Screening and Validation of Interaction Proteins for Peanut Receptor Protein Kinase AhASRK1

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Abstract

Receptor-like kinases (RLKs) are core molecules in plant cellular signal transduction, and the phosphorylation status of their intracellular domains (CDs) and their binding to downstream proteins are key steps in initiating signal transduction cascades. AhASRK1 is a receptor-like kinase that regulates aluminum sensitivity in peanut. To elucidate the molecular regulatory mechanism by which AhASRK1 participates in the aluminum stress response, this study used the intracellular domain of AhASRK1 (AhASRK1-CD) as bait protein to screen a normalized peanut cDNA library using yeast two-hybrid (Y2H) technology. Meanwhile, site-directed mutagenesis was used to construct constitutively non-phosphorylated and constitutively phosphorylated mutants to investigate the regulatory role of key phosphorylation sites in protein interactions. The results showed that: (1) A total of 67 candidate interacting proteins were obtained through primary screening and rescreening by yeast two-hybrid analysis, covering the polygalacturonase inhibitor/non-catalytic subunit family, EMSY-LIKE protein family, blue copper protein superfamily, XTH gene family, Bax Inhibitor-1 family, and others. (2) The candidate interacting proteins were mainly enriched in pathways such as structural constituents of the ribosome, cell wall biosynthesis, and xyloglucan metabolism. (3) After key phosphorylation sites in AhASRK1-CD were simulated as constitutively non-phosphorylated (T-A mutation), the interaction with some candidate proteins was lost, whereas simulated constitutive phosphorylation (T-D mutation) did not affect the interactions. This study identified the interacting protein components of AhASRK1-CD and confirmed that key phosphorylation modifications in the intracellular domain serve as an important switch mediating the binding of AhASRK1 to downstream target proteins, providing key candidate genes and a theoretical basis for further elucidating the molecular pathways by which AhASRK1 regulates the aluminum stress response in peanut.

Full Text

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Screening and Validation of Interaction Proteins for Peanut Receptor Protein Kinase AhASRK1

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Abstract: Receptor-like kinases (RLKs) are core molecules in plant cell signal transduction. The phosphorylation status of their cytoplasmic domain (CD) and their binding to downstream proteins are key steps in initiating signal-transduction cascade responses. AhASRK1 is a receptor-like kinase that regulates aluminum sensitivity in peanut. To elucidate the molecular regulatory mechanism by which AhASRK1 participates in the peanut response to aluminum stress, this study used the cytoplasmic domain of AhASRK1 (AhASRK1-CD) as bait protein and employed yeast two-hybrid (Y2H) technology to screen a peanut normalized cDNA library; meanwhile, site-directed mutagenesis was combined to construct constitutively non-phosphorylated mutants and constitutively phosphorylated mutants to explore the regulatory roles of key phosphorylation sites in protein interactions. The results showed that: (1) after initial screening and rescreening by yeast two-hybrid, a total of 67 candidate interacting proteins were obtained, covering families such as the multiple polygalacturonase inhibitor/non-catalytic arabinogalactan family, the EMSY-LIKE protein family, the blue copper protein superfamily, the XTH gene family, and the Bax Inhibitor-1 family. (2) The candidate interacting proteins were mainly enriched in pathways such as glycoprotein structural composition, cell-wall biosynthesis, and xyloglucan metabolism. (3) After the key phosphorylation sites of AhASRK1-CD were simulated as constitutively non-phosphorylated (T-A mutation), the ability to interact with some candidate proteins was lost, whereas after simulation of constitutive phosphorylation (T-D mutation), the interactions were not affected. This study clarified the interacting protein components of AhASRK1-CD and confirmed that key phosphorylation modifications in the cytoplasmic domain are an important switch mediating the binding of AhASRK1 to downstream target proteins, providing key candidate genes and a theoretical basis for subsequent in-depth elucidation of the molecular pathway by which AhASRK1 regulates the aluminum-stress response in peanut.

Keywords: peanut; receptor-like kinase; AhASRK1-CD; yeast two-hybrid; phosphorylation

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Screening and validation of interaction proteins for peanut receptor protein kinase AhASRK1

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Abstract: Receptor-like kinases (RLKs) are core molecules in the plant cell signal transduction network. The phosphorylation status of its cytoplasmic domain (CD) and its binding to downstream proteins constitute the pivotal steps in initiating the signal transduction cascade. AhASRK1 is a receptor-like kinase that regulates aluminum sensitivity in peanuts. To elucidate the molecular regulatory mechanism by which the AhASRK1 in peanuts participates in the

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aluminum stress response, the intracellular domain of AhASRK1 (AhASRK1-CD) was used as the bait protein to screen the normalized peanut cDNA library via yeast two-hybrid (Y2H) assay. Simultaneously, using site-specific mutation technology, we constructed a persistently non-phosphorylated mutant and a persistently phosphorylated mutant to investigate the regulatory role of key phosphorylation sites in protein-protein interactions. The results were as follows: (1) Through initial and secondary yeast two-hybrid screening, a total of 67 candidate interacting components are obtained, including the polygalacturonase inhibitor/non-catalytic subunit family, EMSY-LIKE protein family, blue copper protein superfamily, XTH gene family, and Bax Inhibitor-1 family. (2) Candidate interacting proteins are mainly enriched in the structural constituent of ribosome, cell wall biogenesis, and xyloglucan metabolic process pathways. (3) The persistent non-phosphorylation simulation (T-A mutation) results in loss of interaction capability with certain candidate proteins, whereas the persistent phosphorylation simulation (T-D mutation) does not affect interactions. This study identified the interacting protein components of AhASRK1-CD and verified that key phosphorylation modification of the cytoplasmic domain serves as a critical switch mediating the binding of AhASRK1 to its downstream target proteins. The findings provide candidate key genes and a theoretical basis for further elucidating the molecular pathway by which AhASRK1 regulates peanut responses to aluminum stress.

