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EXPRESSION OF A DROUGHT-RESPONSIVE HELIX-LOOP-HELIX TRANSCRIPTION FACTOR *OsbHLH061* IN RICE (*Oryza sativa* ssp. *japonica*)

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ABSTRACT

Water scarcity poses a significant global challenge for agriculture, growing increasingly severe and widespread. Urgent measures are needed to develop crops that can withstand drought conditions. Recently, the method of transferring genes, particularly transcription factors, has emerged as a promising approach to enhance plant tolerance to stress. Genes are selected based on their differential expression patterns during stress, which can be identified through transcriptome analysis. In this research, the helix-loop-helix transcription factor *OsbHLH061* was identified among up-regulated genes in microarray data derived from mRNA extracted from rice plants exposed to various abiotic stresses. The increased expression of *OsbHLH061* under drought conditions was confirmed by quantitative real-time PCR (qRT-PCR). Its promoter analysis using the GUS reporter gene revealed spatial regulation of *OsbHLH061* in germinating seeds, leaves, and root tips of young seedlings. Overexpression of *OsbHLH061* was achieved by introducing the gene into rice plants under the control of the promoter of *Rab21* in response to abscisic acid 21. However, transgenic plants did not exhibit any significant advantages in drought resistance compared to wild-type plants. These findings suggested the need for a different expression system. Moreover, the lack of significant changes in phenotype despite altering this transcription factor indicated a multi-locus nature of drought resistance associated with this gene.

Keyword: Drought, helix-loop-helix, multi-locus trait, *OsbHLH061*, transcription factor.

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INTRODUCTION

Rice is one of the most important staple foods in the world. However, it consumes a substantial portion, ranging from 34% to 43%, of the world's irrigation water and accounts for withdrawing 24% to 30% of the planet's total freshwater (Surendran et al., 2021). In the climate change conditions, water shortage is very near threatening. Given the rapid expansion of water-deficient agricultural lands, developing drought-tolerant plants becomes increasingly critical. Traditional breeding methods have fallen short in yielding drought-resistant varieties, prompting recent endeavors to turn to genetic engineering. This approach involves the introduction of a select few genes aimed at enhancing the plant's ability to withstand stress (Agarwal et al., 2006; Cushman & Bohnert, 2000). Overexpression of stress-inducible genes in transgenic plants has yielded some promising stress-tolerant phenotypes, underscoring the beneficial roles of these gene products in stress resilience (Cushman & Bohnert, 2000; Bhatnagar-Mathur et al., 2008; Hu et al., 2013; Liu et al., 2013; Yu et al., 2013). Transcription factors (TFs) stand out as especially promising candidates among potential target genes due to their ability to regulate numerous other genes. This suggests that modifying TFs could lead to widespread changes across various genetic pathways (Hussain et al., 2011).

The basic helix-loop-helix (bHLH) transcription factors and their homologs constitute a substantial family within both plant and animal genomes. In the rice genome alone, 167 bHLH genes have been identified, with phylogenetic analysis revealing their organization into distinct, well-supported clades, referred to as subfamilies (Li et al., 2006). While researchers have extensively characterized the functions of bHLH transcription factors in the model plant *Arabidopsis thaliana*, exploring areas such as iron homeostasis and responses to abiotic stresses, similar comprehensive summaries for economically significant crops like rice were lacking until 2023. bHLH family in rice is divided into four categories: plant growth and development; metabolism synthesis; plant signalling, and abiotic stress responses (Zou et al., 2023). Among the well-known members of the bHLH family in rice, six genes have been reported to function in abiotic stress including *OsICE1*, *OsICE2*, *OsbHLH024*, *OsbHLH057*, *OsbHLH130*, and *OsbHLH148* (Zou et al., 2023).

