Nucleotide-binding and molecular interactions of plant disease resistance proteins
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CHAPTER 3

Protein-protein interactions as a proxy to monitor conformational changes and activation states of the tomato resistance protein I-2


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ABSTRACT

Plant resistance proteins (R) are involved in pathogen recognition and subsequent initiation of defence responses. Their activity is regulated by inter- and intramolecular interactions. In a yeast two-hybrid screen we identified two clones (I2I-1 and I2I-2) specifically interacting with I-2, a *Fusarium oxysporum* f. sp. *lycopersici* resistance protein of the CC-NB-LRR family. Sequence analysis revealed that I2I-1 belongs to the Formin gene family (*SlFormin*) whereas I2I-2 has homology to translin-associated protein X (*SlTrax*). *SlFormin* required only the N-terminal CC I-2 domain for binding, whereas *SlTrax* required both I-2 CC and part of the NB-ARC domain. Tomato plants stably silenced for these interactors were not compromised in *I*-2 mediated disease resistance.

When we used extended or mutant forms of I-2 as baits, we observed distinct, and often opposite, interaction patterns for the two interactors. These interaction patterns corresponded with the proposed nucleotide-dependent active or inactive states of the I-2 protein. This supports previously proposed models that active and inactive R proteins adopt distinct conformations associated with ATP binding and hydrolysis. Furthermore, our data shows that these conformational states can be monitored in the yeast system.
INTRODUCTION

The interaction between the soil-born, xylem-colonizing fungus *Fusarium oxysporum f. sp. lycopersici* (Fol) and its host, tomato (*Solanum lycopersicum*) is a model system to study the molecular basis of disease resistance in plants. Tomato plants defend themselves against fungal colonization by the secretion of anti-microbial components, pathogenesis-related (PR) proteins and blocking the xylem vessels by tyloses, pectic gels and gums (Beckman, 2000; Rep et al., 2002). In a susceptible plant, blocking of the xylem vessels by the spreading fungus and the responding plant reduces water flow, thereby leading to wilting and eventually death. Some plants, however, are resistant to particular isolates of Fol. Upon infection they respond faster and hence more effectively, restricting fungal colonisation. This gene-for-gene type of resistance depends on the presence of a dominant resistance (*R*) gene in the plant that recognises a matching fungal avirulence factor (*Avr*) (Flor, 1942). Many Avrs are in fact effectors and therefore gene-for-gene type of resistance is also called effector-triggered immunity or ETI (Jones and Dangl, 2006).

In the tomato - Fol interaction three *R/Avr* pairs have been identified, and their relation is well studied (Simons et al., 1998; Rep et al., 2004; Houterman et al., 2008; Houterman et al., 2009; Takken and Rep, 2010). The resistance gene that mediates defences against race 2 isolates of Fol has been cloned and is called *I-2* (Immunity to race 2) (Simons et al., 1998; Houterman et al., 2009). *I-2* is expressed in root and stem parenchyma cells that are in direct contact with the xylem tissue (Mes et al., 2000). *Avr2* recognition by *I-2* and subsequent defence responses, visible as a hypersensitive response (HR) can be artificially induced in leaves and stems, either by virus-based overexpression of *Avr2* in tomato carrying *I-2*, or in *N. benthamiana* leaves after transient co-expression with *I-2* through agroinfiltration (Houterman et al., 2009). *I-2* belongs to the CC-NB-LRR (CNL) class of R proteins that contains an amino-terminal coil coiled domain (CC), a central nucleotide binding (NB) domain, and a C-terminal domain containing leucine rich repeats (LRRs). The most conserved domain of this class of R proteins is the NB domain, which is part of a larger region called NB-ARC for Nucleotide Binding domain shared by *Apaf-1*, R proteins and Ced-4 (van der Biezen and Jones, 1998). With a purified recombinant form of *I-2*, which lacked the LRR domain, a role of the NB-ARC domain in ATP/ADP binding and ATP hydrolysis has been shown (Tameling
Biochemical analyses of two constitutively active I-2 mutants (S233F and D238E) showed that they were affected in ATP-hydrolysis, but not in ATP/ADP binding, suggesting that these mutants are locked in the ATP bound state. When these mutations were combined with a mutation in the P-loop (K207R) to block nucleotide binding, the autoactivation phenotype was abolished. These observations show that nucleotide binding is required for activation of defence signalling and that the ATP bound state most likely represents the activate state (Tameling et al., 2002; Tameling et al., 2006). Binding of ADP was found to stabilize the I-2-nucleotide complex, which implies that the different nucleotide binding states exhibit different conformations. Based on these observations a molecular switch model was proposed (Tameling et al., 2006). In the “off” state the R protein is tightly bound to ADP. It is assumed that upon Avr perception the conformation of the nucleotide binding pocket changes, resulting in release of ADP. Subsequent binding of ATP results in a second conformational change (“on” state) that allows the protein to activate the downstream defence-signalling cascade. Hydrolysis of the bound ATP by its intrinsic ATPase activity reverts I-2 to the “off” state. In this biochemical model the conformation of the protein is regulated by its nucleotide binding state. To get insight into the conformation of the I-2 NB-ARC domain the crystal structure of the NB-ARC domain from the ADP-bound state of Apaf-1 was used as a template to obtain a 3D model of this domain (Riedl et al., 2005; Takken et al., 2006; van Ooijen et al., 2008 b). The predicted structure allowed mapping of the amino acid residues in I-2 that are most likely involved in nucleotide binding and hydrolysis. Mutations of many of those residues, which are highly conserved in other R proteins, resulted in either a constitutive active- or a loss-of-function phenotype (Tameling et al., 2006; van Ooijen et al., 2008 b). Mutations in the corresponding residues in other R proteins conferred similar phenotypes (Dinesh-Kumar et al., 2000; Tao et al., 2000; Bendahmane et al., 2002; van Ooijen et al., 2008 b). These genetic data further support the above mentioned molecular switch model in which a change in the nucleotide binding state results in a conformational change representing the different activation states (on/off).

In this study, we report the identification of two I-2-interacting (I2I) proteins. We analyzed the functional involvement of these interacting proteins in I-2 mediated defence using stably silenced tomato lines. Furthermore, using yeast two-hybrid (Y2H) we mapped the minimal domains of I-2 that are required for the interaction with these proteins. Notably, we observed
distinct interaction patterns for the various I-2 mutants that correspond to the different proposed nucleotide-dependent conformational states. This indicates that the different nucleotide-dependent conformational changes can be monitored in the yeast system providing direct support for the switch model.

RESULTS

Opposite Y2H interaction patterns of I-2-interacting proteins suggests different conformational states of I-2

Using a near full-length I-2 protein (amino acids 1-872) as bait, an Y2H screen of a Fusarium-tomato interaction cDNA library was carried out. This screen resulted in the identification of two interacting clones (I2I-1 and I2I-2, for I-2 interacting clone-1 and -2 respectively). The interactions were confirmed using full-length I-2 protein (Fig. 1A; upper panel). To test whether these proteins interact with I-2 specifically we also analyzed their ability to interact with two other NB-LRR proteins: Mi-1.2 and Rx. Both Rx, conferring resistance to Potato virus X (PVX) (Bendahmane et al., 1995; Bendahmane et al., 1999), and Mi-1.2, conferring resistance to the nematode Meloidogyne incognita, aphids, psyllid and white flies (Milligan et al., 1998; Rossi et al., 1998; Vos et al., 1998; Nombela et al., 2003; Casteel et al., 2006) are Solanaceous proteins also belonging to the CC-NB-LRR resistance protein class. As shown in Figure 1A, only I-2 interacts with the proteins encoded by the two cDNAs as visualized by the growth on selective medium. All full-length bait proteins are expressed in yeast (Fig. 1A; lower panel). Similar results were obtained for the CC-NB-ARC baits of those three R proteins (Fig. S1). Notably, the CC-NB-ARC bait of the I-2 paralog (I-2C1) showed no interaction with I2I-1 or I2I-2 (Fig. S1). I-2C1 shows 75% sequence identity with I-2 and is encoded by the same gene cluster; however the gene does not confer resistance to F. oxysporum (Simons et al., 1998). Both interactors bind to I-2 but not to Rx, Mi-1.2 or I-2C1, suggesting that these interactions with I-2 are highly specific.