Keywords: peanut, receptor-like kinase, AhASRK1-CD, yeast two-hybrid, phosphorylation

Plants need to rely on precise signal-transduction networks to perceive and respond to external environmental stimuli and endogenous growth signals, thereby maintaining their normal growth and development. Receptor-like protein kinases (RLKs) participate in intercellular and cell-environment signal-

transduction processes mediated by various signaling molecules (Walker & Zhang, 1990). RLKs are one of the largest receptor families in plant epidermal cells; the vast majority are localized on the plasma membrane, and their structure mainly includes an extracellular domain, a transmembrane domain, and an intracellular domain (Stone & Walker, 1995). The extracellular domain is located outside the cell membrane and is mainly responsible for recognizing and binding extracellular signaling molecules; it is the core structure by which receptors perceive external signals. The transmembrane domain is mainly responsible for membrane localization, connecting the extracellular and intracellular structural domains, mediating signal transmission across the membrane, regulating receptor activity, and maintaining protein stability. The intracellular domain is located on the inner side of the cell membrane and has serine/threonine or tyrosine kinase activity; through autophosphorylation and binding with downstream interacting proteins, it initiates intracellular signal-cascade responses, regulates the expression of related genes, and thereby participates in multiple physiological processes such as plant growth and development, immune defense, and responses to adversity (Jose et al., 2020).

RLKs are core membrane receptors by which plants perceive extracellular signals and initiate intracellular cascade responses (Liu et al., 2024), and they mainly accomplish signal transduction cooperatively through phosphorylation modification of intracellular structural domains and specific interactions with downstream proteins. Phosphorylation modification can remodel the spatial conformation of RLKs and activate kinase activity; it is the basis of their functional activation (Liu et al., 2024) and also the core means of regulating activation of RLK intracellular domains and signal transmission. Mutations at key phosphorylation sites often lead to loss of kinase activity or changes in interaction capacity, thereby affecting signal transduction. Studies have shown that the Arabidopsis LysM-RLK receptor kinase CERK1 mediates immune responses triggered by chitin and peptidoglycan. CERK1 itself can be phosphorylated by chitin treatment, but after mutation of its key phosphorylation sites (Thr-479) and (Tyr-428), it completely loses the ability to complement the chitin response of the *cerk1* mutant (Suzuki et al., 2018). After mutation of the activation-loop phosphorylation site of rice OsSERK2, it loses the ability to interact with the main receptor, leading to abnormal brassinolide signaling pathways (Bojar et al., 2014).

Protein interactions mediated by the intracellular domains of RLKs are a core link in signal transmission. They can bind multiple functionally differentiated downstream proteins to form complex regulatory networks, including MAPK kinases and small G proteins involved in signal integration and amplification (Pandey, 2020; Ye et al., 2025), transcription factors such as bZIP, MYB, and WRKY that regulate gene transcription (Li et al., 2021; Ye et al., 2025), as well as ion-transport proteins and antioxidant metabolic enzymes that perform physiological functions. They broadly participate in biological processes such as ion homeostasis and adversity responses (Wang et al., 2024; Xu et al., 2025). The diversification of RLK-interacting proteins endows them with multi-pathway reg-

ulatory capacity, enabling them to precisely regulate plant growth and development and adversity responses, and is an important guarantee for the functional diversification of RLKs. Therefore, mining

interacting proteins of RLKs and investigating the regulatory roles of phosphorylation sites in these interactions are key to elucidating the signal-transduction mechanisms they mediate.

Peanut is an important oil and economic crop in China, with a perennial planting area of approximately 73 million mu, ranking first among all oil crops in total output. According to data from the National Bureau of Statistics, since 2007 the sown area of peanut in the southern peanut-producing region has steadily increased, and its position in the national production pattern has continued to become more prominent. However, this region is dominated by acidic red and yellow soils, and the soil exchangeable aluminum content is relatively high; aluminum toxicity is an important factor restricting the improvement of local peanut yield per unit area (Qin Ruijun and Chen Fuxing, 1999; Wu Meiqing et al., 2022). Based on comparative transcriptomic analysis of peanut root tips from aluminum-tolerant and aluminum-sensitive peanut varieties under aluminum stress, we mined and identified a receptor-like protein kinase with dual specificity for serine/threonine and tyrosine phosphorylation (Xiao et al., 2021; Huang Shuting et al., 2020). Further studies confirmed that the gene-encoded product is localized to the plasma membrane and can mediate aluminum-stress-induced programmed cell death by activating the Ca^{2+}-ROS-MAPK signaling cascade, playing a key regulatory role in the peanut aluminum-toxicity response. According to its functional characteristics, it was named AhASRK1 (aluminum sensitive receptor-like protein kinase 1) (Fu et al., 2025). Previous studies have also identified key phosphorylation sites in the intracellular domain of AhASRK1 (AhASRK1-CD), but the downstream interacting proteins that regulate AhASRK1-mediated aluminum signal transduction remain completely unknown. Whether these core phosphorylation sites regulate interactions between AhASRK1 and downstream proteins has not yet been verified, and whether the modification status of different phosphorylation sites produces differential regulatory effects on downstream candidate interacting proteins, forms functional divergence mechanisms, and thereby mediates the branched transduction and responses of aluminum-stress signaling remains unclear.

