Kinase-dead mutation: A novel strategy for improving soybean resistance to soybean cyst nematode *Heterodera glycines*

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**Abstract**

Protein kinases phosphorylate proteins for functional changes and are involved in nearly all cellular processes, thereby regulating almost all aspects of plant growth and development, and responses to biotic and abiotic stresses. We generated two independent co-expression networks of soybean genes using control and stress response gene expression data and identified 392 differentially highly interconnected kinase hub genes among the two networks. Of these 392 kinases, 90 genes were identified as "syncytium highly connected hubs", potentially essential for activating kinase signalling pathways in the nematode feeding site. Overexpression of wild-type coding sequences of five syncytium highly connected kinase hub genes using transgenic soybean hairy roots enhanced plant susceptibility to soybean cyst nematode (SCN; *Heterodera glycines*) Hg Type 0 (race 3). In contrast, overexpression of kinase-dead variants of these five syncytium kinase hub genes significantly enhanced soybean resistance to SCN. Additionally, three of the five tested kinase hub genes enhanced soybean resistance to SCN Hg Type 1.2.5.7 (race 2), highlighting the potential of the kinase-dead approach to generate effective and durable resistance against a wide range of SCN Hg types. Subcellular localization analysis revealed that kinase-dead mutations do not alter protein cellular localization, confirming the structure–function of the kinase-inactive variants in producing loss-of-function phenotypes causing significant decrease in nematode susceptibility. Because many protein kinases are highly conserved and are involved in plant responses to various biotic and abiotic stresses, our approach of identifying kinase hub genes and their inactivation using kinase-dead mutation could be translated for biotic and abiotic stress tolerance.

**Keywords**
gene co-expression network, *Heterodera glycines*, kinase-dead, protein kinases, soybean
INTRODUCTION

The soybean cyst nematode (SCN; *Heterodera glycines*) is an obligate sedentary endoparasite of the soybean (*Glycine max*) root system and causes significant yield loss (Koenning & Wrather, 2010). The parasitic second-stage juvenile (J2) penetrates the root and migrates intercellularly toward the vascular tissues, where it induces and maintains a metabolically hyperactive feeding site, known as the syncytium. The parasitic juvenile feeds from the syncytium throughout the parasitic stages to maturity as an adult male or female. The current best management strategy to control SCN infection in soybean fields is the use of nematode-resistant cultivars coupled with effective cultural practices. However, the frequent use of the same resistant germplasm has provoked the ability of SCN to adapt to these strategies and readily overcome resistance (Vuong et al., 2013).

Extensive gene expression analysis of SCN-infected soybean plants has revealed massive gene expression changes in signal transduction pathways (Kandoth et al., 2011; Klink et al., 2007, 2009, 2013). Protein kinases, as core components of signal transduction pathways, phosphorylate the majority of cellular proteins, altering their activities, subcellular localization, folding, and macromolecular interactions, thereby affecting a wide range of intracellular signalling pathways (Colcombet & Hirt, 2008; Lehti-Shiu & Shiu, 2012; Tena et al., 2011). Protein kinase genes exist by the hundreds in all plant species in which they have been surveyed and comprise more than 3% of the annotated proteins in plants (Lehti-Shiu & Shiu, 2012; Lehti-Shiu et al., 2009). The protein kinase repertoire or kinome has significantly more members in plants than other eukaryotes, including animals. Recent gene duplication events and high retention rate of duplicates in plants is probably the reason for the increasing kinome growth, and has functional significance (Hanada et al., 2008; Lehti-Shiu & Shiu, 2012; Liu et al., 2015).

Several studies have indicated that extensive expansion of specific protein kinase families such as receptor-like kinase (RLK)/Pelle has also contributed to the large size of plant protein kinase families (Gao & Xue, 2012; Lehti-Shiu et al., 2009; Shiu & Bleecker, 2003; Shiu et al., 2004; Vij et al., 2008; Wei et al., 2014). The RLK/Pelle family has expanded to a significantly greater extent than other kinases and in general constitutes more than 60% of the protein kinases in flowering plants. The large size of this family is attributed to the expansion of a few subfamilies, specifically those bearing leucine-rich repeat (LRR) domains (Lehti-Shiu et al., 2009). Because members of these subfamilies are known to have roles in defence/resistance responses, it has been suggested that the wide expansion is probably a result of adaptation to rapidly evolving pathogens (Lehti-Shiu et al., 2009). In contrast, the RLK subfamilies with developmental functions are conserved in size (Shiu et al., 2004). Functional studies of protein kinases in *Arabidopsis thaliana* during the last two decades have positioned protein kinases as core components of various signalling pathways controlling multiple biological processes (Champion et al., 2004; Colcombet & Hirt, 2008; Gish & Clark, 2011; Morillo & Tax, 2006; Rodríguez et al., 2010; Tena et al., 2011).

Several experimental lines of evidence have suggested that protein kinases may play a role in establishing plant–nematode interactions. First, plant protein kinases regulate numerous cellular processes, including those involved in innate immunity and disease resistance (Mendy et al., 2017; Tena et al., 2011). Second, protein kinases are commonly targeted by pathogen effectors including the cyst nematode effector 10A07 (Hewezi et al., 2015). Third, protein kinase genes are frequently identified among the functional groups of genes with greatest altered expression on SCN infection (Jain et al., 2016; Klink et al., 2007; Kofsky et al., 2021). Thus, dysregulation of kinase functions and signalling can be explored as an effective disease resistance approach. In this context, the robust conservation of the kinase domain in the protein kinase gene superfamily has facilitated the identification of single amino acids critical for the activity of these enzymes (Hanks & Hunter, 1995). Mutations in only one or two sequences of amino acids can completely abolish the activity of these enzymes but do not interfere with substrate recognition, that is, these kinases can be modified to create “kinase-dead mutants” that lack the ability to autophosphorylate or catalyse phosphorylation of targeted proteins but function antagonistically to the wild-type kinase (have dominant-negative effects) (Haruta et al., 2018; Hewezi et al., 2015).

