Journal of Tropical Agriculture 63(4): 180-186, 2025
ISSN 0971-636X (Print), ISSN 0973-5399 (Online)
DOI No.: 10.63599/JTA.2025.1567

The lncRNA13966.1 activates the expression of the WRKY4 transcription factor in black pepper (*Piper nigrum* L.)

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Received on 28 April 2025; received in revised form 20 August 2025, accepted 30 September 2025.

Abstract

Piper nigrum L., commonly known as black pepper, is a commercially important spice crop. The regulatory activities driven by long non-coding RNAs in defense responses remain unexplored in black pepper. Overexpression of lncRNA13966.1 showed upregulation of *WRKY4* transcript, suggesting its potential role in defense. Through bioinformatics analysis, we identified that the intergenic lncRNA13966.1 targets *WRKY4* TF through the *trans*-mode, is localized in the nucleus, and possesses small ORFs.Through RT-qPCR assay, it was shown that *Agrobacterium* mediated transient overexpression of this lncRNA positively regulates *WRKY4* TF.

Keywords: Black pepper, lncRNA, Overexpression,Transient expression, *WRKY*

Introduction

LncRNAs are non-coding RNA transcripts having more than 200 nucleotides, with no protein coding potential.The recent studies have revealed their crucial roles in plants and animals, regulating the gene expression under stress conditions and developmental processes, by exerting their effects at the replication, transcription, translation, and RNA processing (M. Li et al., 2023; Tan et al., 2023; Q. Liu et al., 2024; X. Zhao et al., 2024). Functional roles of lncRNAs are defined according to their mode of action. In *cis*-mode, lncRNAs alter gene expression by interacting with neighbouring genes, while in *trans*-mode, lncRNAs alter the expression of distant target genes (Kung et al., 2013).

WRKY TFs are important regulators in abiotic and biotic stress responses and are identified in multiple plant species. They regulate the expression of target genes through binding to a 'W box' element in promoter regions. They are also reported to perform significant roles in defense responses, either by activating or repressing genes involved in effector-triggered immunity and pattern-triggered immunity pathways (Wani et al., 2021). For example, in response to *Rhizoctonia solani* in rice, *OsWRKY4* acts as a positive regulator in defense response through the jasmonic acid and ethylene signaling pathway. Over expression of *OsWRKY4* resulted in increased expression of jasmonic acid and ethylene responsive pathogenesis-related genes *PR1a*, *PR1b*, *PR5*, and *PR10*/*PBZ1* (Wang et al., 2015).

LncRNAs and *WRKY* are involved in biotic and abiotic stress pathways directly or indirectly in many plants. For example, tomato *WRKY1* transcription factor activates lncRNA33732 and induces respiratory burst oxidase homolog gene expression by increasing ROS production during *Phytophthora infestans* attack (Cui et al., 2019). In a recent study, transcriptome analysis of lncRNA18313 overexpressed wheat plants under cadmium stress showed significant upregulation of seven *WRKY* TFs (Zhao et al., 2025). Cold related grapevine lncRNAs, which are differentially expressed, were predicted to target protein-coding genes involved in stress responses such as *WRKY41* TF, CBF4 TF, and late embryogenesis abundant protein Lea14-A (Wang et al., 2019). Previously, 565 differentially expressed lncRNAs were identified in tobacco during stress response against root-knot nematode (Li et al., 2018).

The *Phytophthora capsici,* causing foot rot, is an oomycete pathogen that causes both aerial and soil infections in black pepper (Lamour et al., 2012). Foot rot disease is one of the major diseasesin this spice crop, causing a significant amount of crop loss worldwide. To date, no cultivar resistant to this disease has been identified, and all parts of black pepper are susceptible to the pathogen (Ravindran, 2000). Even though studies on small RNAs and lncRNAs have been conducted in black pepper (Asha et al., 2016; Asha and Soniya, 2016, 2017; Kumar et al., 2023), the regulatory action of lncRNAs against this pathogen is yet to be revealed.Transcriptome sequencing studies with infected leaf samples, conducted in

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our laboratory, has shown that lncRNA 13966.1 gets differentially expressed under foot rot infection (unpublished).

