

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

3  
4 **Short title**

5 Potato Hormonal Signalling Network  
6

7 **One-sentence summary**

8 Analysis of integrated prior knowledge and ensemble networks highlights a previously  
9 unidentified connection between ethylene and salicylic acid signalling modules in potato.  
10

11 **Author Contributions**<sup>1</sup>  
12

13 **Funding Information**<sup>2</sup>  
14

15 **Authors**

16 Živa Ramsak,<sup>a,3</sup> Anna Coll,<sup>a</sup> Tjaša Stare,<sup>a</sup> Oren Tzfadia,<sup>b,c</sup> Špela Baebler,<sup>a</sup> Yves Van de  
17 Peer,<sup>b,c,d</sup> Kristina Gruden<sup>a</sup>  
18

19 <sup>a</sup> National Institute of Biology, Department of Biotechnology and Systems Biology, 1000  
20 Ljubljana, Slovenia

21 <sup>b</sup> Department of Plant Systems Biology, VIB, 9052 Ghent, Belgium

22 <sup>c</sup> Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent,  
23 Belgium

24 <sup>d</sup> Genomics Research Institute, University of Pretoria, Private bag X20, Pretoria 0028, South  
25 Africa  
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<sup>1</sup> 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.

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<sup>3</sup> Address correspondence to [ziva.ramsak@nib.si](mailto:ziva.ramsak@nib.si)

30 **ABSTRACT**

31

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

48

49 **INTRODUCTION**

50

51 Plants have evolved a multi-layered immune system to cope with potential invasion of  
52 pathogens (Jones and Dangl, 2006). Recognition of invading organisms triggers a rapid  
53 induction of signalling cascades, leading to diverse defence responses (Pieterse et al.,  
54 2012). Effectiveness of these downstream events is crucially dependent on salicylic acid  
55 (SA), jasmonic acid (JA) and ethylene (ET), but other hormones were also shown to play  
56 important roles in plant immunity (Verma et al., 2016). Hormonal signals differ considerably  
57 in timing, quantity and composition, depending on the type of attacker (Blüthgen, 2015).  
58 Crosstalk between hormonal pathways can have antagonistic or synergistic effects and is  
59 largely multi-dimensional (Tsuda and Somssich, 2015). This interconnected plant hormonal  
60 network provides an important regulatory mechanism, granting plants quick adaptation  
61 abilities via intruder-specific alterations (Pieterse et al., 2012). At the molecular level,  
62 crosstalk between signalling pathways with several regulatory feedback loops adds  
63 robustness to the plant immune signalling network (Windram and Denby, 2015). Network  
64 analysis—application of mathematical graph theory approaches—also continues to be  
65 paramount in systems biology investigations of complex systems (Barabási, 2009). This  
66 approach, which involves thorough analyses of critical system properties, facilitates

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

69

70 While heterogeneous technologies of high-content omics allow us to capture snapshots of  
71 the systems, the challenge now lies in integration of knowledge into a coherent systems view  
72 (Hillmer and Katagiri, 2016). Network inference from omics datasets allows us to deduce the  
73 underlying structure of activated processes. However, due to high noisiness, high  
74 dimensionality and low sample sizes of data, this is a non-trivial task (Veiga et al., 2010).  
75 Thus, additional improvements are needed; for example, incorporation of prior knowledge  
76 can greatly improve reconstructed network accuracy, simultaneously reducing noise and  
77 sparsity effects of the source data, without inflating the computational cost (Ghanbari et al.,  
78 2015).

79

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

96

97

98 **RESULTS**

99

100 **Construction of the comprehensive knowledge network**

101

102 Firstly, a previous plant immune signalling model (PIS-v1; Miljkovic et al., 2012) was  
103 expanded with manually curated knowledge from recently published literature. The addition  
104 of 64 *Arabidopsis* genes to the existing model resulted in an expanded PIS-v2 model with  
105 212 biological components (177 genes, 31 metabolites and 4 small RNAs), categorized into  
106 108 component families as defined by Miljkovic et al. (2012). Following the abstraction of the  
107 component families, we added 32 new reactions, with the total number of reactions reaching  
108 111 (Supplemental Data Set 1).