The exploration of plant protein kinases for engineering pathogen resistance has been very limited. This is mainly due to their large number associated with redundant functions and compensatory signalling, making selection of individual protein kinases for functional analysis a real challenge. Therefore, identification of novel targeting approaches to select critical kinases within the kinome is essential to efficiently disrupt signalling cascades driven by aberrant kinase activity induced by plant pathogens to cause diseases. In this context, gene co-expression networks offer genome-scale information and have tremendous potential to identify highly connected genes (hub genes) that are trait-correlated as key regulators (Palumbo et al., 2014; Stuart et al., 2003). When the expression patterns of two or more genes are correlated across multiple tissues and stress conditions, these genes are said to be co-expressed (Wolfe et al., 2005). These co-expression associations are generally inferred from genome-wide gene expression studies with no reference to the mechanisms behind these correlations. However, by comparing control and stress gene co-expression networks, it is possible to identify differentially interconnected hub genes that change the network structures and topology (de la Fuente, 2010; Palumbo et al., 2014).

While plant protein kinases are commonly targeted by pathogen effector proteins (Block et al., 2008; Dou & Zhou, 2012; Hogenhout et al., 2009), including those secreted from SCN (Hewezi & Baum, 2013; Hewezi et al., 2015), identifying and targeting hub kinases or kinome modulators in plants has not yet been explored. In this study, we developed and validated a new approach for identifying bona fide target genes for SCN resistance. This novel approach relies on identifying highly interconnected kinase hub genes to prioritize SCN resistance gene candidates for functional validation using the novel kinase-dead mutation approach. Our functional assays validate our novel approach of identifying functional kinase hub genes that are...
SCN-related and confirm the function of the kinase-dead mutations in enhancing soybean resistance to SCN.

2 | RESULTS

2.1 | Generation of global gene co-expression networks and identification of highly connected kinase hub genes

Differential networking of gene expression has emerged as a powerful approach to detect changes in network structures and topology to identify differentially connected hub genes among two networks that are trait-related (de la Fuente, 2010; Palumbo et al., 2014). We generated two independent co-expression networks of soybean genes using control and stress response gene expression data from publicly available RNA-Seq data sets (Figure 1a,b). The co-expression networks were generated from 394 samples collected under nonstressed conditions and 281 samples collected under various stress conditions (Table S1). These samples were from different tissues, including cotyledon, embryo, seed, whole seedling, root, lateral root, root hair, root tip, nodule, leaf, flower, and pod. The RNA-Seq data of stressed conditions included samples from plants that were subjected to abiotic stresses (CO₂, cold, drought, heat, ozone, salt, pH, iron deprivation, nitrogen deprivation, phosphorous deprivation, and potassium deprivation) or biotic stresses (Aphis glycines, Macrophomina phaseolina, Fusarium oxysporum, Heterodera glycines, Phytophthora sojae, Sclerotinia sclerotiorum, and soybean mosaic virus). Reads per kilobase of transcript per million reads mapped (RPKM) values of all genes across various samples are provided in Table S1. Construction of the networks was performed by determining all pairwise gene expression correlations between individual soybean genes using a Pearson correlation coefficient (PCC) value of 0.70 and a false discovery rate (FDR) less than 0.05. We used PCC because it has been recently proven to be better suited for the construction of global gene co-expression networks than other methods (Liesecke et al., 2018). The co-expression network generated using control gene expression data comprised 61,162 edges (co-expression events) and 8685 nodes (genes) (Figure 1c). Of these 8685 nodes, 806 (9.28%) are putative protein kinases (Figure 1c; orange dots in Figure 1a). Similarly, the co-expression network generated using stress gene expression data comprised 70,037 edges and 9600 nodes (genes) (Figure 1c). Of these 9600 nodes, 887 (9.24%) are kinases (Figure 1c; orange dots in Figure 1b). These results indicate that protein kinases constitute a significant part of gene co-expression nodes.

![Gene co-expression networks of soybean genes under control and stress conditions. (a) and (b) Two independent co-expression networks were generated using control (a) and stress (b) gene expression data. Nodes indicate genes and edges indicate co-expression events between co-expressed genes. Nodes in orange represent protein kinases. (c) Summary table of total numbers of edges, nodes, and kinase nodes in the co-expression networks generated using control and stress gene expression data](image-url)
We next identified kinase hub genes among the 806 and 887 kinase nodes indicated above. A protein kinase was considered a hub if it is co-expressed with at least 25 genes. Also, kinase genes with two-fold differential co-expression events between the two networks were considered as differentially co-expressed hub genes. Using these criteria, we identified 416 and 518 kinase hub genes in the control and stress networks, respectively (Figure 2a). Of these kinase hub genes, 271 were common to both gene co-expression networks, 145 were specific to the control network, and 247 were specific to the stress gene co-expression network (Figure 2a). In total, we identified 392 (145 + 247) differentially highly interconnected kinase hub genes between the two networks. An example of a protein kinase hub gene showing distinct co-expression events between stress and control networks is provided in Figure 2b. The protein kinase gene Glyma.06G145500 has only 26 edges (co-expressed genes) in the control network, whereas it has 309 edges in the stress network (Figure 2b).