Transient expression is a fast and efficient mode of gene expression in which the target gene or transcript gets expressed within 2 to 4 days in the non-heritable somatic tissues (Kaur et al., 2021). Although stable gene expression has been previously reported in black pepper, functional studies of genes and non-coding RNAs using transient expression are limited. Thus, the objective of this study was to functionally validate the role of lncRNA13966.1 through *Agrobacterium* mediated transient overexpression and analysis of the expression of the target gene *WRKY4*.

Materials and methods

Plant sample

Clonally propagated seedlings of black pepper cv. Panniyur-1, which is susceptible to *Phytophthora capsici*, were collected from the College of Agriculture, Vellayani, and maintained within the greenhouse at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India,under natural photoperiod, with a temperature range of 27±2°C, and relative humidity of 75-80%. Plants at three to five leaf stages were selected for RNA isolation and transient transformation.

Bioinformatics analysis of lncRNA13966.1

The coding probability of lncRNA13966. 1 was analyzed using CPC2 (https://cpc2.gaolab.org/). The presence of small open reading frames in lncRNA13966. 1 was determined with the online tool ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The subcellular localization was predicted using the LncLocater2.0 (https://www.csbio.sjtu.edu.cn/bioinf/lncLocator2/). The secondary structure was predicted using the RNAfold Web server.

Extraction of total RNA and synthesis of cDNA

Total RNA was extracted from infiltrated regions of the leaves using the RNeasy Plant mini kit (Qiagen, USA). Using a Colibri Microvolume Spectrometer (Berthold, Germany), absorbance at 260 and 280 nm and their ratios were measured to estimate the quality and quantity of RNA. Through formaldehyde gel electrophoresis, the integrity of the RNA samples were determined. The RNA concentration was calculated based on the 260 nm absorbance value. Absorbance ratios of 260/280 nm in the range of 1.8 to 2.0 and 260/230 nm between 2.0 to 2.2 were confirmed for RNA samples with less contamination of protein or polysaccharide. One microgram of total RNA was used for reverse transcription using the High-Capacity cDNA Reverse Transcription kit (ABI). The cDNA synthesized was stored in a -20°C refrigerator for further cloning and quantitative RT-PCR analysis.

Design of primers for cloning lncRNA

Primers to clone lncRNA13966.1 into the binary vector pBI121 for overexpression were manually designed based on the full-length sequence of the lncRNA13966.1. Briefly, for generating the forward primer, nucleotides from the 5'-3' end were selected, and to its left restriction enzyme site and sitting sequence for *Xba*-I were added. For generating the reverse primer, the last 20 nucleotides were selected and the restriction enzyme site and sitting sequence for *Sac*-I were added to the right side of the primer sequence, followed by generating the reverse complement.The random 6 bases added upstream to the restriction site of both primers were meant for efficient cleavage by the restriction enzymes.The primers were Forward 5'-TAAGGCTCTAGAACCCAATAGTATGTTGCC-3', where the underlined nucleotides arethe incorporated site for *Xba*-I restriction digestion, and Reverse 5'-GCCTTAGAGCTCGTTTGATTCACACTCTA-3', where the underlined nucleotidesare an incorporated site for *Sac*-Irestriction digestion.

Plasmid construct

The overexpression vector was createdusing the binary vector pBI121 under the control of CaMV35S promoter. The lncRNA13966.1 was amplified using the primers generated. The PCR reaction was performed in a 50µL volume consisting of 25µL of 2X Phire HS II master mix (Thermo

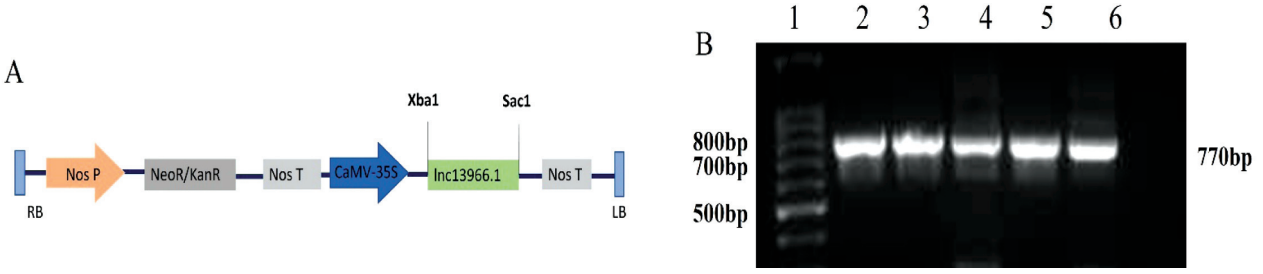

Figure 1. (A) Diagrammatic representation of the expression cassette used for the overexpression of lncRNA13966.1 in black pepper. (B) Gel image showing PCR of the *Agrobacterium* clones showing amplification of lncRNA13966.1. Lane1: 100 bp ladder, lane 2 to 6: PCR amplification of lncRNA13966.1 fragment from the plasmid isolated from the transformed *Agrobacterium* cells.