OsbHLH061 (Os11g0601700) shares the closest phylogenetic relationship with OsbHLH062 and OsbHLH063/IRO3 (Li et al., 2006), both of which are transcription factors involved in iron homeostasis (Zheng et al., 2010). Not long after the study of those 2 TFs, OsbHLH061 is also reported to negatively control the long-distance transport of Fe in plants for adaptation to changing Fe environments and maintain Fe homeostasis in rice (Wang et al., 2022). In terms of how it works, *OsbHLH061* expression increases when there is a high iron level. It also directly interacts with OsPRI1. Together, they form a complex that brings in co-repressors called TOPLESS/TOPLESS-RELATED (OsTPL/TPR). This complex then suppresses the expression of *OsIRO2* and *OsIRO3*. The specific part of OsbHLH061 responsible for this suppression activity is its ethylene-responsive element-binding factor-associated amphiphilic repression (EAR) motif (Wang et al., 2022). This is also in line with another report, in which OsbHLH061 (OsbHLHq11) is found to interact with proteins involved in Fe homeostasis affecting chlorophyll biosynthesis, such as NAS1, IRO2, and OsHRZ1. Therefore, it is proposed that OsbHLH061 has the potential to enhance photosynthetic efficiency by regulating chlorophyll synthesis in rice (Jang et al., 2022), which may subsequently lead to increased drought tolerance (Shin et al., 2020). Moreover, *OsbHLH061* is also found in Nitrogen and/or Water signalling by using a gene regulator network (Shank et al., 2022) suggesting the potential use of this gene in drought tolerance.

A common strategy for the development of plant varieties that are tolerant to drought is to

express the drought-responsive genes under the control of a strong constitutive promoter, such as *35S* from the cauliflower mosaic virus (Peng et al., 2011; Novak et al., 2013; Withanage et al., 2015; Du et al., 2016), *Ubi1* from maize (Hasthanasombut et al., 2011), and *Act1* from rice (Su et al., 1998). However, some energy and metabolites may be wasted due to the biosynthesis of unnecessary RNAs and proteins that are not required under non-stress conditions and these extra metabolites would even have negative effects on plant growth and development under normal conditions (Kasuga et al., 1999; Ito et al., 2006). Thus, stress-inducible promoters should be employed.

Rab21, Response to ABscisic acid 21, was first identified in rice plants subject to water-stress (Mundy and Chua, 1988). Its promoter region has been shown to induce gene expression under abiotic stresses (Rai et al., 2009; Yi et al., 2010; Ganguly et al., 2011; Choe et al., 2013; Teng et al., 2018). Analysis of *Rab21* promoter (Rab21p) fragments of different lengths reveals the variation of induction: a 358-bp promoter fragment results in high basal expression (Rai et al., 2009), yet a longer sequence (1557 bp) resolves this issue (Yi et al., 2010) and is ideally applicable to transgene expression for inducibly controlling drought resistance genes or other functional genes in rice (Teng et al., 2018). Consequently, the 1,556−1,558 bp sequence of Rab21p has been used to produce drought tolerance overexpressed transgenic rice (Kim et al., 2014; Minh-Thu et al., 2018).

In this study, *OsbHLH061* was found among genes that were up-regulated in expression microarray from drought-treated rice. After the expression pattern was confirmed by qRT-PCR, its promoter was analyzed by GUS expression and the gene was over-expressed in rice under Rab21 promoter (1556 bp) with the aim to produce drought tolerant plants.

MATERIALS AND METHODS

Plant growth and stress treatment design

Rice seeds (*Oryza sativa* L. *japonica* cultivar Nakdong, ND) were initially germinated on MS0 agar media (0.25% phytagel) and placed in a growth chamber at 28 ºC, undergoing a cycle of 2 days in darkness followed by 1−2 day(s) in light. Afterward, seedlings were transplanted into soil and cultivated in a greenhouse under a 16-hour light/8-hour dark cycle for 11 days, with 6 seeds per pot (5 × 5 × 6 cm). For inducing acute dehydration, 14-day-old plants were uprooted, immersed in fresh water for 1 day to prevent any transient gene expression due to potential root damage, and then exposed to air-drying conditions in a growth chamber set at 28 ºC, with 50% humidity and continuous fluorescent light of 150 $\mu mol.m^{-2}.s^{-1}$. Throughout this process, roots were shielded by tissue and aluminum foil to mimic their natural subterranean environment with limited air exposure in darkness. Following various time intervals depending on the experiment, leaves and roots were harvested separately, flash-frozen in liquid nitrogen, and stored at -70 ºC until further analysis. For the gradual drought treatment, potted plants were taken out from the water and left to dry in the greenhouse until they wilted. The plants were then rewatered to test for their recovery after drought.

