Identification of the phosphorylation targets of symbiotic receptor-like kinases using a high-throughput multiplexed assay for kinase specificity

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Abstract

Detecting the phosphorylation substrates of multiple kinases in a single experiment is a challenge, and new techniques are being developed to overcome this challenge. Here, we utilized a multiplexed assay for kinase specificity (MAKS) to identify the substrates directly and to map the phosphorylation site(s) of plant symbiotic receptor-like kinases. The symbiotic receptor-like kinases Nodulation Receptor-like Kinase (NORK) and Lysin motif domain-containing receptor-like kinase 3 (LYK3) are indispensable for the establishment of root nodule symbiosis. Although some interacting proteins have been identified for these symbiotic receptor-like kinases, very little is known about their phosphorylation substrates. Using this high-throughput approach, we identified several other potential phosphorylation targets for both these symbiotic receptor-like kinases. In particular, we also discovered the phosphorylation of LYK3 by NORK itself which was also confirmed by pair-wise kinase assays. Motif analysis of potential targets for these kinases revealed that the acidic motif xxxsDxxx was common to both of them. In summary, this high-throughput technique catalogs the potential phosphorylation substrates of multiple kinases in a single efficient experiment, the biological characterization of which should provide a better understanding of phosphorylation signaling cascade in symbiosis.

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INTRODUCTION

Proteins are vital cogs for normal cell functioning. In addition to providing structural support to the cells, proteins are involved in catalysis, transport, storage, or signal transmission (Duan and Walther, 2015). Even in this era of systems biology, studying proteomes is more challenging due to their complex and dynamic nature. A single gene can encode several proteins due to their multi-level regulation, such as alternative splicing, multiple reading frames, and post-translational modifications (PTMs)(Silva-Sanchez *et al.*, 2014). For instance, the human genome, which consists of about 30,000 genes, can encode anywhere between 200,000 to 2 million proteins (Van Eyk and Dunn, 2006). Chief among the mechanisms that increase the functional diversity of the cellular proteome is the PTMs, which are modification(s) either during or after the synthesis of proteins by the ribosome (Silva-Sanchez *et al.*, 2015; Pawson and Scott, 1997). At least 300 different PTMs are known so far, and it is widely believed that PTMs are essential for the biological functioning of synthesized proteins (Silva-Sanchez *et al.*, 2014; Witze *et al.*, 2007).

Protein phosphorylation is the most prominent reversible PTM, catalyzed by two classes of enzymes—protein kinases and phosphatases—that alter the localization, binding, configuration, protein interactions, and complex formations of proteins. Modified proteins, in turn, regulate intracellular biological functions (Bond *et al.*, 2011; Khoury *et al.*, 2011). Protein kinases are one of the largest gene families in eukaryotes (Manning, 2002) and due to recent whole-genome duplication events in flowering plants, this family of genes is significantly larger than in other eukaryotic lineages, suggesting their importance in plant species (Lehti-Shiu and Shiu, 2012). Receptor-like kinases (RLKs) are protein kinases which consist of a ligand-binding extracellular domain, a transmembrane domain, and an intracellular kinase domain. They are involved in plant growth and development, stress response, cell regulation, metabolism, and plant–microbe interactions (Shiu and Bleecker, 2001; Lehti-Shiu and Shiu, 2012; Stone and Walker, 1995). Despite the importance of protein kinases in plants, very few kinases have been characterized functionally. Only a small fraction of the identified plant RLKs have a known function (Shiu and Bleecker, 2001; Gish and Clark, 2011). The identification of potential phosphorylation targets/substrates of these kinases will immensely aid in the functional characterization of these kinases. However, this process is both challenging and limiting. Current approaches for target identification of protein kinases in plants consist of performing a protein–protein interaction assay, such as yeast two-hybrid (Y2H) or co-immunoprecipitation (CO-IP) assays, using the kinase(s) of interest. Protein interaction studies are often followed by pair-wise *in vitro* kinase assays using radiolabeled ATP to determine the phosphorylation status of interacting protein(s).

Although widely used, the major drawback of the classical Y2H screen is false positive or false negative results (Brückner *et al.*, 2009; Phizicky and Fields, 1995). Several factors, such as steric hindrance of a reporter protein, choice of the protein used for fusion in bait or prey, or proteins for which posttranslational modifications is a prerequisite for interaction but that does not occur in yeast cells, may contribute to false negatives. Conversely, high expression of bait or prey proteins, mis-localization of these proteins in compartments not similar to their native cell environment, mis-folding of proteins, and interaction of prey proteins with reporter proteins may lead to false positives (Brückner *et al.*, 2009). Similar to Y2H assays, CO-IPs have been used in thousands of protein–protein interaction studies, but this technique is not very sensitive and often fails to detect transient protein–protein interactions—especially those involving kinases and phosphatases (Phizicky and Fields, 1995; Daniel *et al.*, 2013). Also, the interactors identified may be a part of the larger protein complex and therefore a direct interaction cannot be inferred (Phizicky and Fields, 1995; Daniel *et al.*, 2013). Although not yet common in plant research, chip-based approaches have been used for the identification of phosphorylation substrates of kinases. These include a screening of random peptide libraries containing degenerate penta- or heptapeptides and identifying the putative substrate(s) using radioactivity. Kinome profiling, using chips spotted with consensus kinase substrates selected from the phosphorylated peptides database, Protein Kinase Research, can be used for identifying kinase substrates. This chip consists of substrates from different organisms including bacteria, plants, animals, and fungi, making the identification of substrates specific to plant kinases cumbersome (Ritsema *et al.*, 2007; Ritsema and Peppelenbosch, 2009). Also, positional scanning peptide libraries, where a semi-degenerate 20-mer peptide library with the phospho-acceptor amino acid fixed in the center position, are also used for identifying the phosphorylation motif of plant kinases (Sugiyama and Ishihama, 2016; Sirichandra *et al.*, 2010). However, the approaches mentioned above usually use radioactivity to identify putative targets, and individual phosphorylation sites cannot be identified. Kinase-client assay (KiC assay) is a further improvement in the chip–based technology. In this approach, kinase activity can be quantified by comparing the spectral counts of phosphorylated and non-phosphorylated peptides, and also individual phosphosites can be mapped. Using KiC several putative targets of 17 different kinases were identified, and phosphorylation of *Arabidopsis thaliana* protein phosphatase inhibitor-2 (AtPPI-2) by three different kinases was confirmed (Ahsan *et al.*, 2013). Instead of peptide libraries, arrayed-protein chips, where individual proteins are expressed, purified and immobilized on chips and incubated with kinase of interest are used to identify potential substrate of kinases. This method was utilized for the identification of phosphorylation targets of *Arabidopsis thaliana* mitogen-activated protein kinases (MAPK) in plants (Feilner *et al.*, 2005). Although not impossible, recombinant expression and purification of all the proteins are expensive and difficult. Furthermore, scaling-up of these chip-based libraries (either peptide or protein libraries) are expensive and limits the number of kinases being screened. Most peptide library approaches identify the phosphorylation motif likely to be phosphorylated by the kinases. Since plant genomes are large there may be hundreds if not thousands of proteins with those phosphorylation motifs, screening all those proteins to identify the actual phosphorylation substrate becomes a limiting factor for these approaches (Miller and Turk, 2016).