Next, we set out to identify the minimal fragment of I-2 required for interaction with I2I-1 and I2I-2. For this purpose, we tested five Y2H baits encoding either i) full-length I-2 (I-2FL), ii) the CC-NB-ARC carrying the first 5 LRRs (I-2N+), iii) the CC-NB-ARC domain (I-2N), iv) an CC-NB-ARC variant carrying an internal deletion in its ARC2 sub-domain that
removed the conserved MHD motif (I-2N\textsuperscript{AMHD}) and v) the CC domain alone (I-2CC) (Fig. 1B). As expected both I2I clones interacted with the full-length I-2 protein indicated by a clear growth of the yeast cells on selective medium after five days. Remarkably, a truncation, that removed the 18 C-terminal LRRs (I-2N\textsuperscript{+}), resulted in loss of interaction with I2I-1, but not with I2I-2. When the remaining 5 LRRs were removed (I-2N) I2I-1 regained its ability to interact, whereas I2I-2 lost this ability. For I2I-2 the interaction was recovered when an internal deletion in the ARC2 sub-domain was made (I-2N\textsuperscript{AMHD}), but this deletion compromised the interaction with I2I-1. Truncation of the entire NB-ARC domain resulted once more in an opposite interaction pattern, as the CC domain alone (I-2CC) binds only to I2I-1 (Fig. 1B). These results show that I2I-1 binds preferentially to I-2 baits that do not bind to I2I-2 and \textit{vice versa}. The observation that each bait interacts with at least one I2I-clone proves that all baits are expressed and do accumulate to high enough levels for an interaction, as was confirmed by western blot analyses (data not shown). The opposite interaction patterns observed for both I2I clones suggest that the various I-2 truncations might have different conformations that differ in exposure of their interaction interfaces. For I2I-1 this interaction interface is present in the CC domain whereas I2I-2 apparently interacts with a surface formed by both the CC and NB-ARC domains (the NB-ARC domain alone did not interact with I2I-1 or I2I-2 (data not shown)). Furthermore, it indicates that the presence or absence of domains and subdomains influences these interaction interfaces most likely by intra-molecular interactions.
**I-2 Y2H interactors: SlFormin and SlTrax**

**Fig. 1. Identification of I-2 domains required for Y2H interaction with I2I-1 and I2I-2.**

A) Y2H analyses show interactions of I2I-1 or I2I-2 with full-length I-2 as bait, but not with Rx and Mi-1.2. The baits and preys did not show autoactivity as tested by co-expression with the pACT2 and pAS2-1 empty vectors. The test for activation of the HIS3 marker is shown after 10 days of growth (upper panel). Expression of the bait proteins was confirmed by western blot (WB) analyses using α-Gal4BD antibody detection on total yeast protein extracts (lower panel). B) Y2H analyses of I2I-1 and I2I-2 with various I-2 baits: I-2FL (full-length: 1-1266aa), I-2N+ (1-643aa), I-2N (1-520aa), I-2N\textsuperscript{∆MHD} (1-520aa with deletion in between 484-496aa) and I-2CC (1-170aa). The different I-2 (sub)domains are indicated and coloured: CC domain (orange), NB (red), ARC1 (purple), ARC2 (blue), LRR (green). The test for activation of the ADE2 marker is shown after 5 days of growth; the other two markers (HIS3, LacZ) gave identical results. + : activation of all three selectable markers, - : no activation of these markers. The smallest part of I-2 enabling interaction with I2I-1 is the CC domain and for I2I-2 the CC-NB-ARC domain.

**Differential Y2H interaction patterns coincide with active and inactive I-2 conformations**

Our results suggest that different conformational states of I-2 can be studied by monitoring their Y2H patterns with the two interacting clones. To test this hypothesis we assayed interactions between the two identified I2I clones and I-2 variants that are predicted to have different nucleotide-dependent conformations, notably wild-type I-2 and three mutants: S233F, D283E, and K207R. The nucleotide binding states of these proteins have been determined and linked to *in planta* phenotypes; e.g. autoactivation for I-2\textsuperscript{S233F} and I-2\textsuperscript{D283E} and loss-of-function for I-2\textsuperscript{K207R} (Tameling et al., 2006). Individual mutations were introduced into the two, C-terminally truncated wild type proteins exhibiting differences in their
interaction pattern with the two I2I clones, notably I-2N and I-2N+ (Fig. 1B). Interestingly, I-2N$^{K207R}$ showed an interaction pattern that differed from wild type: the mutant could no longer bind I2I-1, but gained the ability to interact with I2I-2 (Fig. 2A). This confirms that I2I-2 is capable of binding I-2N without LRRs. Both I-2N$^{S233F}$ and I-2N$^{D283E}$ showed an interaction pattern identical to wild type (Fig. 2A). Introduction of the K207R mutation into I-2N+ did not change the interaction pattern (Fig. 2B). When the effect of the individual S233F and D283E mutations in the extended bait was analyzed, we observed an interaction pattern that differed from wildtype: both interacted with I2I-1 (Fig. 2B). Autoactivating mutations in the I-2N+ setting did not affect the interaction with I2I-2.

To conclude, the observed differences in the Y2H interaction patterns between I2I-1 and I2I-2 with the various I-2 truncations and mutants support the hypothesis that the proposed nucleotide-dependent conformational states of I-2 can indeed be monitored by the interaction with the two I-2 interactors in the Y2H system.

![Fig. 2. Y2H interaction of mutated I-2 N-terminal variants with I2I-1 and I2I-2](image_url)

Interaction patterns of mutated I-2N (A) and I-2N+ (B) The test for activation of the ADE2 marker is shown after 1 week of growth for I2I-1 and after 28 hours for I2I-2. The other two auxotrophic markers (HIS3, LacZ) are not shown, but gave identical results. + : activation of all three markers, - : no activation of these markers. None of the bait or prey constructs showed autoactivity. Three mutations in the NB-ARC domain were tested: a nucleotide binding mutant K207R representing the empty state and two ATPase impaired mutants S233F and D283E representing the active state of I-2. In the lower part of the two panels the interaction with wild-type baits are depicted (WT).
Characterization of putative I-2 interactors