On the basis of the above findings, this study used the aluminum-tolerant peanut variety 99-1507 as the experimental material and adopted a yeast two-hybrid mating system. Through bait-vector toxicity and autoactivation assays, screening of a cDNA library from peanut root, stem, and leaf tissues, and verification of interactions involving phosphorylation-site mimic mutations, we sought to explore the following two questions: (1) Which functional proteins can the intracellular domain of AhASRK1 (AhASRK1-CD) specifically bind, and in which physiological pathways are the candidate interacting proteins mainly enriched? (2) Do the T-A (alanine mutation, sustained dephosphorylation) and T-D (aspartic acid mutation, mimicking sustained phosphorylation) modification states

of the core phosphorylation sites of AhASRK1-CD alter the interaction capacity of AhASRK1 with its candidate proteins?

1 Materials and Methods

1.1 Experimental Materials

The yeast strains Y2HGold and Y187, *Escherichia coli* strain DH5α, and the plasmids *pGBKT7(BD)*, *pGBKT7-53*, *pGBKT7-lam*, and *pGADT7-T* were all preserved in this laboratory. The *pGBKT7-AhASRK1-CD* plasmid was constructed in this laboratory. The peanut normalized cDNA (AD-candidate interacting component) library was purchased from Nanjing Zhongding Biotechnology Co., Ltd.; the materials used to construct the cDNA library were roots, stems, and leaves of peanut variety 99-1507 cultured hydroponically for 7 days.

1.2 Yeast Two-Hybrid Assay

1.2.1 Construction of the pGBKT7-AhASRK1-CD Bait Vector and Transformation into Yeast

Using the *pMD19-T-AhASRK1* vector containing the *AhASRK1* gene as the template, and using 5′-atggccatggaggccgaattcCCTTCAATAATGAGTCAACACCAT-3′ and 5′-atgcggccgctgcaggtcgacGCGATCTTCAGATGTTGGAAGAT-3′ as primers, PCR amplification was performed with DNA polymerase LA Taq (LA Taq Version 2.0). After recovery of the amplification product, the target fragment containing *AhASRK1-CD* was obtained. The PCR reaction system was 25 μL, including 1 μL each of the upstream and downstream primers, 12.5 μL Premix Taq, 1 μL template, and nuclease-free water added to 25 μL. The PCR program was set as follows: 94 °C for 5 min; 94 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s, 30 cycles (steps 2–4); 72 °C for 10 min; and storage at 4 °C. The *pGBKT7* plasmid was then double-digested to obtain a linearized vector. The target fragment containing *pGBKT7-AhASRK1-CD* was ligated with the linearized *pGBKT7* vector. The ligation reaction system is shown in Table 1. The above reaction mixture was incubated in a PCR instrument at 37 °C for 30 min, and the ligation product was transformed into *E. coli* DH5α and screened.

Positive clones were selected for PCR identification and sequencing. After correct identification, the yeast strain Y2HGold was transformed by the PEG/LiAc method. The transformants were spread on SD/-Trp plates, cultured for 2–3 d, and positive clones were picked.

Table 1 Carrier construction ligation reaction system

Table 1 The carrier facilitates the connection of the reaction system

Reagent	System (10 μL)
Linearized vector	4 μL
Gel-purified target fragment	3 μL
5×CE Buffer	2 μL
Exnase	1 μL

1.2.2 Assay of autoactivation of the bait protein

A single colony of *pGBKT7-AhASRK1-CD* on SD/-Trp selective medium was picked and placed in 1 mL of 0.9% NaCl solution. From this, 10 μL of the bacterial suspension was taken and diluted 100-fold with 0.9% NaCl solution. Then 100 μL of the above diluted bacterial suspension was spread on SD/-Trp/-His/-Ade selective medium plates and cultured in a 30 °C incubator for 3–5 d.

1.2.3 Assay of toxicity of the bait protein

Single colonies of *pGBKT7-AhASRK1-CD* and *pGBKT7* (empty vector) on SD/-Trp selective medium were separately picked and placed in 1 mL of 0.9% NaCl solution. From each, 10 μL of bacterial suspension was taken and diluted 100-fold with 0.9% NaCl solution. Then 100 μL of each of the above two diluted bacterial suspensions was spread on SD/-Trp medium. Yeast carrying the *pGBKT7* plasmid was used as the blank control, and yeast carrying *pGBKT7-AhASRK1-CD* was used as the experimental group. The plates were cultured in a 30 °C incubator for 3–5 d.