In the current study, we a used a multiplexed assay for kinase specificity (MAKS), previously used in yeast and human stem cells, to identify the phosphorylation substrates and sites of two symbiotic-receptor-like kinases: Lysin motif domain-containing receptor-like kinase 3 (LYK3) and Nodulation receptor-like kinase (NORK) sometimes referred to as Does not make infections2 (DMI2). These proteins are required for legume nodulation and arbuscular mycorrhizal associations in the model legume *Medicago truncatula* (Arrighi *et al.*, 2006; Gabriella Endre *et al.*, 2002; Patrick Smit *et al.*, 2007). This approach provides information about putative targets of kinases which can be further confirmed by a targeted approach. Here we used crude protein extracts as the target instead of a peptide library. The main advantage of MAKS is that in a single experiment, multiple kinases can be monitored for their activity using isobaric tagging technology (Brumbaugh *et al.*, 2014). Also, we can identify phosphorylation substrates without any of the methods used for detecting protein–protein interactions, thus avoiding their drawbacks. Furthermore, the putative targets obtained from our approach are much more relevant than those obtained using peptide library because protein extracts were used instead of peptides. MAKS identified several substrates for both symbiotic receptor-like kinases that we tested. Of particular interest is the phosphorylation of LYK3 by NORK. We confirmed this interaction using pair-wise *in vitro* kinase experiments, indicating that this method can be used to identify the substrates and interaction partners of other receptor-like kinases. Also, we identified the motifs that were most likely to be phosphorylated by these symbiotic receptor-like kinases. In summary, MAKS is a valid and efficient method broadly applicable to many kinases in plant sciences.

RESULTS AND DISCUSSION

Multiplexed assay to identify targets of protein kinases in plants

Phosphorylation/dephosphorylation is a dynamic process and various properties of the phosphorylated proteins, such as low stoichiometry, heterogeneity, along with the dynamic range of the technique used, can affect the coverage detection and quantification of phosphoproteins/peptides (Grimsrud *et al.*, 2010; Jayaraman *et al.*, 2012; Silva-Sanchez *et al.*, 2014; Wenger *et al.*, 2011). Hence there is a continuous need to develop and advance techniques including those related to sample preparation, enrichment, and fragmentation to achieve higher coverage and detection (Grimsrud *et al.*, 2010; Silva-Sanchez *et al.*, 2014). Although research in the last two decades using model legumes has led to the identification of several components of the symbiotic signaling cascade, the underlying mechanism of signal processing from symbiotic receptor-like kinases localized in the plasma membrane to the nucleus is not fully understood. Because of the involvement of several symbiotic receptor-like kinases, we suppose that a symbiotic signaling cascade, mediated by protein phosphorylation and dephosphorylation, might be established, thereby transducing the signals from the plasma membrane to the nucleus. Therefore, this study was conducted to study the phosphorylation specificity of these symbiotic receptor-like kinases with the aim of identifying novel components and substrates of symbiotic receptor-like kinases to dissect the pathway more precisely. We focused on identifying the autophosphorylation sites and substrates for LYK3 and NORK. One of the bottlenecks of studying phosphorylation specificity is the low-throughput nature of traditional *in vitro* kinase assays. Conventionally, kinase assays involve an *in vitro* reaction, after which the transfer of a radiolabeled

phosphoryl group to a given substrate is detected, directly measuring phosphorylation but requiring the use of hazardous materials (Brumbaugh *et al.*, 2014). Also, this method cannot multiplex kinases and substrates or directly localize phosphorylation to a single amino acid when more than one potential site is present. To multiplex kinase assays and identify potential phosphorylation substrates and the phosphorylation sites of the symbiotic receptor-like kinase(s), we used a multiplexed assay for kinase specificity. To our knowledge, this is the first time that this approach is used for plant proteins (Brumbaugh *et al.*, 2014).

A heterologous *Escherichia coli* bacterial expression system was used for the expression of the kinase domains of both these proteins. For this, the kinase domains of LYK3 and NORK were tagged to glutathione s-transferase (GST) and maltose binding protein (MBP) tags, respectively. The expressed kinase domains of LYK3 and NORK were purified using Glutathione Superflow and amylose resin, respectively. In the initial step, crude protein extracts from one week old Medicago seedlings were mixed with these purified candidate kinases plus kinase buffer. As a mock control kinase buffer alone mixed with crude Medicago protein(s) was used (Figure 1). After a 30 minute incubation, each sample was enzymatically digested and differentially labeled with isobaric tags, rendering a unique chemical signature to each peptide and simultaneously linking it to the reaction from which it originated (Thompson *et al.*, 2003). Following labeling, the six samples were combined and enriched for phosphopeptides using immobilized metal affinity chromatography (IMAC). Peptides were separated by reversed-phase (RP) chromatography and introduced to the mass spectrometer, where precursor charges were isolated and subjected to tandem mass spectrometry (MS/MS), providing sequence identity, phosphorylation site localization, and quantitative information in a single scan. During fragmentation, the isobaric tags are cleaved, creating reporter ions. The intensity of these reporter ions can serve as a proxy for phosphorylation abundance following the kinase reaction. The spectrum in Figure 2A shows an MS/MS scan mapped to AsNILLDK^{2+}, a tryptic peptide of cysteine-rich receptor-kinase-like protein. Fragment ions allow the phosphorylation event to be confidently localized to Ser488. The inset depicting the reporter tag mass-to-charge (*m/z*) indicates strong up-regulation of this phosphorylation site in response to the LYK3 kinase. Using this multiplexed assay for kinase specificity approach, we identified multiple putative substrates of both these symbiotic receptor-like kinases, and they fall into various categories, such as receptor-like kinases, storage proteins, RNA binding proteins, transporters, catalytic enzymes, transcription factors, and ubiquitin ligases.

Broad classification of phosphorylated proteins

Tables 1 and 2 present proteins displaying the greatest increase in phosphorylation after incubation with either LYK3 (Table 1) or NORK (Table 2). These proteins have never been characterized in Medicago symbiosis, suggesting that they may be the possible missing links in connecting plasma membrane signals to the nuclear events in the symbiotic signaling cascade. In addition to these proteins, other interesting proteins with significant fold changes (p-value < 0.10) are presented in Supplementary Tables S1 and S2. This compiled list offers some insight into the potential interactors of the tested symbiotic receptor-like kinases.

A striking feature of proteins phosphorylated (directly or indirectly) by both these kinases is the prevalence of proteins directly or indirectly involved in ubiquitination and protein trafficking. Previous studies have shown that both LYK3 and NORK/SYMRK interact with E3 ubiquitin ligases (SYMRK is the ortholog of NORK in the other model legume *Lotus Japonicus*). LYK3 interacts with PUB1, while NORK/SYMRK interacts with PUB1 and the seven in absentia E3-ubiquitin ligase SINA4 and both these interactions negatively affect nodulation (Den Herder *et al.*, 2012; Mbengue *et al.*, 2010; Vernié *et al.*, 2016). This study identifies other ubiquitin ligases that may interact with and act as potential substrates for LYK3 and NORK. It should be mentioned that in this study that PUB1, the characterized interactor and phosphorylation substrate of LYK3 and NORK, was not detected. Nonetheless, we detect several proteins/ligases that are involved in ubiquitination, such as an E3 ubiquitin-protein ligase RGLG2-like protein (Medtr3g086520), a ubiquitin-specific protease family C19 protein (Medtr4g005510), and an ARM repeat RING/U-box protein (Medtr3g466220), as putative targets of LYK3. Similarly, an ubiquitin-conjugating enzyme family protein (Medtr8g015590) and a regulatory component of the 26S proteasome (Medtr8g106120) were found as putative targets of NORK.

