Plant Physiology Preview. Published on June 22, 2018, as DOI:10.1104/pp.18.00450

Network modelling unravels mechanisms of crosstalk between ethylene and salicylate signalling in potato

Short title

Potato Hormonal Signalling Network

One-sentence summary

Analysis of integrated prior knowledge and ensemble networks highlights a previously unidentified connection between ethylene and salicylic acid signalling modules in potato.

Author Contributions [1]

Funding Information [2]

Authors

Živa Ramšak,[a,3] Anna Coll,[a] Tjaša Stare,[a] Oren Tzfadia,[b,c] Špela Baebler,[a] Yves Van de Peer,[b,c,d] Kristina Gruden[a]

[a] National Institute of Biology, Department of Biotechnology and Systems Biology, 1000 Ljubljana, Slovenia

[b] Department of Plant Systems Biology, VIB, 9052 Ghent, Belgium

[c] Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium

[d] Genomics Research Institute, University of Pretoria, Private bag X20, Pretoria 0028, South Africa

[1] K.G. acquired funding and conceived, supervised and designed the research. A.C., T.S. and K.G. designed and conducted the laboratory experiments. Ž.R., O.T. and Y.P. developed the computational analysis. Ž.R., A.C., Š.B. and K.G. interpreted the data. Ž.R., Š.B. and K.G. discussed the results and wrote the manuscript. All authors read and approved the final manuscript.

[2] This work was supported by grants from the Slovenian Research Agency (P4-0165, J4-7636, J7-7303, N4-0026).

[3] Address correspondence to ziva.ramsak@nib.si

ABSTRACT

To develop novel crop-breeding strategies, it is crucial to understand the mechanisms underlying the interaction between plants and their pathogens. Network modelling represents a powerful tool that can unravel properties of complex biological systems. In this study, we aimed to use network modelling to better understand immune signalling in potato. For this, we first built on a reliable Arabidopsis (*Arabidopsis thaliana* L.) immune signalling model, extending it with the information from diverse publicly available resources. Next, we translated the resulting prior knowledge network (20,012 nodes, 70,091 connections) to potato (*Solanum tuberosum* L.) and superimposed it with an ensemble network inferred from time-resolved transcriptomics data for potato. We used different network modelling approaches to generate specific hypotheses of potato immune signalling mechanisms. An interesting finding was the identification of a string of molecular events illuminating the ethylene pathway modulation of the salicylic acid pathway through NPR1 gene expression. Functional validations confirmed this modulation, thus supporting the potential of our integrative network modelling approach for unravelling molecular mechanisms in complex systems. In addition, this approach can ultimately result in improved breeding strategies for potato and other sensitive crops.

INTRODUCTION

Plants have evolved a multi-layered immune system to cope with potential invasion of pathogens (Jones and Dangl, 2006). Recognition of invading organisms triggers a rapid induction of signalling cascades, leading to diverse defence responses (Pieterse et al., 2012). Effectiveness of these downstream events is crucially dependent on salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), but other hormones were also shown to play important roles in plant immunity (Verma et al., 2016). Hormonal signals differ considerably in timing, quantity and composition, depending on the type of attacker (Blüthgen, 2015). Crosstalk between hormonal pathways can have antagonistic or synergistic effects and is largely multi-dimensional (Tsuda and Somssich, 2015). This interconnected plant hormonal network provides an important regulatory mechanism, granting plants quick adaptation abilities via intruder-specific alterations (Pieterse et al., 2012). At the molecular level, crosstalk between signalling pathways with several regulatory feedback loops adds robustness to the plant immune signalling network (Windram and Denby, 2015). Network analysis—application of mathematical graph theory approaches—also continues to be paramount in systems biology investigations of complex systems (Barabási, 2009). This approach, which involves thorough analyses of critical system properties, facilitates

discovery of novel key players or interactions, making it suitable for providing new insights into plant defence specificities (McCormack et al., 2016).

While heterogeneous technologies of high-content omics allow us to capture snapshots of the systems, the challenge now lies in integration of knowledge into a coherent systems view (Hillmer and Katagiri, 2016). Network inference from omics datasets allows us to deduce the underlying structure of activated processes. However, due to high noisiness, high dimensionality and low sample sizes of data, this is a non-trivial task (Veiga et al., 2010). Thus, additional improvements are needed; for example, incorporation of prior knowledge can greatly improve reconstructed network accuracy, simultaneously reducing noise and sparsity effects of the source data, without inflating the computational cost (Ghanbari et al., 2015).

Despite extensive potato-breeding programs, average potato yields still do not reach their physiological potential (Singh, 2008). This is the result of the sensitivity of potato to a wide range of environmental factors. The aim of the current study was to improve understanding of potato immune signalling using network modelling and thus, in the long-term, to provide means for novel crop-breeding strategies directed towards high and sustainable yields. We built on a manually curated plant immune signalling model (Miljkovic et al., 2012), complementing it with knowledge from various public resources, the majority of the available data coming from the model plant *Arabidopsis thaliana*. We also inferred networks using time-resolved transcriptomics data of both compatible and incompatible potato-virus interactions (Stare et al., 2015; Baebler et al., 2014) and superimposed them with our knowledge network. We tested the resulting network for its potential for generating novel hypotheses and show that network analysis revealed a previously unknown connection between ET and SA signalling, namely that activation of the ET signalling module, through ethylene insensitive 3 (EIN3), induces expression of nonexpressor of PR genes 1 (NPR1), an important regulator of SA signalling. This newly identified crosstalk was experimentally validated in potato.

RESULTS

Construction of the comprehensive knowledge network

Firstly, a previous plant immune signalling model (PIS-v1; Miljkovic et al., 2012) was expanded with manually curated knowledge from recently published literature. The addition of 64 Arabidopsis genes to the existing model resulted in an expanded PIS-v2 model with 212 biological components (177 genes, 31 metabolites and 4 small RNAs), categorized into 108 component families as defined by Miljkovic et al. (2012). Following the abstraction of the component families, we added 32 new reactions, with the total number of reactions reaching 111 (Supplemental Data Set 1).

We combined the graph of binary PIS-v2 interactions with three layers of publicly available information: protein-protein interactions (PPIs), transcriptional regulation (TR) and regulation through microRNA (miRNA). This resulted in an *Arabidopsis thaliana* comprehensive knowledge network (AtCKN; Figure 1A), with 20,012 nodes (19,812 genes, 186 miRNA families, 3 metabolites and 11 viral proteins) and 70,091 connections (Supplemental Table 1). Each data layer covers unique gene or miRNA subsets in the entire network, with only six nodes present in all four layers, which indicates that our layer selection was well suited for inclusion (Figure 2).

Use of prior knowledge to improve the plant immune signalling model

To assess the potential of using prior knowledge for the improvement of the mechanistic model of plant immune signalling, we extracted a subnetwork of AtCKN with all components of PIS-v2. This subnetwork consisted of 391 connections between 212 nodes in the fully expanded version or 254 connections between 108 nodes at the level of component families. By comparing the PIS-v2 layer against the remaining layers of AtCKN (PPI, TR, miRNA), we found that 45 connections were present in both subnetworks and 67 only in the PIS-v2, and 142 novel reactions were found from the remaining AtCKN layers. These represent model upgrades, demonstrating the value of dispersed knowledge sources also for the construction of detailed mechanistic models. Inspecting these new connections showed that manual curation is more successful in knowledge extraction within a signalling module (Figure 3B) than between signalling modules (Figure 3A; Supplemental Data Set 2).

Translation of knowledge to potato and integration with experimental data

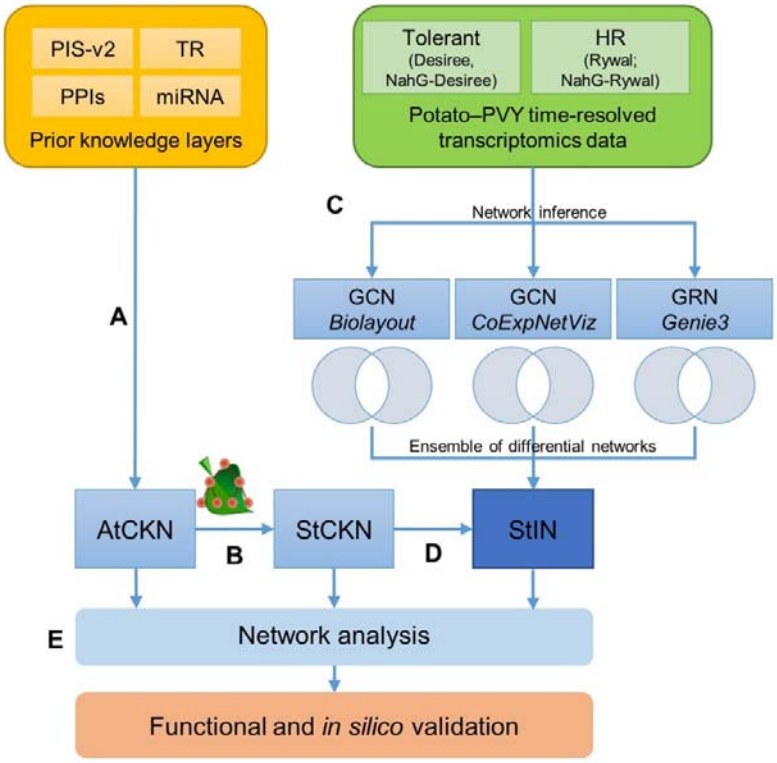

Figure 1: Schematic overview of networks construction and analyses. **A**, Networks of four prior knowledge layers were merged into the Arabidopsis thaliana comprehensive knowledge network (AtCKN). **B**, Orthologous relationships were used to translate from Arabidopsis to potato, forming the Solanum tuberosum comprehensive knowledge network (StCKN). **C**, Starting with two time-resolved transcriptome datasets, gene co-expression networks (GCNs; relationships between co-expressed genes) and gene regulatory networks (GRN; transcription-factor-to-regulated-gene relationships) were inferred using three methods. For each inference method, two subnetworks were generated for mock-inoculated and viral-infected samples. Removing all connections present in all co-expression or gene regulatory networks resulted in differential networks. D, StCKN and differential networks were merged into the potato integrated network (StIN). E, The created networks were analysed using network analysis approaches. PIS-v2 – plant immune signalling model; PPIs – protein-protein interactions; TR – transcriptional regulation; miRNA – regulation via miRNA.