1.2.4 Yeast two-hybrid mating and preliminary identification

Yeast Y2HGold single colonies transformed with the *pGBKT7-AhASRK1-CD* plasmid were inoculated into 50 mL SD/-Trp liquid medium and cultured for 16–20 h until the OD_{600} reached 0.8–1.0. The cells were collected by centrifugation and resuspended in 4–5 mL SD/-Trp liquid medium. Subsequently, they were mixed with the peanut cDNA library in a 1 L conical flask; 45 mL 2×YPDA medium was added, and the mixture was slowly shaken at 30 °C and 50 $r \cdot min^{-1}$ for 20–24 h. After 20 h, mating was observed microscopically. After mating passed the microscopic examination, the bacterial suspension was collected, centrifuged, and resuspended in 10 mL 0.5×YPDA medium. Part of the mating product was diluted and spread on SD/-Trp-Leu double-dropout medium to determine the number of screened library clones; the remainder was spread on SD/-Trp-Leu-His triple-dropout medium. Positive clones growing on the triple-dropout medium were transferred to quadruple-dropout medium (SD/-Trp-Leu-Ade-His+X-α-gal) to identify the mating results.

1.2.5 Point-to-point validation of candidate interacting proteins

Some AD-candidate interacting protein recombinant vectors were transformed into Y187, and positive clones were picked on SD/-Leu. Yeast Y2HGold single colonies transformed with the *pGBKT7-AhASRK1-CD* plasmid were picked and inoculated into SD/-Trp; both were cultured overnight at 30 °C and 220 r·min^{-1}. Subsequently, 10 μL of each of the above bacterial suspensions was taken and co-inoculated into 1 mL 2×YPDA liquid medium, and the cultures were slowly shaken at 30 °C and 50 r·min^{-1} for 20–24 h. The mixed cultures were separately spread on SD/-Trp/-Leu/-His/-Ade+X-α-gal quadruple-

were spread on selection medium lacking the culture base and cultured at 30 °C for 3–5 d.

2 Results and Analysis

2.1 Construction and Identification of the *BD-AhASRK1-CD* Bait Vector

To screen for interacting components of AhASRK1, we constructed a bait vector, *pGBKT7-AhASRK1-CD*, containing the intracellular-domain fragment of the *AhASRK1* gene (*AhASRK1-CD*) (Fig. 1: A). We transformed the *pGBKT7-AhASRK1-CD* plasmid into the Y2HGold strain and performed toxicity and self-activation assays. The results showed that, on SD/-Trp plates, yeast cells transformed with the *AhASRK1-CD* plasmid grew normally, and the colony size was essentially consistent with that of yeast carrying the empty vector; there were no significant differences in colony growth rate or colony diameter, indicating that the vector was not toxic and that AhASRK1-CD was non-toxic (Fig. 1: B). On SD/-Trp/-His/-Ade plates, no colonies appeared for yeast transformed with AhASRK1-CD (Fig. 1: C), indicating that AhASRK1-CD had no self-activation activity and could be used as a bait protein for library screening.

A. PCR detection of the *pGBKT7-AhASRK1-CD* bacterial suspension (M. DL2 000; 1–4. PCR amplification products of *pGBKT7-AhASRK1-CD*); B. Toxicity detection of pGBKT7-AhASRK1-CD; C. Self-activation detection of pGBKT7-AhASRK1-CD.

A. PCR detection of the *pGBKT7-AhASRK1-CD* bacterial suspension (M. DL2 000; 1–4. PCR amplification products of *pGBKT7-AhASRK1-CD*); B. Toxicity detection of pGBKT7-AhASRK1-CD; C. Self-activation detection of pGBKT7-AhASRK1-CD.

Fig. 1 Construction of the bait vector and detection of bait protein toxicity and self-activation

Fig. Construction of the bait vector and detection of bait protein toxicity and self-activation

Fig. 3 AD-candidate interacting components with AhASRK1 one-to-one validation

Figure 1: Fig. 3 AD-candidate interacting components with AhASRK1 one-to-one validation

2.2 Screening of Proteins Interacting with AhASRK1 in a Peanut cDNA Library by Yeast Two-Hybrid Assay

The Y2HGold bait strain carrying the bait expression vector *pGBKT7-AhASRK1-CD* was mated and transformed with the Y187 prey strain carrying the cDNA library. After microscopic examination confirmed the formation of zygotes (Fig. 2: A), the transformation mixture was spread on SD/-Trp-Leu-Ade medium for preliminary screening, and the positive clones obtained from the primary screen were picked and transferred to SD/-Trp-Leu-Ade-His+X-α-gal medium for further screening (Fig. 2: B). A total of 128 positive clones were obtained after screening.

A. Microscopic examination of the conjugation products between the Y2HGold strain transformed with the bait vector and the Y187 strain carrying the library; the arrows indicate typical yeast zygotes;

B. Growth of positive clones on SD/−Trp−Leu−Ade−His+X-α-gal medium.

A. Microscopic examination of the conjugation products between the Y2HGold strain transformed with the bait

vector and the Y187 strain carrying the library, arrows indicate typical yeast zygotes; **B.** Positive clones growing on SD/-Trp-Leu-Ade-His+X-α-gal medium.

Fig. 2 Yeast dual transformation and growth of positive clones

The 128 clones obtained were subjected to plasmid extraction and then tested with AD primers; the results are shown in Fig. 3A. Plasmids capable of amplifying bands were subsequently selected and transformed back into *E. coli*, and transformants resistant to ampicillin were screened, i.e., transformants containing the library plasmids. Plasmids were extracted from these transformants and directly sent for sequencing. After excluding positive clones containing identical genes, a total of 67 candidate interacting components of AhASRK1-CD were obtained (Table 2). Six target genes were selected for one-to-one validation. The results showed that the transformants of all six target clones could grow normally on SD/-Trp-Leu-Ade-His+X-α-gal medium (Fig. 3B).

