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Designing of a LAMP-based sensor in detecting nitrogen-starvation in *Oryza sativa* L. spp. indica

Yokimiko C. David-Torrejos[1] and Mark Christian Felipe R. Redillas[1,*]
[1]*Department of Biology, De La Salle University, 2401 Taft Ave, Malate, Manila, 1004*
**Corresponding Author: mark.christian.redillas@dlsu.edu.ph*

Abstract: Effective nitrogen fertilizer management is critical in agriculture, particularly for optimizing nutrient use efficiency in rice cultivation. This study presents the development of a gene expression-based monitoring system utilizing isothermal amplification to detect endogenous nitrogen levels in *Oryza sativa* L. spp. indica (NSIC Rc 222). The expression profiles of two nitrogen-responsive genes, *OsALN* and *OsUPS1*, were first validated in this local rice variety using real-time RT-PCR. Capitalizing on the genes' antagonistic expression patterns under varying nitrogen conditions, loop-mediated isothermal amplification (LAMP)-based sensors were designed to enable rapid gene expression detection. Using the designed sensor, successful amplification of both genes in response to the presence and absence of nitrogen was achieved, demonstrating its potential for monitoring nitrogen-related gene expression. This study lays the groundwork for a field-deployable molecular tool to support nitrogen monitoring and precision nutrient management in rice cultivation. Further optimization will improve the sensitivity and visualization of amplified products, facilitating more accurate detection and enhanced clarity in result interpretation.

Key Words: ***Oryza sativa*; nitrogen; rice; LAMP; gene expression; molecular sensor**

1. INTRODUCTION

Managing nitrogen fertilizer use is a critical aspect of agricultural farming, where proper nutrient application is essential to maximize its effectiveness in rice cultivation. Various methods have been developed to monitor nitrogen levels in plants, ranging from leaf color charts to handheld devices like the SPAD meter. These methods primarily measure chlorophyll content and correlate it with nitrogen levels in plants. However, a significant drawback of these techniques is their dependence on the plant's delayed response to nitrogen stress (Muñoz-Huerta et al., 2013; Lee et al., 2018; Redillas et al., 2019).

The complex network of nitrogen and carbon metabolism in rice makes it hard to generate a real-time molecular-based monitoring system to track the nitrogen status in rice plants. In a molecular level, various genes and regulatory systems were already reported to have strong correlation with nitrogen stress. These studies include gene expression analysis and transcriptomic studies that reported genes that are highly responsive to the availability of exogenous and endogenous nitrogen supply, generating a pool of candidate genes that can be targets of a molecular-based real-time monitoring sensor (Shin et al., 2018; Yang et al., 2015a; Yang et al., 2015b; Lee et al., 2018). The first molecular sensing system reported to track nitrogen status in rice was established by targeting the expression of two nitrogen sensitive genes that showed an opposite expression when exposed to nitrogen-limited condition (Lee et al., 2018). However, such a strategy can only be done by generating new breeds of transgenic rice that harbor the sensor,

limiting its use to the existing rice varieties.

This study aims to develop a molecular-based diagnostic tool to monitor nitrogen stress in *Oryza sativa*. Specifically, this study aims to observe the expression of reported nitrogen-sensitive genes, *OsALN* and *OsUPS1* in *Oryza sativa* L. NSIC RSC 222 under nitrogen starvation. The diagnostic tool was designed by utilizing the Reverse Transcription Loop-mediated Isothermal Amplification that will detect the expression of both in response to nitrogen starvation. Here, the expression of *OsALN* and *OsUPS1* in a rice variety were evaluated to confirm the expression profile of the two genes, then sets of primers for both genes were designed for isothermal amplification.

2. METHODOLOGY

Plant Material

The *Oryza sativa* L. spp. indica NSIC RSC 222 seeds were obtained from the Philippine Rice Research Institute, Department of Agriculture. The seeds were activated at 50°C for 5 days. Seed germination was performed in a damp tissue paper at 30°C placed in dark area until the radicle or plumule emerges. Then, the seedlings were transferred and grown in a hydroponic solution at 28°C with 16h/8h day and night cycle.

Nitrogen Starvation Treatment

After seed germination, the rice seedlings were allowed to grow further in a plastic container containing a SNAP Hydroponic Solution until the 2nd and 3rd leaf stage prior to nitrogen starvation. The day and night cycle were set to 16h/8h at 6000 µmol m-2 s-1. After 14 days after transplantation in hydroponic set-up, the seedlings were washed and transferred to a nitrogen-free MS media to induce nitrogen starvation. Three biological replicates were collected after 3 days and stored accordingly until further use.