109

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

118

119 **Use of prior knowledge to improve the plant immune signalling model**

120

121 To assess the potential of using prior knowledge for the improvement of the mechanistic  
122 model of plant immune signalling, we extracted a subnetwork of AtCKN with all components  
123 of PIS-v2. This subnetwork consisted of 391 connections between 212 nodes in the fully  
124 expanded version or 254 connections between 108 nodes at the level of component families.  
125 By comparing the PIS-v2 layer against the remaining layers of AtCKN (PPI, TR, miRNA), we  
126 found that 45 connections were present in both subnetworks and 67 only in the PIS-v2, and  
127 142 novel reactions were found from the remaining AtCKN layers. These represent model  
128 upgrades, demonstrating the value of dispersed knowledge sources also for the construction  
129 of detailed mechanistic models. Inspecting these new connections showed that manual  
130 curation is more successful in knowledge extraction within a signalling module (Figure 3B)  
131 than between signalling modules (Figure 3A; Supplemental Data Set 2).

132

133 **Translation of knowledge to potato and integration with experimental data**

134

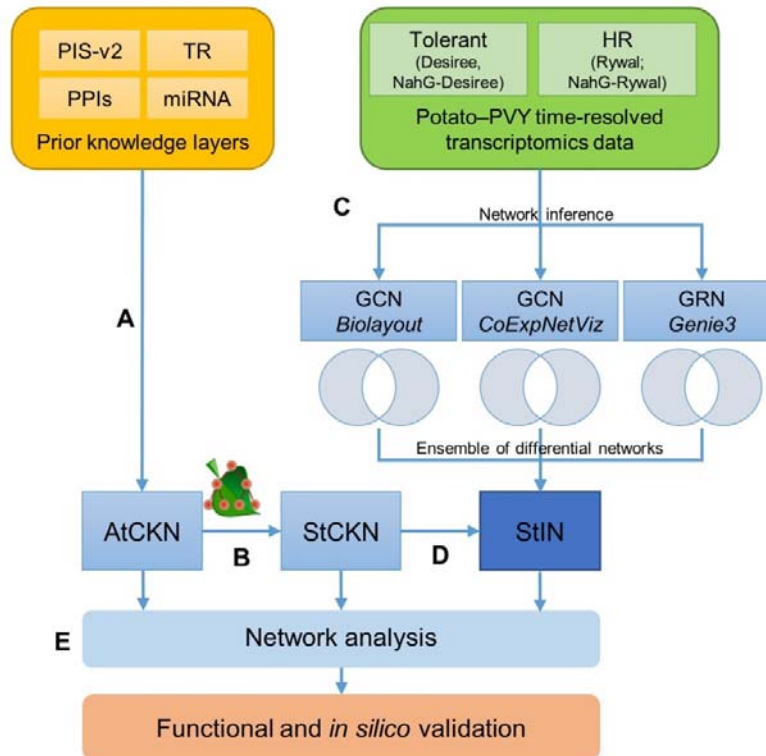


Figure 1: Schematic overview of networks construction and analyses. **A**, Networks of four prior knowledge layers were merged into the *Arabidopsis thaliana* comprehensive knowledge network (AtCKN). **B**, Orthologous relationships were used to translate from *Arabidopsis* to potato, forming the *Solanum 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.

135 Based on predictions of orthologous relationships, we translated AtCKN from *Arabidopsis* to  
 136 potato to form the *Solanum tuberosum* comprehensive knowledge network (StCKN). This  
 137 resulted in an intermediary abstracted network with 9679 nodes (9497 orthologue groups,  
 138 168 miRNA families, 3 metabolites, 11 viral proteins) and 43,393 connections. Next, we  
 139 inferred all combinations between potato genes of the same orthologue group for each  
 140 abstracted connection; this resulted in the StCKN (Figure 1B) having 18,036 nodes (17,855  
 141 genes, 168 miRNA families, 3 metabolites, 11 viral proteins) and 296,834 connections. This  
 142 expansion is the result of a many-to-many relationship of orthologous genes between  
 143 species. But it also includes cases where no homologous gene is found.