Based on predictions of orthologous relationships, we translated AtCKN from Arabidopsis to potato to form the *Solanum tuberosum* comprehensive knowledge network (StCKN). This resulted in an intermediary abstracted network with 9679 nodes (9497 orthologue groups, 168 miRNA families, 3 metabolites, 11 viral proteins) and 43,393 connections. Next, we inferred all combinations between potato genes of the same orthologue group for each abstracted connection; this resulted in the StCKN (Figure 1B) having 18,036 nodes (17,855 genes, 168 miRNA families, 3 metabolites, 11 viral proteins) and 296,834 connections. This expansion is the result of a many-to-many relationship of orthologous genes between species. But it also includes cases where no homologous gene is found.

To identify transcriptional modules in potato that contribute to potato immune signalling in potato virus Y (PVY) infection, we selected datasets profiling temporal response dynamics in potato genotypes displaying either a tolerant or hypersensitive response (108 samples). Out

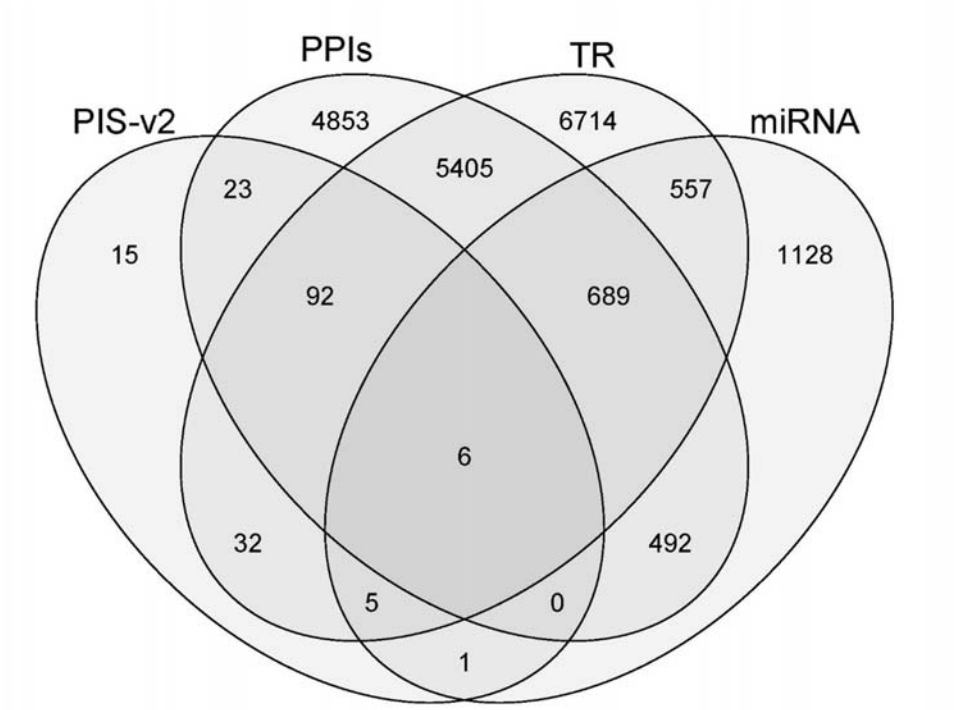

Figure 2: Contribution of the four layers to node coverage in *Arabidopsis thaliana* comprehensive knowledge network (AtCKN). Overlap display between the four layers contributing to AtCKN. PIS-v2 – plant immune signalling model; PPIs – protein-protein interactions; TR – transcriptional regulation; miRNA – regulation via miRNA.

148 of 17,855 potato StCKN genes, 10,920 (61%) had a microarray probe assigned. We used
149 the expression values of these genes to infer a targeted and non-targeted co-expression
150 network and a gene regulatory network: the former, in order to propose genes controlled by
151 the same transcriptional regulatory program, and the latter, to propose potential regulators
152 (Figure 1C).
153

Two types of subnetworks generated (mock-inoculated and viral-infected) allowed us to examine differences between gene connections. Subnetworks of mock-inoculated plants reflecting developmental cues were all of similar size (25,916, 25,910 and 30,570 connections for targeted, non-targeted co-expression and gene regulatory network, respectively). The sizes of subnetworks reflecting plant responses to viral infection were between 56–64% of the sizes of mock-inoculated subnetworks, but again similar to each

other (16,716, 15,993 and 17,204 connections in the same order as above). The difference in connection count per method could be explained by a greater variability of the viral subset (tolerant, hypersensitive), which resulted in a smaller number of inferred connections. Conversely, as developmental profiles of all genotypes share greater similarities, the number of inferred connections was larger in the mock-inoculated subnetwork. Comparison of predicted connections among all three approaches revealed they are largely independent, as

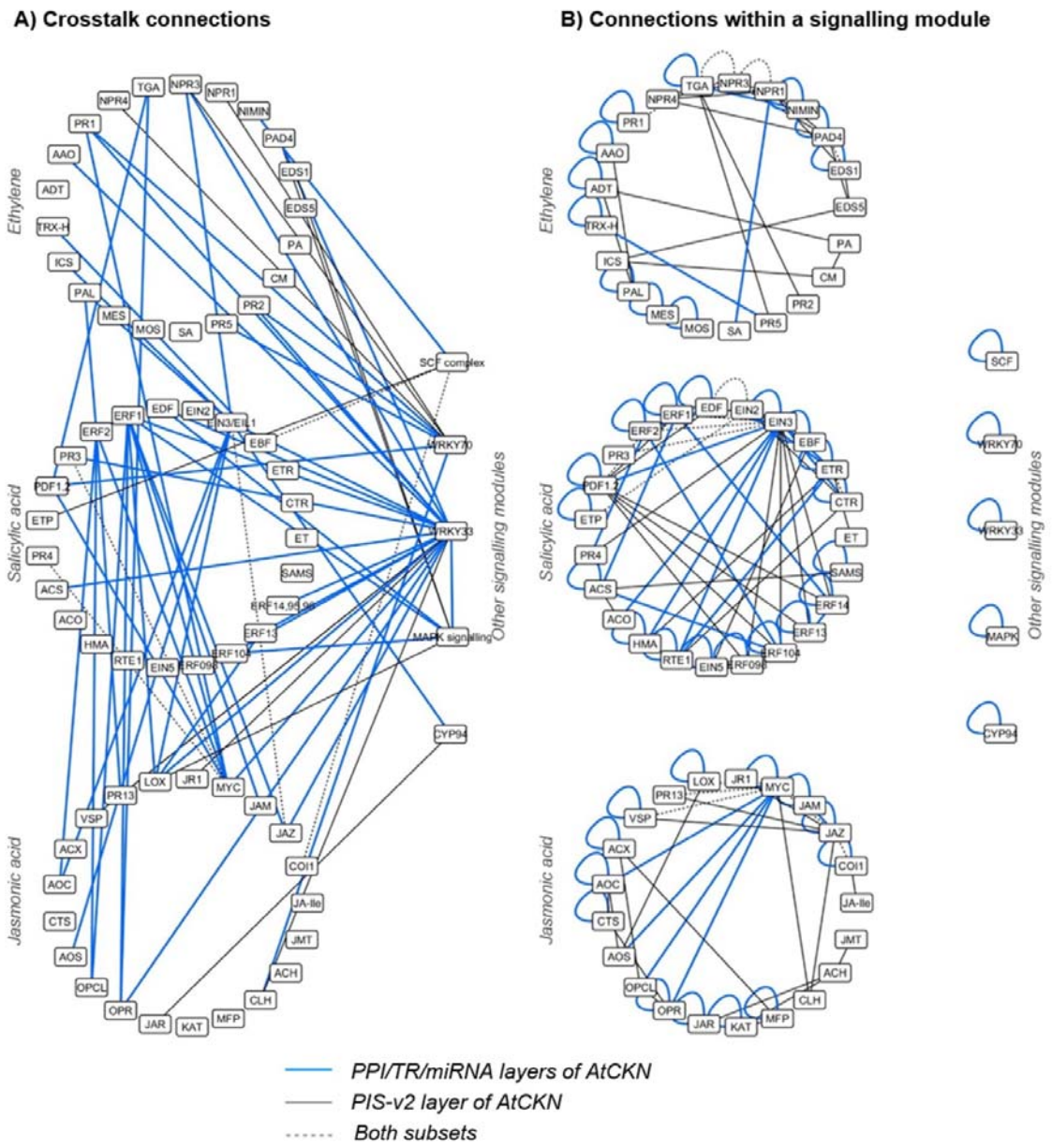

Figure 3: Connections between gene families of three plant hormone immune signalling pathways (ethylene, jasmonic acid, salicylic acid) and other signalling modules. Contribution of the plant immune signalling model (PIS-v2) layer in contrast to that of the remaining AtCKN layers (PPI, protein-protein interaction; TR, transcriptional regulation; miRNA) in terms of **A**, connections between signalling modules (i.e. crosstalk connections) and **B**, connections within a signalling module. Solid blue lines – novel connections present in PPI, TR and miRNA layers of AtCKN; solid grey lines – connections present only in the PIS-v2 layer; dotted grey lines – connections existing in both compared subsets.

the three methods shared only 111 out of 116,391 total unique connections (Supplemental Figure 1).