Total RNA Extraction

Leaf samples were collected from plant samples supplemented with and without nitrogen. The leaves were submerged in RNA later for 24 hours at 4°C, then transferred to -20°C until further use. The total RNA was extracted using the GF-1 Total RNA Extraction Kit (Vivantis Technologies, Malaysia). Initially, leaf tissues were mechanically crushed using a micropestle in a lysis buffer containing ß-mercaptoethanol. Then, the extraction of RNA was performed following the manufacturer's protocol.

Real-time RT-PCR

Extracted RNA samples were quantified using the FLUOstar Omega Microplate reader, wherein the absorbance was measured at 260 and 280 nm to determine the concentration and purity. The qRT-PCR was performed using KAPA SYBR FAST One-Step qRT-PCR Master Mix (2X) Kit and Rotor-Gene Q Real-Time PCR Cycler (Qiagen, Germany). Each PCR reaction contains qPCR Master Mix, 200nM forward and reverse primer, 1X KAPA RT Mix 2.5 ug total RNA. Gene specific primers were listed in Table 1. To normalize the expression, the *OsUBI* gene was used as an internal control. For each technical replicate, three biological samples were pooled and analyzed for quantitative gene expression. Livak method ($2^{-\Delta\Delta Ct}$) was used to analyze the fold change in gene expression analysis.

Table 1. Primer list for the gene expression analysis of the selected genes (Lee et al., 2018)

Primer Name	Sequence (5'-3')
OsALN-F	GGACTACGGTGACGCGGTTA
OsALN-R	CAGCTGCTGCTCTTGTACCA
OsUPS1-F	GCCTGCCTTGGATCTCTTGT
OsUPS1-R	GGAGGTGCTTGGTGAGTTCT
OsUbi1-F	ATGGAGCTGCTGCTGTTCTA
OsUbi1-R	TTCTTCCATGCTGCTCTACC

Loop-mediated Isothermal amplification

The primers were designed using the PrimerExplorer V5, an online web interface available at https://primerexplorer.jp/e/. For each target sequence, a set of six primers will be designed, namely, Forward inner primer, Backward inner primer, Forward primer, Backward primer, Loop forward primer, and Loop backward primer. The RT-LAMP Assay was performed using the WarmStart LAMP Kit (DNA and RNA) by New England BioLabs, Inc. Briefly, a 25µL RT-LAMP Assay was prepared in a 0.2 mL microcentrifuge tubes by mixing 12.5µL WarmStart LAMP 2x Master Mix, 0.5µL Fluorescent dye (50x), 2.5µL LAMP Primer mix (10x), 1µL total RNA sample, and 8.5µL deionized water. The 10X primer mix contains 16µM Forward inner primer, 16µM Backward inner primer, 2µM Forward primer, and 2µM Backward primer. The RT-LAMP assay reaction mixtures were incubated at 65^{0}C for 30 mins. Positive amplification was verified by mixing the amplicon with an intercalating dye (GelRed®) and visualizing under a UV light.

3. RESULTS AND DISCUSSION

A previous study employed a molecular-based approach to develop a sensor capable of monitoring gene expression in response to nitrogen stress. This system targets nitrogen-responsive genes, *OsALN* and *OsUPS1*, to detect dynamic changes on gene expression during nitrogen starvation. The sensor operates through the generation of transgenic lines that express the reporter system, which limits its application to newly engineered plants rather than existing cultivars (Lee et al., 2018). In contrast, the current study aims to develop a molecular sensor leveraging both genes that can be directly applied in local farming systems, including existing crop varieties. Initially, we first evaluated their expression in *Oryza sativa* L. ssp indica NSIC RSC 222, a variety bred by the International Rice Research Institute, to determine if these can be used as targets for a molecular sensor. In a previous report, gene expression analysis showed the antagonistic responses of these genes in *Oryza sativa* L. ssp japonica (Dongjin rice cultivar) when subjected to varying nitrogen conditions (Lee et al., 2018). Figure 1B shows a similar trend of gene expression profile of *OsALN* and *OsUPS1* at the 3rd day from the removal of nitrogen from the hydroponic solution, suggesting the potential of these genes for the sensor development as both genes are involved in nitrogen metabolism in rice (Lee et al., 2018). The *OsALN* gene (Os04g0680400), encoding the enzyme allantoinase, catalyzes the initial step in the catabolism of allantoin, a principal ureide and nitrogen storage compound in rice. Allantoin is primarily synthesized in the roots and subsequently transported to various aerial tissues, where it serves as a mobile reservoir of nitrogen to support growth and development. During high nitrogen demand or nitrogen remobilization, it is broken down into glyoxylate, where ammonium ions are released as by-products (Redillas et al., 2019).