A. AD primer detection after plasmid extraction of AhASRK1 interacting components (**M.** DL5 000; **1-16.** AhASRK1-CD candidate interacting components); **B.** One-to-one validation of AhASRK1-CD with AD-candidate interacting components.

Fig. 3 One-to-one validation of AD-candidate interacting components with AhASRK1

Table 2 Information on AhASRK1-CD interacting proteins

Gene No.	Gene description	Classify
LOC107481320	Polygalacturonase 1 β-like protein 3 in peanut *Arachis duranensis* polygalacturonase 1 β-like protein 3	Cell wall metabolic protein
LOC112770709	Peanut protein EMSY-LIKE3 *Arachis hypogaea* protein EMSY-LIKE 3	Uncharacterized

Gene ID	Protein name	Functional annotation
LOC107478600	Peanut suppressor of mec-8 and unc-52 protein homolog 2 *Arachis duranensis* suppressor of mec-8 and unc-52 protein homolog 2	Uncharacterized Uncharacterized
LOC112794013	Peanut uncharacterized LOC112794013 *Arachis hypogaea* uncharacterized LOC112794013	Uncharacterized Uncharacterized
LOC112709359	Peanut uncharacterized LOC112709359 *Arachis hypogaea* uncharacterized LOC112709359	Uncharacterized Uncharacterized
LOC107634750	Peanut basic blue protein *Arachis ipaensis* basic blue protein	Uncharacterized Uncharacterized
LOC112723002	BI1-like protein in peanut *Arachis hypogaea* BI1-like protein	Poptosis-related protein Poptosis-related protein
LOC107604996	Peanut actin-related protein 2 *Arachis ipaensis* actin-related protein 2	Uncharacterized Uncharacterized

Gene ID	Protein name	Functional annotation
LOC112795837	Peanut endo-1,3; 1,4-β-D-glucanase *Arachis hypogaea* endo-1,3;1,4-β-D-glucanase	Cell wall metabolic protein Cell wall metabolic protein
LOC112715727	Peanut IAA-amino acid hydrolase ILR1-like protein 5 *Arachis hypogaea* IAA-amino acid hydrolase ILR1-like 5	Uncharacterized Uncharacterized
LOC112695211	Peanut pre-mRNA-splicing factor SLU7 *Arachis hypogaea* pre-mRNA-splicing factor SLU7	Uncharacterized Uncharacterized
LOC112734587	Peanut DNA-directed RNA polymerase II subunit RPB7 *Arachis hypogaea* DNA-directed RNA polymerase II subunit RPB7	Uncharacterized Uncharacterized
LOC112723465	Peanut uncharacterized LOC112723465 *Arachis hypogaea* uncharacterized LOC112723465	Uncharacterized Uncharacterized
LOC112800059	Peanut probable xyloglucan endotrans-glucosylase/hydrolase protein 30 *Arachis hypogaea* probable xyloglucan endotrans-glucosylase/hydrolase protein 30	Cell wall metabolic protein Cell wall metabolic protein
LOC107644115	Peanut glutamine synthetase nodule isozyme *Arachis ipaensis* glutamine synthetase nodule isozyme	Uncharacterized Uncharacterized

Gene ID	Protein name	Functional annotation
LOC112695783	Peanut probable pectinesterase/pectinesterase inhibitor 41 *Arachis hypogaea* probable pectinesterase/pectinesterase inhibitor 41	Cell wall metabolic protein Cell wall metabolic protein
LOC107611807	Peanut uncharacterized LOC107611807 *Arachis ipaensis* uncharacterized LOC107611807	Uncharacterized Uncharacterized
LOC112710223	Peanut nucleoside diphosphate kinase 1 *Arachis hypogaea* nucleoside diphosphate kinase 1	Uncharacterized Uncharacterized
LOC107604913	Peanut acetyl-coenzyme A synthetase, chloroplastic/glyoxysomal *Arachis ipaensis* acetyl-coenzyme A synthetase, chloroplastic/glyoxysomal	Uncharacterized Uncharacterized
LOC112766309	Peanut phosphatidate phosphatase PAH2 *Arachis hypogaea* phosphatidate phosphatase PAH2	Uncharacterized Uncharacterized
LOC112696264	Peanut V-type proton ATPase subunit F *Arachis hypogaea* V-type proton ATPase subunit F	Uncharacterized Uncharacterized
LOC112783659	Peanut chlorophyll a-b binding protein CP29.3, located in the chloroplast	Uncharacterized

Gene ID	Description	Functional annotation
LOC112802922	*Arachis hypogaea* chlorophyll a-b binding protein CP29.3, in chloroplastic	Uncharacterized

