1. Koncepcja

Based on the trROSETTA, DeepFRI and Alphafold *in silico* analysis of AT5G45470 and AT5G45480 (for reference, see the npcRNA document), was performed to predict the protein structure, its localization and possible functions. Additionally, cloning was performed to verify the results.

1. Wyniki

Both protein coding sequences at5g45470 and at5g45480 are a part of locus annotated as lncRNA (at5g45472). This lncRNA showed the highest change in expression in RNA seq library, being the only lncRNA overexpressed in all 3 tested groups (E+, N+, NiE+). The expression levels were tested in E+ for selected lncRNAs and at5g45472 showed 4.8 fold increase 12 days upon inoculation (see npcRNA for reference). Additionally, at5g45472 was tested in the presence of Fe, Fe and endophyte, with 3.2-fold and 2.5-fold increases, respectively (Fig. 1a). Moreover, the comparison of expression levels 24 h and 48 h post inoculation wrt 12 days post inoculation showed almost no differences in expression levels (1.9 and 1.8 fold change), therefore suggesting that the levels of at5g45472 are elevated as soon as 24 h past inoculation (Fig. 1b). The expression of these genes was also tested in *A. arenosa* seedlings grown in a presence of HMs and inoculated with endophyte for 16 days (Fig. 1c), but no significant change in expression was observed.

Sequence analysis and model prediction were performed for full protein sequences, as well as sequences with TM fragments removed. Analysis reveals that with shorter sequence, the score value (with score of 0.2 indicating 50% probability of the annotated function) for predicted localisation and function increases (Figs. 2-3). For both proteins, the proposed nuclear localisation was confirmed by confocal imaging of transiently transfected tobacco leaves with recombinant proteins carrying RFP tag on N-terminus (Fig. 4). Results confirm the localization of N-termini inside the cell nucleus.

1. Metoda

**Plant cultivation:**

*A. thaliana*(Col-0) seeds were grown in pot with soil to be used for WT plant transformation with *A. tumefaciens* carrying pK7WG2D.

A. *thaliana* (Col-0) seeds were surface sterilized with 1.5% NaOCl for 2 min, 96% EtOH for 1 min, and 75% EtOH for 2 min, rinsed with deionized water three times, sown in Petri dishes with ¼MS medium and placed in 4°C under darkness for 48 hr. Subsequently, the seeds were transferred to a growth chamber (Panasonic MLR‐352H‐PE, Korea) with a 16‐hr photoperiod, 100 ± 20 21/17°C day/night temperature and 50% humidity. After 10 days, the seedlings were moved to sterile jars with MSR media with no sugar (6 plants per jar). Additionally, 50 uL of resuspended cultures of *S. ruberrimus* grown on PDA plates were added to one group (named **B**), 10 uM of Ni was added to another group (named **C**) and both Ni and *S. ruberrimus* were added to another group (named **D**), with control group (named **A**) growing solely on MSR.

SALK lines: N816425 (at5g45470 mutant) and SALK\_019821 (homozygous mutant of at5g45480) were ordered. They will be grown and used for plant transformation with *A. tumefaciens* carrying pK7WG2D.

**qPCR**

For qPCR, cDNA was obtained from RNA that was reverse transcribed using iScript cDNA Synthesis Kit (BioRad) and the following protocol:

Component and Volume per Reaction:

5x iScript Reaction Mix - 4 µl

iScript Reverse Transcriptase - 1 µl

Nuclease- free water - Variable

RNA template (1000 ng) - Variable

Total volume - 20 µl

Reaction Protocol:

Priming - 5 min at 25°C

Reverse transcription - 20 min at 46°C

RT inactivation - 1 min at 95°C

Primer efficiency was tested and qPCR was performed using the following protocol (with 3 technical replicates):

iSYBR green (Bio-Rad):

SYBR Green Mastermix - 7.5 µl

qPCR primers (10 µM) - 1.5 µl

cDNA (10 ng) - Variable

Nuclease- free water - Variable

Total volume - 15 µl

**Bioinformatic analysis**

Protein sequences from at5g45470 and at5g45480 were subjected to trROSETTA and DeepFRI pipelines. Model sequences and GO terms were obtained.