Scientific), 10µM each of the primers, and 1.5 µL DMSO. The amplification was carried out with an initial denaturation set at 98°C for 35seconds, 30 cycles of 98°C for 5 seconds, 50°C for 5 seconds, and 72°C for 15 seconds, and final extension of 72°C for 1 minute. The obtained PCR amplicon was gel purified using Nucleo Spin Gel and PCR Clean-up kit (Macherey-Nagel), digested with *Xba*-I and *Sac*-I restriction enzymes (NEB), and cloned into the pBI121 vector with CaMV 35S promoter, after the restriction digestion with the same enzymes, replacing the GUS gene. (Figure 1A).

The over expression vector pBI121-13966. 1 and the empty vector pBI121 were introduced into the competent cell (DH5αstrain) of *E. coli* using the heat shock method. Briefly, 500 ng each of pBI121-13966.1 and empty vector were added to 100 ìL of competent cells and kept on ice for 30 minutes. The mixture was placed at 42°C for 90 seconds and transferred to ice for 5 minutes. One microlitre of LB broth was added to the mixture and incubated at 37°C for 1 hour at 180 rpm, and were platedon LB agar medium with 50mg/L kanamycin. After 1 day, colonies were picked and inoculated into 10 mL of LB medium with 50 mg/L kanamycin and grown overnight with a speed of 250 rpm at 37°C. Extraction of the plasmid DNA was performed using the QIAprep Spin Miniprep kit using the manufacturer's instructions. The colonies were analyzed by PCR using the full-length primers for lncRNA13966.1. Forward – 5'ACCCAATAGTATGTTGCC3' and Reverse 5' GTTTGATTCACACTCTA 3' (Figure 1B).

Agrobacterium mediated transienttrans formation

Both the purified vectors pBI121-13966.1 and the empty vector were transferred into *Agrobacterium tumefaciens* (strain GV3103) through the freeze-thaw method. *Agrobacterium* harbouring plasmids were selected on solid LB medium incorporated with rifampicin (25 mg/L) and kanamycin (50mg/L) and were incubated in the dark at 28°C for 2 days at 200 rpm. Later, the initial culture (around 200 µL)was added to 100 mL of LB broth with appropriate antibiotics and incubated until OD_{600} reached 0.8. The cells were later centrifuged and added to *Agrobacterium* infiltration buffer consisting of 15 µM acetosyringone, 10 mM MES (pH 5.6), and 10 mM $MgCl_2$.The resuspended bacteria were grown for 2 hrs. before infiltration. Second leaves from the shoot apex of black pepper plants were used for infiltration. The transient expression was performed through the syringe infiltration method. The abaxial side of the leaves was lightly scraped to enhance infiltration of the inoculum. The infiltration was done using a needleless syringe, and the infiltrated region was labelled. Later, the infiltrated plants were grown under normal conditions.The transient infiltration was conducted in triplicate, from the second leaves of three black pepper vines, and harvested at 48 hpi.

Designing of qRT-PCR primers

Primers for expression analysis of lncRNA and *WRKY4* gene were designed using Integrated DNA Technologies (www.idtdna.com) using the sequences obtained from the RNA-seq results. The quantitative RT-PCR primers used in the present study are provided in Table 1.

Table 1.The qRT-PCR primer sequences used in the study

Primers for qRT-PCR	Sequences (5'-3')
lncRNA13966.1 FP	CCAGCAACAAGAAGAAGGA
lncRNA13966.1 RP	TGAGTCACCCGCTCTATTA
WRKY4 FP	TCCTGCTGTTGATAAACCTGTTA
WRKY4 RP	TGGATGGGTACATTTGTAGTAGC
Pn18S rRNA FP	AGACGAACAACTGCCAAAGC
Pn18S rRNA RP	GCGGAGTCCTAAAAGCAACA