The distinct interaction patterns in yeast are suggestive for a functional involvement of I2I-1 and I2I-2 in I-2 mediated resistance. To learn more about the proteins encoded by these two cDNAs, we set out to clone the full-length cDNAs corresponding to the complete proteins. The insert of clone I2I-1 spans 1084 basepairs (GenBank accession number: AY150043) and contains an ORF of 648 nucleotides as well as a putative 3'UTR and a poly(dA) stretch (19 residues). A BLAST search with this nucleotide sequence in the tomato genome database (http://solgenomics.net) revealed a predicted gene (Solyc07g052730) of which the 3’ end matches 100% with the sequence of I2I-1. The I2I-1 cDNA sequence differs from this gene by 6 nucleotides (originating from the cloning adapter used) at its 5’ end and the a poly(dA) stretch at its 3’ end. To validate the predicted gene model we set out to clone a full-length cDNA. To this end we employed a gene walking approach using a vector-specific and gene-specific primers to PCR-amplify and subclone new sequences from the cDNA interaction library that was also used for the Y2H screens. For the amplification of a complete cDNA we designed one primer (I2I-1-F1) corresponding to the most upstream 5’ sequence obtained from the ‘gene walking’ experiments, and one primer (I2I-2stop-R) corresponding to the 3’ end of the ORF as determined in the I2I-1 clone. This gene-specific primer pair was used to amplify the full-length cDNA. Three independent full-length clones of approximately 3 kb were sequenced. These three sequences were used together with the I2I-1 insert to assemble a consensus I2I-sequence of 3,425 nucleotides excluding the poly (A) stretch (GenBank accession number: LN836738). The assembled consensus sequence contains a 5’ UTR of 172 nucleotides, an open reading frame of 2832 nucleotides encoding a protein of 944 amino acids and a 3’ UTR of 418 nucleotides, and corresponds to gene Solyc07g052730 on tomato chromosome 7 (nucleotides 58,506,937 – 58,513,630). Comparison of the full-length cDNA and the gene revealed a sequence identity of 100% to exon sequences, and confirmed the positions of the first five introns of the gene model predicted by the SOL Genomics Network (http://solgenomics.net). A sixth intron was predicted to start one nucleotide upstream of the stop codon in the cDNA. However, comparing the genomic and cDNA sequences places the start of this last intron in the 3’UTR, notably 12 nucleotides downstream of the stop codon. The gene organization is shown in Fig. 3A.
The amino acid sequence encoded by the Solyc07g052730 ORF is predicted to contain a signal peptide of 35 amino acids (SignalP 3.0). However, the ORF contains an in frame AUG-triplet at codon-position 13 (as well as at position 18). A translational start at the second AUG codon would result in a protein with a signal peptide of 22 amino acids, in eukaryotes a more commonly observed signal peptide length. So, most likely the second AUG is the genuine start codon. Furthermore, the amino acid sequence contains in its C-terminal half a so-called Formin Homology 2 (FH2) domain, a hallmark domain of formins, as well as a transmembrane domain and a proline–rich domain that may correspond to the Formin Homology 1 (FH1) domain (Fig. 3A). From these observations we conclude that Solyc07g052730 encodes a tomato Formin, and hence we refer to this gene, from which interacting clone I2I-1 was derived, as SlFormin. BLAST searches with the complete SlFormin cDNA sequence on the tomato genome revealed the presence of at least 13 Formin homologs (http://solgenomics.net/). Since the I-2 interacting Formin apparently is a member of a larger protein family, we tested whether I-2 also interacts with other Formin homologs. A close Formin homolog, SGN-U583099 (70% homology in the FH2 region) corresponding to Solyc10g006540 (http://solgenomics.net/), was selected and region corresponding to the I2I-1 encoded fragment was analyzed for its ability to interact with I-2 in a Y2H assay. This homolog did not interact with I-2N (data not shown) suggesting a specific interaction of SlFormin with I-2.

The insert of clone I2I-2 spans 732 nucleotides (GenBank accession number: AY150044) and is part of unigene SGN-U575744, an assemblage of 15 tomato ESTs (http://solgenomics.net/). Full-length cDNA was amplified from our cDNA library using primers corresponding to a (putative) start (I2I-2-ATG-F) and end (I2I-2 stop-R) of the ORF of SGN-U57544, respectively, and three full-length, independent clones were sequenced. From the unigene and our newly obtained sequences a consensus was derived of 1274 nucleotides (excluding the poly(dA) stretch present in AY150044) with an ORF encoding a protein of 285 amino acids. A BLAST search with the consensus sequence in the Sol Genomics Network Database indicated Solyc04g05310.2.1 on chromosome 4 as the corresponding gene. The predicted gene-model includes 4 introns, of which one is located in the 3’ UTR 5 nucleotides downstream the stopcodon. The gene encodes a protein of 196 amino acids showing 100% identity with the sequence of amino acids 90-285 of the protein encoded by the consensus.
This strongly suggests that the part of the gene encoding amino acids 1-89 of the ‘consensus’ protein is missing in the tomato genome database. Indeed, an additional search in the database with the first 270 nucleotides of the consensus sequence as query gave no hit at all. Blasting \textit{S. pimpinellifolium} genomic sequences (http://solgenomics.net/) with the consensus identified a gene with 7 introns (Fig. 3B) on contig 1850142 showing nearly 100% identity with the consensus sequence. Comparing the \textit{S. pimpinellifolium} gene with Solyc04g05310.2.1 showed that from more or less the middle of intron 3 onwards the two sequences are nearly the same. A BLAST search with the sequence just upstream Solyc04g05310.2.1 identified an identical sequence in \textit{S. pimpinellifolium} contig 1850142 approximately 6 kb upstream intron 3, suggesting that a 6 kb DNA fragment is missing in the \textit{S. lycopersicum} genomic database.

As mentioned above the consensus ORF encodes a protein of 285 amino acids. However, 36 nucleotides downstream the start of the ORF there is a second AUG. We cannot exclude that this codon is used as translational initiation codon. Both the longer and shorter translation products carry a translin homology domain. Moreover, a potential nuclear export signal (NES) was predicted (amino acids 190-200) (Fig. 3B). The protein has homology to a human protein called translin-associated factor X (TRAX; E value 4e-34) (Aoki et al., 1997). Human TRAX is present in a complex with Translin, which is involved in microtubule-dependent mRNA trafficking and translational repression (Aoki et al., 1997; Cho et al., 2004). The tomato I2I-2 will be referred to as \textit{SlTrax}.
Figure 3. Gene model and predicted domain structure of the I-2 interacting proteins *S*I*Formin* and *S*I*Trax*.
Of both genes the intron-exon structure is depicted by a black line. The yellow boxes represent coding sequences (exons) interrupted by introns (/). The cDNA nucleotide positions of the exons are indicated above the line whereas the length of introns is given below the line. The numbers at the start and the stop refer to the position of the gene in solgenomics database (http://solgenomics.net/). The proteins and their predicted domains are represented by rectangles and the position of the domains is indicated above the figure. The regions required for binding to I-2 are marked by a pink box. **A)** *S*I*Formin*, gene Solyc07g052730 position 58506937-58513630 (http://solgenomics.net/). SP- signal peptide (orange); TM- trans-membrane domain (blue); FH1 and FH2- Formin homology domain 1 (green) and 2 (purple) and **B)** *S*I*Trax*: contig 1850142 (8881-13284) (http://solgenomics.net/). NES- nuclear export signal (orange) and Translin- Translin domain (blue).