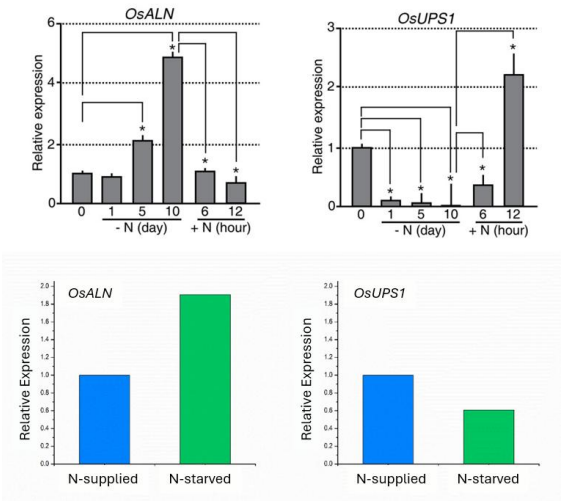

Figure 1. Expression of *OsALN* and *OsUPS1* in the presence and absence of nitrogen. Data obtained from A) Lee et al., 2018 and B) from this study.

In a previous report, the expression of *OsALN* showed a slight decrease in expression after 24 hours, then gradually increased up to 10 days of nitrogen starvation (Figure 1A, Lee et al., 2018). In contrast, the expression of *OsUPS1* was down-regulated when nitrogen becomes limited. This ureide permease 1, encoded by *OsUPS1* (Os12g0503000) gene, is a transporter that is highly expressed during the seedling stage and mature roots, whereas the expression decreases in the leaves as the rice plant matures (Redillas et al., 2019). Previous studies have shown the high response of *OsUPS1* from Japonica rice to the presence or absence of exogenous nitrogen. In these reported studies, the absence of nitrogen significantly downregulated the expression of the *OsUPS1* after 24h of nitrogen starvation (Lee et al., 2018; Redillas et al., 2019).

The Reverse transcription-Loop mediated isothermal amplification has been used as a diagnostic tool for the detection viruses in rice plant (Le et al., 2010; Lai et al., 2018). Hence, this study aimed to extend its application in the development of a molecular-based nitrogen sensor using RT-LAMP as a diagnostic tool. To examine whether the RT-LAMP can monitor the nitrogen status of rice plants, the expression profiles of the *OsALN* and *OsUPS1* were initially confirmed using gene expression analysis. After establishing the expression of both genes, we then designed a LAMP-based molecular sensor that can monitor the endogenous nitrogen in rice seedlings. The sensor was designed using an isothermal amplification method that uses one incubation temperature, which will be later helpful for an on-site field assessment.