Quantitative real-time PCR analysis

Total RNAs were isolated using TriReagent (Molecular Research Center, USA). To synthesize the first strand cDNA, 5 µg of total RNA was reverse-transcribed with the Super Script III First Strand kit (Invitrogen, USA) following the manufacturer's instructions. Gene-specific primers (Table 1) were designed using Primer Designer 4 software (Sci-ed. Software, USA). qRT-PCR was carried out using 2x qRT-PCR Pre-mix with 20x EvaGreen™ (SolGent, Seoul, Korea). The amplification reactions were performed at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 20 seconds, 58 °C for 40 seconds, 72 °C for 20 seconds, in a 20-µL mix containing 1 µL of 20x EvaGreen™, 0.25 µM primers and 10 ng cDNA. qRT-PCR analysis was performed using a Stratagene Mx3000p instrument and Mx3000p software, v2.02 (Stratagene, USA).

The rice *Ubiquitin1* (AK121590) transcript was used as a normalization control, and three technical replicates were analyzed for all quantitative experiments. The statistical analysis was performed using SPSS 16.0 (IBM, USA).

Table 1. Gene specific primers used in this research

Region/gene name	Experiment	Forward primer	Reverse primer
Rab21 (AK121952)	qRT-PCR	5'-CGTCTACCGTGAGAACCACA-3'	5'-GTAGGCGATGAAGCTGATGA-3'
Dip1 (AK070197)	qRT-PCR	5'-GAAGCCGGAAGACGCAACTG-3'	5'-CAACCATGGCCTGGTCTCAC-3'
RbcS (AK121444)	qRT-PCR	5'-ACCACCGACACCGGCGAGAA-3'	5'-TTGCGCTCGGCTAGCTCATC-3'
Ubi (AK121590)	qRT-PCR	5'-ATGGAGCTGCTGCTGTTCTA-3'	5'-TTCTTCCATGCTGCTCTACC-3'
OsbHLH061 (*AK*)	qRT-PCR	5'-GAACAGTCCGCTCTTCTAAC-3'	5'-TCCTGAGGCCGTGACGCATA-3'
OsbHLH061p (Os11g0601700)	Promoter isolation	5'-AAAAAGCAGGCTAAACTCACGCCCACACGA-3'	5'-AGAAAGCTGGGTGCCTCTACAGGGAGGCGA-3'
OsbHLH061 (*AK068017*)	cDNA isolation from library (with attB1, attB2 site)	5'-AAAAAGCAGGCTCCATGCCCCAAGTGAGGAA-3'	5'-AGAAAGCTGGGTCTAGCCCTTTTTTCGACC-3'
Rab21F-attB2	Transgene confirmation	5'-ACCACCGACACCGGCGAGAA-3'	5'-ACCACTTTGTACAAG AAAGCTGGGT-3'
GUS_261F P35S_56R	Transgene confirmation	5'-TTCGATGCGGTCACTCATTA-3'	5'-AGCCTCTCTAACCATCTGTG-3'

Vector construction and transformation in rice

DNA isolation was performed using PCR with specific primers (Table 1). The coding sequence of *OsbHLH061* was obtained from the cDNA library of GreenGene Biotech (Yongin, Korea), while the promoter region, a 2-kb genomic DNA fragment located upstream of its start codon, was isolated from genomic DNA. These sequences were then inserted into binary vectors using the Gateway technique (Invitrogen, USA). In the overexpression construct (Fig. 1a), *OsbHLH061* was regulated by Rab21 promoter, which was known to respond to drought stress and isolated from rice (Mundy & Chua 1988; Reddy et al., 2002). GFP was co-transferred under the drive of Wsi18, a seed-specific promoter (Yi et al., 2011), for the purpose of screening transgenic seeds. The herbicide bialaphos (*bar*) gene was used as a selection marker during culture. The transferred region of the promoter analysis construct contained *GUS* and *bar* expression cassettes (Fig. 1b). The two constructs were introduced into *Agrobacterium tumefaciens* LBA 4404 through triparental mating and then transferred into embryogenic calli derived from mature seeds of *Oryza sativa* cultivar. Nakdong, following previously established protocols (Jang et al., 1999).

Double-headed arrows show the amplicon region in the experiment of transgene confirmation. The transgenic lines, which were transformed with (a) and (b) constructs, were called S and P lines; respectively.

