A plant cell wall-associated kinase encoding gene is dramatically downregulated during nematode infection of potato

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ABSTRACT
Plant cell wall associated kinases (WAKs) and WAK-like kinases (WAKLs) have been increasingly recognized as important regulators of plant immunity against various plant pathogens. However, the role of the WAK/WAKL family in plant-nematode interactions remains to be determined. Here, we analyzed a WAK-encoding gene (Soltu.DM.02G029720.1) from potato (Solanum tuberosum). The Soltu.DM.02G029720.1 encoded protein contains domains characteristic of WAK/WAKL proteins and shows the highest similarity to SIWAKL2 from tomato (S. lycopersicum). We thus named the gene as StWAKL2. Phylogenetic analysis of a wide range of plant WAKs/WAKLs further revealed close similarity of StWAKL2 to three WAK/WAKL proteins demonstrated to play a role in disease resistance. To gain insights into the potential regulation and function of StWAKL2, transgenic potato lines containing the StWAKL2 promoter fused to the β-glucuronidase (GUS) reporter gene were generated and used to investigate StWAKL2 expression during plant development and upon nematode infection. Histochemical analyses revealed that StWAKL2 has specific expression patterns in potato leaf and root tissues. During nematode infection, GUS activity was mostly undetected at nematode infection sites over the course of nematode parasitism, although strong GUS activity was observed in roots adjacent to the infection region. Furthermore, mining of the transcriptomic data derived from cyst nematode infection of Arabidopsis roots identified a few WAK/WAKL genes, including a StWAKL2 homologue, found to be significantly down-regulated in nematode-induced feeding sites. These results indicated that specific suppression of WAK/WAKL genes in nematode-induced feeding sites might be crucial for cyst nematodes to achieve successful infection of host plants. Further studies are needed to uncover the role of WAK/WAKL genes in plant defenses against nematode infection.

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Introduction
Plant cell wall-associated kinases (WAK) and WAK-like kinases (WAKL) are a unique group of receptor-like protein kinases (RLKs) which are involved in many functions in plants, including plant development and plant immunity against pathogen infection.1–3 This group of RLKs contains an extracellular domain with similarity to the vertebrate epidermal growth factor (EGF)-like domain, a transmembrane (TM) domain, and an intracellular serine/threonine protein kinase domain.4 The Arabidopsis (Arabidopsis thaliana) genome encodes five WAKs and twenty-two WAKLs.5 Arabidopsis WAK/WAKL genes have distinct and yet overlapping expression patterns, some of which are required for cell elongation and plant development.2 Recently, genome-wide analysis and characterization of the WAK/WAKL gene family has been reported for many other plant species including tomato (Solanum lycopersicum) and cotton (Gossypium hirsutum).6,7

WAK/WAKL proteins have been increasingly recognized as important contributors to disease resistance against bacterial and fungal pathogens.8 Transgenic plants overexpressing AtWAK1 are more resistant to the necrotrophic pathogen Botrytis cinerea.8 AtWAKL22, another member of the Arabidopsis WAK/WAKL family, is identified to encode a novel type of disease-resistance protein that confers resistance to a broad spectrum of Fusarium races.9 Consistently, Arabidopsis (Col-0 ecotype) mutated in AtWAKL22 was more susceptible to Fusarium infection.9 In rice, a few OsWAK genes are revealed to be important regulators in rice resistance against the blast fungus Magnaporthe oryzae. Studies have revealed that several OsWAK genes including OsWAK14, OsWAK25, OsWAK90, and OsWAK91 were upregulated upon infection by the blast fungus.10,11 Rice lines mutated in OsWAK14 or OsWAK91 were all more susceptible to the blast fungus, whereas lines with OsWAK91 overexpression showed increased disease resistance.10 OsWAK91 was further shown to be involved in ROS production and defense gene expression during pathogen infection.10 In tomato, SIWAK1 plays an important role in plant immunity against the bacterial pathogen Pseudomonas syringae pv. tomato,3 WAK/WAKL genes involved in disease resistance have also been identified in other plant species including maize and cotton.1,7,14,15
Little is known about the WAK/WAKL gene family in potato. Potato is an economically important crop, but its production is threatened by various potato plant pathogens including potato cyst nematodes (PCN; *Globodera rostochiensis* and *G. pallida*). Cyst nematodes are soil-borne root pathogens. These endoparasitic nematodes actively interfere with host defenses, primarily through the action of their secreted effector proteins, to ensure the establishment of feeding cells within roots from which they drain the needed nutrients that ultimately results in disease symptoms.\(^{16,17}\) Studies have revealed that some plant RLKs, such as CLAVATA1 and RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), are required for cyst nematode parasitism.\(^{16,18}\) Our initial search of potato RLKs led to the identification of the *SolDu.DM.02G029720.1* gene. Primary sequence analysis indicated that *SolDu.DM.02G029720.1* encodes a WAK/WAKL protein. Although the WAK/WAKL gene family has been studied in many plant species and under both biotic and abiotic conditions, knowledge on the function of potato WAK/WAKL genes as well as a role of WAK/WAKL genes in plant-nematode interactions are mostly lacking. In this study, we analyzed the *SolDu.DM.02G029720.1* gene from potato and investigated its expression under normal plant growth conditions and upon nematode infection through the utilization of promoter-GUS lines.