Table 2. Designed LAMP primers for *OsALN* and *OsUPS1*

Primer name	Sequence (5'-3')
OsALN_2-6_F3	GCTCTGCTTGATGGACACAT
OsALN_2-3_B3	CATAGCAGGCCTTTCGCT
OsALN_2_4_6_FIP	GCCTCCCCATGCCCTTAAGAAGTTGAGCTCAGACCATTCACC
OsALN_2_3_BIP	CCTGTAACATGGTCGCATGGGACCACCATGATGCCAGTTGAT
OsALN_3_5_FIP	GCCTCCCCATGCCCTTAAGAAGCAGACCATTCACCATCAGCT
OsALN_4_5_B3	AGCATAGCAGGCCTTTCG
OsALN_4_5_BIP	CCTGTAACATGGTCGCATGGGACTCCACCATGATGCCAGTT
OsALN_6_B3	TGCAAGCATAGCAGGCC
OsALN_6_BIP	CCTGTAACATGGTCGCATGGGAGCTCCACCATGATGCCAG
OsUPS1_1_F3	CGGAGACGCCTAATTTCCTC
OsUPS1_1_B3	TGATCCGGCCATCGAGAA
OsUPS1_1_FIP	AAGATGCCACCCGACATCGCGCTCACCCAGATGCAGGAC
OsUPS1_1_BIP	CGCAGTACGGGTGGGCGTTCCCTATGACAACCTTCAGGC
OsUPS1_2_F3	CGGCCTACCTGAAGGATTG
OsUPS1_2_B3	AGGAGAGTGTAAGTCCGCC
OsUPS1_2_FIP	TGGAACGCATTGCCAAGGCCGAAGGGGAGGGATCTTGCTC
OsUPS1_2_BIP	TATGCTGCTGCTGACGCTGTCCATCTCCGGTACTCGCCGAA
OsUPS1_3_F3	CGGCCTACCTGAAGGATTG
OsUPS1_3_B3	CCCCAGGAGAGTGTAAGTCC
OsUPS1_3_FIP	CTGGAACGCATTGCCAAGGCGAAGGGGAGGGATCTTGCT
OsUPS1_3_BIP	CTGACGCTGTCCAGGCGTTGCGACGATCTCCGGTACTCG
OsUPS1_4_F3	TGGTGGTGTACACGGCAT
OsUPS1_4_B3	GCCATGAACTGGAACGCAT
OsUPS1_4_FIP	TGGGGTGGTAGAGGAACCAGATCTTCTACTTCTCCCTCGCCT
OsUPS1_4_BIP	GTCGCTCACGGCCTACCTGACAAGGCCACACACCATGC

In this method, the sensor aims to target the expression of both genes in response to the availability of nitrogen. The observed antagonistic response from both genes will serve as a cross-reference to check the expression of both genes when nitrogen is insufficient from the growth medium. Briefly, LAMP primers were designed targeting a 200-250 bp regions in both genes (Figure 2). Since the target of this sensor are the expression profiles of the two genes, the sequences were used as a template for the design of the LAMP primers. Table 2 lists all the LAMP primers generated from PrimerExplorer V5. Each primer set was tested for the amplification of the two genes using the extracted total mRNA as template from the nitrogen-supplied and nitrogen-starved rice seedlings. These samples were collected from the third day after removing nitrogen from the growth medium. LAMP assays using the designed primer sets were performed and the results were shown in figure 2.

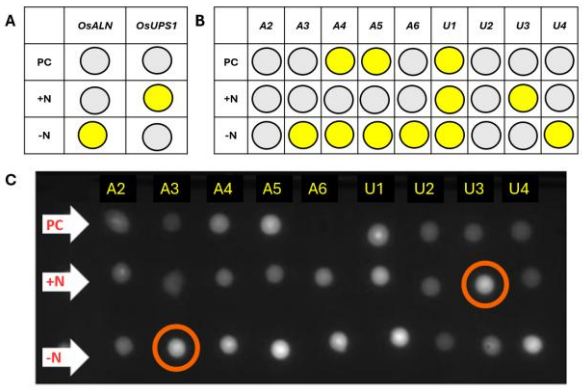

Figure 2. Visualization of the LAMP products with GelRed® under a UV light. (PC)-positive control at 0H, (+N)-with nitrogen at 3D, (-N)-without nitrogen conditions at 3D. A) Expected Result B) Diagram of the obtained result C) Actual fluorescence of samples under UV light

Using the gene expression profile in figure 1 as reference, it is expected to have amplification of the *OsALN* only on nitrogen-starved rice seedlings, whereas no expression should be observed from the nitrogen-supplied rice seedlings. This can be observed using primer sets OsALN_3, OsALN_4, OsALN_5, and OsALN_6. In contrast, there should be no amplification product for *OsUPS1* from the nitrogen-starved rice seedling and positive amplification for this gene should be observed on the nitrogen-supplied rice seedling, which can only be observed using primer UPS1_3. To detect the presence of the amplified products without the need to analyze in an agarose gel electrophoresis, samples were mixed with an intercalating dye to make them fluoresce when viewed under a UV light.

4. CONCLUSIONS

Development of a molecular-based sensor by targeting the expression of nitrogen-sensitive genes was demonstrated in this study as a proof of concept, where the expressions of the *OsALN* and *OsUPS1* were targeted using total RNA as templates of the isothermal gene amplification. The expression was initially monitored using qRT-PCR to test the responses of the genes in the presence and absence of nitrogen. Upon confirmation of their antagonistic responses, the molecular sensor was designed based on the principle of LAMP. The designed system in this study have shown the possibility of using gene expression as targets of the sensors. Subsequent efforts should focus on enhancing its sensitivity and improving the visualization of amplicons to advance its analytical performance

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