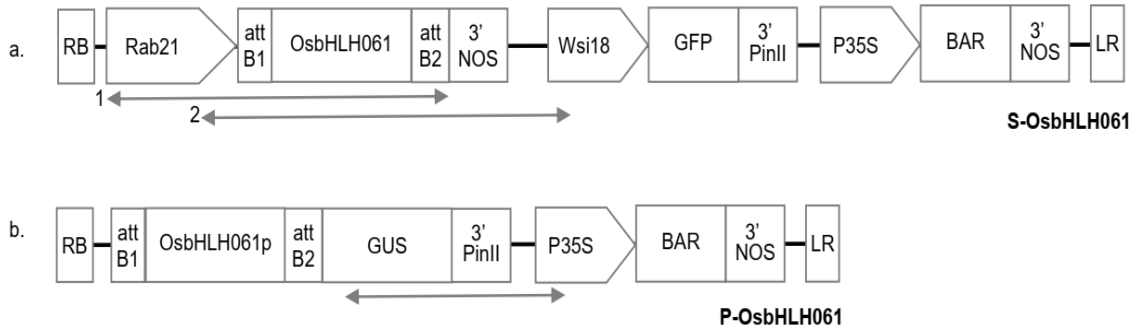

Figure 1. Vector construction to make transgenic rice for stress inducible expression (a) and promoter analysis (b) of *OsbHLH061*

Genomic DNA preparation and confirmation of transgene

Genomic DNA (gDNA) was extracted from leaf samples using DNAzolES (Molecular Research Center, USA) following the manufacturer's instructions with slight modifications. The presence of transgenes was confirmed by PCR using gene-specific primers (Table 1) positioned as indicated in Figure 1. PCR reactions were carried out in a 20-µL solution containing 1 µL rice gDNA, 0.25 pM gene-specific primers, and 10 µl of 2x PCR master mix (Intron Biotechnology, USA). The PCR program consisted of an initial denaturation step at 94 ^{o}C for 2 minutes, followed by 30 cycles of denaturation at 95 ^{o}C for 30 seconds, annealing at 55 ^{o}C for 30 seconds, extension at 68 ^{o}C for 30 seconds, and a final extension step at 68 ^{o}C for 10 minutes. Subsequently, 5 µL of the PCR reaction mixture was analyzed by gel electrophoresis on a 1.5% agarose gel.

Promoter analysis by GUS staining

Rice seeds were germinated and grown in a greenhouse as previously described. Samples were collected at different development stages due to experimental requirements. Histochemical localization of β-glucuronidase (GUS) was examined using 5-bromo-4-chloro-3-indolyl β-D-glucuronidase (X-gluc) based on a standard protocol (Bomblies, 2000).

GFP visualization

T1 seeds of non-transgenic and transgenic lines were manually peeled and attached to a piece of carton whose surface was covered by double-sided tape, then visualized under an Olympus FluoView FV1000 confocal microscope (Olympus, Japan). The GFP-labeled seeds were then picked out and germinated to produce T2 plants.

RESULTS

Change of *OsbHLH061* transcription factors during acute dehydration

Transcription factors are key components in understanding how genes respond to stress signals, as many previous studies have indicated (Shinozaki & Yamaguchi-Shinozaki, 2000; Agarwal et al., 2006; Wang et al., 2008; Hussain et al., 2011; Lata et al., 2011; Hu et al., 2013; Liu et al., 2013; Wang et al., 2021). Among 29 groups affected by drought, bHLH is the 2nd topmost strongly affected groups (Minh-Thu, 2014). Os11g0601700/LOC_Os11g38870/*OsbHLH061* (Li et al., 2006) was selected for further study.

To examine gene expression in both leaves and roots during stress, we conducted an acute dehydration in which plants were removed from soil and air-dried in a growth chamber for 0.5, 2, 6, and 12 hours. Compared to the Standard Evaluation System for Rice in drought issued by IRRI (2002), the leave rolling degree at time points 0.5, 2, 6, and 12 hours can be classified into 0, 3, 7 and 9; respectively (Minh-Thu et al., 2013). In this study, the expression of markers known to be induced, *Rab21* and *Dip1*, or repressed, *RbcS*,

by water deficit (Mundy & Chua, 1988; Reddy et al., 2002; Oh et al., 2005; Ali & Komatsu, 2006) were compatible with the degree of leaf rolling during drought. At 0.5 hours, the degree of leaf rolling remained at 0, indicating an insignificant effect of dehydration on leaf appearance. Both *RbcS* and *Rab21* showed no significant changes (Figs. 2b, d), while only *Dip1* was nearly 2-fold upregulated (Fig. 2c). At 2 hours, when the drought severity reached degree 3, all markers showed dramatic changes: *Dip1* increased 176-fold, *Rab21* increased 6-fold, and *RbcS* decreased by 3-fold. Similarly, when the drought in leaves reached level 7 (at 6 hours) and 9 (at 12 hours), *Dip1* and *Rab21* were still induced and *RbcS* was more repressed (Figs. 2b-d). The changing pattern of *Dip1* and *Rab21* in roots was similar to that in leaves with a significant increase observed starting at 2 hours (Figs. 2c, d). In the case of *RbcS*, its relative expression was less than 0.1 compared to *Ubiquitin* in all time points (Fig. 2b) and this was expected because *RbcS* was a photosynthetic tissue-specific gene (Sakoda et al., 2020).