**Generation of stably silenced *S*I*Formin* and *S*I*Trax* tomato lines.**
The distinct interaction patterns observed in our Y2H analyses of *S*I*Formin* and *S*I*Trax* with the I-2 bait proteins suggest active binding - or release - of these interactors depending on the nucleotide-dependent conformational state of I-2. The interaction patterns make both interacting proteins prime candidates to be involved in I-2 mediated resistance. To test if *S*I*Formin* and *S*I*Trax* are involved in I-2 mediated resistance towards Fol we created transgenic tomato lines in which expression of *S*I*Formin* or *S*I*Trax* was knocked-down using post-transcriptional gene silencing.
Fig. 4. *Sl*Formin and *Sl*Trax expressions levels in Formin and Trax RNAi tomato plants
Transcript levels were determined by real-time qPCR relative to *α*-tubulin in roots of five-week-old tomato plants transformed with the empty vector EV (red) or silenced for A) *Sl*Formin (blue) or B) *Sl*Trax (green). Depending on RNAi construct (3’ or 5’ end) four lines per gene were analysed: 3a, 3b, 5a, 5b. Average transcription levels for two biological and two technical replicas and SD are shown. C) Formin5b plants are smaller and yellowish compared to empty vector transformed tomato plants (EV). The photograph shows a five-weeks-old representative Formin5b and EV plant. D) Necrotic lesions at the edge of a leaf from a Formin5b plant.
RNAi silencing constructs were designed to silence simultaneously the genes of interest as well as GUS reporter gene. The latter can be transiently expressed in leaves of stable transformants by agroinfiltration to simplify screening for plants exhibiting strong silencing (Wroblewski et al., 2007; Ament et al., 2010; Krasikov et al., 2010). For each gene we created two constructs that target either the 3’ or the 5’ end of the transcribed gene sequence, respectively. For each transformation at least ten independent transformants per construct were generated. Of these transformants around 80% was diploid and strong GUS silencing was found in 40-60% of the cases. Lines having a single copy insertion and showing GUS silencing were self-pollinated, and progeny homozygous for the transgene was selected. Two transgenic lines were chosen for each (5’ and 3’ end) RNAi construct and were named 5a, 5b, 3a and 3b, respectively. Consequently SfFormin or SfTrax transcript levels in roots were quantified using qPCR. All selected stably silenced tomato lines showed a significant reduction in relative expression of the targeted genes as compared to the control line transformed with an empty vector (Fig. 4A and 4B). The strongest silencing was observed for Formin3a and Formin5b lines; up to 90% reduction in SfFormin expression levels (Fig. 4A). Of the SfTrax RNAi lines Trax5a and 5b showed the strongest reduction (80%) in SfTrax expression levels (Fig. 4B). Interestingly, constructs targeting the SfTrax 5’ end exhibited stronger silencing than the 3’ silencing constructs, which did not exceed 50% (Fig. 4B). Remarkably, both silenced plants containing the 5’ construct for SfFormin, exhibited clearly aberrant phenotypes. The plants were smaller, yellowish and the leaf edges show small necrotic lesions (Fig. 4C and 4D). The 3’ silencing inducing constructs for SfFormin did not exhibit these phenotypes. Also none of the SfTrax silenced lines exhibited a visible phenotype. For the subsequent experiments the two lines for each gene that showed the highest silencing level were selected: Formin3a and 5b; Trax5a and 5b.

**Fusarium bioassay on RNAi SfFormin and SfTrax lines**

Generation of silenced SfFormin and SfTrax tomato plants materialized possibilities to test whether decreased expression influenced I-2 function. First, we tested the response of the silenced plants to *F. oxysporum* (Fol) infection. Ten-day-old seedlings of the silenced and empty vector transformed (EV) tomato lines were infected with a race 2 isolate of Fol (Fol007). This race lacks *Avr1*, but expresses *Avr2* and hence is avirulent on this *I-2*
containing cultivar (Mes et al., 1999). The severity of disease development is scored by the disease index that corresponds to the number of brown, infected xylem vessels in the stem (Rep et al., 2004). In a resistant cultivar, like cv. Motelle (EV) none or few plants showed brown xylem vessels resulting in a disease index of 0 or occasionally 1 (Fig. 5A). Resistance towards a race 2 isolate is so efficient that water and Fol007 treatments are normally equivalent in their disease scores (data not shown; (Houterman et al., 2009)). As shown in Figure 5A, no significant differences in disease indexes were observed between controls and silenced lines inoculated with Fol007. Hence, silencing of neither SlFormin nor SlTrax compromised I-2 mediated defence responses towards a race 2 isolate of Fol (Fol007). Inoculation of this isolate on susceptible tomato plants (cv. C32) caused wilt disease, confirming that the inoculum was infectious (data not shown). We also inoculated RNAi SlFormin, SlTrax and EV tomato lines with a race 3 isolate of Fol (Fol029) that carries Avr3 and is virulent on Motelle (1, I-2, i-3) (Marlatt, 1996; Rep et al., 2004). All plants were severely diseased (Fig. S2), which shows that disease development and hence susceptibility is not affected. Taken together, no significant change in either disease resistance or susceptibility was observed upon Fol infection in SlFormin or SlTrax silenced plants.

**PVX::Avr2 screen on SlFormin and SlTrax RNAi lines**

As the above described bioassays did neither confirm nor exclude the possibility that SlFormin or SlTrax is involved in I-2 function, we next examined the ability of the silenced lines to respond to Avr2. To express Avr2 in tomato plants we employed the Potato Virus X (PVX)-based expression system (Takken et al., 2000). A recombinant viral replicon carrying the Avr2 transgene was cloned into a binary vector that can be introduced in a plant by toothpick Agrobacterium-mediated inoculation. Systemic spread of the PVX::Avr2 virus, which expresses Avr2, triggers defence responses in an I-2 carrying plant. Induction of defence is visible as a hypersensitive response (HR) that spreads from the inoculated cotyledons to the non-inoculated leaves (Houterman et al., 2009). The prediction was, that if SlFormin or SlTrax is involved in recognition or down-stream signalling of I-2, it could affect the timing or the extent of the HR in the silenced tomato lines inoculated with PVX::Avr2. To quantify differences in HR development we devised a scale from 1 (HR development only on
primary inoculated leaf) to 4 (extensive systemic HR and strong curling of the systemic leaves) (Fig. S3).

![Bioassay](image)

**Fig. 5. Silencing of SlFormin or SlTrax showed no detectable influence on I-2 mediated resistance to Fusarium oxysporum.**

A) Ten-day-old seedlings of empty vector (EV) and SlFormin or SlTrax silenced tomato lines were infected with race 2 *Fusarium oxysporum* f. sp. *lycopersici* (Fol007). The disease index was determined for the indicated number (n=) of plants at 21 days post inoculation. Distribution of plants over the different disease indexes is depicted. Anova analysis reveals no significant differences in the distribution of plants over the different disease indexes between silenced (Formin3a, Formin5b, Trax5a, Trax5b) and non-silenced (EV) tomato lines upon infection with Fol007. The presented data represent two independent experiments. 

B) Four-weeks-old tomato plants were tooth-pick inoculated with Agrobacterium carrying PVX::Avr2. Development of HR as a result of Avr2 recognition by endogenous I-2 was quantified after 10 days post inoculation. The graph illustrates the percentage of plants with an HR intensity ranging from scale 1 to 4 for each line. Anova analysis indicates that the Formin5b line shows a significantly higher HR intensity upon PVX::Avr2 inoculation. The presented data represent two independent experiments. For an illustration of the HR intensity scale see Fig. S3.
As a negative control we used PVX::Avr2^{R/H}, which expresses a variant of Avr2 that is not recognized by I-2 and hence does not trigger HR on an I-2 plant (Houterman et al., 2009). PVX::Avr2^{R/H} was used to exclude that the virus itself induced cell death in this screen (data not shown). Ten-days-post inoculation (10 dpi) of PVX::Avr2 on empty vector (EV) control plants, around half of them showed a class 1 HR, while the rest showed a response of 2 or more (Fig. 5B). This intermediate stage of HR development on the controls suggested that 10 dpi is an appropriate time-point to score for enhanced or reduced HR development on the silenced lines. The one line that showed significant differences in the HR distribution was the Formin5b line. Most of the Formin5b plants showed an HR score over 1, the majority residing in group 4. The same plants, when non-inoculated, are yellowish, small and have necrotic spots on their leaf edges (Fig. 4C and 4D). Since appearance of necrotic lesions (HR) was used to discriminate the groups, a phenotypic influence of Formin5b phenotype affecting the scoring cannot be excluded. Furthermore, the retarded growth of Formin5b plants might facilitate systemic spreading of the virus resulting in faster HR development. Nevertheless, our results indicate a trend that both S/Formin silenced lines, regardless of their visible phenotypes, exhibited an enhanced HR development (Fig. 5B).