2.2 Identification of highly connected kinase hub genes that change expression in SCN feeding site

Highly connected hub genes have been shown to play key roles in the biology of lower organisms (yeast, fly, and worm) and higher organisms (mice and humans) (Vidal et al., 2011). Identifying hub genes that change the topology of signalling pathways in the feeding site of SCN is expected to lead to identifying crucial targets for genetic manipulation. We reasoned that highly connected kinase hubs with a key role in facilitating plant susceptibility to SCN should exhibit differential expression in the syncytium relative to nonsyncytial cells. Therefore, we compared the gene list of highly interconnected kinase hubs specified above (392 genes) with a reference list of 6903 syncytium differentially expressed genes (Ithal et al., 2007; Kandoth et al., 2011; Klink et al., 2009, 2010). Interestingly, we identified 90 protein kinases as “syncytium highly connected hubs” potentially central to modulating the structure and function of kinase signalling pathways in the nematode feeding sites (Figure 2c and Table S2). Out of these 90 kinase hub genes, 40 were highly interconnected in the control network and 50 were highly interconnected in the stress network. Subcellular localization prediction of these kinases provided suggestions for extracellular, cytoplasmic, or nuclear localizations (Table S2), suggesting a role of these hubs in signal perception and transmission to the nucleus to control gene expression of downstream targets.

2.3 Overexpression of syncytium kinase hub genes in wild-type form significantly enhanced soybean susceptibility to SCN Hg Type 0 (race 3)

If syncytium kinase hub genes have functions in mediating soybean susceptibility to SCN, one can reasonably postulate that ectopic expression of wild-type variants would increase plant susceptibility to SCN. To this end, we overexpressed the wild-type coding sequences of five syncytium highly connected kinase hub genes (Glyma.13G150000, Glyma.12G056000, Glyma.18G141500, Glyma.04G222800, and Glyma.14G026300) using the soybean hairy root system and challenged the transgenic roots with SCN. These five kinase hub genes were selected because they showed varied levels of connectivity (between 33 and 622 co-expression events) and belong to various kinase subfamilies. The five selected kinases encode leucine-rich repeat protein kinase family proteins (Glyma.13G150000 and Glyma.04G222800), a malectin/receptor-like protein kinase family protein (Glyma.14G026300), an...
AGC (cyclic AMP-dependent, cGMP-dependent and protein kinase C) protein kinase (Glyma.12G056000), and a cysteine-rich receptor-like protein kinase (Glyma.18G141500). These five kinases also exhibited differential expression in the SCN-induced syncytium. Glyma.04G222800, Glyma.14G026300, and Glyma.12G056000 were up-regulated in the SCN syncytium on average by 9.7-, 3.26-, and 2.15-fold, respectively, whereas Glyma.13G150000 and Glyma.18G141500 showed 4.86- and 7.26-fold down-regulation in the syncytium, respectively (Ithal et al., 2007; Klink et al., 2009, 2010). Several transgenic hairy root plants overexpressing these five kinase hub genes under the control of a soybean ubiquitin promoter were generated in the SCN-susceptible cultivar Williams 82. Transgenic roots were identified and selected using the green fluorescent protein (GFP). Transgenic hairy roots expressing the empty vector containing the GFP marker gene were used as control. Reverse transcription quantitative PCR (RT-qPCR) quantification of the expression levels of wild-type variants of these five genes in three biological samples of the transgenic hairy roots revealed a more than 5-fold increase in the expression of these kinases in the transgenic hairy roots as compared with the control roots expressing the empty binary vector (Figure S1a). Both transgenic and control plants were inoculated with 3000 eggs of SCN (HG type 0 race 3) in three biologically independent experiments each with at least five plants. Five weeks after inoculation the number of cysts per plant was counted and used to calculate the female index. The female index was calculated by dividing the average number of cysts on the transgenic hairy root lines by the average number of cysts on the control plants, which was set to 100%. Data represent mean ± SE of three replicates each with five plants. Asterisks indicate statistically significant differences from control plants ($p < 0.05$) as determined by analysis of variance.
2.4 | Overexpression of kinase-dead variants of syncytium kinase hub genes significantly enhanced soybean resistance to SCN Hg Type 0 (race 3)

We next examined whether overexpression of inactive variants of the five selected syncytium kinase hub genes (Glyma.13G150000, Glyma.12G056000, Glyma.18G141500, Glyma.04G222800, and Glyma.14G026300) would function antagonistically to the wild-type variants, resulting in increased soybean resistance to SCN. The kinase-dead variants were generated by mutating the two conserved lysine residues in the ATP-binding pocket and the substrate-binding pocket to arginine (Figure S2). These lysine residues interact directly with ATP and mutation of these residues leads to a classical “kinase-dead” mutant (Haruta et al., 2018; Hewezi et al., 2015; Zhou et al., 2018). Because the main function of protein kinases is the phosphorylation of specific substrates, kinase-dead mutations are expected to confer loss-of-function phenotypes. The transgenic hairy roots overexpressing kinase-dead variants were not visibly different from the empty vector-transgenic plants. RT-qPCR quantification of the expression levels of the kinase-dead variants in three biological samples of the transgenic hairy roots revealed a more than 6-fold increase in the expression of these kinases in the transgenic hairy roots relative to the control roots expressing the empty vector (Figure 5b). The transgenic hairy root plants were inoculated with SCN (Hg Type 0) in three independent experiments and susceptibility levels were determined as described above. Interestingly, the transgenic hairy roots overexpressing syncytium kinase hub genes in inactive forms showed a significant reduction in SCN susceptibility. Overexpression of Glyma.13G150000 in inactive form reduced the female index by 36% of the control, whereas Glyma.12G056000, Glyma.18G141500, and Glyma.04G222800 reduced the female index by about 50%. Glyma.14G026300 showed the most significant impact with 67% reduction in the female index (Figure 3b). These results confirm the structure-function of the kinase-dead mutation in generating loss-of-function phenotypes causing significant decrease in nematode susceptibility.

2.6 | Kinase-dead mutations did not alter protein cellular localization

The reduced nematode susceptibility phenotype of kinase-dead overexpressing plants implies that the inactive variants localize to the same cellular compartment as the wild-type variants to function antagonistically to the wild-type proteins and generate loss-of-function effects. Therefore, we fused the coding sequences of wild-type and kinase-dead variants of the five syncytium kinase hub genes upstream of the enhanced yellow fluorescent protein (eYFP) reporter and under the control of the CaMV 35S promoter. Then, we determined the subcellular localization of these fusions in onion epidermal cells using particle bombardment. As shown in Figure 5, the five syncytium kinase hub fusions, both in wild-type and inactive kinase forms, were localized to the plasma membrane and/or cytoplasm. These data not only confirm the subcellular localization predictions of these kinases (Table S2), but also indicate that kinase-dead mutations do not alter the cellular localization of these kinases.