Dip1, *Rab21 and RbcS* were stress markers, *Ubiquitin* (AK121590) was normalization control. L/R0, 0.5, 2, 6, 12 were leaves and roots collected at 0, 0.5, 2, 6, 12 h after the onset of stress treatment. Different characters showed statistical differences at p = 0.05 (Duncan test, n = 3).

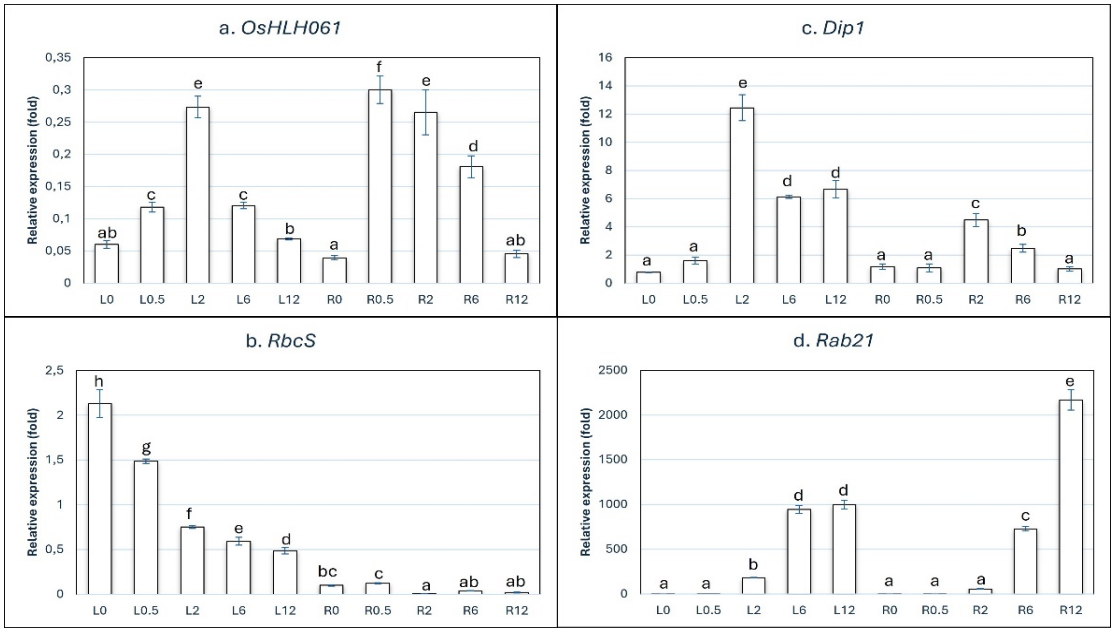

Figure 2. Expression pattern of *OsbHLH061* during acute dehydration in rice

OsbHLH061 was up-regulated in both leaves and roots during acute dehydration with more sustainable induction detected in roots (Fig. 2a). After 0.5 hours of acute dehydration, the gene increased 2-fold in the leaf and 7-fold in the root, respectively. Thus, the induction of *OsbHLH061* was earlier compared to *Rab21* or *RbcS*, and similar to *Dip1* in leaves. However, the relative intensity of *OsbHLH061* was not as high as *Dip1*. *OsbHLH061* expression reached its peak at 2 hours in both leaves and roots, gradually declined by 6 hours and returned to normal at 12 hours. This suggested the function of *OsbHLH061* in early response of plants to drought and it might have more roles in roots than in leaves.