Gene ID	Description	Functional annotation
LOC112802922	*Arachis hypogaea* heavy metal-associated isoprenylated plant protein 39	Uncharacterized
LOC107470461	*Arachis duranensis* phosphatidylinositol 4-kinase β 1	Uncharacterized
LOC130935578	*Arachis stenosperma* cysteine proteinase mucunain-like	Uncharacterized
LOC107625448	*Arachis ipaensis* homoserine kinase	Uncharacterized
LOC107622328	*Arachis ipaensis* ubiquitin-60S ribosomal protein L40	Translation ribosomal protein
LOC107631140	*Arachis ipaensis* probable ATP synthase 24 kDa subunit, mitochondrial	Uncharacterized
LOC107619114	*Arachis ipaensis* 60S ribosomal protein L27	Translation ribosomal protein
LOC112696909	*Arachis hypogaea* AP-1 complex subunit σ-1	Uncharacterized
LOC112779704	*Arachis hypogaea* translationally-controlled tumor protein homolog	Uncharacterized
LOC112699067	*Arachis hypogaea* GPI mannosyltransferase 3	Uncharacterized
LOC130981081	*Arachis stenosperma* uncharacterized LOC130981081	Uncharacterized
LOC112720025	*Arachis hypogaea* uncharacterized LOC112720025	Uncharacterized
LOC112775874	*Arachis hypogaea* F-box protein At2g02240	Uncharacterized
LOC112708162	*Arachis hypogaea* calcium-binding protein CML37	Uncharacterized
LOC112754741	*Arachis hypogaea* peroxidase 44	Uncharacterized

Gene ID	Description	Functional annotation
LOC112696997	*Arachis hypogaea* uncharacterized LOC112696997	Uncharacterized
LOC112802575	*Arachis hypogaea* Cullin-1	Uncharacterized
LOC112772549	*Arachis hypogaea* small ribosomal subunit protein eS27y	Translation ribosomal protein
LOC112790884	*Arachis hypogaea* short-chain dehydrogenase TIC32, in chloroplastic	Uncharacterized
LOC112777265	Peanut homolog protein 1 of vesicle protein-sorting-associated protein 24	Uncharacterized

Locus	Protein annotation	Functional category
	Arachis hypogaea vacuolar protein sorting-associated protein 24 homolog protein 1	Uncharacterized
LOC112787409	Peanut UDP-glucuronic acid decarboxylase 6 *Arachis hypogaea* UDP-glucuronic acid decarboxylase 6	Cell wall metabolic protein
LOC112765162	Peanut 26S proteasome non-ATPase regulatory subunit 7 homolog protein A *Arachis hypogaea* 26S proteasome non-ATPase regulatory subunit 7 homolog protein A	Uncharacterized
LOC112797775	Peanut large ribosomal subunit protein eL34 *Arachis hypogaea* large ribosomal subunit protein eL34	Translation ribosomal protein

Locus	Protein annotation	Functional category
LOC112801916	Peanut glucuronokinase 1 *Arachis hypogaea* glucuronokinase 1	Cell wall metabolic protein
LOC112730543	Peanut bifunctional L-3-cyanoalanine synthase/cysteine synthase 1 (mitochondrial origin) *Arachis hypogaea* bifunctional L-3-cyanoalanine synthase/cysteine synthase 1(from mitochondrial)	Uncharacterized
LOC112782646	Peanut GDSL esterase/lipase At1g28590 *Arachis hypogaea* GDSL esterase/lipase At1g28590	Uncharacterized
LOC112789792	An as-yet uncharacterized long non-coding RNA (lncRNA) and non-coding RNA (ncRNA) in peanut *Arachis hypogaea* uncharacterized long non-coding RNA (lncRNA) and non-coding RNA (ncRNA)	Uncharacterized
LOC107635379	Peanut membrane-anchored ubiquitin-fold protein 2 *Arachis ipaensis* membrane-anchored ubiquitin-fold protein 2	Uncharacterized
LOC112777473	Peanut large ribosomal subunit protein eL27 *Arachis hypogaea* large ribosomal subunit protein eL27	Translation ribosomal protein

Locus	Protein annotation	Functional category
LOC112801849	Peanut miracle fruit protein/sweet protein Miraculin *Arachis hypogaea* miracle fruit protein/sweet protein Miraculin	Uncharacterized
LOC112799990	An as-yet uncharacterized long non-coding RNA (lncRNA) in peanut *Arachis hypogaea* uncharacterized long non-coding RNA (lncRNA)	Uncharacterized
LOC112790211	Probable lactoylglutathione lyase in peanut, distributed in chloroplasts *Arachis hypogaea* probable lactoylglutathione lyase, in chloroplastic	Antioxidant and stress-related enzyme
LOC112803087	Peanut glutathione reductase, cytosolic *Arachis hypogaea* glutathione reductase, cytosolic	Antioxidant and stress-related enzyme
LOC112715030	Peanut ATP-dependent (S)-NAD(P)H-hydrate dehydratase *Arachis hypogaea* ATP-dependent (S)-NAD(P)H-hydrate dehydratase	Uncharacterized
LOC112800800	Peanut peptidyl-prolyl cis-trans isomerase Pin1 *Arachis hypogaea* peptidyl-prolyl cis-trans isomerase Pin1	Uncharacterized

Gene ID	Annotation	Classification
LOC112744989	A 14-3-3-like protein A in peanut *Arachis hypogaea* 14-3-3-like protein A	Uncharacterized Uncharacterized
LOC112709742	Peanut metal transporter Nramp1 *Arachis hypogaea* metal transporter Nramp1	Ion transport protein Ion transport protein
LOC112744054	Peanut K(+) efflux antiporter 2, located in chloroplasts *Arachis hypogaea* K(+) efflux antiporter 2, in chloroplastic	Ion transport protein Ion transport protein
LOC107614197	Peanut trafficking protein particle complex subunit 3 *Arachis ipaensis* trafficking protein particle complex subunit 3	Uncharacterized Uncharacterized
LOC112728675	Peptide methionine sulfoxide reductase B5 in peanut *Arachis hypogaea* peptide methionine sulfoxide reductase B5	Antioxidant and stress-related enzyme Antioxidant and stress-related enzyme
LOC112702442	An as-yet uncharacterized protein in peanut *Arachis hypogaea* uncharacterized protein	Uncharacterized Uncharacterized
LOC112801469	Probable aquaporin PIP1-2 in peanut *Arachis hypogaea* probable aquaporin PIP1-2	Ion transport protein Ion transport protein
LOC112775840	Peanut large ribosomal subunit protein eL28y *Rachis hypogaea* large ribosomal subunit protein eL28y	Translational ribosomal protein Antioxidant and stress-related enzyme