2.7 | Co-expressed genes of syncytium kinase hubs are enriched in various biological processes and metabolic pathways

We next examined whether genes co-expressed with each of the five selected syncytium kinase hubs are enriched in specific biological processes and metabolic pathways. Gene Ontology (GO) analysis of Glyma.04G222800 co-expressed genes revealed enrichment in terms related to response to nematode, defence response, cell wall organization, hormone regulation, and transmembrane transport (Figure 6a). Glyma.12G056000 co-expressed genes were enriched in terms involved in the indole acetic acid (IAA) biosynthetic process, protein phosphorylation, and transmembrane transport (Figure 6a). Glyma.14G026300 co-expressed genes were enriched in terms associated with transmembrane transport (Figure 6a).

KEGG pathway analysis of genes co-expressed with the five selected syncytium kinase hubs revealed enrichment in pathways with a possible role in controlling plant response to nematode infection, including the MAPK signalling pathway, hormone signal
transduction, inositol phosphate metabolism, and protein processing and ubiquitination (Figure 6b). Together, these analyses suggest that genes co-expressed with the syncytium kinase hubs share biological functions and may also be co-regulated during SCN infection.

3 | DISCUSSION

The large number of protein kinase-encoding genes in plant genomes, along with their redundant functions, often impede efforts for selecting single protein kinase genes for functional studies. In this regard, differential networking of gene expression has the potential to identify differentially highly interconnected kinase hub genes among two or more gene co-expression networks that are trait-related (de la Fuente, 2010; Palumbo et al., 2014). In this study, we constructed two independent soybean co-expression networks of control and stress gene expression data. We found that protein kinases represented more than 9% of all detected nodes despite the fact that the soybean protein kinase gene family constitutes only 4.67% of all predicted protein-coding genes (Liu et al., 2015). This finding reflects the high tendency of protein kinases to function as highly interconnected hubs in gene co-expression networks. Importantly, the network analysis resulted in the identification of 392 differentially connected kinase hub genes among the two networks. This gene list represents bona fide targets for functional studies of kinase genes with functions related to biotic or abiotic stresses.

Among this gene list, several kinases have been documented to play important roles in regulating plant responses to biotic or abiotic stress conditions. For instance, the kinase hub gene GmSnRK1.1

![FIGURE 4 Overexpression of kinase-dead variants of syncytium kinase hub genes significantly increased soybean resistance to soybean cyst nematode (SCN) Hg Type 1.2.5.7 (race 2). (a) and (b) Female index of transgenic hairy root plants overexpressing wild-type (a) or kinase-dead variants (b) of five kinase hub genes (Glyma.13G150000, Glyma.12G056000, Glyma.18G141500, Glyma.04G222800, and Glyma.14G026300) inoculated with SCN Hg Type 1.2.5.7 (race 2) relative to the transgenic hairy root plants expressing the empty vector (control). The female index was determined by dividing the average number of cysts on the transgenic hairy root lines by the average number of cysts on the control plants, which was set to 100%. Data represent mean ± SE of three replicates each with five plants. Asterisks indicate statistically significant differences from control plants (p < 0.05) as determined by analysis of variance.](image)
Glyma.08G240300 has been reported to positively regulate soybean resistance to P. sojae (Wang et al., 2019). Glyma.10G298400, another kinase hub, is a homolog of the Arabidopsis gene encoding PBS1 protein kinase, which is targeted and cleaved by the Pseudomonas syringae pv. glycinea effector protein AvrPphB, leading to disease resistance (Helm et al., 2019). Among the 392 kinase hub genes, we also found HAK1 (Glyma.11G200300), which has been recently shown to be involved in soybean resistance to herbivores (Uemura et al., 2020). Also, a function for the kinase hub gene GmWNK1 (Glyma.20G145800) in soybean osmotic stress tolerance through abscisic acid-mediated signalling has been demonstrated (Wang et al., 2010). Furthermore, 31 Arabidopsis orthologs of the identified kinase hub genes have been implicated in biotic or abiotic stress responses (Table S3), providing additional evidence for the key functions that these kinase hub genes may play in stress responses.

Of the 392 kinase hub genes, 90 were found to change expression in the SCN feeding site and therefore were considered as "syncytium highly connected hubs". These kinase hub genes probably constitute the core components of signal transduction pathways in the syncytium. Notably, 54 of these 90 kinases belong to the RLK-Pelle gene family, which is known to function in regulating plant responses to biotic stresses (Chen et al., 2003; Gish & Clark, 2011; Lehti-Shiu et al., 2009; Liu et al., 2015; Wrzaczek et al., 2010). The syncytium highly connected hubs also included kinases belonging to the AGC, CAMK (calcium/calmodulin-dependent protein kinases), CMGC (cyclin-dependent kinases [CDKs], mitogen-activated protein kinases [MAPkinaes], glycogen synthase kinases [GSK] and CDK-like kinases), and NEK (Never in mitosis A [NIMA]-related kinases) gene families. Members of these gene families have been reported to be involved in plant defence responses and regulation of programmed cell death (Garcia et al., 2012; Lehti-Shiu & Shiu, 2012; Rademacher & Offringa, 2012).
The strong conservation of the kinase domain among kinome members has allowed the identification of critical amino acids required for the activity of these enzymes. Mutations in one or two conserved lysine residues in the ATP-binding pocket and/or the substrate-binding pocket can completely abolish the activity of these enzymes but do not interfere with substrate recognition or binding to other proteins (Hewezi et al., 2015). As a result, protein kinases can be modified to generate kinase-dead mutants that have no enzymatic activity but have dominant-negative effects in sequestering the activity of the substrates/interactors, causing a loss-of-function phenotype. It has been shown that overexpression of a kinase-dead variant of an *Arabidopsis* protein kinase (IPK), which interacts with the cyst nematode effector protein 10A07, functions antagonistically to the wild-type kinase and produces dominant-negative effects of decreased plant susceptibility to nematode infection (Hewezi et al., 2015). The dominant-negative effect of the kinase-dead variant was mediated through physical interactions between the inactive variant and the effector protein (Hewezi et al., 2015).