To elucidate perturbations in network topologies in plant immunity, we extracted differential networks by removing any connections shared between the mock-inoculated and viral-infected treatments. The differential networks were merged as new layers with StCKN into a

Solanum tuberosum integrated network (StIN; Figure 1D) with 402,277 connections between 19,801 nodes (19,619 genes, 168 miRNA families, 3 metabolites, 11 viral proteins).

To validate our approach of StIN construction, we compared interactions covered by selected layers of biological information against a gold standard, a set of highly reliable reactions from the manually curated plant immune signalling model (Table 1). The PPI layer covered 50% of all reactions identified by manual literature curation in our PIS-v2 model. The TR layer had even greater concordance with the gold-standard reactions (80%). On the other hand, connections resulting from gene regulatory network inference covered only 20% of interactions in the gold standard. We must, however, note that some PIS-v2 model connections might be triggered in non-viral infections instead.

Integrated network-driven hypotheses: Ethylene is modulating NPR1 gene expression

First, we analysed the topologies of the generated networks, namely AtCKN, StCKN and StIN. AtCKN showed some bias towards high-degree nodes, a direct result of two included datasets from ChIP-Seq experimental data (Supplemental Table 1). On the other hand, expansion to all potato genes performed for StCKN and StIN distorted the network topological indices (many-to-many phylogenetic relationships). Further network analyses (Figure 1E) aimed at targeted identification of novel crosstalk connections between receptors and transmitters of seven plant hormonal pathways (Supplemental Table 2). Due to topology distortion in translated potato networks, we performed the initial search in AtCKN, afterwards analysing the connections in StCKN.

One of the most interesting findings was the shortest path from the ethylene pathway transmitter EIN3 to SA receptor NPR1. In AtCKN, we identified several shortest paths of 3-step length, involving 32 genes and 61 connections (Figure 4A; Supplemental Data Set 3). One-third of these were binding (PPI) connections, and the remainder, transcriptional regulations. In StCKN, we searched for walks (length 3) from EIN3 to NPR1, and then superimposed co-expression and gene regulatory network connections from StIN (Figure 4B; Supplemental Data Set 3). The potato EIN3 to NPR1 walk subnetwork included 32 genes, 57 StCKN and 48 experimentally inferred connections. Searching for walks of a specific length was required to ease comparisons, as the shortest path between EIN3 and NPR1 after translation was of 2-step length (Figure 4C).

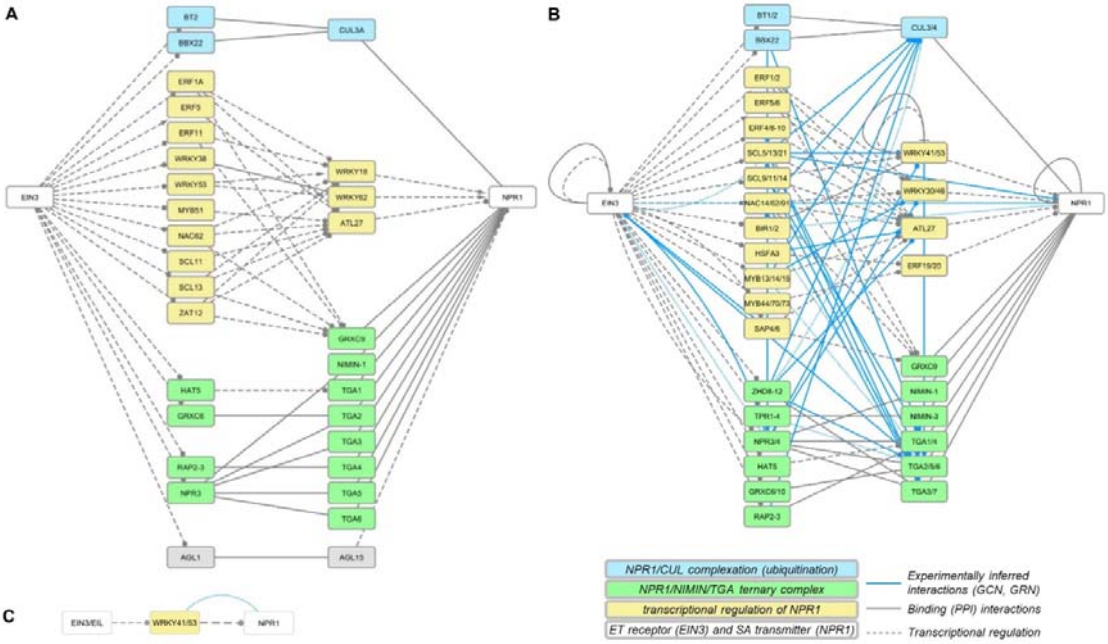

Figure 4: Results of shortest paths or walks from EIN3 (ET) towards NPR1 (SA). **A**, Shortest path (3-steps) in AtCKN; **B**, walk of length 3 in StCKN; **C**, shortest path (2-steps) in StCKN. Line type and colour indicate the interaction type: binding (solid grey), regulation by transcription factors (dashed grey), co-expression (dotted blue) and gene regulatory (solid blue). Target arrow indicates the action of the connection: activatory (arrow), inhibitory (T), unknown (circle) or undirected in the case of binding or co-expression (no arrow). Gene group identifiers corresponding to these images are given in Supplemental Data Set 3.

The majority of the binding-type connections in potato EIN3-NPR1 walk can be attributed to the formation of a ternary complex between NPR1, NIMIN1 and TGA factors (Figure 4, green), which in turn modulates PR1 gene expression (Weigel et al., 2005). The other set of binding-type connections relates to complexation of NPR1 with cullin (Figure 4, blue), which is important for plant immunity regulation (Spoel et al., 2009). The remaining shortest paths indicate potential transcriptional regulation of NPR1 through ET signalling (Figure 4, yellow). As expected, superimposed connections from the transcriptomics data for potato were also denser in this area (Figure 4B, blue connections). The first step of all shortest paths describing transcriptional regulation includes several ethylene-responsive factors (ERFs) and specific members of the C2C2, GRAS, NAC, MYB and HSF families. These transcription factors then target two WRKY transcription factors, in particular, WRKY18 and WRKY62, where the former was shown to have an important role in plant responses to bacterial and fungal pathogens (Chen et al., 2010; Xu et al., 2006), besides another ERF and an ATL.

Superimposing inferred connections based on transcriptional profiles of potato response to viral infection, although incomplete, confirmed potential regulation of NPR1 gene activity through several WRKY (WRKY30/46, WRKY41/53), zinc-finger (SAP4/6) and MYB

(MYB13/14/15, MYB44/70/73) transcription factors (Figure 4B). Interestingly, when searching in a reduced StIN with only transcriptional layer connections, we predicted regulation of NPR1 through ERF (ERF2a), WRKY (WRKY34, WRKY41/53), MYB (MYB18/19, MYB52/54) and bHLH (bHLH84/84) transcription factors (Supplemental Figure 2).

For further evaluation of these findings, we scanned Arabidopsis and potato promoters of NPR1 gene for known *cis*-regulatory elements. Apart from containing known conserved motifs for light and development responses, both promoters contain motifs specific for several hormones (abscisic acid, gibberellic acid, JA and SA; Supplemental Data Set 4). In terms of general stress responses, both promoter regions contain binding sites for responses to heat, drought and defence. In addition, we detected a wounding motif, MYB and WRKY-binding motifs in the potato promoter only.

Experimental validation of transcriptional regulation of NPR1 by ET

To validate our network-generated hypothesis, we tested the transcriptional regulation of NPR1 following the induction of the ET pathway in potato to show the potential of such translation of knowledge to a crop. We additionally checked for the potential of SA signalling module to participate in this process. Thus, we induced the SA signalling module by 2,6-dichloroisonicotinic acid (INA; a functional analogue of SA that is not accessible to degradation by salicylate hydroxylase, NahG) while either leaving the ET module active or blocking its activity (treatment with 1-methylcyclopropene; 1-MCP). Alternatively, we tested the regulatory potential of the ET module, while SA signalling was blocked by using transgenic plants expressing NahG, which degrade any internally produced SA (Figure 5A). Induction of ACO4 gene expression was used as a marker of efficient activation of the ET signalling module and PR1b as a marker of SA signalling module activation.