Besides these, several proteins that are involved in trafficking were identified as the potential targets for both of these tested symbiotic receptor-like kinases. For instance, two ARF GTPase activators Medtr6g027300 and Medtr6g086630 are putative targets of LYK3 and NORK, respectively. ARF family proteins are involved in the synthesis of transport vesicles on the donor membrane (Nielsen *et al.*, 2008). Also, LYK3 potentially phosphorylates a clathrin heavy chain protein (Medtr5g082900), whereas a sorting nexin 2B (Medtr5g073280), a vacuolar protein sorting protein (Medtr4g056480), and an SH3 domain protein (Medtr8g100105) are putative targets of NORK. Recently a clathrin heavy chain protein CHC1 which interacts with ROP6, an interaction partner of NFR5 (an ortholog of LYK3 in another model legume *Lotus japonicus*) has been implicated in nodulation and Medtr5g082900 may play a similar role in Medicago (Wang *et al.*, 2015). Taken together these symbiotic-receptor-like kinases phosphorylate proteins involved in ubiquitination or vesicle trafficking either directly or indirectly. There may be ubiquitination-based degradation or receptor endocytosis, which in turn regulates cell signaling by determining the abundance of receptors that are available at any given time (Haffani *et al.*, 2004; Hartmann *et al.*, 2008; Vanoosthuyse *et al.*, 2003; Moling *et al.*, 2014).

Potential phosphorylation targets of LYK3

Klaus-Heisen *et al.* (2011) demonstrated that phosphorylation of the activation loop is essential for the kinase activity of LYK3 which in turn is required for its biological functions. Phosphorylation sites such as Ser-471, Ser-523, Thr-268, Thr-319, Thr-472, Thr-475, and Thr-520, previously identified and characterized by Klaus-Heisen *et al.* (2011), were also identified in this study. Also, other potential sites such as Ser-307, Ser-323, Ser-584, Thr-311, and Tyr-559 were also identified, the characterization of which will doubtless provide further insights into the biological activity of LYK3.

Among the putative targets *trans*-phosphorylated by LYK3, Cysteine-rich receptor-kinase-like protein (Medtr1g021635) exhibited the highest fold change (12.05). Besides this, a

MAP triple kinase (Medtr4g075410), a serine/threonine kinase HT1-like protein (Medtr4g106980), and a cyclin-dependent kinase (Medtr8g080190) are the other kinases putatively phosphorylated by LYK3. Also, Medtr5g032060, a kinase interacting protein (KIP1-like), is a potential target of LYK3 (Table S1). Although the role of some receptor-like kinases and protein kinases is extensively characterized in symbiotic signaling, the above-mentioned putative targets of LYK3 have not yet been characterized in symbiotic signaling. The use of multiplexed assay for kinase specificity has enabled us to identify these potential targets of LYK3, and the role of some of them in symbiotic signaling will be investigated.

MAP kinase cascades operate via a phosphorelay mechanism, and in symbiotic signaling, the role of a MAP double kinase (SIP2, which interacts with NORK/SYMRK) has been established before (Chen *et al.*, 2012). However, to our knowledge, no MAP triple kinase has been implicated directly in early symbiotic signaling so far, and it would be interesting to investigate the relationships between SIP2 and our MAP triple kinase. The characterization of this newly identified MAP triple kinase should further our knowledge of this pathway.

At least eight different cyclin-dependent kinases are known in plants that have been implicated in cell cycle transition (Tank and Thaker, 2011). These kinases are activated by cyclins, with which they form a complex and phosphorylate target proteins, thereby facilitating cell cycle transition (Tank and Thaker, 2011). In Medicago, cyclins and cell cycle switch components have been implicated in both the early stages of infection and late stages of nodule development.

PIN genes are implicated in auxin transport and knocking down several PIN genes decreased nodulation in Medicago, implying a crucial role of auxin transport in nodulation (Huo *et al.*, 2006). In our current study, an auxin efflux carrier, *MtPIN2* (Medtr4g127100), was identified as a putative target of LYK3. A significant reduction in nodule numbers was observed in prior studies using the RNA interference lines of Mt*PIN2* possibly due to impaired auxin transport (Grunewald *et al.*, 2009; van Noorden *et al.*, 2007; Huo *et al.*, 2006).

Potential phosphorylation targets of NORK

Similar to LYK3 the kinase activity of SYMRK/NORK is affected by its phosphorylation status (Yoshida and Parniske, 2005). At least 37 phosphorylation sites of NORK were identified in this study including three sites (Ser-754, Thr-760, and Thr-593) identified using Q-ToF/MS and characterized by Yoshida *et al.* (2005). The biological implications of these sites need to be ascertained.

In addition to the NORK phosphorylation sites, we also detected several putative *trans*-phosphorylation targets of NORK. A trehalose-6-phosphate synthase domain protein (Medtr4g080160) and a peptidylprolyl *cis*–*trans* isomerase (Medtr3g037570) are among the top putative targets exhibiting phosphorylation changes upon incubation with NORK. In addition to their role in metabolism, sugars are also implicated in signal transduction in particular in stress responses (Fernandez *et al.*, 2010; Ponnu *et al.*, 2011). Although the bulk of trehalose is synthesized by the microbial symbiont, plants also produce trace amounts of

trehalose, and in Medicago, *Phaseolus vulgaris*, and *Glycine max*, trehalose accumulation has been detected in nodules (Brechenmacher *et al.*, 2010).

In addition to these proteins, it is interesting to note that NORK also phosphorylates LYK3 which will be discussed later in detail.

Potential phosphorylation targets of LYK3 and NORK

At least 13 different proteins were found to be a common putative target of both LYK3 and NORK, indicating that both these symbiotic receptor-like-kinases act through some common signaling intermediates, as expected during legume–rhizobium symbiosis (Table 3). Among these 13 proteins, 3 of them, a putative ATP/GTP-binding family protein (Medtr5g042000), a drug resistance transporter-like ABC domain protein (Medtr8g014360), and a P-loop nucleoside triphosphate hydrolase superfamily protein (Medtr8g099065) were phosphorylated at the same residues, Ser-975, Ser-848, and Ser-200, respectively, by both LYK3 and NORK. This result is consistent with the observation by Ahsan *et al.*, 2013 that AtPPI–2 Ser–140 can be phosphorylated by three different kinases.

P-loop nucleoside triphosphate hydrolase is chiefly implicated in programmed cell death, response to biotic (disease), and abiotic stresses in plants (Leipe *et al.*, 2004). As they are a key component of the 26S proteasome complex, they may play a role in protein degradation and turnover (JM *et al.*, 2002).

In the model legume, Lotus at least 91 ABC proteins were detected, and an *AtPDR12*-like gene was upregulated upon inoculation with symbiotic rhizobial bacteria (Sugiyama *et al.*, 2006). It is hypothesized that these ABC transporters, along with the PIN family of genes, play a role in polar auxin transport which affects nodulation (Sugiyama *et al.*, 2006).

A dynamin-2B-like protein, Medtr4g030140, is a putative phosphorylation target of both these symbiotic-receptor-like kinases. Dynamins or dynamin-related proteins (DRP) are vesicles transporting proteins implicated in ligand-induced endocytosis in mammalian systems and plants (Smith *et al.* 2014). It is possible that the dynamin-2B-like protein (Medtr4g030140) potentially phosphorylated by both these symbiotic receptor-like-kinases may play a similar role in symbiosis which needs to be investigated (Smith *et al.*, 2014).