DISCUSSION

I-2 Y2H interaction patterns support the molecular switch model

Upon screening an Y2H tomato-Fusarium cDNA library two proteins, S/Formin and S/Trax, were found to interact with I-2, a Fol R protein of the CC-NB-LRR class. Each interactor showed distinct binding preferences for specific truncated and/or mutated forms of I-2 (Fig. 1B and 2). The interaction pattern of S/Formin and S/Trax differed depending on the I-2 truncation or mutation analysed, and was often found to be opposite. Apparently, I-2 adopts at least two conformations, a S/Formin- or a S/Trax-binding conformation. Some I-2 baits, such as the full-length I-2 and the I-2N+ ATPase mutants bind both interactors. They could represent a third conformation. Alternatively, they could represent a mixture of the two I-2 conformations that are present at different steady state levels in yeast. The exclusive ability to interact either with S/Formin or with S/Trax correlated with the active (I-2^{S233F} and I-2^{D283E})
or inactive/resting ($I-2^{K207R}$) I-2 state (Tameling et al., 2006). The observed interaction patterns of the mutants (Fig. 2) suggest that the $S/\text{Formin}$-binding conformation represents the active I-2 state, while the $S/\text{Trax}$-binding conformation corresponds to the resting or inactive state. Since extension of I-2N with 5 LRRs shifts the equilibrium towards the $S/\text{Trax}$-binding, we conclude that the N-terminal LRR stabilizes the resting state.

The hypothesis that the LRR domain acts as a negative regulatory module for R proteins is in agreement with earlier reports (reviewed by (Lukasik and Takken, 2009). For instance deletion of the LRR domain, as exemplified for RPS5 (Ade et al., 2007) and RPP1A (Weaver et al., 2006) or swapping LRR subdomains with those of homologous proteins, as shown for Rx (Rairdan and Moffett, 2006) and Mi-1.2 (Hwang et al., 2000; Hwang and Williamson, 2003) results in constitutive activate R proteins. These autoactive mutants induce defence signalling in the absence of the corresponding pathogen. Moreover, the region required for negative regulation of Rx (Rairdan and Moffett, 2006) was pinpointed at the interface between the N-terminal part of the LRR and the ARC2 subdomain. Similarly, in our Y2H assays deletion of the complete LRR domain led to an interaction with $S/\text{Formin}$, most likely representing the activated state. Either deletion of the MHD-motif in the ARC2 subdomain or adding the first 5 LRRs that are predicted to interact with the ARC2 subdomain, abolished its ability to bind $S/\text{Formin}$ and allowed the protein to interact with $S/\text{Trax}$ indicative for the resting inactive state.

Another argument in support of a regulatory role for the ARC2 subdomain is that an internal deletion abolished the interaction of I-2 with $S/\text{Formin}$, which suggests that the deleted part is important for the protein to adopt the activated state. Among the 12 aa that were deleted is the conserved “MHD” motif. Point mutations in this highly conserved motif confer either an autoactive or a loss-of-function phenotype for I-2, as well as for other R proteins indicating its importance for R protein function (Takken et al., 2006; van Ooijen et al., 2008 b). For Mi-1.2 and I-2 the MHD motif has been proposed to fulfil the function of a sensor II motif, which regulates subdomain interactions and coordination of the bound nucleotide to control R protein activity (van Ooijen et al., 2008 b). Delimiting the smallest interacting domain of I-2 brought another interesting observation. The active I-2 conformation seems to have an surface exposed on the CC domain that makes interaction possible with $S/\text{Formin}$, while in the inactive state this surface is buried as a consequence of which $S/\text{Formin}$ binding is not
I-2 Y2H interactors: SlFormin and SlTrax

possible. Apparently, activation of the R protein results in exposure of this surface, which would require relaxation of the intramolecular interaction between the CC and NB-ARC-LRR domain. This model fits that proposed for Rx where release of its CC domain from the remainder of the protein was observed upon recognition of its cognate Avr (Moffett et al., 2002). Although the intramolecular interaction of the Rx CC with the NB-ARC-LRR domain was shown previously, it remained elusive to which specific domain the CC binds. Based on our findings these CC-mediated intramolecular interactions might involve the NB-ARC domain as, depending on the NB-ARC domain truncations or mutations, opposite interaction patterns for SlFormin and SlTrax were observed. Presence of both the NB and ARC1 subdomains apparently shields the SlFormin-interaction regions of the CC, and exposes the SlTrax binding surface.

Taken together, our observations fit a refined “switch model” for R protein activation (Lukasik and Takken, 2009). This model is supported by biochemical data for I-2 and M (Tameling et al., 2002; Tameling et al., 2006; Williams et al., 2011), which indicate that its activated state is ATP-bound, while the resting state is represented by a stable ADP-bound condition. In our Y2H assays, with SlTrax and SlFormin, we used the same loss-of-function and autoactivating mutants that were used in our previous biochemical studies. Indeed our findings support the hypothesis that I-2 adopts different conformations depending on its nucleotide bound (Tameling et al., 2006). We here link I-2 activity to at least two different conformations; a SlFormin/ATP bound active state, which requires an intact MHD motif and a SlTrax/ADP-empty resting state that is stabilized by the LRR domain.

The concept that interaction patterns of interacting proteins in Y2H could be used as a proxy to study conformational changes in R proteins might be more generic. For example for RPM1, conferring resistance to Pseudomonas syringae, five proteins interacting with its N-terminus have been reported (TIP49a (Holt et al., 2002), RIN2 and 3 (Kawasaki et al., 2005), RIN13 (Al-Daoude et al., 2005) and RIN4 (Mackey et al., 2002). Like for I-2, their interactions in an Y2H assay required distinct regions of the RPM1 N-terminus. Consistent with our findings, the interaction patterns changed depending on the NB-ARC domain truncation analyzed. It would be interesting to check for these interactors whether their binding abilities correlate with the proposed activation state of the protein, which can be
mimicked by the specific point mutations in the NB-ARC domain like we used in our analyses.

The differences in Y2H interaction patterns for specific R protein variants are not unique for RPM1 and I-2. Many proteins interacting with either the N-terminal CC or TIR domain did no longer interact when the bait was extended to encompass the NB subdomain (NRIP1 (Caplan et al., 2008), WRKY (Shen et al., 2007) and RIN4 (Mackey et al., 2002)). Alternatively, also for interacting proteins that required the NB subdomain their ability to interact is often affected by the length of the bait (RIN2 and 3 (Kawasaki et al., 2005); TIP49a (Holt et al., 2002); RIN13 (Al-Daoude et al., 2005)). The trend is that even though the minimal interacting region is present in a specific bait, often no interaction is found in longer baits. A trivial explanation could be that large baits cannot enter the yeast nucleus. However, in our studies we find that full-length I-2 protein is able to interact with at least Sl/Formin and Sl/Trax. These observations imply that specific variations in interaction patterns could be associated with different R protein folding states and hence can serve as markers to reflect their conformation.

