[2].

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2. Tarkowská D: Plants are Capable of Synthesizing Animal Steroid Hormones.*Molecules*. 2019; **24** (14). PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant hormones research, plant signaling molecules, ultra-trace analysis of

naturally-occurring compounds

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.