144

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

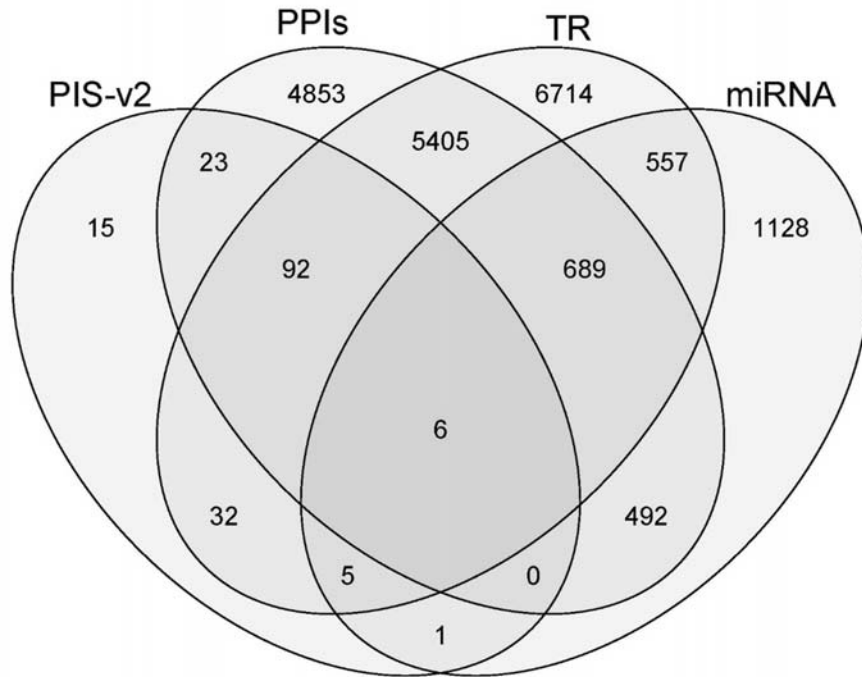


Figure 2: Contribution of the four layers to node coverage in *Arabidopsis thaliana* comprehensive knowledge network (AtCKN). Overlap display between the four layers contributing to AtCKN. PIS-v2 – plant immune signalling model; PPIs – protein-protein interactions; TR – transcriptional regulation; miRNA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