Gene ID	Annotation	Classification
LOC112784025	Peanut potassium transporter *Arachis hypogaea* potassium transporter 2	Ion transport protein Ion transport protein
LOC112792498	Probable xyloglucan endotransglucosylase/hydrolase protein 28 in peanut *Arachis hypogaea* probable xyloglucan endotransglucosylase/hydrolase protein 28	Cell-wall metabolic protein Cell wall metabolic protein

Note: The classification information is organized based on gene annotation results.

Further enrichment analysis was performed on the screened candidate interaction groups. As shown in Figure 4, the candidate interaction groups were mainly enriched in the structural composition of ribosomes, cell-wall biogenesis, and xyloglucan metabolism, indicating that these candidate interacting proteins may mainly participate in metabolic pathways such as the ribosomal translation pathway, the cell wall, and xyloglucan.

X-value represents the P-value; Y-value represents the GO term.

Fig. 4 Enrichment analysis of AhASRK1-CD candidate interacting proteins

2.3 Effect of mutations at key phosphorylation sites on the interaction capacity of AhASRK1-CD

Based on the previous experimental results on key autophosphorylation sites of AhASRK1-CD, we performed site-directed mutagenesis of the key phosphorylation sites, T-A and T-D, and constructed the corresponding bait vectors, namely $pGBKT7$-$AhASRK1^{T-A}$-CD and $pGBKT7$-$AhASRK1^{T-D}$-CD. The mutant plasmids were transformed into Y2HGold and subsequently subjected to one-to-one verification with the above six AD-candidate interacting proteins. The results showed that $pGBKT7$-AhASRK1-CD and $pGBKT7$-$AhASRK1^{T-D}$-CD, when co-cultured with the AD-candidate interacting proteins, could grow normally on SD/-Trp-Leu-Ade-His+X-α-gal medium. However, after Ah1 was co-cultured with $pGBKT7$-$AhASRK1^{T-A}$-CD, the mixed culture could grow normally on SD/-Trp-Leu-Ade-His+X-α-gal medium; whereas after the remaining AD-candidate interacting proteins Ah2, Ah3, Ah4, Ah5, and Ah6 were co-cultured with $pGBKT7$-$AhASRK1^{T-A}$-CD, the mixed cultures could not grow normally on SD/-Trp-Leu-Ade-His+X-α-gal medium (Fig. 5). This indicates

that AhASRK1 differentially regulates interactions with downstream proteins through key phosphorylation sites.

A–F. Growth of mutant AhASRK1 co-cultured with six AD-candidate interacting proteins on SD/-Trp-Leu-Ade-His+X-α-gal medium.

A–F. Growth of mutant AhASRK1 co-cultured with six AD-candidate interacting proteins on SD/-Trp-Leu-Ade-His+X-α-gal medium.

Fig. 5 Point-to-point validation of mutant AhASRK1 and its AD-candidate interaction partners

3 Discussion and Conclusion

Our previous studies showed that AhASRK1 mediates aluminum-stress-induced programmed cell death by activating the Ca^{2+}-ROS-MAPK signaling cascade (Fu et al., 2025). Based on prediction from the functional characteristics of receptor-like protein kinases, the aluminum-sensitive function of AhASRK1 depends on its interactions with multiple intracellular target proteins, through which it transmits signals by regulating target-protein phosphorylation levels and altering protein stability, thereby modulating the aluminum-stress response pathway. The screening results for candidate interacting proteins confirmed the above prediction: AhASRK1 is not targeted to a single downstream component, but exerts its regulatory function through interactions with proteins of different functional types (Table 2). This indicates that it may accomplish multipathway signal transduction and downstream pathway branching control with the aid of multiple interacting proteins, a mode of action consistent with reported regulatory features of receptor-like kinases (Ye et al., 2025; Zhang et al., 2025). Further enrichment analysis showed that AhASRK1 may amplify the toxic effect of aluminum ions on peanut roots by regulating the dynamic balance and remodeling processes of the root cell wall, thereby negatively regulating aluminum tolerance in peanut.

As the primary toxic target of aluminum ions, the cell wall may undergo changes in structure and metabolism that constitute a core step in the negative regulatory role of AhASRK1 (Kopittke et al., 2015). The candidate interacting proteins of AhASRK1 were significantly enriched in two major pathways: cell-wall construction and xyloglucan metabolism. Among them, the enrichment of cell-wall metabolic proteins such as pectinesterases and polygalacturonase inhibitors suggests that AhASRK1 regulates the activities of enzymes such as pectin-modifying enzymes through protein interactions, thereby changing the methyl-esterification status of cell-wall pectin. Activation of pectinesterases promotes pectin demethylesterification and greatly increases the adsorption-binding sites for Al^{3+} in the cell wall (Sun et al., 2016); meanwhile, polygalacturonase inhibitors can reduce pectin degradation (Ferrari et al., 2012), maintaining sufficient pectin carriers to immobilize aluminum ions. AhASRK1 may act jointly with both types of proteins so that more Al^{3+} is immobilized in the peanut root-tip cell wall under acidic conditions. After aluminum accumulates in large

amounts at the root tip, it damages cell-wall structure, inhibits root growth, and induces intracellular oxidative injury, ultimately markedly aggravating the toxic effect of aluminum on peanut root tips.