VAMP-associated protein (Medtr5g012420) mostly implicated in vesicle trafficking is also a putative target of both of these symbiotic receptor-like kinases. (Murray *et al.*, 2011). In Medicago, Vapyrin (VPY), has been implicated in both legume–rhizobia and arbuscular mycorrhizal symbioses (Pumplin *et al.*, 2010; Murray *et al.*, 2011). It is reasonable to speculate that Medtr5g012420, phosphorylated by both LYK3 and NORK, may be involved in late stages of symbiosis.

Overall the multiplexed assay for kinase specificity approach has led to the identification of several putative targets of LYK3 and NORK, respectively (both localized and non-localized) with more than 1-fold change in phosphorylation and a significant *p*-value. In total, we have identified about 842 phosphopeptides being phosphorylated by both these receptor-like kinases (Figure 2D and supplementary data file 1). While this approach is high throughput, it is also possible that we may have some false positives, similar to those frequently observed

with the yeast-two-hybrid approach. The use of cell lysates as the experimental material enables two proteins that are otherwise in different compartments of the cell and with minimal opportunity to interact, to come into proximity with each other and interact, leading to false positives. Also, when protein kinases are removed from their native cell environment, they may lose their regulatory mechanisms, which can also result in non-specific phosphorylation of targets. Furthermore, it is also possible that the kinase of interest may activate other downstream kinases, so there is a possibility of misinterpreting the indirect targets of phosphorylation from downstream kinases as direct phosphorylation substrates for the kinases of interest. Also, background signals, add to the false positives as several active kinases are present in the cell lysates. To address the extent of false positives, we compared the phosphopeptides obtained in this study with our previous large-scale phosphoproteomic analysis of Medicago in which we identified 13293 phosphopeptides specific to symbiotic signaling (Rose *et al.*, 2012, and supplementary data file 1). We found that 696 phosphopeptides overlapped significantly between these studies and 146 phosphopeptides were unique to this work (Figure 2D, and supplementary data file 1). This high level of overlap indicates that a majority of the phosphopeptides identified using our MAKS approach are indeed related to symbiotic signaling, and the degree of false positives may be low. Despite these limitations multiplexed assay for kinase specificity is a powerful tool for the large-scale identification of phosphorylation substrates of multiple kinases under multiple conditions, and as a proof of concept of this technology's applicability to plants, we confirmed the phosphorylation of LYK3 by NORK using a pair-wise *in vitro* kinase assay.

Pair-wise *In vitro* kinase assay confirms LYK3 phosphorylation by NORK

Among the proteins phosphorylated by NORK, LYK3 showed the maximum number of hits and exhibited the 6th highest fold change. Furthermore, the role of *LYK3* and *NORK* in the symbiotic signal transduction pathway has been extensively characterized, and both these genes are essential for the establishment of legume nodulation symbiosis with rhizobia (Arrighi *et al.*, 2006; G Endre *et al.*, 2002; P Smit *et al.*, 2007). Also, *NORK* is essential for the formation of a symbiotic association with arbuscular mycorrhizal fungi (Delaux *et al.*, 2013; G Endre *et al.*, 2002). Because of their significance in symbiotic signaling and also to validate the results of multiplexed assay for kinase specificity approach in which we could not localize the site of LYK3 phosphorylation by NORK, we wanted to confirm the phosphorylation of LYK3 by NORK using a traditional *in vitro* kinase assay technique.

Both LYK3 and NORK exhibit auto-phosphorylation activity (Arrighi *et al.*, 2006; Yoshida and Parniske, 2005). Therefore, wild-type versions of these kinases cannot be used to test the *trans*-phosphorylation of one by the other. A glycine to glutamic acid substitution at the 334th position of the LYK3 kinase domain abolished its kinase activity *in vitro* which in turn affected nodule formation *in planta* (Klaus-Heisen *et al.*, 2011). In the case of NORK, a mutant allele (R38) with a non-synonymous substitution converting glycine to glutamic acid at the 794th position had no effect on mycorrhization of plants but impaired the ability of plants to form nodules (nod⁻, Myc⁺)(G Endre *et al.*, 2002). It was hypothesized that the kinase activity was abolished in these mutants affecting the nodulation. Here we demonstrate that the kinase activity is indeed abolished in the R38 mutant's version of NORK (Figure 3) and used the kinase-inactive versions of these symbiotic receptor-like kinases to test the

trans-phosphorylation of NORK by LYK3 and vice versa. Because the LYK3 and NORK kinase domains are approximately the same sizes, the LYK3, and NORK kinase domains were tagged with GST and MBP, respectively, to differentiate them based on their size on an SDS-PAGE gel. As expected, the wild-type versions of LYK3 and NORK exhibited auto-phosphorylation (Figure 3A and 3B) and *trans*-phosphorylation (as indicated by the phosphorylation of casein) activities (Figure 3A and 3B). Furthermore, their corresponding mutants lacked auto-phosphorylation activity (Figure 3A and 3B). In a reaction mixture of wild-type LYK3 and NORK(G794E), a band corresponding to the autophosphorylation of LYK3 was observed. However, no band corresponding to the size of NORK was observed, indicating that LYK3 indeed failed to trans-phosphorylate the NORK mutant (Figure 3C). In the reaction mixture involving NORK-wild-type and LYK3(G334E), a band corresponding to the size of LYK3 was observed in addition to the expected NORK band (Figure 3C). Because LYK3(G334E) lacks autophosphorylation activity, the detection of the LYK3 band implies that LYK3 is indeed trans-phosphorylated by NORK. We identified that sites Ser-269, Ser-273, Ser-307, Ser-323 and Ser-471 of LYK3 (as a control only mLYK3 was used) were phosphorylated by NORK using Mass spectrometry, thereby validating the results obtained using the multiplexed assay for kinase specificity approach.

Motif analysis

Phosphorylation motifs were identified from localized phosphorylation sites using the Motif-x algorithm (Schwartz and Gygi, 2005). Phosphorylated peptides were centered on the phosphorylated residue and aligned to the *Arabidopsis thaliana* database. A central character of S (serine), 20 occurrences, and a significance threshold of 0.000001 were specified. The human IPI database was used as the background dataset to normalize the score against a random distribution of amino acids. Using these settings, two phosphorylation motifs were identified in the LYK3 treated plants (Figure 2B), and one phosphorylation motif was identified in the NORK treated plants (Figure 2C). The acidic motif xxxsDxxx was common to both kinases studied (Figure 2B and 2C). However, as indicated before some of these phosphorylation substrates may not be the direct targets of these symbiotic RLKs and may be the targets of some downstream kinase(s) activated by these symbiotic RLKs.