Analysis of lncRNA expression by qRT-PCR

Total RNA was extracted from the transformed and control plants using the Qiagen RN easy Plant Mini kit, and the cDNA was generated with the High-Capacity cDNA Reverse Transcription kit. The qRT-PCR reaction consisted of a 10 µL reaction that contained 5 µL of Power SYBR™ Green PCR Master Mix (ABI, Life Technologies, USA), 1 µL of cDNA template (20ng/µL), and forward and reverse primers (5 pMol each). Quantitative PCR was performed on the Applied Biosystems QuantStudio 5 RealTime PCR system under the conditions: an initial melting for 15 seconds at 95°C (40 cycles), annealing for 15 seconds at 60°C, followed by elongation at 72°C for 30 seconds. *Piper nigrum* 18s rRNA was utilized as the endogenous control. The $2^{-\ddot{A}\ddot{A}Ct}$ analysis was performedto calculatethe comparative gene expression (Livak and Schmittgen, 2001). For expression analysis, three technical replicates of three biological samples each of control and overexpression conditions were assessed. Statistical significance of the relative expression level was calculated by unpaired t-test using GraphPad Prism 6.

Results and discussion

Characteristics of lncRNA13966.1 in black pepper

In our transcriptome sequencing study to assess the differential expression of genes during foot rot infection in black pepper, we had identified lncRNA13966.1, a 770 nucleotide long intergenic lncRNA, which was predicted to act in a *trans*-manner with the target *WRKY4*, based on the Pearson correlation coefficient value (unpublished). We predicted the subcellular localization of the lncRNA13966.1 using Lnclocater, since it is an important criterion determining the function of alncRNA (Cao et al., 2018; Chen, 2016). The prediction results revealed that the lncRNA13966.1 transcripts are primarily localized in the

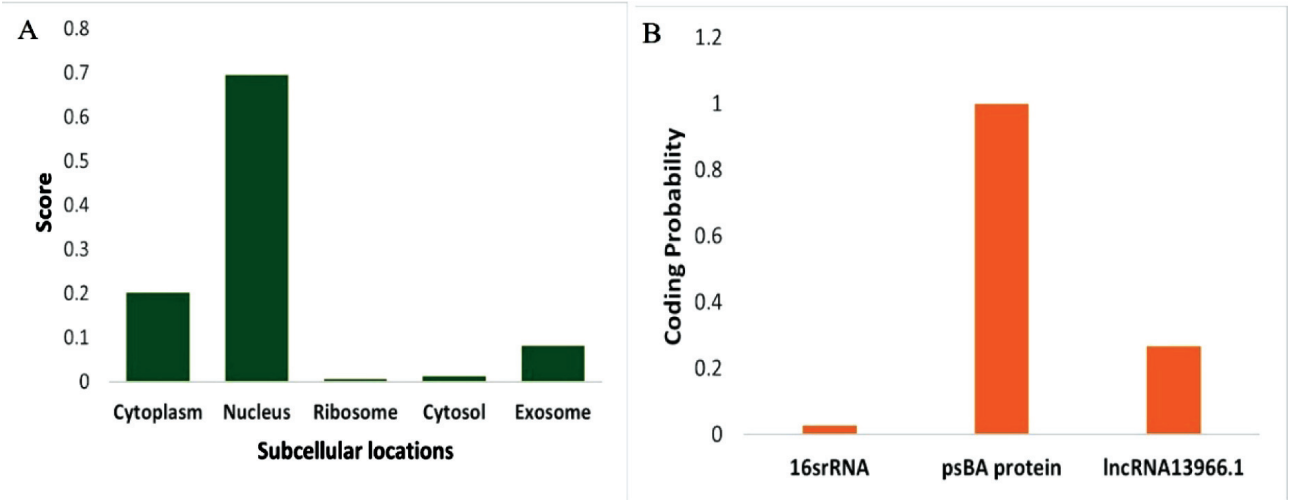

Figure 2. Subcellular localization and coding potential of lncRNA13966.1. (A) Prediction of subcellular localization of lncRNA13966.1 using lncLocator. (B) Coding potential of lncRNA13966.1 determined using the CPC2 program. The psBA protein-coding gene is depicted as an example of a coding RNA, and 16S rRNA is an example of non-coding RNA.

nucleus (Figure 2A). Generally, lncRNAs are considered non-protein-coding, even though new studies have identified the potential of lncRNAs to code for small peptides through small ORFs present in them (Sruthi et al., 2022). Using CPC2, the coding probability of lncRNA 13966.1 was investigated. The coding probability obtained was 0.26, which suggested little or no coding capacity for this lncRNA compared to the gene sequences (Fig. 2B). Further, using the ORF finder tool, we predicted four ORFs with the potential to generate small peptides with start codon ATG, ranging in length from 31 to 90 amino acids (Table 2). These prediction results have supported the non-coding property of the lncRNA13966.1.