Significant upregulation ($p < 0.05$, Student's *t*-test) of NPR1 gene expression after ET treatment substantiated our network-generated hypothesis in potato plants (Figure 5B, ET treatment). Strong induction of the PR1b gene after ET treatment additionally confirmed regulation of the SA signalling module by ET. Induction of PR1b by ET was even stronger than its expected induction by SA signalling (Figure 5B, INA treatment). Tight interaction between both modules was also confirmed by ACO4 induction by both ET and INA treatment. When SA signalling was blocked (using NahG transgenic plants, Figure 5C), the induction of NPR1 gene expression by ET was similar to that in non-transgenic plants, confirming that the string of events leading to activation is not dependent on SA. All other

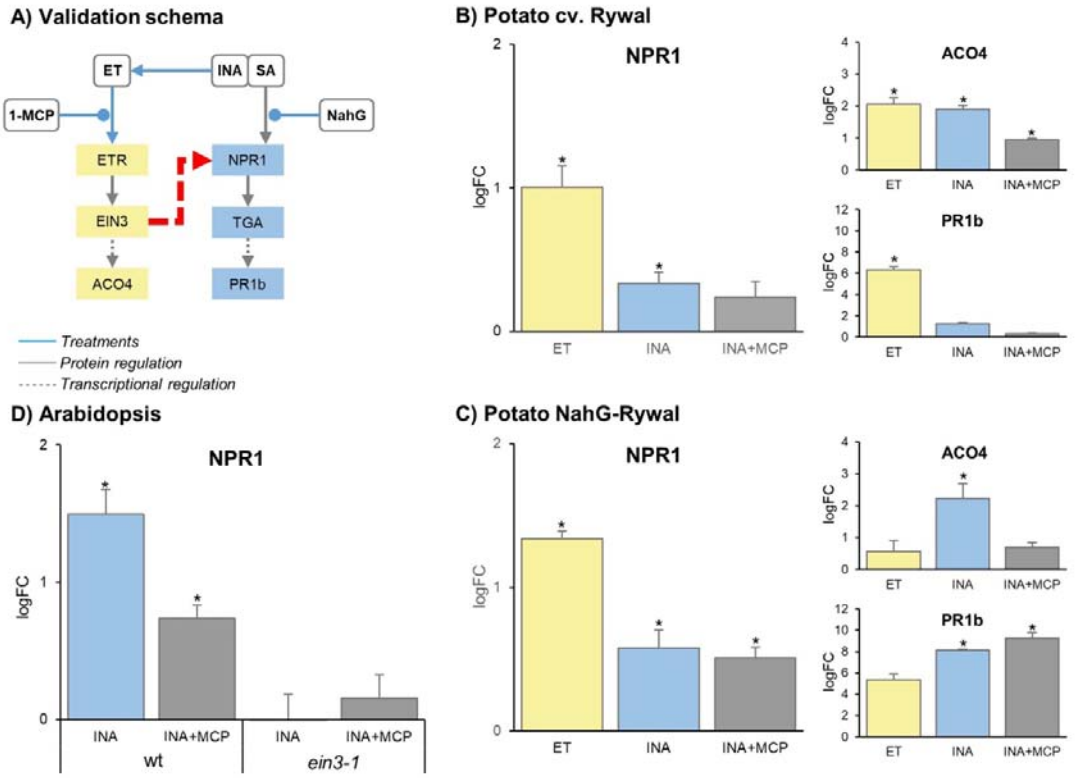

Figure 5: Validation of direct transcriptional regulation of NPR1 by the ethylene (ET) signalling module in potato leaves. **A**, Schema of the underlying biological pathways of ET (yellow) and salicylic acid (SA; blue), with interactions on the protein or transcriptional level (solid and dashed lines, respectively). Interactions can activate (arrow) or inhibit (circle) downstream signalling events. Blue lines denote treatments of the functional validation (ET, INA, 1-MCP) or NahG plants (deficient in SA signalling). The red dashed arrow denotes our tested hypothesis. **B**, Plants belonging to potato cv. Rywal and **C**, its transgenic line NahG-Rywal were treated with ETs (yellow), INA (SA analogue; blue) or a combination of INA with 1-MCP (ET inhibitor; INA+MCP; grey). Log2 fold changes in gene expression in treated and control plants are shown (*- $p<0.05$; n=3; Student's t-test) for ACO4 (ET signalling marker), PR1b (SA signalling marker) and NPR1 (SA signal transmitter). **D**, Arabidopsis thaliana Col-0 wt and ein3-1 mutant were treated with INA or INA+MCP, and log2 fold change in gene expression in treated and control plants for NPR1 (*- $p<0.05$, n=6; Student's t-test) is shown. Error bars denote standard error of biological replicates. Note different y-axis scales for different genes. Results of the second independent potato experiment are provided in Supplemental Figure 3.

module crosstalk observed in non-transgenic plants was also confirmed in the SA-depleted plants.

The direct role of EIN3 in this crosstalk was evaluated using the Arabidopsis ET-insensitive mutant *ein3-1*. The results show that SA triggers expression of the NPR1 gene but that this induction is blocked in the *ein3-1* mutant and diminished if the activation of ETR receptor is blocked by 1-MCP (Figure 5D).

DISCUSSION

Plants have evolved a complex immune system to defend themselves against diverse pathogens and herbivores. This plant immune signalling network with its tightly interconnected signalling modules ensures a timely, precise and effective response to attackers (Coolen et al., 2016). Many regulatory mechanisms are buffered by the network, rendering them undetectable by traditional genetic approaches of single-gene null-mutant analyses (Hillmer et al., 2017), thus making network modelling algorithms invaluable tools to expand our understanding of plant immunity (Windram and Denby, 2015).

An ideal network model would encompass all components of the biological system and all interactions between them. However, due to limited knowledge and data availability, this is currently not possible. To circumvent this problem, researchers studying plant immune signalling have adopted various approaches. Bottom-up approaches based on manual literature curation lead to detailed and accurate models (Naseem et al., 2012; Miljkovic et al., 2012), but their extensiveness is limited. However, the majority of published research builds on networks inferred entirely from experimental data (e.g. Ebrahim et al., 2016; Vermeirssen et al., 2014), or a combination of network inference with prior knowledge (e.g. Jiang et al., 2016; Sabaghian et al., 2015), which can substantially simplify the computational burden of network inference (Windram and Denby, 2015).

In contrast to other efforts of prior knowledge integration (Dai et al., 2016), in our study, we have based our knowledge network on the manually built, highly reliable model of plant immune signalling (Miljkovic et al., 2012) and complemented it with data from various publicly available databases or datasets for Arabidopsis which were published as supplements of manuscripts. Therefore, our results represent the most current and comprehensive knowledge network of immune signalling and related processes in Arabidopsis (see Data availability section of Methods). Compared to data for immune signalling in this model species, those for immune signalling in crop species are much sparser; therefore, the translation of knowledge is essential for crop resistance breeding. It has been shown that knowledge can be transferred cross-species based on orthology, with higher reliability transfer between related species, whereas translation from di- to monocotyledons is less predictive (Lee et al., 2015b). Molecular network rewiring leads to functional divergence and thus plays a central role in speciation (Chae et al., 2012). The speed of network rewiring depends on several factors, including the type of interaction, with transcriptional regulatory networks having one of the fastest rewire speeds (Shou et al., 2011). However, functional modules often experience evolutionary cohesiveness (Chae et

al., 2012), which can be a basis for network translation, as in our case. Because of the sparsity of network data for potato, any new information from these evolutionarily conserved modules alone is extremely valuable. We have translated the AtCKN to StCKN and subsequently inferred networks from time-resolved experimental data on potato–virus interaction. To alleviate unknowns arising from speciation and/or evolution related events and to include the dynamics of potato response to PVY, we applied different methods to construct both co-expression and gene regulatory networks (Figure 1). Such ensemble solutions have been shown to match or outperform single methods, particularly in revealing the true underlying network structure (Vermeirssen et al., 2014).

To assess the validity of our approach, we compared interactions covered by different layers of biological information against a gold standard, i.e. a set of reactions from the manually curated plant immune signalling model (Table 1). We show a coverage of 58% of known interactions in the newly built network. Biologically more relevant, our integrated network approach predicted 142 additional connections between components of the manually built PIS model, which shows the potential of our integrated network approach in generating new testable hypotheses about biological systems (Figure 3).

As the comparison of the PIS model with other knowledge sources revealed low coverage of connections between signalling modules in the literature (Figure 3; Amar and Shamir, 2014), we tested the power of our newly built model to identify crosstalk connections. Intuitively, a regulatory pathway is unlikely to repeatedly pass a node. Given that shortest paths are only a subset of simple paths in graph theory, they do not contain any repeated nodes and could hence represent the most optimal explanation of interdependence between two analysed nodes (Shih and Parthasarathy, 2012). Walks of specific length become of use in the case of between-species translations, which was also the case in our potato network searches. We have indeed discovered an interesting novel transcriptional regulation connection between ET and SA signalling modules, where EIN3 regulates the transcription of the NPR1 gene (Figure 4). Further, we confirmed this predicted mechanism of crosstalk by performing both *in silico* promoter analysis (Supplemental Data Set 4) and a set of experiments in potato and Arabidopsis (Figure 5; Supplemental Figure 3), showing that short paths and walks from network analysis allow for discovery of the underlying signalling pathways.