Promoter analysis

Among six lines of promoter analysis, P14 was not transgenic because its PCR

result was similar to the non-transgenic plant Nakdong (Fig. 3a). With the remaining 5 lines, the GUS staining experiment was carried out with five-day (5-d) and fourteen-day (14-d) seedlings, which represented the germination and young seedlings stage. 5d seedlings showed blue color in their seeds and the tips of their leaves. On the other hand, GUS color was found in almost the whole plant in 14-d, especially strong in leaves and root tip but not in seed (Fig. 3b). The function of *OsbHLH61* in regulating chlorophyll in rice (Jang et al., 2022) might explain the strong expression of this gene in young seedling leaves. Furthermore, its expression in the roots, particularly in the root tips (Fig. 3b), aligns with findings by Wang et al. (2022) and suggests that *OsbHLH061* may have roles in additional aspects of rice development.

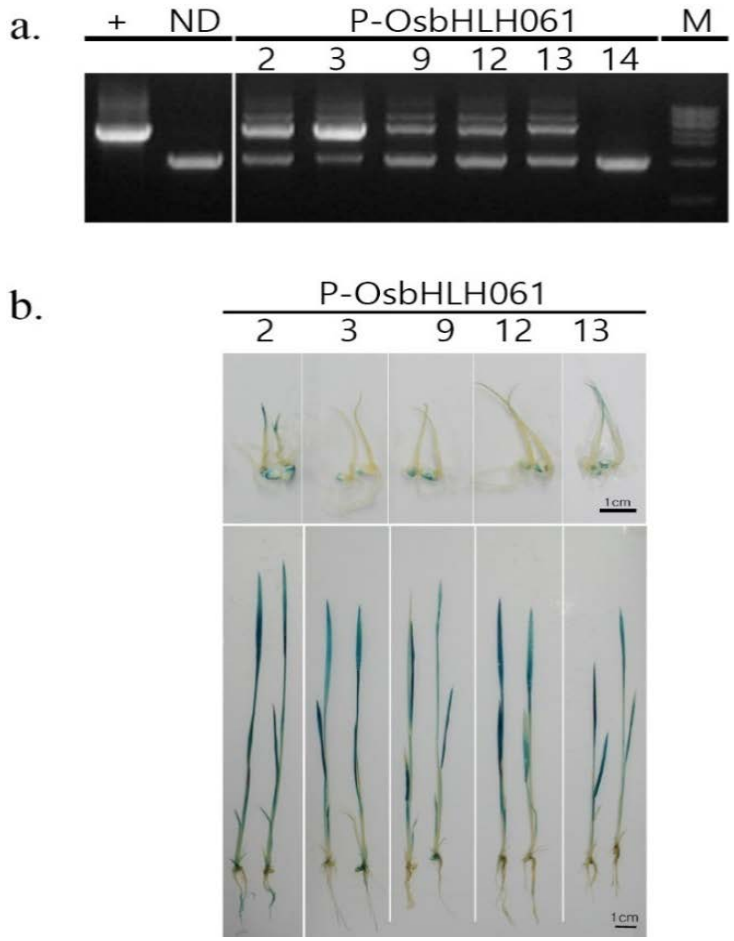

Figure 3. Expression of GUS in plants transformed with OsbHLH061 promoter. (a). Confirmation of *GUS* gene in transgenic lines. +: positive control (plasmid), ND: non-transgenic control (rice cult. Nakdong), M: marker, wells with numbers: transgenic lines; (b). GUS expression in-four-day (4d) (upper) and two-week (2w) (lower) seedlings

Overexpression of OsbHLH061 in rice

From left to right were Nakdong (non-transgenic plant), S3, S11 and S14.

In this study, *OsbHLH061* was constructed in a vector, which also included a Wsi18-GFP-PinII expression cassette. Wsi18 was found to be predominantly active in the

whole grain during seed development (Yi et al., 2011). Thus, it was used for the screening of transgenic lines at seed stage. There were six of the S-OsbHLH061 lines showed GFP expressions in seeds (Fig. 4a), suggesting those are OsbHLH061 overexpression lines. Moreover, the intactness of the whole cassette for OsbHLH061 overexpression was then confirmed by the presence of 2 bands with expected size in all lines, except S2 (Fig. 4b). This result implied that S2 was transformed with GFP but OsbHLH061 was missing. This explained the low expression of *OsbHLH061* in S2 compared to ND (Fig. 4c). According to the relative expression of *OsbHLH061* in S lines compared to ND (Figs. 4c, d), 3 lines selected to test the drought resistance were S3, S11 and S14.