We assessed the impact of the kinase hub genes on SCN parasitism of soybean plants by overexpressing five syncytium kinase hub genes in wild-type and kinase-dead forms. We reasoned that overexpression of kinase-dead variants of syncytium highly interconnected kinase hub genes would interrupt the signalling networks and alter expression of downstream genes that mediate SCN susceptibility, resulting in soybean resistance to SCN. Consistent with this hypothesis, the wild-type variants of these kinases enhanced plant susceptibility to SCN to various degrees whereas the kinase-dead variants produced the opposite effects of significant decreases in plant susceptibility. Our results showing that overexpression of kinase-dead variants of *Glyma.18G141500*, *Glyma.13G150000*, and *Glyma.04G222800* enhanced plant resistance to both Hg Type 0 and Hg Type 1.2.5.7 suggest that these kinases are involved in essential...
signalling pathways required for both Hg types to infect soybean plants. The finding that Glyma.14G026300 and Glyma.12G056000 significantly enhanced plant resistance against Hg Type 0 but not Hg Type 1.2.5.7 suggests that these two kinases mediate signalling processes that are Hg type-specific. The susceptibility assays also revealed that overexpression of wild-type variants of Glyma.13G150000, Glyma.18G141500, Glyma.04G222800, and Glyma.14G026300 exerted distinct impacts on soybean responses to Hg Type 0 and Hg Type 1.2.5.7 (Figures 3 and 4). This finding suggests that these kinases may also modulate signalling pathways specific to each Hg type. This suggestion is in agreement with the finding that various Hg types regulate common and unique sets of genes on soybean infection (Hosseini & Matthews, 2014; Ithal et al., 2007; Mazarei et al., 2011; Zhang & Song, 2017). In this regard, identifying syncytium kinase hub genes involved in essential signalling processes required for soybean compatibility with SCN parasitism could be used to generate effective and durable resistance against a wide range of SCN HG types.

The syncytium kinase hub gene Glyma.14g026300 is a close homolog of the Arabidopsis leucine-rich repeat receptor-like kinase (LRR-RLK) with extracellular malectin-like domain 1 (LIK1; AT1G07650), which has been reported to induce cell death when overexpressed in its active form in Nicotiana benthamiana leaves (Li et al., 2020). The impact of the kinase hub gene Glyma.13g150000, a homolog of Arabidopsis PXY-correlated (PXC1, At2G36570), on soybean susceptibility to SCN could be related to its possible role in secondary cell wall formation (Wang et al., 2013). Dramatic changes in the structure and composition of the plant cell wall commonly occur during syncytium formation and development (Bohlmann & Sobczak, 2014; Zhang et al., 2017). Several reports have demonstrated the positive role of various CYSTEINE-RICH RLK proteins in disease resistance via activation of defence responses and reactive oxygen species (ROS) signalling (Acharya et al., 2007; Bourdais et al., 2015; Idanheimo et al., 2014; Kimura et al., 2020; Yadeta et al., 2017). In contrast, our overexpression of an inactive variant of the CYSTEINE-RICH RLK Glyma.18g141500 produced a significant reduction in plant susceptibility to SCN, suggesting a negative function in disease resistance. Similar to our finding, loss-of-function mutants of the Arabidopsis cysteine-rich RLK CRK20 increased plant resistance to P. syringae pv. tomato DC3000 (Edeleri et al., 2011).

Together, our functional assays not only validate our approach of identifying functional kinase hub genes that are trait-related but also confirm the structure–function of the kinase-dead mutation in producing loss-of-function effects. The loss-of-function phenotypes could be due to the ability of catalytically inactive kinases to bind to the substrates and interacting proteins as previously reported (Haruta et al., 2018; Hewez et al., 2015; Liu et al., 2016). Because many protein–protein associations are transient and phosphorylation-dependent (De Bodt et al., 2009; Nintemann et al., 2017; Nishi et al., 2011), retention of interacting proteins could be critical for the inactive kinases to produce the dominant negative effects of increasing soybean resistance to SCN. Considering the fact that plant pathogens generally target common signal transduction pathways to infect host plants and cause disease (Cao et al., 2019; Langin et al., 2020), it is plausible that engineered inactive kinases would provide resistance against other pathogens. This possibility needs to be further investigated. Also, many protein kinases are involved in plant responses to various biotic and abiotic stresses, and hence our approach of identifying kinase hubs genes and their inactivation using kinase-dead mutation could be translated for biotic and abiotic stress tolerance. From an application point of view, kinase hub gene candidates can be inactivated using targeted mutation and genome editing methods (e.g., the CRISPR/Cas9 system) or by identifying single nucleotide polymorphisms (SNPs) in the kinase domain.

4 | EXPERIMENTAL PROCEDURES

4.1 | Data acquisition and RNA-Seq analysis

RNA-Seq samples were downloaded from the NCBI Sequence Read Archive (Leinonen et al., 2011). The SRA files were then converted into FASTQ files. A total of 806 RNA-Seq data sets of various soybean tissues were downloaded comprising 483 data sets from soybean plants growing under nonstressed conditions and 323 data sets from soybean plants growing under various stressed conditions. Higher sequencing depth of the RNA-Seq samples ensured accurate measurement of the gene expression, particularly for genes with low expression (Balloz et al., 2015; Li et al., 2014). Therefore, to include samples with higher sequencing depth in the gene co-expression network, we filtered out RNA-Seq samples with fewer than 10 million reads. Low quality reads and adapter sequences were trimmed using Trimmomatic (Bolger et al., 2014). The high-quality reads were then mapped to the soybean reference genome (Wm82. a2. v. 1) using TopHat v. 2.0.14 (Trapnell et al., 2009). We also filtered out RNA-Seq samples with mapping efficiency of less than 70%, as previously described (Giorgi et al., 2013; van Dam et al., 2018). The number of reads mapped to each protein-coding gene was counted using HTSeq (Anders et al., 2015). Genes with counts of less than 10 in more than 90% of the samples were removed as previously described (Langfelder & Horvath, 2008).