Evidence on the importance of ET in plant immune signalling is emerging from several perspectives (Broekgaarden et al., 2015). For example, ET biosynthesis was found to be crucial for induction of programmed cell death during the interaction of *Nicotiana umbratica* with *Alternaria alternata* (Mase et al., 2012) and *Pseudomonas syringae*-triggered

Arabidopsis susceptibility to herbivory (Groen et al., 2013). Several experiments have shown that SA can modulate ET signalling (Caarls et al., 2016; Guan et al., 2015; Zander et al., 2014; Van der Does et al., 2013). This regulation is, in most cases, implicated in the context of JA/SA antagonism (Caarls et al., 2015; Derksen et al., 2013; Robert-Seilaniantz et al., 2011). Only a few studies indicate that ET might be an important regulator of SA signalling. It was shown that EIN3 transcription factors directly target the promoter of ICS2, negatively regulating SA biosynthesis (Chen et al., 2009). On the other hand, Frye et al. (2001), Mikkelsen (2003) and Leon-Reyes et al. (2009) have shown ET potentiation of PR1 gene expression in Arabidopsis. We observed the same effects in our potato experiments (Figure 5). Chromatin immunoprecipitation sequencing of EIN3 targets revealed NPR3 promoter as its direct target (Chang et al., 2013), providing further evidence that the two modules are connected.

Most studies involving NPR1 as a master regulator of SA signalling have focused on its posttranslational modifications or loss-of-function effects. ET modulation of NPR1 role in JA/SA antagonism was studied by performing a series of experiments with the *npr1* mutant, which did not allow identification of the effects of transcriptional, translational and posttranslational regulation (Leon-Reyes et al., 2009). Further studies showed the importance of proteasome-mediated degradation of NPR1 (Ding et al., 2016; Saleh et al., 2015; Fu et al., 2012) and nuclear import (Lee et al., 2015a; Kovacs et al., 2015; Fu et al., 2012) for effective SA perception. To our knowledge, no study performed so far has focused on the regulation of NPR1 gene expression.

Detailed inspection of our integrated network shows several potential transcription regulation paths from EIN3 to NPR1 (Figure 4B), which involve a cascade of one or two transcription factors. Some of the transcription factors are well-characterised (ERF and WRKY), while some were not investigated in detail (ATL27 or bHLH), or at least not in relation to immune signalling (MYB). As our *in silico* analyses of the NPR1 promoter identified WRKY and MYB transcription factor-binding motifs, these are the most likely candidates for signal transduction. Considering different experiments, including ours, we reason, that apart from regulation of NPR1 activity on the protein level, transcriptional regulation of the NPR1 gene also contributes to immune signalling in plants.

CONCLUSIONS

We conclude that integration of prior knowledge and experimental datasets followed by network modelling is useful for hypothesis generation, as suggested previously (Medeiros et al., 2015). Network analysis results thus help us to understand the complex interactions and the information flow between a causal and affected gene within a system of interest. However, one must note that, while network analysis is useful in helping us understand the organisation and information-processing capabilities of the system, its results are still a static view of the system (Chae et al., 2012). Additionally, connectivity between components does not automatically imply that signals are propagated through them. In order to understand how this organisation enables differential responses based on particular trigger, changes in time and space after receiving the stimuli must be observed. Thus, to understand all emerging properties of immune signalling, network analysis should be combined with dynamic modelling.

METHODS

Network-based knowledge integration

First, a previously inferred plant immune signalling model (Miljkovic et al., 2012) was upgraded by adding manually curated reactions from the recent literature (forming PIS-v2). Further, we transformed the model reactions to a graph of binary interactions, forming the first knowledge layer. Next, we retrieved additional binary connections from different public resources representing additional knowledge layers (full descriptions in Supplemental Table 1); protein-protein interactions (PPI) from databases AtPIN and STRING-v10, two yeast two-hybrid experiments and three experiments on plant-pathogen interactions; transcriptional regulation (TR) from atRegNet and ATRM, two ChIP-Seq experiments and one predicted dataset; regulation through miRNA (miRNA) from miRTarBase, PMRD and PNRD.

These prior knowledge layers were integrated into the *Arabidopsis thaliana* comprehensive knowledge network (AtCKN; Figure 1A). Reliability ranks were assigned to each connection between two nodes in AtCKN (Supplemental Table 1). To translate the network from Arabidopsis to potato, a union of three orthologue clusterings was used (available in the GoMapMan database as OCD_all (Ramšak et al., 2014; www.gomapman.org/exports/). Only connections where both Arabidopsis nodes had a defined orthologue in potato were kept in the *Solanum tuberosum* comprehensive knowledge network (StCKN; Figure 1B).

Network inference from experimental datasets

Two microarray datasets profiling temporal response dynamics in potato genotypes with a tolerant (GEO:GSE58593; Stare et al., 2015) or hypersensitive (GEO:GSE46180, Baebler et al., 2014) response to viral infection were used. Microarray features (microarray probes) were translated to potato gene models (Ramšak et al., 2014). For potato genes covered by several microarray features, one was selected as representative microarray feature based on the maximum number of differentially expressed time points per feature between mock-inoculated and viral-infected samples (FDR corrected p-values <0.05). When several such features were present, the ones with highest log fold change (logFC) and highest average expression across time points were prioritized.

Three network inference methods were applied to the gene expression data. Non-targeted co-expression networks were inferred with BioLayout (Theocharidis et al., 2009), afterwards

running the Markov clustering algorithm (Van Dongen, 2008) to divide the graph into discrete subsets. Pearson correlation coefficients (PCCs) were calculated on gene expression of representative microarray features for 156 PIS-v2 potato genes and their 2548 first neighbours in StCKN (PCC ≥ 0.98, top 1 percentile). Targeted co-expression networks were inferred with CoExpNetViz (Tzfadia et al., 2016), calculating co-expression values using mutual information and PCCs (percentiles set between 1 and 99). As bait, 156 potato genes of PIS-v2 were used against all 17,171 representative microarray features. Inference with Genie3 (Huynh-Thu et al., 2010) was performed on the same subset of microarray features as for non-targeted co-expression network inference (weight ≤ 6×10^{-3}, top 1 percentile). Thresholds for BioLayout and GENIE3 were determined empirically, so that the resulting networks followed the scale-free and small-world properties of complex networks. Each method was used to generate a mock-inoculated and a viral-infected subnetwork, from data for 16 biological samples each (Figure 1C). By removing any connections shared between networks from both treatments (mock-inoculated and viral-infected), a differential network was created for each inference type. Finally, binary interactions in StCKN and both differential networks were merged to create the *Solanum tuberosum* integrated network (StIN).

Validation of the network construction approach

To assess and estimate the importance and contributions of various knowledge layers in StIN, a gold standard (set of reliable connections) was constructed from manually curated PIS-v2. Genes were grouped into so-called component families (see Miljkovic et al. (2012) for representation levels), and connections compared at this level of abstraction. We kept 37 reactions, where all components had both an Arabidopsis and a potato orthologue. StCKN prior knowledge layers (PPI, TR, miRNA) and differential networks were then compared against the gold standard.

Network analyses

NetworkAnalyzer (Doncheva et al., 2012) was applied to calculate graph indices and MCODE (Bader and Hogue, 2003) to search for highly interconnected subgraphs in the constructed networks. Pajek (Batagelj and Mrvar, 1998) was applied to search for shortest paths and walks between all combinations of 14 manually selected genes known to be involved in plant signalling (specifically receptors and transmitters for seven plant hormones (Supplemental Table 2). For all network visualisations, Cytoscape (Shannon et al., 2003) was used.

In silico promoter analyses

Sequences 1500 nt upstream of the NPR1 gene translation start site were extracted for Arabidopsis (Araport 11) and potato (SpudDB) and scanned for known *cis*-regulatory elements with TRANSFAC (Matys, 2003) and PlantCARE (Lescot et al., 2002).

Plant growth and treatments

Potato (*Solanum tuberosum* L.) cv. Rywal and its transgenic line NahG-Rywal, which is deficient in SA accumulation (by expressing SA hydroxylase; (Baebler et al., 2014)) were cultivated as previously described (Baebler et al., 2009). *Arabidopsis thaliana* (L.) Heynh ecotype Columbia (Col-0) wild-type (wt) and mutant *ein3-1*, with reduced responsiveness to ethylene (Chao et al., 1997; TAIR germplasm ID CS8052), were grown in soil under long-day conditions as described for potato. Treatments were performed on 4-week-old plants. For SA treatments, plants were sprayed with 300 µM INA (2,6-dichloroisonicotinic acid, Aldrich, St Louis, Missouri, USA) dissolved in ethanol; control plants were sprayed with 1% ethanol solution. For ET treatments, plants, sealed in airtight clear plastic containers, were treated with 50 ppm ET (Messer, Bad Soden am Taunus, Germany); control plants were sealed in identical containers without ET. To inhibit the ET signalling pathway, plants were first treated with SmartFresh (containing 0.14% 1-methylcyclopropene, 1-MCP; AgroFresh, Inc., Philadelphia, Pennsylvania, USA) according to the manufacturer's protocol; this was followed by INA and 1-MCP treatment after 2 h. Plant leaves were sampled 24 h after treatment and immediately frozen in liquid nitrogen (3–6 plants per treatment and genotype). The experiment was performed twice for potato and once for Arabidopsis.