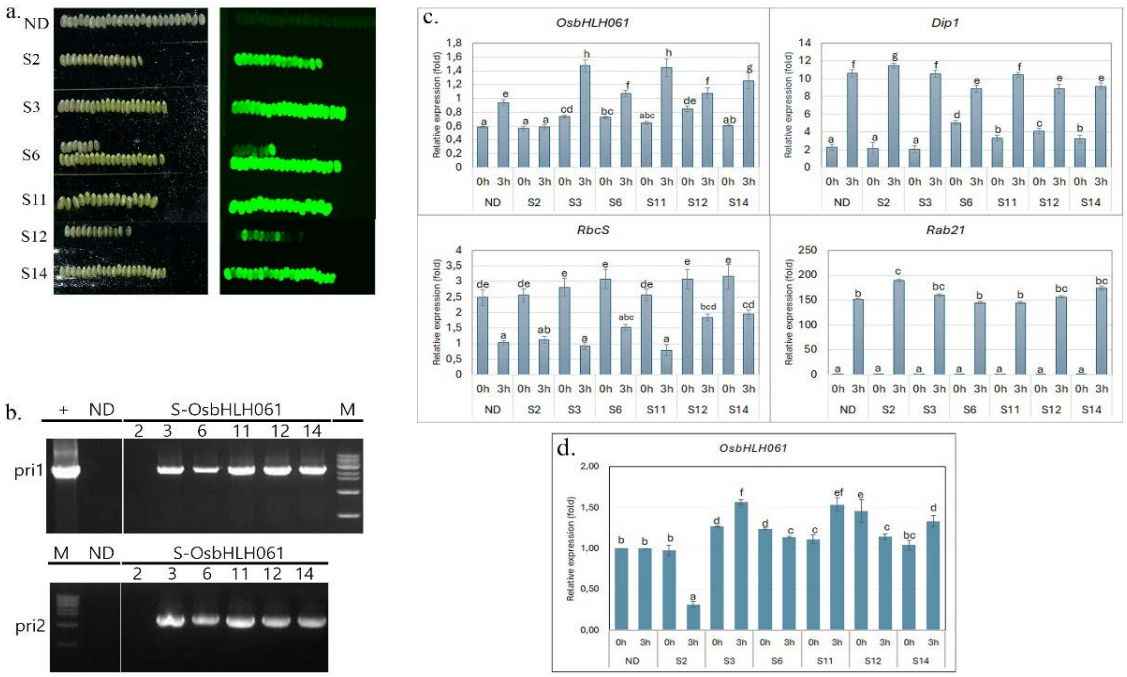

Figure 4. Overexpression of *OsbHLH061* in rice. (a). T1 seed selection by GFP expression; (b). Confirmation of exogenous Rab21-*OsbHLH061* region in transgenic. +: positive control (plasmid), ND: non-transgenic control (rice cult. Nakdong), M: marker, wells with numbers: transgenic lines, the position of pri1 and pri2 were shown in Figure 1a; (c). Expression of *OsbHLH061* in the leaf of non-transgenic and transgenic lines under acute dehydration. *Dip1, Rab21 and RbcS* were stress markers, *Ubiquitin* (AK121590) was normalization control. 0, 3 h: leaves collected at 0 hour and 3 hour after onset of stress treatment. ND: non-transgenic control (rice cult. Nakdong). S2-14: overexpression lines of *OsbHLH061*; (d). Relative expression of *OsbHLH061* in transgenic lines compared to ND. Expression levels of *OsbHLH061* in ND were set to 1.00, and relative expression in transgenic lines was calculated by comparison to ND at corresponding time points. Different characters in (c) and (d) showed statistical differences at p = 0.05 (Duncan test, n = 3)

A potted drought experiment was conducted using 14-day-old seedlings. After 3 days of drought, most leaves in S3 and S11 were tightly rolled and curled (Fig. 5), corresponding to degree 7 of leaf rolling in the Standard Evaluation System for Rice (IRRI, 2002) or 6 hours of acute dehydration (described above). S14 performed better, with leaves forming a deep V-shape but remaining upright (Fig. 5), similar to degree 3 or 2 hours

of acute dehydration. However, this stage was comparable to ND. Drought treatment was halted at this point to prevent irreparable damage to the plants. After 2 days of rewatering, ND and S12 recovered well, with nearly 90% survival rates, while S3 and S11 showed less than 20% survival (data not shown). Therefore, overexpression of *OsbHLH061* driven by the *Rab21* promoter did not improve drought tolerance in rice plants.