The function of the interacting proteins in I-2 mediated resistance

The R protein interactors mentioned above are functionally involved in disease resistance (Holt et al., 2002; Mackey et al., 2002; Al-Daoude et al., 2005; Kawasaki et al., 2005; Shen et al., 2007; Caplan et al., 2008). The function of Trax in plants is unknown and to our knowledge this protein has not been linked to disease resistance signalling before. In mammals TRAX regulates, together with Translin, translation by microtubule-dependent trafficking of mRNAs (Jaendling and McFarlane). Sl/Formin homologs are nucleating factors necessary for actin polymerization and stress fibre formation (Ingouff et al., 2005). Operative assembly or disassembly of cytoskeleton filaments is required for proper plant growth, disruption of this process in RNAi Formin5 plants could explain their reduced stature (Fig. 4C). Depolarization and cytoskeleton reorganization in plants has also been linked to decreased non-host resistance towards fungal pathogens, like Blumeria graminis and Colletotrichum in Arabidopsis (Yun et al., 2003; Shimada et al., 2006). Furthermore, mycotoxins such as cytochalasins, trigger actin depolarization that causes disease and induce programmed cell death PCD (Franklin-Tong and Gourlay, 2008). Plant actin cytoskeleton
I-2 Y2H interactors: SIFormin and SITrax

hence seems to be involved in regulation of PCD and HR (Franklin-Tong and Gourlay, 2008), and disturbed actin polymerisation in the Formin5 silenced S. lycopersici lines might explain the formation of necrotic lesions on its leaf edges.

The two I-2 interactors identified are predicted to have a different localization in the cell. SIFormin contains a signal peptide and a transmembrane domain, which implies membrane localization (Fig. 3A). Such a localisation would be similar as that observed for the orthologous class I of Arabidopsis Formins: Fh6, Fh4, Fh8 and Fh5 (Favery et al., 2004; Deeks et al., 2005; Ingouff et al., 2005). SITrax carries a nuclear export signal, similar to its animal counterparts, which suggests that it might shuttle between nucleus and cytoplasm (Jaendling and McFarlane, 2010) (Fig. 3B). If I-2 in planta interacts with these proteins it implies either that distinct (membrane, cytoplasm and nucleus) pools of I-2 protein exist, or that dynamic shuttling of I-2 between these two subcellular compartments occurs. Recent data indicates that NB-LRR R proteins indeed localize in different sub-cellular compartments and that this bipartite localization is important for their function (MLA10 - (Shen et al., 2007), N - (Burch-Smith et al., 2007), Rx - (Slootweg et al., 2011; Tameling et al., 2011) RPS4- (Wirthmüller et al., 2007) and SNC1- (Zhu et al., 2010)). Whether I-2 activation, its ability to trigger defence, and its ability to interact with the I2I clones, are related to a specific subcellular location (nucleus for SITrax or plasma mebrane for SIFormin) remains to be investigated.

Functional involvement of SIFormin and SITrax in I-2-mediated resistance was assayed using the silenced tomato lines created in this study (Fig. 4-5). No compromised I-2-mediated resistance nor a change in susceptibility in Fol bioassays (Fig. 5A and Fig. S2) was observed. This could imply that SIFormin and SITrax are not involved in I-2 mediated resistance or that their involvement could not be detected using our experimental set-up. Possibly, involvement of SIFormin and SITrax was masked by redundancy of these genes in the host. However, we do not favour this explanation as SITrax is a single copy gene, and an interaction between Formins and I-2 was only detected for this homolog and not for a closely related one (data not shown). Alternatively, the silencing levels in the knock-down might be insufficient to confer a phenotype. For both genes the relative expression levels were reduced by 80-90% (Fig. 4), but we cannot exclude that the residual expression levels are sufficient for successful resistance signalling. To resolve this issue knock-out lines have to be created and tested in bioassays. An
alternative explanation for our results is that resistance is not compromised but enhanced, which would be undetectable in our Fol bioassays. To test this last hypothesis, Avr2 was systemically expressed in the silenced tomato lines using PVX::Avr2 and onset and severity of HR was quantified. The PVX::Avr2 assay revealed a statistically significant enhancement of I-2-mediated responses to Avr2 for the Formin5b line and a similar tendency for the Formin3a line (Fig. 5B). Unfortunately, we cannot exclude the possibility that the Formin5b phenotype affected PVX spreading and HR quantification, so these data have to be interpreted with care. Nevertheless, if the tendency towards increased I-2-triggered HR is indeed correlated with reduced \( S/I \)Formin expression levels, two explanations apply. Either \( S/I \)Formin could be involved in perception of Avr2, possibly as a guardee (Van der Biezen and Jones, 1998). We do not favour this hypothesis as we did not find an interaction between I2I-1 and Avr2 in an Y2H assay (data not shown) nor seems \( S/I \)Formin a virulence target for Fol, as the silenced lines did not show altered susceptibility to the virulent race 3 isolate (Fig. S1). An alternative explanation is that \( S/I \)Formin acts downstream of I-2 and is involved in negative regulation of HR or defence. Future experiments should therefore focus on establishing the putative function and the role of \( S/I \)Formin and \( S/I \)Trax in I-2 mediated resistance.

Taken together, functional involvement in I-2 mediated resistance for the two interactors could be confirmed nor disproved. However, their interaction pattern was found to correlate with the proposed activation states of I-2, making these interactors excellent markers to investigate I-2 activation-dependent conformational changes. Our findings suggest that Y2H assays using N-terminal interactors can be used to monitor conformational changes in R proteins. For I-2, at least two conformational states appear to be present in vivo.

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MATERIAL AND METHODS

Yeast two-hybrid (Y2H) constructs and protein expression.

Bait constructs:

Construction of the tomato-Fusarium cDNA library and I-2 baits used for screening I-2 FL and I-2N: CC-NB-ARC have been described before (de la Fuente van Bentem et al., 2005). The bait used for the library screen was constructed by subcloning the Ncol/SacI fragment of I-2 FL in the Y2H bait vector pAS2-1 (CLONTECH Laboratories, Palo Alto, CA, USA), resulting in bait CC-NB-ARC-LRR1-12 (amino acids 1-872). Bait I-2N+: CC-NB-ARC-LRR1-5 (amino acids 1-643) was constructed by cloning the Ncol/PstI fragment into pAS2-1. Bait I-2NAMHD: CC-NB-ARCAMHD was obtained via exonuclease activity after digestion of the Tth111-I restriction site in bait I-2 CC-NB-ARC. After re-ligation and sequencing the codons encoding amino acids 485-495 appeared to be deleted. Bait I-2CC (amino acids 1-168) was constructed by cloning the Ncol/EcoRV fragment of I-2 into pAS2-1.

The I-2N mutants: CC-NB-ARC (S233F, D283E) were described before as I-2 FL (S233F, D283E) clones in pGreen1K vector (Tameling et al., 2006). Ncol/XhoI fragments of those pGreen vectors were used to create I-2 FL baits with corresponding mutations in pAS2-1 digested with Ncol/SalI. Afterwards, I-2 FL baits (S233F, D283E) were digested with PstI to release a fragment thereby resulting in deletion of LRR5-29 and creation of I-2N+:S233F, I-2N+:D283E in pAS2-1.

To create the I-2N: CC-NB-ARC bait with the K207R mutation, an Ncol/XhoI fragment from pGEX-KG construct with I-2N:K207R (Tameling et al., 2002) was cloned into pAS2-1 that was digested with Ncol and SalI. Replacing the Ncol/BamHI fragment of this clone with the pAS2-1: I-2N+ made the bait I-2N+:K207R.

The Rx CC-NB-ARC and Rx full-length coding region in pBAD was obtained from Wageningen University (Hans Keller). An Ncol/EcoRI fragment of Rx CC-NB-ARC was first ligated into a pGEXT4-1 vector digested with the same enzymes. Vector pGEXT4-1 was previously modified to contain extra restriction sites for Ncol, Xbal and BglII. This plasmid encodes Rx amino acids 1 to 426 which is fused N-terminally to GST. Bait Rx: CC-NB-ARC was created following the same strategy where an Ncol/EcoRI fragment from pGEXT4-1: Rx CC-NB-ARC was cloned into pAS2-1 vector digested with Ncol and EcoRI. To create full-length Rx bait, firstly a Ncol/Smal fragment of full-length Rx from pBAD were exchanged with Ncol/Pmel fragment of pGEXT4-1. This plasmid encodes full-length Rx protein fused N-terminally to GST and C-terminally to HIS tag. Then a Ncol/SalI fragment was ligated to pAS2.1 vector digested previously with the same enzymes.