4.2 | Co-expression network analysis

Read counts of RNA-Seq samples were normalized using the variance stabilizing transformation function of DESeq2 (Love et al., 2014). Pairwise Pearson’s correlation coefficients (PCCs) of each protein kinase with all expressed protein-coding genes were computed using the R package psych. To enhance the robustness of the network, we constructed multiple networks by using random sets of samples from the data, as recently recommended by van Dam et al. (2018). This would ensure the removal of pairwise correlations that occur due to specific bias rather than due to biologically relevant interaction (van Dam et al., 2015). To this end, the
pairwise correlations between the kinases and all soybean genes were computed 500 times by randomly selecting 90% of the samples. Gene pairs were considered co-expressed only if they co-expressed 450 times in the 500 randomized tests. Only gene co-expression events with positive PCC values were considered as co-expressed (Jordan et al., 2004; Vandepoele et al., 2009). A protein kinase was considered as a hub if it was co-expressed with at least 25 genes and a protein kinase was considered to be differentially co-expressed if the gene showed at least a two-fold difference in the number of co-expression events between the stress and control networks. The co-expression network was visualized using Cytoscape v. 3.7.2 (Shannon et al., 2003).

### 4.3 | GO enrichment analysis

GO terms enrichment analysis was performed using agriGO (Tian et al., 2017) with Fisher’s exact test and Bonferroni multtest adjustment with a significance cut-off value of 0.05. KEGG analysis was performed by submitting protein sequences to the online database KOABAS. Pathways with adjusted $p < 0.05$ were considered as statistically significant (Xie et al., 2011).

### 4.4 | Subcellular localization prediction and validation

Subcellular localizations of protein kinases were predicted using the online database WoLF PSORT (Horton et al., 2007). To validate the predicted subcellular localization in planta, the coding sequences of the protein kinases (both wild-type and kinase-dead) were PCR-amplified using forward and reverse primers containing restriction enzyme sites (Table S4). The PCR products were digested and purified. After digestion, the purified PCR products were fused to the N-terminus of the eYFP reporter gene in the pSAT6-EYFP-N1 vector. The sequences of all the constructs were confirmed by sequencing. These constructs were introduced into onion epidermal cells by biolistic bombardment as previously described (Hewezi et al., 2010). After bombardment, epidermal peels were incubated at 26°C for 16–24 h in the dark. The subcellular localization of the fused proteins was visualized using the EVOS FL Auto Cell Imaging System (Life Technologies). The subcellular localization assay was repeated twice, each with three replicates.

### 4.5 | Generation of transgenic soybean hairy roots overexpressing wild-type and kinase-dead variants

The wild-type coding sequences of five protein kinase genes were PCR-amplified using gene-specific primers containing Ascl or Swal and BamHI or AvrII restriction sites in the forward and reverse primers, respectively. PCR products of these protein kinases were digested, purified, and cloned into the pG2RNAi2 vector by replacing the GUS linker. The kinase-dead variants of the protein kinases were synthesized by mutating the conserved lysine residue in the ATP-binding site and substrate-binding site to arginine. The kinase-dead variants were PCR-amplified, digested, purified, and cloned into the pG2RNAi2 vector under the control of the Gmubi promoter by replacing the GUS linker. All constructs were verified by sequencing. The binary vector contains the GFP reporter gene under the control of the CaMV 35S promoter to enable the transgenic hairy roots to be identified. All wild-type and kinase-dead variants of the five kinases cloned into the pG2RNAi2 binary vector as well as the empty pG2RNAi2 vector were transformed into Agrobacterium rhizogenes K599 by the freeze-thaw method and used to generate transgenic hairy roots. Composite transgenic hairy root plants were generated by injecting A. rhizogenes containing different binary constructs in the hypocotyl of 5-day-old Williams 82 seedlings as recently described by Rambani et al. (2020). The inoculated soybean seedlings were maintained in Percival reach-in plant growth chambers at 26°C with a 16 h light/8 h dark cycle. After 3 weeks, transgenic hairy roots strongly expressing GFP were selected using a dissecting stereomicroscope equipped with a GFP filter (SZX12; Olympus). Soybean plants with three transgenic hairy roots were selected for SCN susceptibility assays as described below. Primers used for constructing the binary vectors are provided in Table S4.

### 4.6 | Nematode infection assays

Transgenic hairy root plants were transferred to Cone-tainers filled with steam-sterilized sand mixed with top soil at 3:1 ratio. The composite plants were then inoculated with 2500 eggs of SCN Hg Type 0 (race 3) or Hg Type 1.2.5.7 (race 2). Five weeks postinoculation, the cysts were extracted from each plant separately and counted using a stereomicroscope. Cyst counts were used to calculate the female index as a percentage of the average number of cysts counted on the overexpression plants relative to those counted on the control plants expressing the empty vector. The experiment was conducted using a randomized complete block design with five biological replicates and repeated three times. Statistically significant differences between the transgenic overexpression lines and the control plants expressing the empty vector were determined using analysis of variance (ANOVA) with $p < 0.05$.