Conclusion

Strategies targeting the activity of a specific kinase often require the use of phosphorylation-specific antibodies to determine the phosphorylation status of specific residues of protein kinases or their substrates, but the information gained from such studies is not necessarily quantitative. Traditionally, a biochemical approach to kinase activity has been the radioisotope filtration binding assay, where radiolabeled phosphate is incorporated into the kinase substrate. Kinase phosphoryl transfer activity, which is proportional to the amount of phosphorylated substrate, is detected. This method is not well-suited for high throughput screening of multiple phosphorylation targets, as numerous wash steps are required. Additionally, there are inherent dangers associated with the use of radioisotopes. Alternatively, MS-based phosphopeptide analysis provides a high-throughput alternative for identifying, localizing, and quantifying phosphorylation sites on a single amino acid, though it can be difficult to determine the kinase responsible for this activity. Recently, an MS-based assay to determine phosphorylation rates, utilizing synthetic peptides with known

phosphorylation sites combined with cell lysate serving as a kinase source, was described (Yu *et al.* 2009). Although the phosphorylation rate of up to 90 different phosphorylation sites was determined with this method, it requires an extensive library of synthetic peptides, and cannot directly determine the activating kinase. A strategy employing stable isotope labeling allows the quantitative comparison of a control and kinase phosphorylated substrate, but is limited to the analysis of one or two samples (Singh *et al.*, 2012). The MS-based *in vitro* kinase activity used here utilizes isobaric tag technology, allowing the analysis of up to 10 samples within a single experiment (Brumbaugh *et al.*, 2014). This multiplexing capability allowed us to combine replicates of both kinase-treated and non-treated controls within a single experiment, allowing for statistical analysis of phosphorylation events and increasing the experimental throughput.

In the present study, proteins involved in ubiquitination or those associated with the ubiquitination pathway are among the major proteins phosphorylated by both these symbiotic receptor-like kinases. Vesicle trafficking, regulated by ubiquitination, directs the movement of receptor-like kinases back and forth from the plasma membrane (Furlan *et al.*, 2012). Recent experimental evidence demonstrates that cell signaling and endocytic recycling of membrane proteins are closely associated events rather than two independent processes (Scita and Di Fiore, 2010). Phosphorylation of several proteins related to ubiquitination in our study suggests a possible depletion of signal transduction by protein degradation via the 26S proteasome pathway or receptor endocytosis culminating in the sorting of receptors to another destination. Previous studies have demonstrated the alteration in the localization of LYK3 upon treatment with rhizobia and the interaction of LYK3 and SYMRK with E3 ubiquitin ligases PUB1 and SINA4, respectively, but the underlying mechanism is not understood (Haney *et al.*, 2011). Our current work, due to its inherent high throughput nature, has identified several potential phosphorylation candidates for these symbiotic receptor-like kinases which would otherwise be time-consuming and cumbersome to identify using the conventional methods. The characterization of these candidates should lead not only to the further understanding of the function of individual components but also in deciphering the mechanism of signal transduction from the plasma membrane to the nucleus in symbiotic signaling.

MATERIALS AND METHODS

In vitro protein purification and site-directed mutagenesis

The kinase domain of LYK3 was cloned into the pGEX-6P-1 vector (Amersham Biosciences, Piscataway, NJ, USA) as described in Arrighi *et al.*, (2006). For the NORK kinase domain, a sequence-verified construct was recombined using the LR recombination reaction (Invitrogen, La Jolla, CA, USA). A maltose binding protein (MBP)::*NORK* (KD) construct was generated in the Gateway® vector pVP16 (Center for Eukaryotic Structural Genomics, Madison, WI, USA). The QuickChangeII® site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA.) was used to induce point mutations on *pGEX-6p-1::LYK3* (kinase domain) and *pVP16:: NORK* (kinase domain). The following primers were used: LYK3G334EF (5′-TCAAGGTGGATTTGAAGCTGTCTATT) and LYK3G334ER (5′-CATAATAGACAGCTTCAAATCCACCTT) for LYK3 and NORKR38F

(5′-CTTGAAATTGTAAGCGAACGGGAACC) and NORKR38R (5′-TTGAGAGGTTCCCGTTCGCTTACAAT) for NORK. The sequence-verified constructs were used for protein expression and purification.

In vitro kinase assays

An *in vitro* kinase assay was performed following the protocol that was described by Arrighi *et al.*, (2006). Briefly, the aforementioned purified proteins were checked by SDS–PAGE and quantified by Bradford assay (Stoscheck,1990). Then, 1 µg of protein in 20 µl of kinase buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol, 600 nM ATP, 110 nM [10 mCi] [γ-^{32}P]ATP) was incubated at 30°C for 1.5 hours. The reaction was stopped by boiling with SDS-PAGE sample buffer at 95°C for 5 minutes and analyzed by SDS-PAGE. The gels were dried, and the phosphorylated proteins were analyzed by autoradiography (Fujifilm, New Berlin, WI, USA).

Protein extraction

Protein was obtained from 7-day old seedlings of Medicago through chloroform/methanol extraction following the protocol used in Rose *et al.*, 2012. One volume of chloroform and three volumes of water were added to the plant extract. The solution was vortexed and then centrifuged for five minutes (4,696× *g*, 4°C). Following centrifugation, the top layer was removed and discarded using a serological pipette. Three volumes of methanol were subsequently added, and the solution was vortexed and centrifuged for five minutes (4,696× *g*, 4°C). The supernatant was removed and discarded using a serological pipette. The resulting protein pellet was washed three times by vortexing with ice-cold 80% acetone followed by five minutes of centrifugation (10,000× *g*). The pellet was dried on ice for 30 minutes and stored at −80°C.

Protein lysis and kinase reaction

The protein pellets were resuspended in ice-cold lysis buffer containing 8 M urea, 40 mM Tris (pH 8), 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium orthovanadate, 6 mM sodium pyrophosphate, 1× mini ethylenediaminetetraacetic acid-free protease inhibitor (Roche Diagnostics) and 1× phosSTOP phosphatase inhibitor (Roche Diagnostics, Indianapolis, IN, USA). The lysate protein concentration was measured by bicinchoninic acid assay (Thermo Pierce, Rockford, IL, USA). A total of twelve 1 mg aliquots of protein lysate were prepared. These aliquots were divided for two separate experiments, with each experiment comprising three control samples and three samples that were treated with the kinase of interest. Each aliquot was resuspended in a kinase reaction buffer containing 5 mM ATP, 5 mM $MgCl_2$, 50 mM Tris (pH 7.7) and 7.5 mM β-glycerophosphate. Purified NORK or LYK3 was added to half of the lysate aliquots at a 1:75 kinase:protein ratio. Kinase was omitted from the other lysate samples. The lysate was incubated with the kinase reaction buffer at room temperature for thirty minutes.

Protein digestion and isobaric tagging

Each aliquot was reduced using 5 mM dithiothreitol and incubated for 30 minutes at room temperature. Free thiols were alkylated with 15 mM iodoacetamide (IAA) in the dark for 30

minutes. The alkylation reaction was quenched with 5 mM dithiothreitol. The urea concentration was diluted to 1.5 M with 50 mM Tris (pH 8.0). The proteins were digested with trypsin (Promega, Madison, WI, USA) at a 1:50 enzyme:protein ratio and were incubated at ambient temperature overnight. Following digestion, the peptides were acidified with trifluoroacetic acid and desalted over a tC18 Sep-Pak (Waters, Milford, MA, USA). TMT labeling was carried out according to the manufacturer's instructions (Thermo Pierce). Following tagging, the proteins were mixed in a 1:1:1:1:1:1 ratio according to BCA results, with three channels serving as controls and three channels that were treated with either LYK3 or NORK kinase.