In case of Mi1.2: CC-NB-ARC bait, a Ncol/BsmI fragment was cut out from plasmid pKG6210 containing the Mi1.2 gene (www.keygene.com) and cloned into pAS2-1 digested with Ncol/Smal. The resulting plasmid was digested with Ncol, filled with Klenow polymerase and re-ligated in order to adjust the sequence into the frame (161- 896aa). To create the full-length Mi1.2 bait several intermediate plasmids have been constructed. Firstly, following amplification on pKG6210 (www.keygene.com) with primers FP181 (egg gat cac gtt att tcg atc acc ggt atg c), FP182 (ggg gta ccc ggc tat ttc ttt acc gac atc), FP183 (ggg gtac ccc gtt gtg aca aat cgg cgg tg), FP184
(ctg gtc gac cta ctt aaa taa ggg gat att ctt ctg) and three-point ligation BamHI/KpnI/Sall, NB-ARC-LRR fragment of Mi-1.2 gene has been cloned into pUC vector, previously digested with BamHI/Sall. Then, a XhoI/MscI fragment of pKG6210 (www.keygene.com) has been cloned into this plasmid digested with the same enzymes.

The resulting vector has been used to create bait NB-ARC-LRR in pAS2.1 plasmid by ligation of its BamHI/Sall fragment into pAS2.1 digested with the same enzymes. To adjust the sequence into the frame this plasmid was digested additionally with NcoI, filled with Klenow polymerase and re-ligated. An AgeI/SalI fragment of resulting vector has been exchanged with an AgeI/SalI fragment from described above Mi1.2: CC-NB-ARC bait to create bait expressing almost full-length protein that is missing only first 161aa. To fill the gap of first 161aa and create final bait expressing full-length Mi1.2 protein, a PCR fragment amplified by FP194 (cat gcc atg gaa aaa cga aaa gat att gaa g) and FP180 (ggg gta ccg agt tga aac aga ggt aag ac) on pKG6210 (www.keygene.com) was ligeted to this vector after NcoI digests. Since vectors carrying full-length Mi1.2 gene are unstable in E.coli, the resulting vector has been cloned directly to yeasts.

For the I-2 C1: CC-NB-ARC bait we used a cosmid containing this homolog (cosmid A29 (Simons et al., 1998) as starting material. Using primers F I-2C1 (CAG ATT TGA GCC ATG GAG ATT GG) and R I-2C1 (GGG CCG ACA TTG TTC CAA CAT ATG) and Pfu DNA polymerase (Stratagene) a DNA fragment was amplified that encoded amino acids 1-526 from I-2C1. The fragment was cloned into the pAS2-1 vector using the same restriction sites as used for the corresponding I-2 fragment (de la Fuente van Bentem et al., 2005).

**Prey construct:**

The insert of SfFormin homolog SGN-U583099 was amplified using primers: FM13 (TTC CCA GTC ACG ACG TTG T) and RHom (CCG AAT TCG TAT ACG AGC TGC CCG TGC). PCR products were digested with EcoRI and XhoI restriction enzymes and ligated into the pACT2 vector (CLONTECH Laboratories, Palo Alto, CA, USA).

For all bait and prey constructs western blot analysis confirmed that the expected chimeric proteins were produced in the yeast host PJ69-4a. For these blots yeast cells were collected and proteins were extracted as described in the Clontech yeast protocol book (http://www.clontech.com/images/pt/PT3024-1.pdf). Protein samples were loaded on SDS–PAGE gels for immunoblotting. Blots were probed using αGal4DB for bait proteins or with αGal4AD for prey proteins (Clontech) antibodies followed by goat anti-mouse antibodies conjugated to Horseradish peroxidase (Pierce).

**Yeast two-hybrid (Y2H) assays and –library screen**

The Y2H assays and –library screens were performed as described (de la Fuente van Bentem et al., 2005). The nearly full-length I-2 bait (1-872aa) was used to screen the tomato-Fusarium interaction cDNA library and 7.106 yeast transformants were tested for growth on MM–HWL plates. After two days of growth at 30°C, the plates were replica-plated to MM -AWL and MM -HWL selective plates, and to MM -WL plates. The original plate was subjected to an X-gal staining procedure (Duttweiler, 1996) for detection of the LacZ marker. A second
series of selective plates (MM-AWL, MM-HWL and MM-WL) was made from the first MM-WL replicate. After 5 days of growth at 30°C, the growth on the second series of selective plates was determined.

**Cloning of SlFormin and SlTrax:**
To obtain a full-length cDNA sequence of SlFormin we performed primer-walking using gene- and plasmid-specific primers on the tomato-<i>Fusarium</i> interaction cDNA library as template (de la Fuente van Bentem et al., 2005). After 3 steps, we identified the full-length coding region that was amplified with the primers combination: I2I-1-F1 (AGG GGC TTC AAT CCA TCT G) and I2I-1-stop-R (CAG TCG ACC TAC GGG CTT GAG CTC TCG T). PCR fragments were cloned into pGEMT-easy (Promega) and three independent clones were sequenced. The sequence consensus was compared to the genomic sequence present in the SOLdatabase (SL1.00sc05390_84.1.1) (http://solgenomics.net/; SGN Tomato Combined). In case of SlTrax, the full-length cDNA was directly amplified from the cDNA library (de la Fuente van Bentem et al., 2005) with primers: I2I-2-ATG-F (ATG GCT TCA AAA CCC CAG CGC) and I2I-2-R (TTC AAT GTC TGG CAT GCC CAA). The forward primer was designed on the ATG codon of SGN-U575744 sequence (http://solgenomics.net/) of which C-terminal part of coding region was identical to the insert in I2I-2. PCR fragments were cloned into pGEM-T easy (Promega). After sequencing three independent clones, the consensus was compared to SGN-U575744.

All PCRs were performed with Pfu (Fermentas).

**Design of RNAi construct.**
The RNA interfering hairpin constructs were produced by fusing part of the target gene to a fragment of the <i>GUS</i> gene (Wroblewski et al., 2007; Tomilov et al., 2008). Briefly, around 300-400 bp fragments covering 3’ or 5’ end of the gene-coding region was amplified with primers in which a SfiI restriction enzyme cleavage site was introduced. The specific primer sets for SlFormin were: Formin 3’ F1 Sfi-I (ATG GCC ATG TAG GCC GTC CTG AGT CTT TGC AAG) and Formin 3’ R1 Sfi-I (ATG GCC AGA GAG GCC GAC AGT GAG AGG CTG TGG); Formin 5’ F1 Sfi-I (ATG GCC ATG TAG GCC CGA TTA GGG GCT TCA ATC) and Formin 5’ R1 Sfi-I (ATG GCC AGA GAG GCC GAA TCC ATA CTT GCT CGG) (15 bp adapters containing the SfiI cleavage site are underlined). For SlTrax the used primers combinations were: Trax 3’ F1 Sfi-I (ATG GCC ATG TAG GCC CTG CAG TTT TGT GCG TGA) and Trax 3’ R1 Sfi-I (ATG GCC AGA GAG GCC TGC CCA ACA GTG GAT AAC); Trax 5’ F1 Sfi-I (ATG GCC ATG TAG GCC CAG CGC ATT CTG CAC TTG) and Trax 5’ R1 Sfi-I (ATG GCC AGA GAG GCC GAA CCC CAG GAG AAT ATG C). The obtained fragments were fused to the <i>GUS</i> gene and introduced into the binary vector pGollum to create an inverted repeat structure as described before (Wroblewski et al., 2007; Ament et al. 2010; Krasikov et al. 2010).