The core members of the xyloglucan metabolic pathway are XTH family proteins, which are responsible for cell-wall loosening and remodeling and directly determine root elongation capacity (Guo et al., 2022). Under aluminum stress, imbalance in xyloglucan metabolism inhibits root growth (Li et al., 2024). The interaction between AhASRK1 and XTH family proteins may regulate the function of XTH-catalyzed xyloglucan remodeling, reducing the plasticity and extensibility of root-tip cell walls. Under Al^{3+} stress, the negative effects caused by blocked cell-wall remodeling are further intensified, significantly exacerbating the inhibition of root growth induced by aluminum stress. Together, the above results suggest that AhASRK1 can simultaneously regulate two key processes —cell-wall pectin modification and xyloglucan remodeling—and, through the coordinated action of multiple cell-wall regulatory pathways, enhance the toxic effect of aluminum stress on peanut roots.

In addition to acting on cell-wall metabolic pathways, AhASRK1 may also target structural components of the ribosome, reducing the root system' s own aluminum-detoxification capacity by disrupting cellular protein translation. Previous studies have demonstrated that aluminum toxicity inhibits normal protein translation in plant roots and reduces the accumulation of functional proteins related to antioxidation and aluminum tolerance (Wang et al., 2013; Rangu et al., 2018). Accordingly, we speculate that AhASRK1 may bind ribosomal proteins and thereby regulate the structural stability of the ribosome, obstructing normal mRNA translation and further inhibiting the synthesis of functional proteins related to antioxidation and aluminum tolerance. Because root cells lack sufficient protective proteins, aluminum stress–induced root-cell damage is aggravated. Therefore, we infer that AhASRK1 mainly targets the cell wall, increasing aluminum immobilization in root tips through pectin-metabolism proteins while also binding XTH proteins to regulate xyloglucan remodeling and inhibit root elongation; in addition, by interacting with ribosomal proteins, it obstructs translation of functional proteins and lowers cellular antioxidant capacity. Through the coordinated action of multiple pathways, it aggravates aluminum-induced injury to the peanut root system.

Phosphorylation modification is a key regulatory mode for activation of the cytoplasmic domains of RLKs. Its key phosphorylation sites not only affect the activity of the kinase itself, but may also, through differential phosphorylation, achieve branching and fine regulation of different downstream signals. In *Arabidopsis thaliana*, the co-receptor receptor-like kinase BAK1 simultaneously participates in the regulation of brassinosteroid signal transduction, innate immunity, and cell death.

(Schwessinger et al., 2011), LR receptor-like protein kinase DPY1 can determine, through the phosphorylation status of different sites in its intracellular domain, whether to activate the growth-regulatory pathway or the stress-response path-

way (Zhao et al., 2023). In this study, after the key phosphorylation site of AhASRK1-CD was simulated as persistently non-phosphorylated by the T-A mutation, its ability to interact with some candidate proteins was lost; whereas interactions under simulated persistent phosphorylation (T-D) were unaffected (Fig. 5). Because phosphorylation modification can reshape the local spatial conformation of proteins by introducing negatively charged phosphate groups, stabilizing the charge and spatial matching of the interaction interface, if a phosphorylation site lies within the binding region between a kinase and a target protein, mutating it to alanine will completely eliminate the phosphorylation modification at that site, disrupt the folding of the interaction interface, reduce intermolecular binding affinity, and ultimately block protein interaction (Yan et al., 2012). Accordingly, we speculate that the key phosphorylation site in this study is located within the interaction region between AhASRK1 and specific target proteins, and that the loss of phosphorylation modification caused by the T-A mutation induces conformational disorder at the interaction interface, which is the core reason for the disappearance of protein interactions.

This differential interaction pattern suggests that AhASRK1 may mediate changes in its own conformation through the status of key phosphorylation sites, differentially regulate the affinity and recruitment efficiency of different interacting proteins, and thereby achieve diversion and fine regulation of downstream signals. We infer that the negative regulation of peanut aluminum tolerance by AhASRK1 is not limited to activation or suppression of a single pathway; rather, with dynamic switching of phosphorylation at key sites as the molecular basis, it distributes signals among multiple aluminum-response pathways, and multiple pathways synergistically amplify aluminum-toxicity effects, weakening aluminum tolerance in peanut.

In summary, this study preliminarily screened 67 proteins that interact with AhASRK1 and clarified that AhASRK1 differentially regulates its interactions with interacting proteins through key phosphorylation sites. This provides important experimental evidence for further exploration of the signaling pathway mediated by AhASRK1 and its molecular mechanism in the peanut aluminum-stress response. Subsequent studies should further functionally validate the candidate interacting proteins and clarify their specific roles in the AhASRK1-mediated aluminum-stress response. In addition, the interacting proteins screened by yeast two-hybrid technology still require further validation through experiments such as in vitro pull-down and in vivo bimolecular fluorescence complementation to ensure the authenticity and reliability of the interactions; this is also one of the key directions for subsequent research.

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