### 4.7 | RNA isolation and RT-qPCR analysis

To quantify the expression levels of kinase hub genes in transgenic hairy root plants, total RNA was extracted from 500 mg of root tissues following Verwoerd et al. (1989). The total RNAs were then treated with DNase I (Invitrogen) following the manufacturer’s instructions. RT-qPCR was performed in the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) using Verso SYBR Green One-Step RT-qPCR Rox mix (Thermo Scientific) following the manufacturer’s instructions. To normalize the mRNA expression level of
various kinase genes, soybean ubiquitin (Glyma.20G141600) was used as internal control. Primers used for qPCR quantification assays are shown in Table S4.

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CONFLICT OF INTEREST
Tarek Hewezi and Sarbottam Piya have intellectual property related to the study.

DATA AVAILABILITY STATEMENT
The data supporting the findings of this study are provided in the manuscript and supporting information.

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REFERENCES
Acharya, B.R., Raina, S., Maqbool, S.B., Jagadeeswaran, G., Mosher, S.L., Appel, H.M. et al. (2007) Overexpression of CRK13, an Arabidopsis cysteine-rich receptor-like kinase, results in enhanced resistance to Pseudomonas syringae. The Plant Journal, 50, 488–499.

Anders, S., Pyl, P.T. & Huber, W. (2015) HTSeq-a Python framework to work with high-throughput sequencing data. Bioinformatics, 31, 166–169.

Ballouz, S., Verleyen, W. & Gillis, J. (2015) Guidance for RNA-seq co-expression network construction and analysis: safety in numbers. Bioinformatics, 31, 2123–2130.

Block, A., Li, G., Fu, Z.Q. & Alfano, J.R. (2008) Phytopathogen type III effector weaponry and their plant targets. Current Opinion in Plant Biology, 11, 396–403.

Bohmann, H. & Sobczak, M. (2014) The plant cell wall in the feeding sites of cyst nematodes. Frontiers in Plant Science, 5, 89.

Bolger, A.M., Lohse, M. & Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics, 30, 2114–2120.

Bourdais, G., Burdiak, P., Gauthier, A., Nitsch, L., Solojarvi, J., Rayapuram, C. et al. (2015) Large-scale phenomics identifies primary and fine-tuning roles for CRKs in responses related to oxidative stress. PLoS Genetics, 11, e1005373.

Cao, F.Y., Khan, M., Taniguchi, M., Mirmiran, A., Moeder, W., Lumba, S. et al. (2019) A host–pathogen interactome uncovers phytopathogenic strategies to manipulate plant ABA responses. The Plant Journal, 100, 187–198.

Champion, A., Kreis, M., Mockaitis, K., Picaud, A. & Henry, Y. (2004) Arabidopsis kinome: after the casting. Functional and Integrative Genomics, 4, 163–187.

Chen, K., Du, L. & Chen, Z. (2003) Sensitization of defense responses and activation of programmed cell death by a pathogen-induced receptor-like protein kinase in Arabidopsis. Plant Molecular Biology, 53, 61–74.

Colcombet, J. & Hirt, H. (2008) Arabidopsis MAPKs: a complex signaling network involved in multiple biological processes. Biochemical Journal, 413, 217–226.

van Dam, S., Craig, T. & de Magalhaes, J.P. (2015) GeneFriends: a human RNA-seq-based gene and transcript co-expression database. Nucleic Acids Research, 43, D1124–1132.

van Dam, S., Vosa, U., van der Graaf, A., Franke, L. & de Magalhaes, J.P. (2018) Gene co-expression analysis for functional classification and gene–disease predictions. Briefings in Bioinformatics, 19, 575–592.

De Bodt, S., Proost, S., Vandepoele, K., Rouze, P. & Van de Peer, Y. (2009) Predicting protein–protein interactions in Arabidopsis thaliana through integration of orthology, gene ontology and co-expression. BMC Genomics, 10, 288.

Dou, D. & Zhou, J.M. (2012) Phytopathogen effectors subverting host immunity: different foes, similar battleground. Cell Host & Microbe, 12, 484–495.

Ederli, L., Madeo, L., Calderini, O., Gehring, C., Moretti, C., Buonarrio, R. et al. (2011) The Arabidopsis thaliana cysteine-rich receptor-like kinase CRK20 modulates host responses to Pseudomonas syringae pv. tomato DC3000 infection. Journal of Plant Physiology, 168, 1784–1794.

de la Fuente, A. (2010) From “differential networking”—identification of dysfunctional regulatory networks in diseases. Trends in Genetics, 26, 326–333.

Gao, L.L. & Xue, H.W. (2012) Global analysis of expression profiles of rice receptor-like kinase genes. Molecular Plant, 5, 143–153.

Garcia, A.V., Al-Yousif, M. & Hirt, H. (2012) Role of AGC kinases in plant growth and stress responses. Cellular & Molecular Life Sciences, 69, 3259–3267.

Giorgi, F.M., Del Fabbro, C. & Licausi, F. (2013) Comparative study of RNA-seq and microarray-derived coexpression networks in Arabidopsis thaliana. Bioinformatics, 29, 717–724.

Gish, L.A. & Clark, S.E. (2011) The RLK/Pelle family of kinases. The Plant Journal, 66, 117–127.

Hanada, K., Zou, C., Lehti-Shiu, M.D., Shinozaki, K. & Shiu, S.-H. (2008) Importance of lineage-specific expansion of plant tandem duplicates in the adaptive response to environmental stimuli. Plant Physiology, 148, 993–1003.

Hanks, S.K. & Hunter, T. (1995) Protein kinases 6. The euukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. The FASEB Journal, 9, 576–591.

Haruta, M., Gaddameedi, V., Burch, H., Fernandez, D. & Sussman, M.R. (2018) Comparison of the effects of a kinase-dead mutation of Arabidopsis FERONIA on ovule fertilization and root growth. FEBS Letters, 592, 2395–2402.

Helm, M., Qi, M., Sarkar, S., Yu, H., Whitham, S.A. & Innes, R.W. (2019) Engineering a decy substrate in soybean to enable recognition of the soybean mosaic virus Nla protease. Molecular Plant-Microbe Interactions, 32, 760–769.