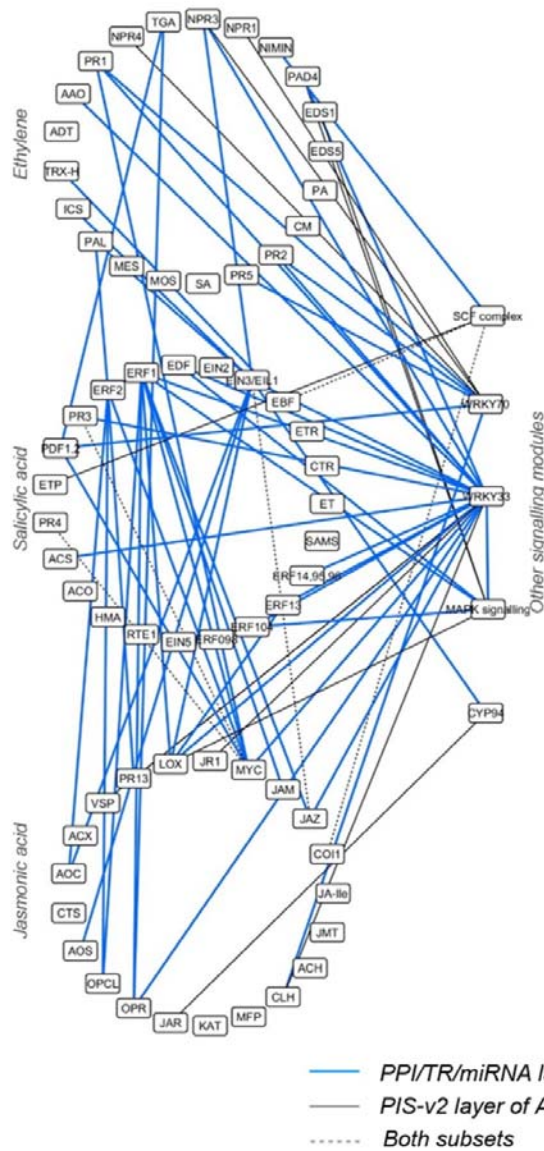
153

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

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



### A) Crosstalk connections



### B) Connections within a signalling module

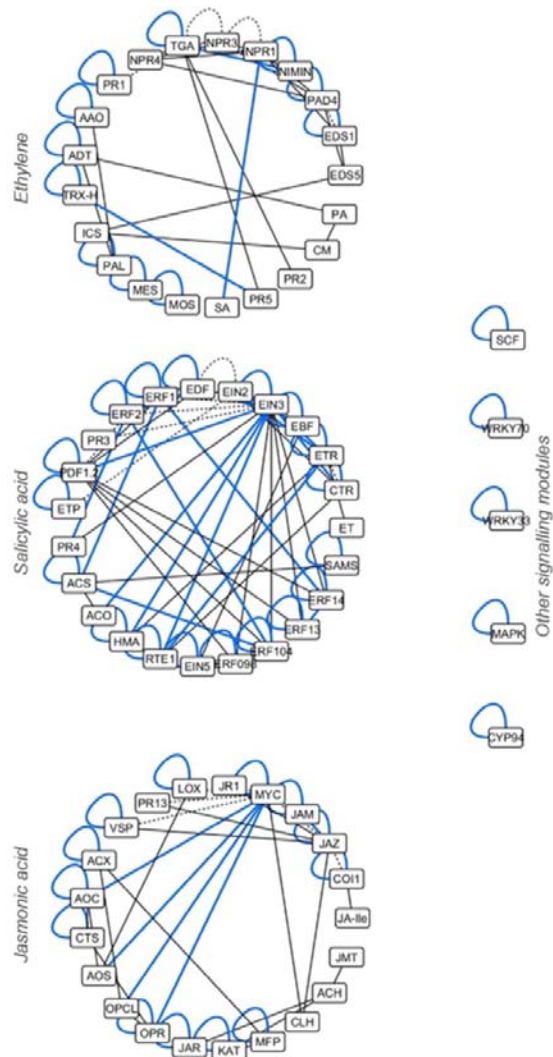


Figure 3: Connections between gene families of three plant hormone immune signalling pathways (ethylene, jasmonic acid, salicylic acid) and other signalling modules. Contribution of the plant immune signalling model (PIS-v2) layer in contrast to that of the remaining AtCKN layers (PPI, protein-protein 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.

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

168

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

172 *Solanum tuberosum* integrated network (StIN; Figure 1D) with 402,277 connections between  
173 19,801 nodes (19,619 genes, 168 miRNA families, 3 metabolites, 11 viral proteins).

174

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

183

#### 184 **Integrated network-driven hypotheses: Ethylene is modulating NPR1 gene** 185 **expression**

186

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

196

197 One of the most interesting findings was the shortest path from the ethylene pathway  
198 transmitter EIN3 to SA receptor NPR1. In AtCKN, we identified several shortest paths of 3-  
199 step length, involving 32 genes and 61 connections (Figure 4A; Supplemental Data Set 3).  
200 One-third of these were binding (PPI) connections, and the remainder, transcriptional  
201 regulations. In StCKN, we searched for walks (length 3) from EIN3 to NPR1, and then  
202 superimposed co-expression and gene regulatory network connections from StIN (Figure  
203 4B; Supplemental Data Set 3). The potato EIN3 to NPR1 walk subnetwork included 32  
204 genes, 57 StCKN and 48 experimentally inferred connections. Searching for walks of a  
205 specific length was required to ease comparisons, as the shortest path between EIN3 and  
206 NPR1 after translation was of 2-step length (Figure 4C).