Strong cation exchange fractionation

Strong cation exchange (SCX) fractionation was performed at a flow rate of 3.0 ml/minute using a polysulfoethylaspartamide column (9.4 × 200 mM, PolyLC) on a Surveyor LC quaternary pump. The tagged samples were resuspended in buffer A and separated using the following gradient: 0–2 minutes, 100% buffer A; 2–5 minutes, 0–15% buffer B; and 5–35 minutes, 15–100% buffer B. Buffer B was then held at 100% for 10 minutes. The column was washed with buffer C and water and re-equilibrated after each use. The buffer compositions that were used were as follows: buffer A: 5 mM KH_2PO_4, 30% acetonitrile (pH 2.6); buffer B: 5 mM KH_2PO_4, 30% acetonitrile, 350 mM KCl (pH 2.6); and buffer C: 50 mM KH_2PO_4, 500 mM KCl (pH 7.5). All of the fractions were collected by hand, frozen, lyophilized and desalted over a tC18 Sep-Pak.

Phosphopeptide sample preparation

The peptide fractions were enriched for phosphopeptides using immobilized metal affinity chromatography (IMAC) with metal beads (Qiagen, Valencia, CA, USA). Following re-equilibration with water, the beads were incubated with 40 mM ethylenediaminetetraacetic acid (pH 8.0) for 30 minutes with shaking. Ethylenediaminetetraacetic acid was removed by three successive water washes, and the beads were then incubated with 100 mM $FeCl_3$ for thirty minutes while shaking. The beads were then washed with four rounds of 80% acetonitrile and 0.1% trifluoroacetic acid. The peptides were resuspended in 80% acetonitrile and 0.1% trifluoroacetic acid and incubated with the beads for one hour with shaking. Non-phosphorylated peptides were removed by washing the beads with 80% acetonitrile and 0.1% trifluoroacetic acid. The phosphorylated peptides were eluted from the beads with 50% acetonitrile, 0.7% NH_4OH and immediately acidified with 4% formic acid (FA).

Liquid chromatography-tandem mass spectrometry

Online reversed-phase chromatography was performed using a NanoACQUITY UPLC system (Waters, Milford, MA, USA). The peptides were loaded onto a 75 μm inner diameter, 360 μm outer diameter bare fused silica capillary that was packed with 10 cm of Magic C18 particles (Bruker-Michrom) for twelve minutes at a flow rate of 1 μl/minute. The peptides were then eluted onto a 5-μm inner diameter, 360-μm outer diameter analytical column that was packed with 17 cm of Magic C18 particles. The peptides were separated over a 90-minutes gradient by ramping from 2% to 35% acetonitrile, 0.2% FA at a flow rate of 0.3 μl/minute.

The eluting peptide cations were converted to gas-phase ions by electrospray ionization and analyzed on a Thermo Orbitrap Elite. Survey scans of peptide precursors from 350 to 1500 m/z were performed at 60K resolution (at 400 m/z) with a 1×10^6 ion count target. The dynamic exclusion duration was set to 45 s, with a maximum exclusion list of 500 and with an exclusion width of 0.5 Th below and 1.5 Th above the selected average mass. Tandem MS was performed with higher-energy C-trap dissociation fragmentation with a normalized collision energy of 35. The analysis was performed in the Orbitrap at a resolution 15K. The MS^2 ion count target was set to 1×10^5 with a maximum injection time of 350 msec.

Database searching

All of the MS/MS data were analyzed using the Coon OMSSA Proteomics Software Suite (COMPASS)(Wenger *et al.*, 2011). The spectra were searched using the Open Mass Spectrometry Search Algorithm (OMSSA)[2] against a concatenated target-decoy database consisting of Medicago protein sequences that were downloaded from UniProt. Tryptic peptides were created *in silico* allowing up to three missed cleavages. The mass tolerance was set to 20 ppm for precursors and 0.01 Th for fragment ions. The carbamidomethylation of cysteines and the TMT 6-plex on lysine and N-terminus were searched as fixed modifications. The oxidation of methionine, the TMT 6-plex on tyrosine, the phosphorylation of tyrosine and the phosphorylation with a neutral loss on serine and threonine were searched as variable modifications. The results were filtered to 1% FDR at the unique peptide level. The TMT-labeled peptides were quantified using TagQuant according to previously published procedures (Wenger *et al.*, 2011). The peptides were combined into protein groups and filtered to 1% FDR. The proteins were quantified by summing the intensity of the reporter ion tags of each channel for each protein. Phosphinator software was used to localize the phosphosites. If the site(s) of phosphorylation can be mapped to a specific amino acid residue with absolute certainty, it is termed 'localized phosphorylation', whereas if the site(s) of phosphorylation cannot be determined with absolute certainty often due to the presence of multiple potential phosphorylation residues in close proximity (even though we are confident that the peptide is phosphorylated), it is termed 'non-localized phosphorylation'. Following phosphosite localization, all of the quantitative measurements were log_2-transformed and mean-normalized. The fold changes between the conditions were determined by averaging the protein-normalized values for each condition and calculating the difference of averages. The *p*-values were then calculated using Microsoft Excel using Student's *t*-test Complete lists of localized and non-localized phosphorylation sites identified in this study, using both these symbiotic-receptor-like kinases, along with fold-changes in comparison to the control are presented in Supplementary data file 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance Statement

Multiplexed assay for kinase specificity, a recently developed radioisotope-independent high-throughput technique, was used to identify and map the phosphorylation substrates of up to 10 kinases in a single experiment. Using this technique for plants, we identified potential phosphorylation substrates of symbiotic receptor–like kinases, and these substrates might be the missing link connecting plasma membrane events to nuclear events during symbiotic signal transduction.
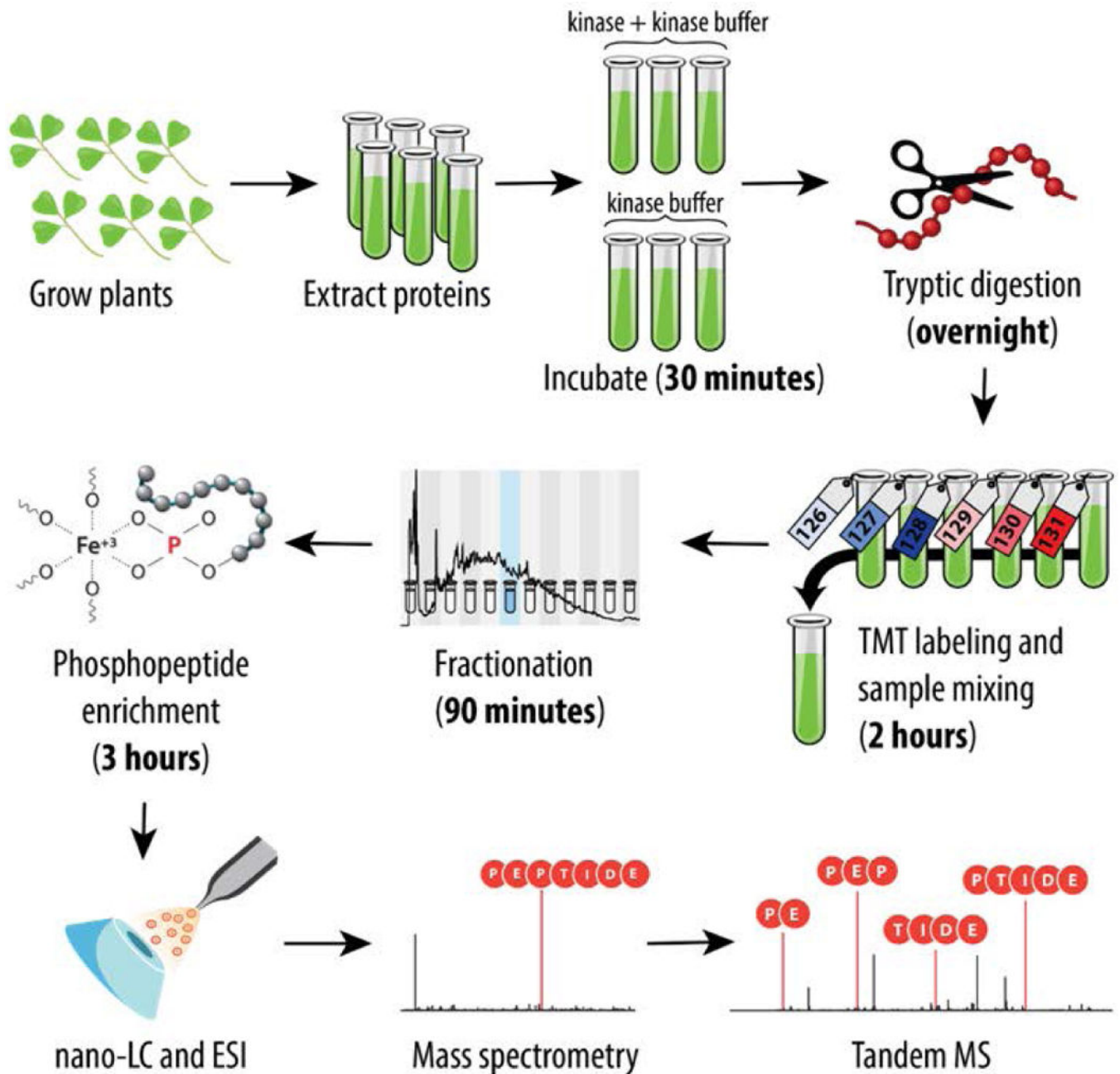
Figure 1. Overview of the analysis strategy