Table 2. Prediction of four ORFs in lncRNA 13966.1 from reading frames 2 and 3 using ORFfinder online tool. The longest ORF was identified to code for a 90 amino acid long polypeptide.

ORF Number	Reading frame	Strand	Start	End	Length of amino acid
1	2	Direct	11	283	90
2	2	Direct	356	451	31
3	2	Direct	497	661	54
1	3	Direct	600	770	57

The secondary structures of lncRNAs are known to affect their downstream functions (Qin et al., 2020). Further, we used the online RNA fold web server to predict the secondary structure of lncRNA13966.1. The secondary structure consisted of several stem loop structures (Fig.3). The MFE (Minimum Free Energy) value predicted for lncRNA13966.1 was -187.60 kcal/mol. A lower MFE value obtained suggested that the predicted structure is stable.

Over expression of lncRNA13966.1

WRKY TFs are important players in plant defense responses. Previously, it was reported that in *Arabidopsis, WRKY3* and *WRKY4* are key regulators of defense responses. These TFs positively regulate plant resistance against the necrotrophic fungal pathogen *Botrytis cinerea*, while *WRKY4* negatively influences plant resistance in response to the biotrophic pathogen *Pseudomonas syringae* (Lai et al., 2008). In our transcriptome study we found that the differentially expressed lncRNA13966.1 targeted the *WRKY4* gene in a *trans*-manner. To functionally verify the lncRNA13966.1, transient overexpression was conducted using syringe infiltration in black pepper leaves at 48 hpi. The introduction of *Agrobacterium* harbouring pBI121-13966.1 plasmid into black pepper leaves resulted in significant upregulation of