207

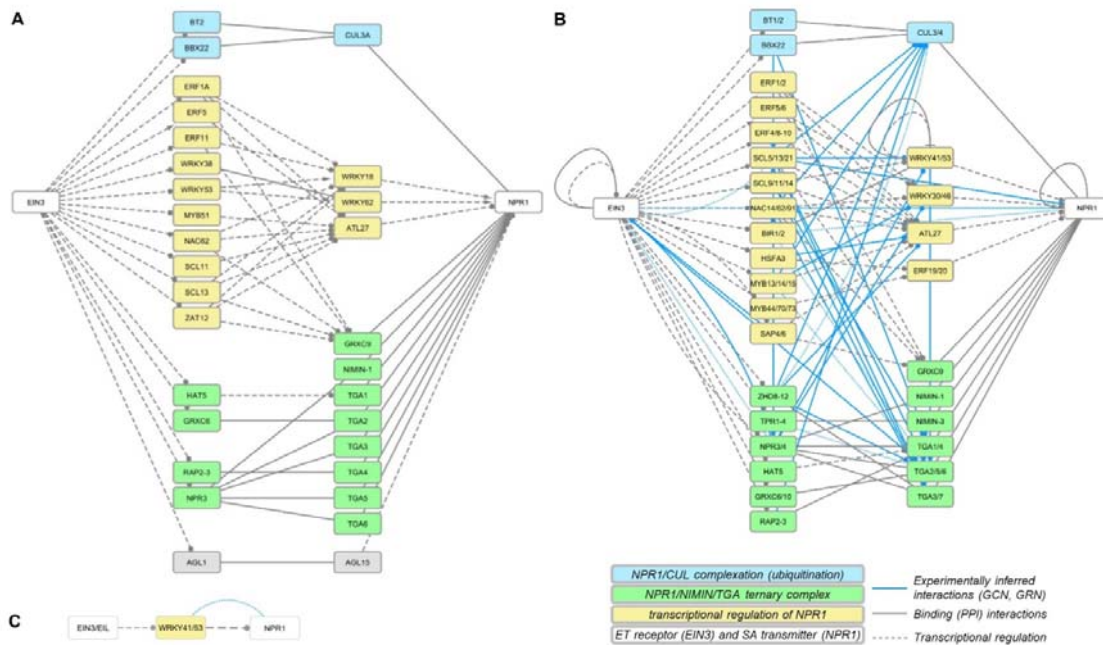


Figure 4: Results of shortest paths or walks from EIN3 (ET) towards NPR1 (SA). **A**, Shortest path (3-steps) in AtCKN; **B**, walk of length 3 in StCKN; **C**, shortest path (2-steps) in StCKN. Line type and colour indicate the interaction type: binding (solid grey), regulation by transcription factors (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.

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

222

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

226 (MYB13/14/15, MYB44/70/73) transcription factors (Figure 4B). Interestingly, when  
227 searching in a reduced StIN with only transcriptional layer connections, we predicted  
228 regulation of NPR1 through ERF (ERF2a), WRKY (WRKY34, WRKY41/53), MYB  
229 (MYB18/19, MYB52/54) and bHLH (bHLH84/84) transcription factors (Supplemental Figure  
230 2).