**Callus formation**

Six seedlings (7 day-old) were fragmented (roots, shoots and leaves) and tissues were placed onto callus inducing medium (CSH protocols).

**CDS cloning**

For cloning, cDNA was obtained from RNA that was reverse transcribed using iScript cDNA Synthesis Kit (BioRad) and the following protocol:

Component and Volume per Reaction:

5x iScript Reaction Mix - 4 µl

iScript Reverse Transcriptase - 1 µl

Nuclease- free water - Variable

RNA template (1000 ng) - Variable

Total volume - 20 µl

Reaction Protocol:

Priming - 5 min at 25°C

Reverse transcription - 20 min at 46°C

RT inactivation - 1 min at 95°C

Then cDNA was amplified using Kapa HIFI polymerase and attB-containing primers for At5g45470 (Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGTGGAAGTGATACCAAA, Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTACTTGCCTATGATGAGTTTGG) and at5g45480 (Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGTGAACGCAATCCCAAA, Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTACTTGCCTATAATCAGTTTGGC):

**Kapa HiFi HotStart ReadyMix PCR Kit (Roche)**

Component and Volume per Reaction:

2x KAPA HiFi HotStart ReadyMix - 12.5 µl

10 µM Forward Primer - 0.75 µl

10 µM Reverse Primer 0.75 µl

PCR-grade water - Variable

Template DNA - Variable

Total volume - 25 µl

PCR cyclin protocol:

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Temperature | Duration | Cycles |
| Initial denaturation | 95°C | 3 min | 1 |
| Denaturation | 98°C | 20 sec | 35 |
| Annealing | 60 °C | 15 sec |
| Extension | 72°C | 3 min |
| Final extension | 72°C | 3 min | 1 |

For expression with C-terminal reporter fusion primers not containing stop codon were used.

**Gateway cloning**

Purified PCR products (At5g45470 and at5g45480 with and without stop codons) were subjected to the BP/LR Gateway cloning protocol, using pDONR/Zeocin as a donor vector and pP7WGR2D, pP7RWG2D and pK7WGR2 (over expression, no fusion tag). For verification of donors, colony PCRs were performed using gene specific primers. Colonies of *E. coli* DH5 alpha carrying a destination plasmid with an insert were selected and plasmids were purified using Roche kit (Roche).

Next, destination vector plasmids with inserts were inserted in competent *A. tumefaciens* strains and colonies were grown on LB plates with 50 ug/ml spectinomycin. For verification of donors, colony PCRs were performed using gene specific primers and colonies were grown in 50 mL YEB with 50 ug/ml rifampicin and spectinomycin.

**Tobacco transformation**

*A. tumefaciens* colonies were grown in 50 mL YEB with selective antibiotics at 30oC in shaker for 48 h (until OD ~ 1.0). Cultures were centrifuged at 1,000 g for 10 min and resuspended in 50 mL infiltration medium (250 mg/L glucose, 2 mM MES, 2 mM Na3PO4, 100 uM acetosyringone) twice. Then, resuspension was loaded into syringe and bottom side of tobacco leaves was infiltrated. For negative control, blank infiltration medium was used. After transformation, plants were kept in sealed bags to maintain humidity and confocal observations were performed at 20-24 and 42-48 hours past transformation.

1. Figury:

**a)**

**b)**

c)

Fig. 1. Expression levels (tested by qPCR) results of a) Fe and Fe-endophyte treated roots b) 24 h and 48 h past inoculation (wrt 12 days) and c) *A. arenosa* with HMs and E+ (16 days). N=3 biological repeats except for c (n=1).

**a)**



**b)**



**c)**

**![Graphical user interface, application

Description automatically generated with medium confidence]()**

**Figure 2.** DeepFRI analysis (CC database) of a) full protein, b and c) at5g45470 sequences without TM fragments.



**Figure 3.** DeepFRI analysis (MF database) of at5g45470 without TM fragments.

A picture containing graphical user interface

Description automatically generated

**Figure 4.** Confocal images of tobacco leaves transfected with a) no construct, b) RFP-AT5g45470 20 h post transfection, c) RFP-AT5g45470 40 h post transfection, d) RFP-AT5g45480 16 h post transfection, e) RFP-AT5g45480 40 h post transfection.