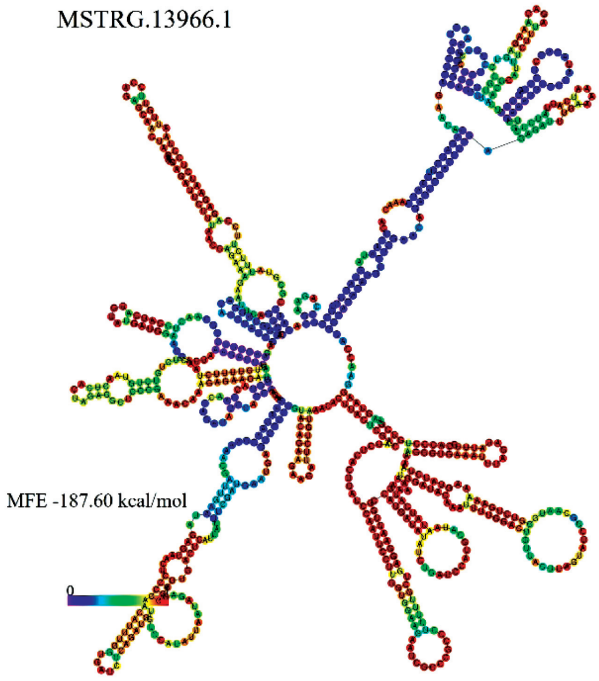

Figure 3. Secondary structure of lncRNA13966.1 predicted using RNAFold

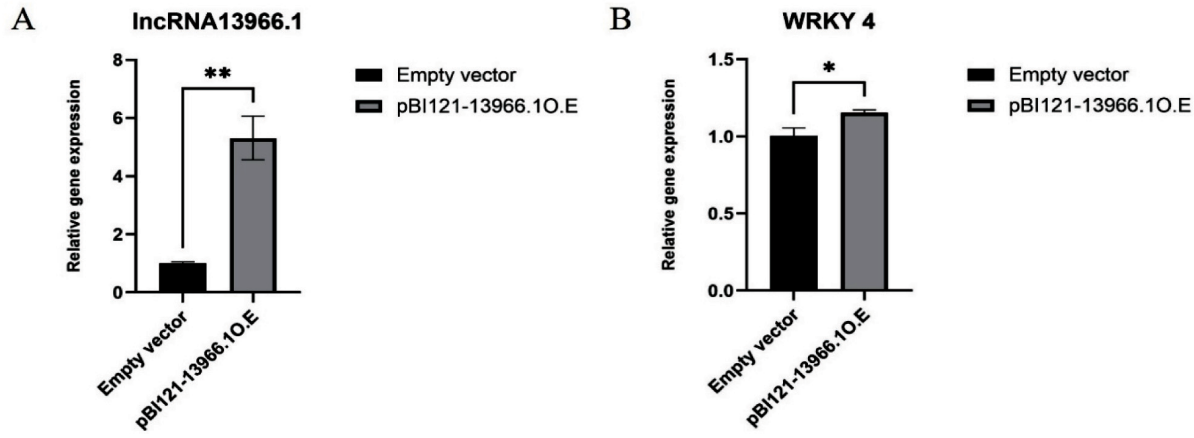

Figure 4. Quantitative real-time PCR expression data of lncRNA13966.1 and *WRKY4* gene in the empty vector pBI121 and overexpression vector pBI121-13966.1 transformed black pepper leaves.

lncRNA13966.1. The expression of lncRNA13966.1 in transiently infiltrated leaves was significantly upregulated compared to the control samples infiltrated with the empty vector, having a log2 fold change ratio of 5.31 and p value less than 0.05, as indicated by the RT-qPCR data (Fig. 4A). Further, the expression of the target gene *WRKY4* was also examined using RT-qPCR (Fig. 4B).The results indicated significant upregulation of *WRKY4*in lncRNA13966.1 overexpressed samples compared to the control, having a log2 fold change ratio of 1.15 and p value less than 0.05. Although the upregulation of the *WRKY4* gene showed only a slight upregulation in lncRNA13966.1 overexpressing samples, the significance level ($p<0.05$) indicates that the lncRNA causes a potential regulatory effect.

In the present study, we employed *Agrobacterium* mediated syringe infiltration method to understand the function of lncRNA. Earlier research in different model and non-model plants has used this technique for studying the regulatory role of lncRNAs in stress responses caused by pathogens (Jiang et al., 2019; Iwakawa et al., 2021; Tan et al., 2023).

Transient expression is cost-effective and allows for rapid gene expression compared to stable gene expression methods, and has emerged as an alternative to stable transformation (Liu et al., 2020; Tyurin et al., 2020). Recent investigations have highlighted the significant roles of lncRNAs in regulating gene expression through different mechanisms, such as regulationat transcriptional and post-transcriptional levels and chromatin structure alterationsduring plant growth and development, environmental and pathogen induced stress responses (Csorba et al., 2014; Zhao et al., 2018; Duan, 2020; Meng et al., 2021; Wang et al., 2021). Consistent with these findings, the current study has validated that the expression of lncRNA13966.1 caused a significant effect on the target gene *WRKY4* expression. However, further studies are required to understand the exact molecular mechanism through which lncRNA13966.1 regulates the *WRKY4* expression and its effect on black pepper resistance against foot rot disease. Previously, several *WRKY* TFs have been identified as important components of plant immunity inmultiple plants (Yu et al., 2001; Lai et al., 2008; Wang et al., 2015; Aamir et al., 2017; Hussain et al., 2018). Understanding the influence of lncRNAs on the alteration of *WRKY*TF expression can help identify other important regulators acting in the plant defense pathway, and such insight can be useful in regulating defense response against pathogens.

Conclusion

In the present study, we successfully overexpressed the lncRNA13966.1 in black pepper leaves via *Agrobacterium* mediated transient overexpression. The real time expression analysis revealed higher expression of *WRKY4* in lncRNA13966.1-pBI121 infiltrated samples compared to the control samples, which suggests a potential regulatory role for the lncRNA13966.1 through *WRKY4* TF. Moreover, the subcellular localization, secondary structure, and coding potential of the lncRNA13966.1 were determined using bioinformatics tools. These results provide a preliminary understanding of the function of lncRNA13966.1 in gene regulation during biotic stress in black pepper. Further studies involving molecular interaction between lncRNA13966.1, *WRKY4* TF, and the downstream targets are essential to substantiate the preliminary results obtained in this study.

Acknowledgements

We acknowledge Dr. T. Makesh Kumar, Principal Scientist, ICAR- Central Tuber Crops Research Institute, Thiruvananthapuram, for kindly providing the pBI121 binary vector used in this study.

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