231

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

239

#### 240 **Experimental validation of transcriptional regulation of NPR1 by ET**

241

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

253

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

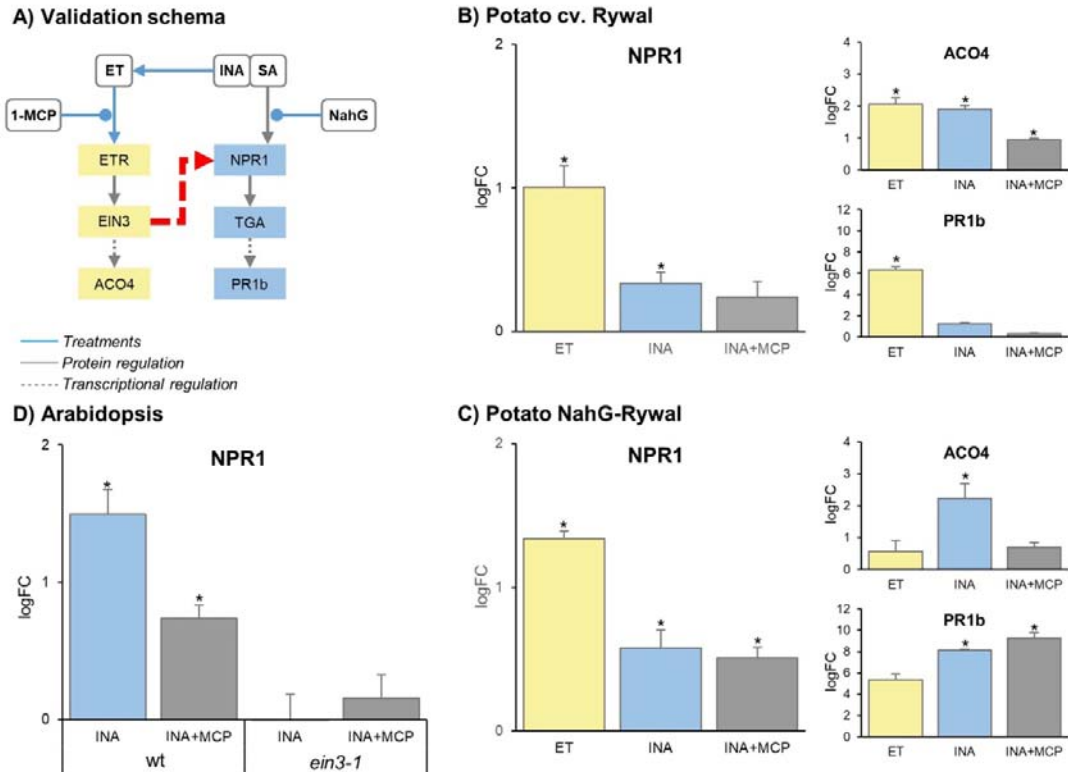


Figure 5: Validation of direct transcriptional regulation of NPR1 by the ethylene (ET) signalling module in potato leaves. **A**, Schema of the underlying biological pathways of ET (yellow) and salicylic acid (SA; blue), with interactions on the protein or transcriptional level (solid and dashed lines, 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). Log<sub>2</sub> 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<sub>2</sub> 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.

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

265

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

270

271

## 272 DISCUSSION

273  
274 Plants have evolved a complex immune system to defend themselves against diverse  
275 pathogens and herbivores. This plant immune signalling network with its tightly  
276 interconnected signalling modules ensures a timely, precise and effective response to  
277 attackers (Coolen et al., 2016). Many regulatory mechanisms are buffered by the network,  
278 rendering them undetectable by traditional genetic approaches of single-gene null-mutant  
279 analyses (Hillmer et al., 2017), thus making network modelling algorithms invaluable tools to  
280 expand our understanding of plant immunity (Windram and Denby, 2015).

281  
282 An ideal network model would encompass all components of the biological system and all  
283 interactions between them. However, due to limited knowledge and data availability, this is  
284 currently not possible. To circumvent this problem, researchers studying plant immune  
285 signalling have adopted various approaches. Bottom-up approaches based on manual  
286 literature curation lead to detailed and accurate models (Naseem et al., 2012; Miljkovic et al.,  
287 2012), but their extensiveness is limited. However, the majority of published research builds  
288 on networks inferred entirely from experimental data (e.g. Ebrahim et al., 2016; Vermeirssen  
289 et al., 2014), or a combination of network inference with prior knowledge (e.g. Jiang et al.,  
290 2016; Sabaghian et al., 2015), which can substantially simplify the computational burden of  
291 network inference (Windram and Denby, 2015).

292  
293 In contrast to other efforts of prior knowledge integration (Dai et al., 2016), in our study, we  
294 have based our knowledge network on the manually built, highly reliable model of plant  
295 immune signalling (Miljkovic et al., 2012) and complemented it with data from various  
296 publicly available databases or datasets for Arabidopsis which were published as  
297 supplements of manuscripts. Therefore, our results represent the most current and  
298 comprehensive knowledge network of immune signalling and related processes in  
299 Arabidopsis (see Data availability section of Methods). Compared to data for immune  
300 signalling in this model species, those for immune signalling in crop species are much  
301 sparser; therefore, the translation of knowledge is essential for crop resistance breeding. It  
302 has been shown that knowledge can be transferred cross-species based on orthology, with  
303 higher reliability transfer between related species, whereas translation from di- to  
304 monocotyledons is less predictive (Lee et al., 2015b). Molecular network rewiring leads to  
305 functional divergence and thus plays a central role in speciation (Chae et al., 2012). The  
306 speed of network rewiring depends on several factors, including the type of interaction, with  
307 transcriptional regulatory networks having one of the fastest rewire speeds (Shou et al.,  
308 2011). However, functional modules often experience evolutionary cohesiveness (Chae et