Gene expression analysis

Leaf samples (~100 mg) were homogenized with the FastPrep Instrument (MP Biomedicals, Santa Ana, California, USA). Total RNA extraction, DNAse treatment, RNA quality control and reverse transcription were performed as previously described (Baebler et al., 2009). For potato, expression was measured using high-throughput quantitative PCR (qPCR) for nonexpresser of PR genes 1 (NPR1), pathogenesis-related protein 1b (PR1b) and aminocyclopropanecarboxylate oxidase 4 (ACO4) genes. The cytochrome oxidase (COX) and elongation factor 1 (EF-1) genes were used as endogenous controls. TATAA PreAmp GrandMaster® Mix (TATAA Biocenter AB, Göteborg, Sweden) was used for cDNA pre-amplification (2 dilutions per sample) according to the manufacturer's specifications. Gene expression analysis of the samples was conducted in Fluidigm BioMark™ HD System Real-

Time PCR (Fluidigm, San Francisco, California, USA) using 48.48 Dynamic Arrays IFC. The sample reaction mix contained pre-amplified sample DNA (10-fold diluted), DNA Sample Loading Reagent (Fluidigm, San Francisco, California, USA) and FastStart Universal Probe Master (Rox; Roche, Basel, Switzerland). The assay reaction mix included the Assay Loading Reagent (Fluidigm, San Francisco, California, USA) and a mix of 2.5 µM TaqMan probe and 9 µM forward and reverse primers. IFC Controller (Fluidigm, San Francisco, California, USA) was used to prime and load the IFC according to the manufacturer's protocol and under standard PRC reaction conditions. A second independent potato experiment was performed and the expression of the same genes was analysed using QuantStudio 7 Flex Real-Time PCR System (ThermoFisher Scientific, Waltham, Massachusetts, USA). Reactions were set as described before (Baebler et al., 2014). For *A. thaliana* samples, expression of NPR1 was analysed and normalized to the expression of COX as described above for the second potato experiment. Detailed information on all qPCR assays performed is presented in Supplemental Table 3. For relative gene expression quantification using standard curve, quantGenius (Baebler et al., 2017; http://quantgenius.nib.si) was used. To determine differences in gene expression between treated and control sample groups, Student's *t*-test was performed.

Data Availability

Microarray transcriptomics data are available from Gene Expression Omnibus (GSE58593, GSE46180). The AtCKN network is available from NDEx (Pratt et al., 2015; http://www.ndexbio.org/#/) with the uuid 67507c30-995f-11e7-a10d-0ac135e8bacf.

Accession Numbers

Sequence data from this article can be found in the NCBI Gene data library under Gene ID 842733 (AT1G64280; AtNPR1), XM_006357647 (Sotub07g011600.1.1; StNPR1), NM_001288166 (Sotub09g006090.1.1, StPR1b).

Supplemental data

Supplemental Figure 1: Comparison of predicted connections for three selected network inference algorithms.

Supplemental Figure 2: Shortest path search from EIN3 to NPR1 in *Solanum tuberosum* integrated network (StIN).

Supplemental Figure 3: Results of the replicated experiment for validation of direct transcriptional regulation of NPR1 by the ethylene (ET) signalling module in potato leaves.

Supplemental Table 1: Contribution of four knowledge layers to the built *Arabidopsis thaliana* comprehensive knowledge network (AtCKN).

Supplemental Table 2: List of selected plant hormone pathway receptors and transmitters.

Supplemental Table 3: Primers and probes used for functional validation in Arabidopsis and potato and their properties according to MIQE guidelines.

Supplemental Data Set 1: Plant immune signalling model, version 2 (PIS-v2).

Supplemental Data Set 2: Comparison of contributions in the PIS model and AtCKN subnetwork.

Supplemental Data Set 3: Gene connections for network analysis results between EIN3 and NPR1.

Supplemental Data Set 4: Results of in silico regulatory element search for AtNPR1 and StNPR1.

ACKNOWLEDGMENTS

This work was supported by grants from the Slovenian Research Agency (P4-0165, J4-7636, J7-7303, N4-0026). We thank Katja Stare and Lidija Matičič for excellent technical assistance.

TABLES

Table 1: *Solanum tuberosum* integrated network (StIN) validation by comparing connection existence in selected data layers against the gold standard. Connections grouped on their reaction effect types (activation, binding, inhibition) and their interaction type (B – protein binding, T – transcriptional regulation). Compared layers include partial *Solanum tuberosum* comprehensive knowledge network (StCKN) (protein-protein interaction, PPI; transcriptional regulation, TR) and two differential networks (merged targeted and non-targeted co-expression and gene regulatory). Coverage of a gold-standard reaction (on the component family level) is indicated by + (reaction present in the layer), +n (reaction present in the layer, but not in the gold standard), – (reaction not present in the layer) or n.a. (not a relevant comparison, e.g. transcriptional regulation cannot validate a protein-binding connection). Co-expression results were not included in validation as they represent co-regulation of genes and not transcriptional regulation.

Gold-standard connections				Validation of StIN			
Group	**Node1**	**Node2**	**Type**	**PPI**	**TR**	**Co-expression**	**Gene regulatory**
Activation	MPK	LOX	B	–	n.a.	n.a.	n.a.
	MPK3	EDS1/PAD4	B	–	n.a.	+	n.a.
	MPK6	EDS1/PAD4	B	–	n.a.	n.a.	n.a.
	EIN2	EIN2	B	+	n.a.	n.a.	n.a.
	EIN2	EIN3(like)	B	–	n.a.	n.a.	n.a.
	RTE1	CTR	B	–	n.a.	n.a.	n.a.
	EDS1/PAD4	EDS5	B	–	n.a.	n.a.	n.a.
	EDS5	ICS	B	–	n.a.	+	n.a.
	NPR	MOS	B	–	n.a.	n.a.	n.a.
	MYC	PR3	T	n.a.	+	–	–
	MYC	PR4	T	n.a.	+	–	–
	EIN3(like)	EBF	T	n.a.	+	+	+
	EIN3(like)	ERF/EDF	T	n.a.	–	–	–
	EIN3(like)	PR3	T	n.a.	+	–	–
	EIN3(like)	PR4	T	n.a.	+	–	–
	ERF/EDF	PDF1.2	T	n.a.	–	–	–
	MYC	JAZ	T	n.a.	+	–	–
	MYC	LOX	T	n.a.	+	+	+
	TGA	PR1	T	n.a.	+	–	–
Protein complex formation	CTR	ETR	B	+	n.a.	n.a.	+n
	COI1	SCF	B	+	n.a.	n.a.	n.a.
	EBF	SCF	B	+	n.a.	n.a.	n.a.
	RBX	CUL	B	+	n.a.	n.a.	n.a.
	EDS1	PAD4	B	+	n.a.	n.a.	n.a.
	GSNO	NPR	B	–	n.a.	n.a.	n.a.
	NPR	TGA	B	+	n.a.	+n	+n
Inhibition	JAZ	EIN3(like)	B	–	n.a.	n.a.	n.a.
	EIN5	EBF	B	–	n.a.	+	n.a.
	ETR/CTR	EIN2	B	+	n.a.	n.a.	n.a.
	JAM	MYC	B	–	n.a.	n.a.	n.a.
	JAZ	MYC	B	+	n.a.	n.a.	n.a.
	COI1	JAZ	B	+	n.a.	n.a.	n.a.
	EBF	EIN3(like)	B	+	n.a.	+	n.a.
	NIMIN	NPR	B	+	n.a.	n.a.	n.a.
		Confirmed reactions (%)		50	80	20	
		Novel reactions (No.)		0	2	2	

FIGURE LEGENDS

Figure 1: Schematic overview of networks construction and analyses. **A**, Networks of four prior knowledge layers were merged into the *Arabidopsis thaliana* comprehensive knowledge network (AtCKN). **B**, Orthologous relationships were used to translate from Arabidopsis to potato, forming the *Solanum tuberosum* comprehensive knowledge network (StCKN). **C**, Starting with two time-resolved transcriptome datasets, gene co-expression networks (GCNs; relationships between co-expressed genes) and gene regulatory networks (GRN; transcription-factor-to-regulated-gene relationships) were inferred using three methods. For each inference method, two subnetworks were generated for mock-inoculated and viral-infected samples. Removing all connections present in all co-expression or gene regulatory networks resulted in differential networks. **D**, StCKN and differential networks were merged into the potato integrated network (StIN). **E**, The created networks were analysed using network analysis approaches. PIS-v2 – plant immune signalling model; PPIs – protein-protein interactions; TR – transcriptional regulation; miRNA – regulation via miRNA.