Six plant replicates were harvested, and proteins were extracted through a chloroform/methanol precipitation. Three of the replicates were incubated with a kinase reaction buffer (controls), while the other three replicates were incubated with a kinase reaction buffer and a purified kinase (either LYK3 or NORK). The resultant proteins were lysed, and trypsin digested, and each replicate was TMT-labeled and combined. Samples were fractionated, then analyzed *via* nanoLC-MS/MS. Estimated time period for some of the major steps is represented in brackets below each step.
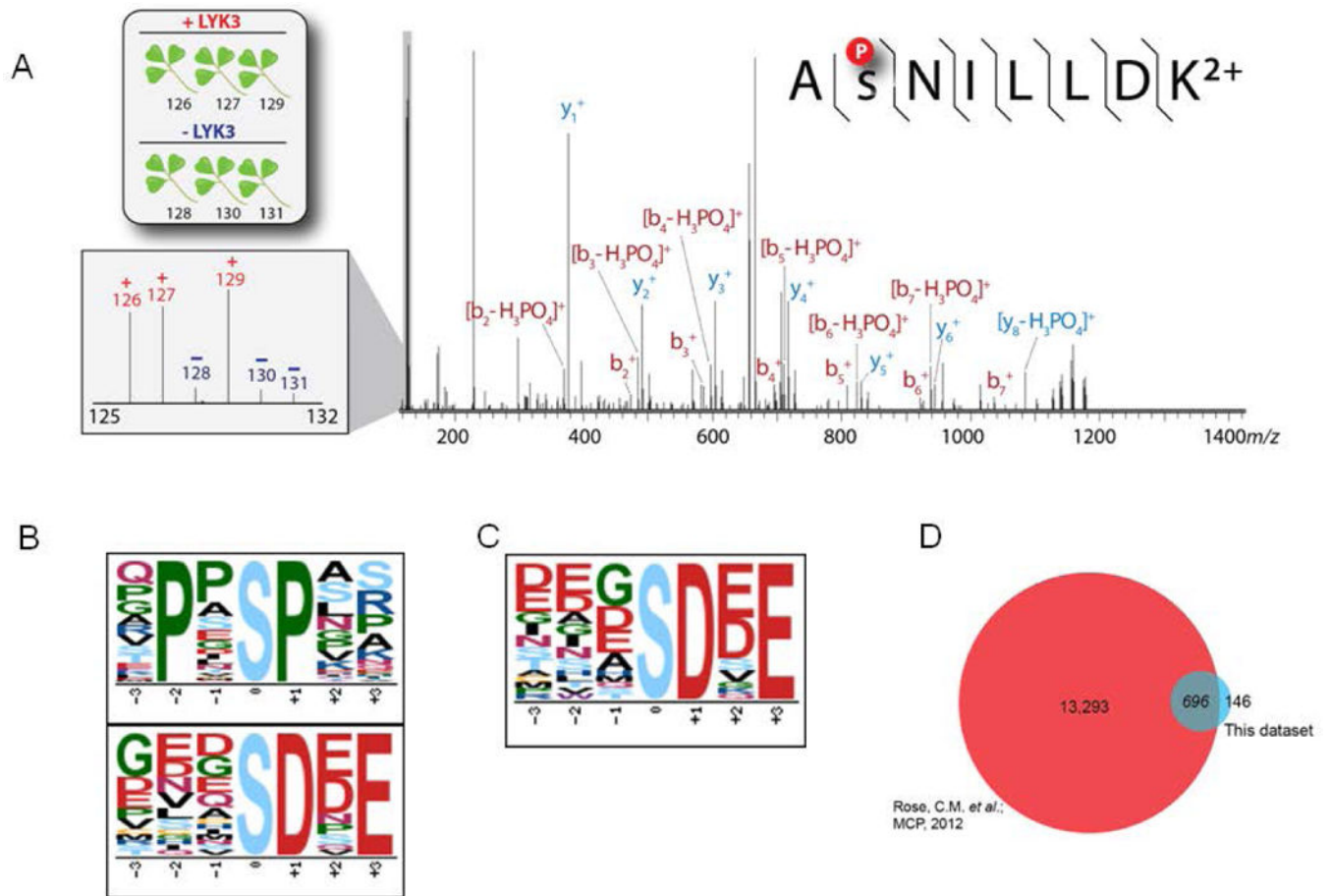
Figure 2. Identification of phosphorylation sites for the substrates of symbiotic receptor-like kinases