**Plant transformation and selection of transgenic plants.**
<i>S. lycopersicum</i> cv. Motelle was transformed with the silencing constructs described above using Agrobacterium mediated transformation using our optimized protocol for plant transformation (Ament et al. 2010; Krasikov et
Transformants were selected as described (Ament et al. 2010; Krasikov et al. 2010). In short, the presence and number of the T-DNA insertions in primary transformants was assessed by analyzing the presence of the neomycin phosphotransferase gene (*NPTII*) using PCR followed by Southern blotting using the *NPTII* gene as probe (data not shown). Efficiency of gene silencing in T0 parents and T1 progeny was assayed by screening for reduced *GUS* expression. Reduction in *GUS* expression was visualized by a histochemical *GUS* staining (Jefferson et al., 1987) of transgenic plants agroinfiltrated with the pTFS40:GUS vector (Jones et al., 1992; Wroblewski et al., 2005) (data not shown).

Per silencing construct, two lines (called a and b) that showed the highest level of *GUS* silencing were selected for further analysis. In these lines *Sl*Formin or *Sl*Trax silencing efficiency was measured using quantitative-PCR (qPCR).

**RNA isolation, cDNA synthesis and quantitative PCR.**

Total RNA was isolated from roots of five four-week-old seedlings using Trizol LS reagent (Invitrogen) followed by a chloroform extraction and isopropanol precipitation. Additional RNA purification was performed using RNaseasy mini-columns (Qiagen) and contaminating DNA was removed with DNase (Fermentas). The concentration of total RNA was estimated on agarose gel and by using a NanoDrop ND-1000 spectrophotometer. cDNA was synthesized from 8 µl total RNA using M-MuLV Reverse Transcriptase (Fermentas) as described by the manufacturer in a 20 µl reaction. PCRs were performed in an ABI 7500 Real-Time PCR system (Applied biosystems) using Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). 20 µl PCR reactions contained 0.25 µM of each primer, 0.1 µL ROX reference dye and 20 ng cDNA. The cycling program was set to 2 min 50°C, 5 min 95°C, 45 cycles of 15 sec 95°C and 1 min 60°C. Primers used were F6qFormin (ACA TGC GGA ACA GGA CAT TA) and R6qFormin (AAA GAG ACG CAA GCC TTC AT) for *Sl*Formin amplification and F1qTrax (GAG GTT AGC AAT CGG TCG AA) and R1qTrax (TCC ATC TCT GGG GCA ATA AG) for *Sl*Trax amplification. Amplification with selected primers sets was confirmed for linearity with standard cDNA dilution series and analysis of primers melting curves. Expression level was normalized to the expression of α-tubulin (TC170178) detected with primers qTubL (CAG TGA AAC TGG AGC TGG AA) and qTubR (TAT AGT GGC CAC GAG CAA AG) and compared to relative expression in an EV line (Empty Vector transformed tomato line). Two independent RNA isolations followed by two cDNA syntheses represented two biological replicas. Each was tested twice with q-PCR.

**Fusarium bioassays.**

Ten-days-old tomato seedlings of the silenced lines and an empty vector control line were inoculated with either an avirulent isolate of *Fusarium oxysporum* f. sp. *lycopersici* (race 2 Fol007) or with a virulent isolate (race 3 Fol029) (Rep et al., 2005) using the root dip method (Wellman, 1939). Mock-inoculated seedlings served as controls. Inoculations and scoring were performed as described (Rep et al., 2004). Briefly, 21 days after inoculation plant-weight above the cotyledons was measured and the disease index was determined. The disease
I-2 Y2H interactors: SlFormin and SlTrax

index is correlated to the extent of browning of vessels, which is illustrated on a scale from 0 (no symptoms, none of the vessels brown) to 4 (wilt disease; all vessels brown or plant dead). One-way ANOVA and pair-wise comparison with Student’s t-test for the weight measurements and the non-parametrical Man-Whitney test for the disease index was performed using GraphPad Prism 5.0 software. The experiment was performed twice, using 40-60 plants per line.

PVX screen
Creation of the binary PVX:Avr2 and PVX:Avr2<sup>6H</sup> constructs and their transformation to A. tumefaciens GV3101 was described before (Houterman et al., 2009). Toothpick inoculation of three-week-old tomato plants was performed as described (Takken et al., 2000; Houterman et al., 2009). Inoculated and systemic leaves were scored at 8, 10 and 12 days after inoculation for development of necrotic symptoms. The hypersensitive response (HR) was quantified on an arbitrary scale from 0 - 4: 0- no HR; 1- only inoculated cotyledons show necrosis, plant does not display visible HR on systemic leaves; score 2- HR started to develop on systemic leaves; score 3- HR is more developed on systemic leaves compare to score 2, upper leaves started to curl down; score 4- extensive systemic HR and strong curling of the leaves (Fig. S3). The experiment was repeated twice, which allowed analysis of ± 35 plants per line. One-way Anova and pair-wise comparison with the non-parametrical Man-Whitney test was performed using GraphPad Prism 5.0 software.

SUPPLEMENTARY FIGURES

Fig. S1. Specificity of interaction in between I2I-1 or I2I-2 and CC-NB-ARC baits of I-2, Rx, Mi-1.2 and I-2C1.
Y2H analyses showed interactions of I2I-1 or I2I-2 with the I-2 bait containing the CC-NB-ARC (1-520aa), but not with similar bait constructs containing the CC-NB-ARC domains of I-2C1 (1-526aa), Rx (1-426aa) or Mi-1.2 (161- 896aa). None of the bait and prey proteins showed autoactivity as tested by co-expression with pACT2 and pAS2.1 empty vectors. Activation of the HIS3 marker is shown after 10 days of growth (upper panel). Expression of baits proteins was confirmed by western blot (WB) analysis using an αGal4BD antibody for detection on total yeast protein extracts (lower panel).
Fig. S2. Bioassay with race 3 *Fusarium oxysporum* f. sp. *lycopersici*

Ten-day-old seedlings of empty vector (EV) transformed and SIFormin or SITrax silenced tomato lines were infected with race 3 *Fusarium oxysporum* f. sp. *lycopersici* (Fol029), which is virulent on the EV control line. The disease index was determined 21 days post inoculation. The distribution of plants over the different disease indexes is depicted. Anova analysis reveals no significant differences in disease indexes between silenced (Formin3a, Formin5b, Trax5a, Trax5b) and non-silenced (EV) tomato lines upon infection with Fol007. Data represent two independent experiments with the number of plants indicated (n=).

Fig. S3. PVX::Avr2 HR intensity scale in tomato.

Photographs represent a zoomed view on a systemic leaf (upper panel) and an entire tomato plant (lower panel) of representative individual I-2 containing cv. Motelle plants inoculated with PVX::Avr2. Appearance of the hypersensitive response (HR) as a result of recognition of Avr2 by I-2 was scaled based on visual scoring. Score: 1- only inoculated cotyledons show necrosis, plant does not display visible HR on systemic leaves; score 2- HR started to develop on systemic leaves; score 3- HR is more developed on systemic leaves compare to score 2, upper leaves started to curl down; score 4- extensive systemic HR and strong curling of the leaves.
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I-2 Y2H interactors: SIFormin and SITrax


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