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

318

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

326

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

341

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

346 Arabidopsis susceptibility to herbivory (Groen et al., 2013). Several experiments have shown  
347 that SA can modulate ET signalling (Caarls et al., 2016; Guan et al., 2015; Zander et al.,  
348 2014; Van der Does et al., 2013). This regulation is, in most cases, implicated in the context  
349 of JA/SA antagonism (Caarls et al., 2015; Derksen et al., 2013; Robert-Seilaniantz et al.,  
350 2011). Only a few studies indicate that ET might be an important regulator of SA signalling. It  
351 was shown that EIN3 transcription factors directly target the promoter of ICS2, negatively  
352 regulating SA biosynthesis (Chen et al., 2009). On the other hand, Frye et al. (2001),  
353 Mikkelsen (2003) and Leon-Reyes et al. (2009) have shown ET potentiation of PR1 gene  
354 expression in Arabidopsis. We observed the same effects in our potato experiments (Figure  
355 5). Chromatin immunoprecipitation sequencing of EIN3 targets revealed NPR3 promoter as  
356 its direct target (Chang et al., 2013), providing further evidence that the two modules are  
357 connected.

358

359 Most studies involving NPR1 as a master regulator of SA signalling have focused on its  
360 posttranslational modifications or loss-of-function effects. ET modulation of NPR1 role in  
361 JA/SA antagonism was studied by performing a series of experiments with the *npr1* mutant,  
362 which did not allow identification of the effects of transcriptional, translational and  
363 posttranslational regulation (Leon-Reyes et al., 2009). Further studies showed the  
364 importance of proteasome-mediated degradation of NPR1 (Ding et al., 2016; Saleh et al.,  
365 2015; Fu et al., 2012) and nuclear import (Lee et al., 2015a; Kovacs et al., 2015; Fu et al.,  
366 2012) for effective SA perception. To our knowledge, no study performed so far has focused  
367 on the regulation of NPR1 gene expression.

368

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

378

379

## 380 CONCLUSIONS

381



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

394

395

## 396 **METHODS**

397

### 398 **Network-based knowledge integration**

399

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

409

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

417

418

### 419 **Network inference from experimental datasets**

420

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

430

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

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

450

### 451 **Validation of the network construction approach**

452

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

460

### 461 **Network analyses**

462

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

470

### 471 ***In silico* promoter analyses**

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

475

### 476 **Plant growth and treatments**

477

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

494

### 495 **Gene expression analysis**

496

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

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

524

### 525 **Data Availability**

526

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

530

### 531 **Accession Numbers**

532

533 Sequence data from this article can be found in the NCBI Gene data library under Gene ID  
534 842733 (AT1G64280; AtNPR1), XM\_006357647 (Sotub07g011600.1.1; StNPR1),  
535 NM\_001288166 (Sotub09g006090.1.1, StPR1b).

536

### 537 **Supplemental data**

538

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

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

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

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

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

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

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

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

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

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

558

559

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561

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564 assistance.

565 **TABLES**

566

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

579

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	

580

581

582 **FIGURE LEGENDS**

583

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

598

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

603

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

612

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



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

620

621 Figure 5: Validation of direct transcriptional regulation of NPR1 by the ethylene (ET)  
622 signalling module in potato leaves. **A**, Schema of the underlying biological pathways of ET  
623 (yellow) and salicylic acid (SA; blue), with interactions on the protein or transcriptional level  
624 (solid and dashed lines, respectively). Interactions can activate (arrow) or inhibit (circle)  
625 downstream signalling events. Blue lines denote treatments of the functional validation (ET,  
626 INA, 1-MCP) or NahG plants (deficient in SA signalling). The red dashed arrow denotes our  
627 tested hypothesis. **B**, Plants belonging to potato cv. Rywal and **C**, its transgenic line NahG-  
628 Rywal were treated with ETs (yellow), INA (SA analogue; blue) or a combination of INA with  
629 1-MCP (ET inhibitor; INA+MCP; grey). Log<sub>2</sub> fold changes in gene expression in treated and  
630 control plants are shown (\*- p<0.05; n=3; Student's *t*-test) for ACO4 (ET signalling marker),  
631 PR1b (SA signalling marker) and NPR1 (SA signal transmitter). **D**, *Arabidopsis thaliana* Col-  
632 0 wt and *ein3-1* mutant were treated with INA or INA+MCP, and log<sub>2</sub> fold change in gene  
633 expression in treated and control plants for NPR1 (\*- p<0.05, n=6; Student's *t*-test) is shown.  
634 Error bars denote standard error of biological replicates. Note different y-axis scales for  
635 different genes. Results of the second independent potato experiment are provided in  
636 Supplemental Figure 3.

637

638

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