**Results and discussion**

**Sequence analysis of *SolDu.DM.02G029720.1***

The *SolDu.DM.02G029720.1* gene is predicted to encode a protein of 703 amino acids which contains an N-terminal signal peptide and displays features of WAK/WAKL proteins, including an extracellular EGF-like domain near the transmembrane region and an intracellular serine/threonine protein kinase domain (Figure 1a). To better understand the relationship of the *SolDu.DM.02G029720.1* encoded protein with other plant WAK/WAKL proteins, we performed a phylogenetic analysis that includes all the Arabidopsis and tomato WAK/WAKL proteins and several other plant WAK/WAKL proteins shown to have a role in plant resistance against pathogen infection.\(^{2,10,14}\) The phylogenetic analysis revealed that the *SolDu.DM.02G029720.1* encoded protein is clustered with SIWAKL2 and SIWAKL5 from tomato, GhWAK77 from cotton, AtWAKL14 and AtWAKL21 from Arabidopsis, and OsWAK25 and OsWAKL21.2 from rice (Figure 1b). The *SolDu.DM.02G029720.1* protein showed the closest similarity to tomato SIWAKL2 (98% similarity), followed by GhWAK77 (73% similarity), SIWAKL5 (72% similarity), and OsWAK25 (68% similarity) and OsWAKL21.2 (55% similarity) from cotton, tomato, and rice, respectively. The *SolDu.DM.02G029720.1* protein has 58% and 63% similarity with AtWAKL14 and AtWAKL21, respectively, from Arabidopsis. As the *SolDu.DM.02G029720.1* encoded protein has the highest similarity with SIWAKL2, we thus name the gene as StWAKL2.

**Tissue-specific expression of StWAKL2 in potato**

To gain insights into the potential regulation and function of *StWAKL2*, we initially analyzed the promoter region (2790 base pairs) upstream of the translation start site of *StWAKL2* to identify putative cis-acting elements using PlantCARE.\(^{19}\) Many phytohormone-responsive regulatory elements associated with auxin (AuxRE and TGA-element), methyl jasmonate (CGTCA-motif and TGACG-motif), abscisic acid (ABRE), and salicylic acid (SA) (TCA-element) were identified (Table 1). Stress-responsive regulatory elements, including TC-rich repeats and the WUN-motif involved in wounding and pathogen responsiveness, as well as elements associated with anaerobic induction (ARE) and drought inducibility (MBS) were also identified. Moreover, several light-responsive elements (I-box, G-box, TCT-motif, and AE-box) were found in *StWAKL2* promoter (Table 1). These results indicated that *StWAKL2* might have an important role in responses to hormones and stress signals.

To further investigate the expression profile of *StWAKL2*, we cloned the 2790-bp promoter sequence of *StWAKL2* (Genbank accession number OK135347) into a binary vector to generate a *StWAKL2pro-GUS* construct, which was subsequently transformed into potato. Histochemical analysis of the obtained transgenic potato lines indicated that *StWAKL2* expression occurs in a tissue-specific manner. In tissue-cultured plantlets, GUS activity was primarily observed in leaves and roots (Figure 2a). A closer examination of the leaf tissue revealed specific GUS activity in stomatal guard cells and trichomes (Figure 2b-c). The rice *OsWAK11* gene was also found to be specifically expressed in leaf trichomes.\(^{20}\) Almost no GUS staining was observed in stems (Figure 2a). Within roots, GUS activity was observed throughout the vasculature starting near the zone of maturation, but no GUS activity was detected in the root apical meristem region (Figure 2d-e). In Arabidopsis, unlike *AtWAK* genes which are predominately expressed in green tissues (stems and leaves), *AtWAKL* genes are mainly expressed in roots.\(^2\) However, among the seven *AtWAKL* genes (*AtWAKL1-AtWAKL7*) that were characterized using promoter-GUS lines, none of them showed a similar expression pattern in roots as *StWAKL2*. *StWAKL2* might have an important function in leaf guard cells and trichomes as well as in root vasculature due to its specific expression in these tissues.