Figure 2: Contribution of the four layers to node coverage in *Arabidopsis thaliana* comprehensive knowledge network (AtCKN). Overlap display between the four layers contributing to AtCKN. PIS-v2 – plant immune signalling model; PPIs – protein-protein interactions; TR – transcriptional regulation; miRNA – regulation via miRNA.

Figure 3: Connections between gene families of three plant hormone immune signalling pathways (ethylene, jasmonic acid, salicylic acid) and other signalling modules. Contribution of the plant immune signalling model (PIS-v2) layer in contrast to that of the remaining AtCKN layers (PPI, protein-protein interaction; TR, transcriptional regulation; miRNA) in terms of **A**, connections between signalling modules (i.e. crosstalk connections) and **B**, connections within a signalling module. Solid blue lines – novel connections present in PPI, TR and miRNA layers of AtCKN; solid grey lines – connections present only in the PIS-v2 layer; dotted grey lines – connections existing in both compared subsets.

Figure 4: Results of shortest paths or walks from EIN3 (ET) towards NPR1 (SA). **A**, Shortest path (3-steps) in AtCKN; **B**, walk of length 3 in StCKN; **C**, shortest path (2-steps) in StCKN. Line type and colour indicate the interaction type: binding (solid grey), regulation by transcription factors (dashed grey), co-expression (dotted blue) and gene regulatory (solid blue). Target arrow indicates the action of the connection: activatory (arrow), inhibitory (T),

unknown (circle) or undirected in the case of binding or co-expression (no arrow). Gene group identifiers corresponding to these images are given in Supplemental Data Set 3.

Figure 5: Validation of direct transcriptional regulation of NPR1 by the ethylene (ET) signalling module in potato leaves. **A**, Schema of the underlying biological pathways of ET (yellow) and salicylic acid (SA; blue), with interactions on the protein or transcriptional level (solid and dashed lines, respectively). Interactions can activate (arrow) or inhibit (circle) downstream signalling events. Blue lines denote treatments of the functional validation (ET, INA, 1-MCP) or NahG plants (deficient in SA signalling). The red dashed arrow denotes our tested hypothesis. **B**, Plants belonging to potato cv. Rywal and **C**, its transgenic line NahG-Rywal were treated with ETs (yellow), INA (SA analogue; blue) or a combination of INA with 1-MCP (ET inhibitor; INA+MCP; grey). Log_2 fold changes in gene expression in treated and control plants are shown (*- $p<0.05$; n=3; Student's *t*-test) for ACO4 (ET signalling marker), PR1b (SA signalling marker) and NPR1 (SA signal transmitter). **D**, *Arabidopsis thaliana* Col-0 wt and *ein3-1* mutant were treated with INA or INA+MCP, and log_2 fold change in gene expression in treated and control plants for NPR1 (*- $p<0.05$, n=6; Student's *t*-test) is shown. Error bars denote standard error of biological replicates. Note different y-axis scales for different genes. Results of the second independent potato experiment are provided in Supplemental Figure 3.

Parsed Citations

Amar, D. and Shamir, R. (2014). Constructing module maps for integrated analysis of heterogeneous biological networks. Nucleic Acids Res. 42: 4208–4219.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Bader, G.D. and Hogue, C.W.V. (2003). An automated method for finding molecular complexes in large protein interaction networks. BMC Bioinformatics 4: 27.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Baebler, Š. et al. (2014). Salicylic acid is an indispensable component of the Ny-1 resistance-gene-mediated response against Potato virus Y infection in potato. J. Exp. Bot. 65: 1095–1109.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Baebler, Š., Krečič-Stres, H., Rotter, A., Kogovšek, P., Cankar, K., Kok, E.J., Gruden, K., Kovač, M., Žel, J., Pompe-Novak, M., and Ravnikar, M. (2009). PVYNTN elicits a diverse gene expression response in different potato genotypes in the first 12 h after inoculation. Mol. Plant Pathol. 10: 263–275.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Baebler, Š., Svalina, M., Petek, M., Stare, K., Rotter, A., Pompe-Novak, M., and Gruden, K. (2017). quantGenius: implementation of a decision support system for qPCR-based gene quantification. BMC Bioinformatics 18: 276.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Barabási, A.-L. (2009). Scale-Free Networks: A Decade and Beyond. Science (80-.). 325: 412–413.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Batagelj, V. and Mrvar, A. (1998). Pajek – Program for Large Network Analysis. Connections 21: 47–57.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Blüthgen, N. (2015). Signaling output: it's all about timing and feedbacks. Mol. Syst. Biol. 11: 843–843.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Broekgaarden, C., Caarls, L., Vos, I.A., Pieterse, C.M.J., and Van Wees, S.C.M. (2015). Ethylene: traffic controller on hormonal crossroads to defense. Plant Physiol. 169: 2371–2379.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Caarls, L., Van der Does, D., Hickman, R., Jansen, W., Van Verk, M.C., Proietti, S., Lorenzo, O., Solano, R., Pieterse, C.M.J., and Van Wees, S.C.M. (2016). Assessing the Role of ETHYLENE RESPONSE FACTOR Transcriptional Repressors in Salicylic Acid-Mediated Suppression of Jasmonic Acid-Responsive Genes. Plant Cell Physiol. 58: 266–278.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Caarls, L., Pieterse, C.M.J., and Van Wees, S.C.M. (2015). How salicylic acid takes transcriptional control over jasmonic acid signaling. Front. Plant Sci. 6: 170.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Chae, L., Lee, I., Shin, J., and Rhee, S.Y. (2012). Towards understanding how molecular networks evolve in plants. Curr. Opin. Plant Biol. 15: 177–184.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Chang, K.N. et al. (2013). Temporal transcriptional response to ethylene gas drives growth hormone cross-regulation in Arabidopsis. Elife 2013: 1–20.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997). Activation of the ethylene gas respons pathway in Arabidopsis by the nuclear protein ethylene insensitive 3 and related proteins. Cell 89: 1133–1144.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Chen, H., Lai, Z., Shi, J., Xiao, Y., Chen, Z., and Xu, X. (2010). Roles of arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress. BMC Plant Biol. 10: 281.
Pubmed: Author and Title

Google Scholar: Author Only Title Only Author and Title

Chen, H., Xue, L., Chintamanani, S., Germain, H., Lin, H., Cui, H., Cai, R., Zuo, J., Tang, X., Li, X., Guo, H., and Zhou, J.-M. (2009). ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 Repress SALICYLIC ACID INDUCTION DEFICIENT2 Expression to Negatively Regulate Plant Innate Immunity in Arabidopsis. Plant Cell 21: 2527–2540.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Coolen, S. et al. (2016). Transcriptome dynamics of Arabidopsis during sequential biotic and abiotic stresses. Plant J. 86: 249–267.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Dai, X., Li, J., Liu, T., and Zhao, P.X. (2016). HRGRN: A Graph Search-Empowered Integrative Database of Arabidopsis Signaling Transduction, Metabolism and Gene Regulation Networks. Plant Cell Physiol. 57: e12(1-7).
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Derksen, H., Rampitsch, C., and Daayf, F. (2013). Signaling cross-talk in plant disease resistance. Plant Sci. 207: 79–87.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Ding, Y., Dommel, M., and Mou, Z. (2016). Abscisic acid promotes proteasome-mediated degradation of the transcription coactivator NPR1 in Arabidopsis thaliana. Plant J. 86: 20–34.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Van der Does, D., Leon-Reyes, A., Koornneef, A., Van Verk, M.C., Rodenburg, N., Pauwels, L., Goossens, A., Körbes, A.P., Memelink, J., Ritsema, T., Van Wees, S.C.M., and Pieterse, C.M.J. (2013). Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. Plant Cell 25: 744–61.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Doncheva, N.T., Assenov, Y., Domingues, F.S., and Albrecht, M. (2012). Topological analysis and interactive visualization of biological networks and protein structures. Nat. Protoc. 7: 670–85.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Van Dongen, S. (2008). Graph Clustering Via a Discrete Uncoupling Process. SIAM J. Matrix Anal. Appl. 30: 121–141.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Ebrahim, A., Brunk, E., Tan, J., O'Brien, E.J., Kim, D., Szubin, R., Lerman, J.A., Lechner, A., Sastry, A., Bordbar, A., Feist, A.M., and Palsson, B.O. (2016). Multi-omic data integration enables discovery of hidden biological regularities. Nat. Commun. 7: 13091.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Frye, C.A., Tang, D., and Innes, R.W. (2001). Negative regulation of defense responses in plants by a conserved MAPKK kinase. Proc. Natl. Acad. Sci. 98: 373–378.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Fu, Z.Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., Mohan, R., Spoel, S.H., Tada, Y., Zheng, N., and Dong, X. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature 486: 228–32.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Ghanbari, M., Lasserre, J., and Vingron, M. (2015). Reconstruction of gene networks using prior knowledge. BMC Syst. Biol. 9: 84.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Groen, S.C. et al. (2013). Pathogen-Triggered Ethylene Signaling Mediates Systemic-Induced Susceptibility to Herbivory in Arabidopsis. Plant Cell 25: 4755–4766.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Guan, R., Su, J., Meng, X., Li, S., Liu, Y., Xu, J., and Zhang, S. (2015). Multilayered Regulation of Ethylene Induction Plays a Positive Role in Arabidopsis Resistance against Pseudomonas syringae. Plant Physiol. 169: 299–312.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Hillmer, R.A. and Katagiri, F. (2016). Toward predictive modeling of large and complex biological signaling networks. Physiol. Mol. Plant Pathol. 95: 77–83.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Hillmer, R.A., Tsuda, K., Rallapalli, G., Asai, S., Truman, W., Papke, M.D., Sakakibara, H., Jones, J.D.G., Myers, C.L., and Katagiri, F. (2017). The highly buffered Arabidopsis immune signaling network conceals the functions of its components. PLoS Genet. 13: e1006639.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Huynh-Thu, V.A., Irrthum, A., Wehenkel, L., and Geurts, P. (2010). Inferring Regulatory Networks from Expression Data Using Tree-Based Methods. PLoS One 5: 1–10.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Jiang, Z., Dong, X., and Zhang, Z. (2016). Network-Based Comparative Analysis of Arabidopsis Immune Responses to Golovinomyces orontii and Botrytis cinerea Infections. Sci. Rep. 6: 19149.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Jones, J.D.G. and Dangl, J.L. (2006). The plant immune system. Nature 444: 323–329.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Kovacs, I., Durner, J., and Lindermayr, C. (2015). Crosstalk between nitric oxide and glutathione is required for NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1)-dependent defense signaling in Arabidopsis thaliana. New Phytol. 208: 860–872.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Lee, H.-J., Park, Y.-J., Seo, P.J., Kim, J.-H., Sim, H.-J., Kim, S.-G., and Park, C.-M. (2015a). Systemic Immunity Requires SnRK2.8-Mediated Nuclear Import of NPR1 in Arabidopsis. Plant Cell 27: 3425–3438.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Lee, T., Kim, H., and Lee, I. (2015b). Network-assisted crop systems genetics: Network inference and integrative analysis. Curr. Opin. Plant Biol. 24: 61–70.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Leon-Reyes, A., Spoel, S.H., De Lange, E.S., Abe, H., Kobayashi, M., Tsuda, S., Millenaar, F.F., Welschen, R.A.M., Ritsema, T., and Pieterse, C.M.J. (2009). Ethylene modulates the role of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 in cross talk between salicylate and jasmonate signaling. Plant Physiol. 149: 1797–809.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P., and Rombauts, S. (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res. 30: 325–327.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Mase, K., Mizuno, T., Ishihama, N., Fujii, T., Mori, H., Kodama, M., and Yoshioka, H. (2012). Ethylene Signaling Pathway and MAPK Cascades Are Required for AAL Toxin–Induced Programmed Cell Death. Mol. Plant-Microbe Interact. 25: 1015–1025.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Matys, V. et al. (2003). TRANSFAC(R): transcriptional regulation, from patterns to profiles. Nucleic Acids Res. 31: 374–378.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