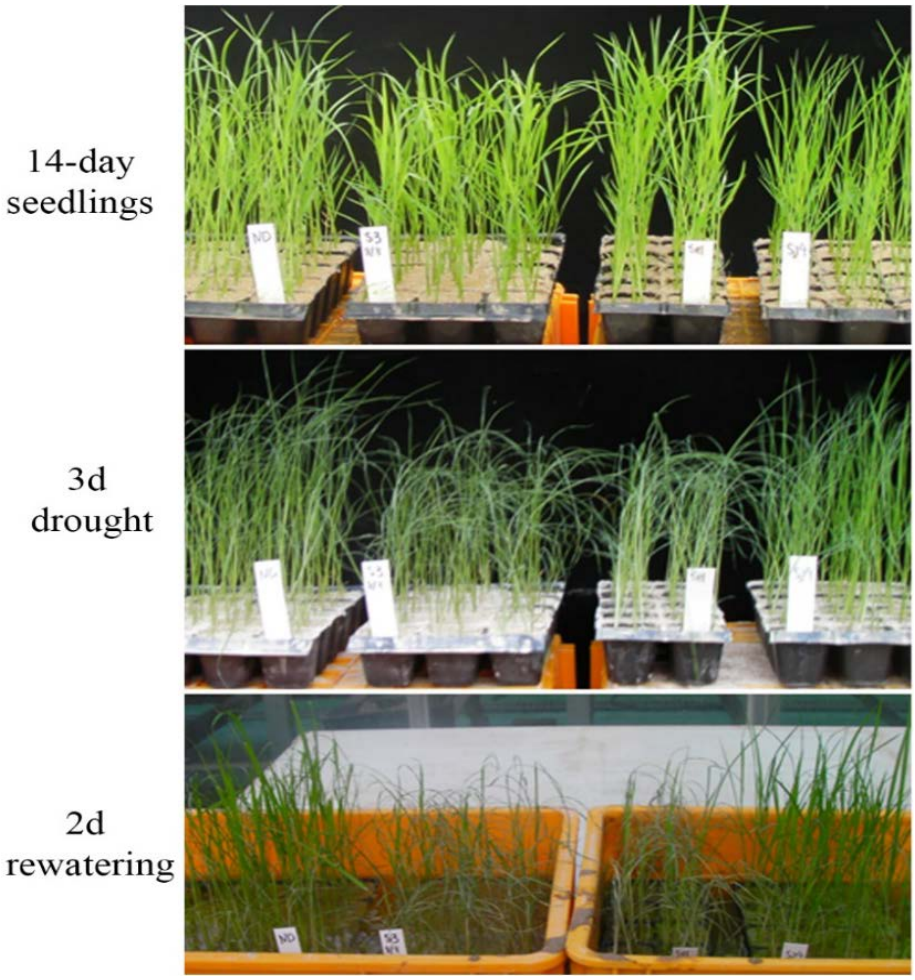

Figure 5. Drought treatment on OsbHLH061 overexpression rice

DISCUSSION

Our primary screening in non-transgenic rice showed that *OsbHLH061* was induced by drought (Fig. 2). This was in line with the previously proposed function of OsbHLH061 in drought tolerance (Shin et al., 2020; Shank et al., 2022). However, we did not observe any drought resistance effects in rice plants overexpressing *OsbHLH061*. This finding highlights the complex nature of drought response in plants in general, especially in rice. It may be the effect of a multi-loci trait because drought tolerance is a complex quantitative trait controlled by multiple genetic loci and susceptible to environmental influences (Wang et al., 2021). The fact that *OsbHLH061* was previously reported to be one of many loci contributing to *Xanthomonas oryzae* pv. *Oryza (Xoo) resistance (Korinsak* et al., *2021*) suggested its possible involvement in another multi-trait context.

Additionally, the choice of the stress-inducible promoter Rab21 may not have been optimal for *OsbHLH061* expression in this study. Theoretically, *OsbHLH061* was expected to exhibit higher up regulation by drought in over-expression lines compared to non-transgenic plants due to the function of Rab21 promoter. However, the observed relative expression of *OsbHLH061* at 3 hours of drought stress was higher in three lines (S3, S11, and S14) but lower in two other lines (S6 and S12) (Fig. 4d). Therefore, to effectively produce drought-resistant rice utilizing *OsbHLH061*, it may be necessary to employ a different expression system or adopt a multi-loci strategy.

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