(A) Tandem mass spectrum of the phosphorylated peptide to AsNILLDK2+, a tryptic peptide of cysteine-rich receptor-kinase-like protein. Reporter tags are present in the low mass region (inset). The intensity of these reporter tags can be used to determine the amount of kinase-directed phosphorylation. Here, the phosphorylation intensity of the LYK3 treated channels (126, 127 and 129 m/z) show an increase in abundance compared to the control channels (128, 130, 131 m/z). Motif analysis of Medicago lysate treated with either (B) LYK3 or (C) NORK purified kinase. (D) Comparison of phosphopeptides from our previous work to the current work. Previously we identified 13293 phosphopeptides specific to symbiotic signaling in Medicago (Rose et al., 2012) and the current studies a total of 842 phosphopeptides were identified out of which 696 overlapped with our previous work and 146 were unique to this study.
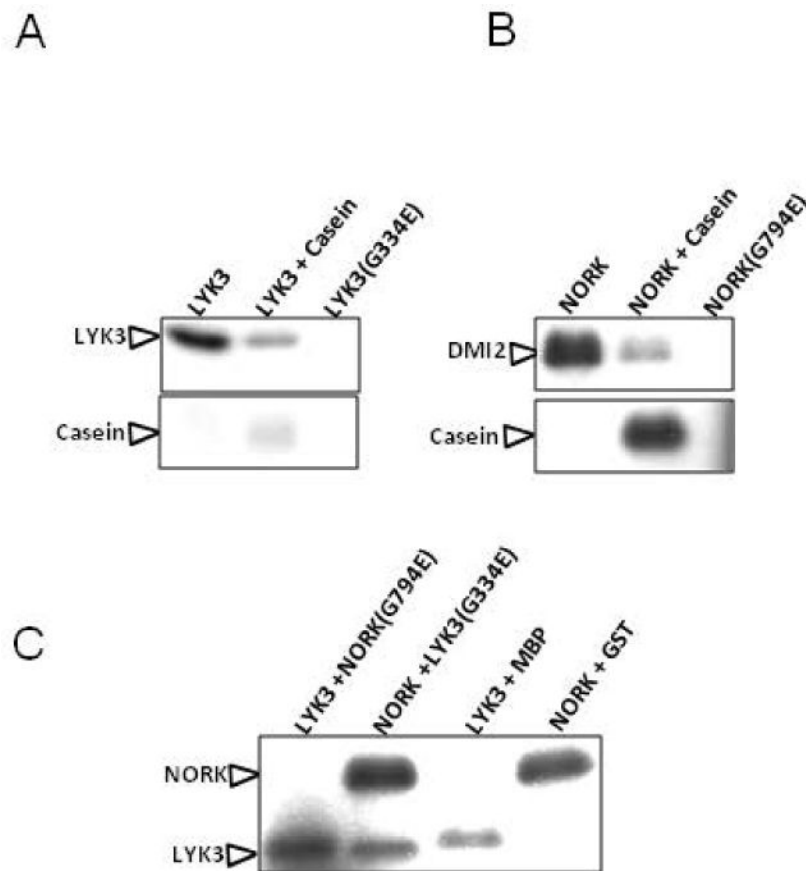
Figure 3. *In vitro* **kinase assay demonstrating the phosphorylation of LYK3 by NORK**
Auto phosphorylation and transphosphorylation activity of LYK3 tagged with GST(indicated by phosphorylation of casein) along with the lack of kinase activity for the mutant version (G334E) of LYK3 (A), autophosphorylation and transphosphorylation activity of NORK tagged with MBP (indicated by phosphorylation of casein) along with the lack of kinase activity for the mutant version (G794E) of NORK (B), lack of phosphorylation of NORK by LYK3 indicated by absence of band corresponding to the size of mutant NORK and a band corresponding to the size of LYK3 in a LYK3 + mutant NORK reaction mix, phosphorylation of LYK3 by NORK indicated by presence of band corresponding to the size of mutant LYK3 and a band corresponding to the size of NORK in a mutant LYK3 + NORK reaction mix, no non-specific phosphorylation indicated by a band corresponding to LYK3 alone in a LYK3 + purified MBP alone reaction mix or a band corresponding to NORK alone in a NORK + purified GST reaction mix (C).

Table 1

A list of the top 15 putative phosphorylation targets of LYK3

The table contains both localized and non-localized proteins calculated at 1% FDR with a p-value less than 0.10. Non-Localized phosphorylation sites are represented by (NL).

Gene ID	Annotation	Phospho-isoform	Fold Change (linear)
Medtr1g021635	Cysteine-rich receptor-kinase-like protein	S488	12.05
Medtr8g089695	Transmembrane protein; putative	T189	11.28
Medtr3g086520	E3 ubiquitin-protein ligase RGLG2-like protein	S395	4.72
Medtr4g061610	Nonphototropic hypocotyl protein	S152	4.07
Medtr4g074930	RNA recognition motif-RBD protein	T368	3.15
Medtr3g098420	110 kDa 4SNc-tudor domain protein	(NL)	3.05
Medtr4g075410	MAP kinase kinase kinase	S409	2.66
Medtr4g125330	Hypothetical protein	S167	2.63
Medtr8g089785	Plant-specific eukaryotic initiation factor	S464	2.58
Medtr4g074930	RNA recognition motif; a.k.a. RRM; RBD protein	(NL)	2.48
Medtr7g097000	Legumin A2	(NL)	2.38
Medtr4g052540	Fission ELM1 protein	S11	2.32
Medtr4g106980	Serine/Threonine-kinase HT1-like protein	(NL)	2.26
Medtr5g065490	Transport inhibitor response protein; putative	(NL)	2.25
Medtr4g005510	Ubiquitin-specific protease family C19 protein	(NL)	2.14

Table 2

A list of the top 15 putative phosphorylation targets of NORK

The table contains both localized and non-localized proteins calculated at 1% FDR with a *p*-value less than 0.10. Non-Localized phosphorylation sites are represented by (NL).

Gene ID	Annotation	Phospho-isoform	Fold Change (linear)
Medtr4g081675	S-locus lectin kinase family protein	(NL)	5.56
Medtr2g028340	Elongation factor Tu GTP-binding domain protein	(NL)	5.06
Medtr1g103550	DUF1677 family protein	S100	2.74
Medtr4g083230	Transcription factor Pur-alpha-like protein	(NL)	2.69
Medtr6g077870	OPT family oligopeptide transporter	S395	2.68
Medtr5g086130	LysM receptor kinase K1B (LYK3)	(NL)	2.68
Medtr5g012420	VAMP-associated protein	(NL)	2.41
Medtr1g072610	Glycinin G4	S549	2.26
Medtr0168s0110	Plant/T23J7-180 protein; putative	S296	2.25
Medtr5g095120	Receptor-like protein	(NL)	2.25
Medtr7g058460	Transcriptional corepressor leunig-like protein	S347	2.17
Medtr7g096990	Legumin A2	S51	2.14
Medtr3g084870	Lipoprotein	S229	1.96
Medtr4g080160	Trehalose-6-phosphate synthase domain protein	S5	1.96
Medtr4g030140	dynamin-2B-like protein	(NL)	1.95

Table 3

A list of putative phosphorylation targets of both NORK and LYK3

The table contains both localized and non-localized proteins calculated at 1% FDR with a *p*-value less than 0.10. Non-Localized phosphorylation sites are represented by (NL). The proteins are listed in ascending order by gene ID.

Gene ID	Annotation	Phospho-isoform		Fold Change (linear)	
		LYK3	NORK	LYK3	NORK
Medtr1g072600	Legumin storage protein	(NL)	(NL)	1.15	1.45
Medtr1g103400	Vicilin 47 kDa protein	Y639	S391	1.15	1.41
Medtr3g006760	Serrate RNA effector molecule-like protein	S364	S15	1.19	1.43
Medtr3g098420	110 kDa 4SNc-tudor domain protein	(NL)	(NL)	3.05	1.37
Medtr4g030140	Dynamin-2B-like protein	(NL)	(NL)	1.53	1.95
Medtr4g108140	IQ calmodulin-binding motif protein	(NL)	(NL)	1.79	1.23
Medtr5g012420	VAMP-associated protein	(NL)	(NL)	1.12	2.41
Medtr5g042000	ATP/GTP-binding family protein; putative	S975	S975	1.16	1.63
Medtr6g477980	Argonaute protein 1A	(NL)	(NL)	1.33	1.37
Medtr7g018610	Nuclear matrix constituent-like protein	(NL)	(NL)	1.34	1.67
Medtr7g097000	Legumin A2	S77	T436	1.31	1.23
Medtr8g014360	Drug resistance transporter-like ABC domain protein	S848	S848	1.16	1.60
Medtr8g099065	P-loop nucleoside triphosphate hydrolase superfamily protein	S200	S200	1.21	1.18