McCormack, M.E., Lopez, J.A., Crocker, T.H., and Mukhtar, M.S. (2016). Making the right connections: Network biology and plant immune system dynamics. Curr. Plant Biol. 5: 2–12.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Medeiros, D.B., Daloso, D.M., Fernie, A.R., Nikoloski, Z., and Araújo, W.L. (2015). Utilizing systems biology to unravel stomatal function and the hierarchies underpinning its control. Plant. Cell Environ. 38: 1457–1470.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Mikkelsen, M.D., Petersen, B.L., Glawischnig, E., Jensen, A.B., Andreasson, E., and Halkier, B.A. (2003). Modulation of CYP79 Genes and Glucosinolate Profiles in Arabidopsis by Defense Signaling Pathways. Plant Physiol. 131: 298–308.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Miljkovic, D., Stare, T., Mozetič, I., Podpečan, V., Petek, M., Witek, K., Dermastia, M., Lavrač, N., and Gruden, K. (2012). Signalling Network Construction for Modelling Plant Defence Response. PLoS One 7: e51822.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Naseem, M., Philippi, N., Hussain, A., Wangorsch, G., Ahmed, N., and Dandekar, T. (2012). Integrated Systems View on Networking by Hormones in Arabidopsis Immunity Reveals Multiple Crosstalk for Cytokinin. Plant Cell 24: 1793–1814.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pieterse, C.M.J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S.C.M. (2012). Hormonal Modulation of Plant Immunity. Annu. Rev. Cell Dev. Biol. 28: 489–521.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pratt, D. et al. (2015). NDEx, the Network Data Exchange. Cell Syst. 1: 302–305.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Ramšak, Ž., Baebler, Š., Rotter, A., Korbar, M., Mozetič, I., Usadel, B., and Gruden, K. (2014). GoMapMan: integration, consolidation and visualization of plant gene annotations within the MapMan ontology. Nucleic Acids Res. 42: D1167–D1175.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Robert-Seilaniantz, A., Grant, M., and Jones, J.D.G. (2011). Hormone Crosstalk in Plant Disease and Defense: More Than Just JASMONATE-SALICYLATE Antagonism. Annu. Rev. Phytopathol. 49: 317–343.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Sabaghian, E., Drebert, Z., Inzé, D., and Saeys, Y. (2015). An integrated network of Arabidopsis growth regulators and its use for gene prioritization. Sci. Rep. 5: 17617.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Saleh, A., Withers, J., Mohan, R., Marqués, J., Gu, Y., Yan, S., Zavaliev, R., Nomoto, M., Tada, Y., and Dong, X. (2015). Posttranslational Modifications of the Master Transcriptional Regulator NPR1 Enable Dynamic but Tight Control of Plant Immune Responses. Cell Host Microbe 18: 169–182.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: A software Environment for integrated models of biomolecular interaction networks. Genome Res. 13: 2498–2504.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Shih, Y.-K. and Parthasarathy, S. (2012). A Single Source k-Shortest Paths Algorithm to Infer Regulatory Pathways in a Gene Network. Bioinformatics 28: i49–i58.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Shou, C., Bhardwaj, N., Lam, H.Y.K., Yan, K.K., Kim, P.M., Snyder, M., and Gerstein, M.B. (2011). Measuring the evolutionary rewiring of biological networks. PLoS Comput. Biol. 7.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Singh, H.P. (2008). Policies and strategies conducive to potato development in Asia and the Pacific region. In Workshop to Commemorate the International Year of Potato - 2008, M.K. Papademetriou, ed (Bangkok, Thailand), pp. 11–17.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Spoel, S.H., Mou, Z., Tada, Y., Spivey, N.W., Genschik, P., and Dong, X. (2009). Proteasome-Mediated Turnover of the Transcription Coactivator NPR1 Plays Dual Roles in Regulating Plant Immunity. Cell 137: 860–872.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Stare, T., Ramšak, Ž., Blejec, A., Stare, K., Turnšek, N., Weckwerth, W., Wienkoop, S., Vodnik, D., and Gruden, K. (2015). Bimodal dynamics of primary metabolism-related responses in tolerant potato-Potato virus Y interaction. BMC Genomics 16: 716.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Theocharidis, A., Van Dongen, S., Enright, A.J., and Freeman, T.C. (2009). Network visualization and analysis of gene expression data using BioLayout Express(3D). Nat. Protoc. 4: 1535–1550.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Tsuda, K. and Somssich, I.E. (2015). Transcriptional networks in plant immunity. New Phytol. 206: 932–947.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Tzfadia, O., Diels, T., De Meyer, S., Vandepoele, K., Aharoni, A., and Van de Peer, Y. (2016). CoExpNetViz: Comparative Co-Expression

Networks Construction and Visualization Tool. Front. Plant Sci. 6: 1–7.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Veiga, D.F.T., Dutta, B., and Balázsi, G. (2010). Network inference and network response identification: moving genome-scale data to the next level of biological discovery. Mol. Biosyst. 6: 469–480.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Verma, V., Ravindran, P., and Kumar, P.P. (2016). Plant hormone-mediated regulation of stress responses. BMC Plant Biol. 16: 86.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Vermeirssen, V., De Clercq, I., Van Parys, T., Van Breusegem, F., and Van de Peer, Y. (2014). Arabidopsis Ensemble Reverse-Engineered Gene Regulatory Network Discloses Interconnected Transcription Factors in Oxidative Stress. Plant Cell 26: 4656–4679.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Weigel, R.R., Pfitzner, U.M., and Gatz, C. (2005). Interaction of NIMIN1 with NPR1 modulates PR gene expression in Arabidopsis. Plant Cell 17: 1279–1291.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Windram, O. and Denby, K.J. (2015). Modelling signaling networks underlying plant defence. Curr. Opin. Plant Biol. 27: 165–171.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Xu, X., Chen, C., Fan, B., and Chen, Z. (2006). Physical and Functional Interactions between and WRKY60 Transcription Factors. Plant Cell 18: 1310–1326.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title

Zander, M., Thurow, C., and Gatz, C. (2014). TGA Transcription Factors Activate the Salicylic Acid-Suppressible Branch of the Ethylene-Induced Defense Program by Regulating ORA59 Expression. Plant Physiol. 165: 1671–1683.
Pubmed: Author and Title
Google Scholar: Author Only Title Only Author and Title