Hewezi, T. & Baum, T.J. (2013) Manipulation of plant cells by cyst and root-knot nematode effectors. Molecular Plant-Microbe Interactions, 26, 9–16.

Hewezi, T., Howe, P.J., Maier, T.R., Hutson, R.S., Mitchum, M.G., Davis, E.L. et al. (2010) Arabidopsis spermidine synthase is targeted by an effector protein of the cyst nematode Heteroder a schachtii. Plant Physiology, 152, 968–984.

Hewezi, T., Juvala, P.S., Piya, S., Maier, T.R., Rambani, A., Rice, J.H. et al. (2015) The cyst nematode effector protein 10A07 targets and recruits host posttranslational machinery to mediate its nuclear trafficking and to promote parasitism in Arabidopsis. The Plant Cell, 27, 891–907.

Hogenhout, S.A., Van der Hoorn, R.A., Terauchi, R., Harada, H., Adams-Collier, C.J. et al. (2007) WoLF PSORT: protein localization predictor. Bioinformatics, 23, 3259–3267.

Hoeijmakers, J.H. & Kast, W. (2011) DNA damage, repair, and cancer: a life of(ly) choices. Science, 331, 1310–1314.

Horton, P., Park, K.-J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C.J. et al. (2007) WolF PSORT: protein localization predictor. Nucleic Acids Research, 35, W585–587.

Hosseini, P. & Matthews, B.F. (2014) Regulatory interplay between soybean root and soybean cyst nematode during a resistant and susceptible reaction. BMC Plant Biology, 14, 300.
Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z. et al. (2017) agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. *Nucleic Acids Research*, 45, W122–W129.

Trapnell, C., Pachter, L. & Salzberg, S.L. (2009) TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics*, 25, 1105–1111.

Uemura, T., Hachisu, M., Desaki, Y., Ito, A., Hoshino, R., Sano, Y. et al. (2020) Soy and *Arabidopsis* receptor-like kinases respond to polysaccharide signals from *Spodoptera* species and mediate herbivore resistance. *Communications Biology*, 3, 224.

Verwoerd, T.C., Dekker, B.M.M. & Hoekema, A. (1989) A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Research*, 17, 2362.

Vidal, M., Cusick, M.E. & Barabasi, A.L. (2011) Interactome networks and human disease. *Cell*, 144, 986–998.

Vij, S., Giri, J., Dansana, P.K., Kapoor, S. & Tyagi, A.K. (2008) The receptor-like cytoplasmic kinase (OsRLCK) gene family in rice: Organization, phylogenetic relationship, and expression during development and stress. *Molecular Plant*, 1, 732–750.

Vuong, T., Jiao, Y., Shannon, J. & Nguyen, H. (2013) Nematode resistance in soybean. In: Varshney, R. & Roberto, T. eds, *Translational genomics for crop breeding*. Vol. 1. Hoboken, NJ: John Wiley & Sons, Inc., pp. 95–124.

Wang, J., Kucukoglu, M., Zhang, L., Chen, P., Decker, D., Nilsson, O. et al. (2013) The *Arabidopsis* LRR-RLK, PXC1, is a regulator of secondary wall formation correlated with the TDF/PXY/TDR-WOX4 signaling pathway. *BMC Plant Biology*, 13, 94.

Wang, L.E., Wang, H., He, S., Meng, F., Zhang, C., Fan, S. et al. (2019) GmSnRK1.1, a sucrose non-fermenting-1(SNF1)-related protein kinase, promotes soybean resistance to *Phytophthora sojae*. *Frontiers in Plant Science*, 10, 996.

Wang, Y., Suo, H., Zheng, Y., Liu, K., Zhuang, C., Kahle, K.T. et al. (2010) The soybean root-specific protein kinase GmWNK1 regulates stress-responsive ABA signaling on the root system architecture. *The Plant Journal*, 64, 230–242.

Wei, K.F., Wang, Y.M. & Xie, D.X. (2014) Identification and expression profile analysis of the protein kinase gene superfamily in maize development. *Molecular Breeding*, 33, 155–172.

Wolfe, C.J., Kohane, I.S. & Butte, A.J. (2005) Systematic survey reveals general applicability of “guilt-by-association” within gene coexpression networks. *BMC Bioinformatics*, 6, 227.

Wrzaczek, M., Brosché, M., Salojärvi, J., Kangasjärvi, S., Idänheimo, N., Mersmann, S. et al. (2010) Transcriptional regulation of the CRK/DUF26 group of receptor-like protein kinases by ozone and plant hormones in *Arabidopsis*. *BMC Plant Biology*, 10, 95.

Xie, C., Mao, X., Huang, J., Ding, Y., Wu, J., Dong, S. et al. (2011) KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acid Research*, 39, W316–W322.

Yadeta, K.A., Elmore, J.M., Creer, A.Y., Feng, B., Franco, J.Y., Rufian, J.S. et al. (2017) A cysteine-rich protein kinase associates with a membrane immune complex and the cysteine residues are required for cell death. *Plant Physiology*, 173, 771–787.

Zhang, H. & Song, B.H. (2017) RNA-seq data comparisons of wild soybean genotypes in response to soybean cyst nematode (*Heterodera glycines*). *Genomics Data*, 14, 36–39.

Zhang, L., Lilley, C.J., Imren, M., Knox, J.P. & Urwin, P.E. (2017) The complex cell wall composition of syncytia induced by plant parasitic cyst nematodes reflects both function and host plant. *Frontiers in Plant Science*, 8, 1087.

Zhou, J.G., Liu, D.R., Wang, P., Ma, X.Y., Lin, W.W., Cheng, S.X. et al. (2018) Regulation of *Arabidopsis* brassinosteroid receptor BRI1 endocytosis and degradation by plant U-box PUB12/PUB13-mediated ubiquitination. *Proceedings of the National Academy of Sciences of the United States of America*, 115, E1906–E1915.

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