**StWAKL2 is dramatically suppressed during PCN infection of potato**

Prior to this study, knowledge on the involvement of the WAK/WAKL gene family in plant-nematode interactions was lacking. To evaluate a role of *StWAKL2* in nematode parasitism, transgenic *StWAKL2pro-GUS* potato lines were infected with potato cyst nematode *Globodera rostochiensis* and assayed for GUS activity over a time-course of nematode infection. Interestingly, almost no GUS staining was detected at nematode infection sites from the early to late stages of nematode infection, although strong GUS staining was detected in root tissues adjacent to the infection region (Figure 3a-c). We further used RT-qPCR to verify *StWAKL2* expression in response to nematode infection. As expected, the expression of *StWAKL2* was found to be dramatically reduced during nematode parasitism compared to that in uninfected potato roots (Figure 3d). The results likely correlate with the observation of the suppression of *StWAKL2* expression at nematode infection sites.
infection sites using StWAKL2pro-GUS lines. We also analyzed the transcriptomic data generated by Szakasits et al. (2009) on the study of cyst nematode infection of Arabidopsis roots. Among the twenty-one WAK/WAKL genes found to be expressed in Arabidopsis roots, five of them were revealed to be regulated in response to nematode infection. Interestingly, all the five WAK/WAKL genes including AtWAKL14, a StWAKL2 homologue, showed significant down-regulation in nematode-induced feeding cells when compared to their expression in the normal root tissue (Table S1). Together, these results indicated that specific suppression of WAK/WAKL genes in nematode-induced feeding sites might be crucial for cyst nematode to achieve successful infection of host plants. Our phylogenetic analysis revealed that in addition to the four WAKL genes from Arabidopsis and tomato, StWAKL2 has close similarity with GhWAK77 from cotton and OsWAK25 and OsWAKL21.2 from rice (Figure 1b). All the three WAK genes have been implicated in plant immunity against bacterial and fungal pathogens. The cotton GhWAK77 gene was significantly and constantly upregulated during infection by the soil borne fungus *Verticillium dahliae*. Silencing GhWAK77 compromised cotton resistance to *V. dahliae*. It was suggested that GhWAK77 might function in SA- and JA (jasmonate acid)-mediated signaling pathways to regulate cotton resistance against *V. dahliae*. Both OsWAK25 and OsWAKL21.2 were upregulated during pathogen infection. Furthermore, overexpression of OsWAK25 resulted in elevated expression of several defense-related genes and increased plant resistance to the bacterial pathogen *Xanthomonas oryzae* and the blast fungus *Magnaporthe oryzae*. Both StWAKL2 and AtWAKL14 are grouped with GhWAK77, OsWAK25, and OsWAKL21.2 based on the
Table 1. Putative cis-acting regulatory elements present in the StWAKL2 promoter.

| Type of responsiveness                        | Number of cis-acting elements |
|-----------------------------------------------|-------------------------------|
| Auxin responsiveness                          | 1                             |
| AuxRE                                        |                               |
| TGA-element                                   | 1                             |
| Methyl jasmonic acid (MeJA) responsiveness    | 1                             |
| CGTCA-motif                                   |                               |
| TGACG-motif                                   | 1                             |
| Abscisic acid (ABA) responsiveness            |                               |
| ABRE                                          | 1                             |
| Salicylic acid (SA) responsiveness           | 1                             |
| TCA-element                                   |                               |
| Defense and stress responsiveness             | 1                             |
| TC-rich repeats                               |                               |
| Wound responsiveness                         | 1                             |
| WUN-motif                                     |                               |
| Anaerobic induction and drought inducibility  |                               |
| ARE                                          | 2                             |
| MBS                                          | 1                             |
| Light responsiveness                         |                               |
| I-box                                         | 1                             |
| G-box                                        | 1                             |
| TCT-motif                                     | 2                             |
| AE-box                                       | 1                             |

phylogenetic analysis (Figure 1b). Due to this close relatedness, we hypothesize that StWAKL2 and AtWAKL14 may have a critical role in plant defenses against nematode infection. It would be interesting to determine if overexpression of StWAKL2 or AtWAKL14 would render host plants more resistant to cyst nematode infection.

In summary, we have analyzed a specific StWAKL2 gene from potato through sequence comparison with a wide range of plant WAK/WAKL genes and used StWAKL2pro-GUS lines to reveal the tissue-specific expression and the negative regulation of StWAKL2 during cyst nematode infection of potato. This study indicated for the first time that active regulation of specific members of the WAK/WAKL gene family might be critical for nematode parasitism of host plants.

**Materials and methods**

**Nematode culture and plant materials**

The potato cyst nematode *Globodera rostochiensis* was propagated on potato (*Solanum tuberosum*) and nematode infection assays on potato plantlets were conducted as previously described.\(^{22,23}\) Potato cv. Désirée was used for generating transgenic plants.\(^{24}\)

**Sequence analysis of Soltu.DM.02G029720.1 encoded protein (named StWAKL2)**

The SMART (simple modular architecture research tool) database (http://smart.embl-heidelberg.de/) and NCBI-CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) were used to identify domains in Soltu.DM.02G029720.1 encoded protein sequence.

Phylogenetic analysis of plant WAK/WAKL proteins and characterization of the StWAKL2 promoter region

Phylogenetic analysis of StWAKL2 with all the WAK/WAKL proteins from Arabidopsis and tomato and several other plant WAK/WAKL proteins demonstrated to have a role in plant defenses was performed as previously described.\(^{25}\) The

![Figure 2. Tissue-specific expression of StWAKL2 in potato. Transgenic potato lines expressing StWAKL2pro-GUS was generated and used to determine StWAKL2 expression in potato tissues. Strong GUS staining was observed primarily in leaf and root tissues (a). A close examination of leaf and root tissues revealed GUS staining in leaf guard cells and trichomes (b-c) as well as throughout the root vasculature near the zone of maturation with no GUS staining in the root apical meristem (d-e). Scales bars = 1 cm in (a) and 200 μm in (b-e), respectively.](image-url)
PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to search for cis-acting elements in the StWAKL2 promoter region (~2790 bp to −1 bp).

Expression construct and potato transformation

A 2790-bp sequence upstream of the start codon of StWAKL2 (Soltu.DM.02G029720.1) was amplified from potato (cv Désirée) genomic DNA by PCR using primers St2g23450_1-pro-SaII-F (5′-CTCGTCGACCTGTAACAGCTTAGATCAACAC-3′) and St2g23450_1-pro-BamHI-R (5′-GAGAGATTCGCTGATATCAAT-3′), and cloned into the binary vector pBI101.2, at the SaII and BamHI sites, to generate the StWAKL2pro-GUS construct. Transgenic potato lines expressing StWAKL2p-GUS were generated as described.24,27

Histochemical GUS assay and nematode staining

Shoot tops cut from in vitro-grown transgenic potato plantlets were cultivated either in glass-tubes or in six-well plates containing the proper medium. Two weeks after growth, plantlets were either used for GUS staining to investigate the spatial expression of the StWAKL2 gene or used for nematode infection. Roots at 3, 10, and 21 days post inoculation (dpi) of nematodes were collected and used for GUS staining followed by nematode staining. Non-infected plantlets and nematode-infected roots were infiltrated with GUS substrate buffer and incubated for 12–14 h at 37°C. Stained roots were subsequently performed for infected roots collected at 3 dpi and 10 dpi. Stained roots were mounted on glass slides and visualized with a Nikon Eclipse TS100 inverted microscope.

StWAKL2 expression during nematode infection

mRNA from uninfected potato roots and G. rostochiensis infected root segments containing nematodes at 3, 10, and 21 days post inoculation (dpi) was extracted and used for RT-qPCR as previously described. Primes StWAKL-2_1308 F (5′-AAAGTCCACAGTGATGATGG-3′) and StWAKL-2_1526 R (5′-GCCCTTTCTCTCTGATGCT-3′) were used to target StWAKL2, and primers StRPN7_F (5′-GAGGGGAGG AATGCAGAT-3′) and StRPN7_R (5′-TCCATCTGCACTGCTTACC-3′) were used to target the potato 26S proteasome subunit gene StRPN7 (Soltu.DM.07G005010.1) that served as an endogenous reference for data analysis. The RT-qPCR data were obtained from three independent experiments, with three technical replicates for each cDNA sample.

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