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Molecular characterization of *OsCURT1A* from upland rice in response to osmotic stress

Bernadette Toni[1], Nurulhikma Md Isa[2], Cheng Seng Tan[2], Ismanizan Ismail[1,2], Zamri Zainal[1,2*]

[1]Institute of System Biology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
[2]School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

***Corresponding author: zz@ukm.edu.my**

Abstract

CURT1 proteins in *Arabidopsis thaliana* have been reported to be important for inducing grana curvature. Currently, we have identified transcript encoding *CURT1A* from *Oryza sativa* cv. indica through RNA-seq analysis and characterised using heterologous system in Arabidopsis. The *OsCURT1A* gene shares 80% of its amino acid sequence with Arabidopsis *AtCURT1A.* Phylogenetic analysis revealed that the *OsCURT1A* is also closely related to the CURT1 proteins in other choloroplast- conatining organisms. *In silico* analysis of *OsCURT1A* promoter shows that several *cis-elements* related to stress are present in the 5' upstream from the coding region. Under normal conditions, there were no notable changes in the phenotype and chlorophyll a;b ratio between three Arabidopsis genotypes, which were overexpressed (*35S::OsCURT1A)*, T-DNA insertional mutant line (*atcurt1a)*, and Wild type (Col-0). However, overexpression of *OsCURT1A* under salinity condition demonstrate high chlorophyll a:b compared to Col-0, whereas, the lack of *atcurt1a* gene in the mutant line showed reduced chlorophyll a:b ratio. These results indicate that the *OsCURT1A* might have a function as salt-stress related gene, which may indirectly regulate the chlorophyll a:b ratio. Therefore, *OsCURT1A* can be used as a potential candidate for salinity stress tolerance in crop improvement.

Keywords: Upland rice, drought, stress-responsive genes, curvature thylakoid, *Arabidopsis thaliana.*
Abbreviations: NaCl_Sodium chloride; PEG_Polyethylene glycol; RT-qPCR_Real-Time Quantitative PCR.

Introduction

Rice is the most consumed cereal grain, and it is grown on 9% of the world's arable land. In Asia, rice accounts for 50-80% of the Asian population's calorie intake (Thiruneelakandan & Subbulakshmi, 2014). As the world population continues to increase, it is expected that the production of rice will increase to serve the demand (Godfray, 2010). Therefore, it is very crucial that rice production must be sustainable to ensure food security, as well as the economic survival of millions of small rice farmers. Currently, the great challenge posed against sustaining rice cultivation is climate change. Current climate prediction models indicate an increase in the frequency of droughts, flooding, and high temperature conditions, known as heat waves (Mittler and Blumwald, 2010).

There are four rice ecosystems which are: irrigated, rain-fed lowland, deep-water, and rain-fed upland (Bernier et al., 2008). The frequent and unpredictable drought conditions in upland areas cause upland rice to be more readily exposed to water deficit stress compared to the other rice ecosystems. However, our understanding regarding the underlying genetic mechanism employed by upland rice during water-deficit conditions is still very limited. Understanding the mechanisms of how the upland rice responds to stress will provide significant opportunities in developing new varieties of stress-tolerant rice.

Current advancements in RNA sequencing technologies (RNA-seq) provide high-throughput methods for identifying stress-related genes and give us better insight into, and a deeper understanding of, the abiotic stress mechanism (Deyholos, 2010). Abiotic stress-inducible genes are divided into two major groups, which are functional and regulatory proteins (Hirayama and Shinozaki, 2010). Functional proteins are directly involved with stress tolerance, such as detoxification, osmolyte biosynthesis, regulating water uptake, and transport. Whereas the second group, which is made of regulatory proteins, is involved in signalling and gene expression regulation such as ARF (ADP-ribosylation factor), GRP (glycine-rich RNA binding protein), CBF2, CBF3, and AP2 domain-containing proteins, which were induced under salt and/or drought conditions (Wang et al., 2007 and Wu et al., 2005).

Several studies reported that the photosynthetic system is thought to be one of the primary biological components which can easily be affected by water deficit (Chaves et al., 2002). This argument was supported by studies on the changes in the organelle's structure which indirectly affect photosynthesis (Chaves et al., 2009). Recently, it was found that curvature thylakoid 1 (CURT1) proteins in *Arabidopsis thaliana* are responsible for maintaining grana curvature by regulating the stacking and destacking of the thylakoid membrane (Armbruster et al., 2013). Apart from its importance in maintaining the thylakoid membrane, CURT,

which is the orthologue of *AtCURT1A* in *Synechosystis* sp. 6803, was also found to have a role in the response to osmotic stress (Heinz et al., 2016).

In this study, we attempt to characterise *OsCURT1A*, which is the orthologue of *AtCURT1A* in *O. sativa*. *OsCURT1A* was proposed as a drought-responsive gene from our previous transcriptome analysis of PEG-treated Kuku Belang (SRP074520), and was deposited in the GenBank (MF140272) (Toni et al., 2017). The transcript level of *OsCURT1A* in Kuku Belang rice treated with 20 % PEG-8000 under hydroponic conditions, and 200 mM of NaCl were evaluated using RT-qPCR. To further understand the function of *OsCURT1A*, transgenics *Arabidopsis* which were harbouring over-expressed *OsCURT1A (35S::OsCURT1A)* were produced. Besides *35S::OsCURT1A*, *A. thaliana atcurt1a* T-DNA knockout mutant was employed in this study to understand its function.

Results

In silico analysis of OsCURT1A encodes thylakoid membrane protein

The data obtained from the transcriptome analysis of the 20% PEG-treated Kuku Belang seedlings (Toni et al., 2017) has identified a drought-related full-length transcript with a size of 471bp encodes for thylakoid membrane protein. This gene was then selected for further characterisation. The transcript with accession number MF140272, known as *O. sativa* curvature thylakoid 1A (*OsCURT1A*), has a high homology with *A. thaliana* curvature thylakoid 1A (*AtCURT1A*), with an ~80% amino acid sequence similarity. Analysis using the rice database shows that *OsCURT1A* is located in the sixth chromosome. Only one transcript was identified with the size of 845 bp, consists of four exons and three introns. *OsCURT1A* is predicted to encode 156 amino acids with a theoretical molecular weight of 16 kDa and a PI of 9.92. The secondary structure of OsCURT1A was predicted to consist of five α-helices and one β-strand (Fig. 1), with two transmembrane regions identified residing at the amino acid positions of Thr_{84} to Ile_{106} and Leu_{111} to Leu_{133}, (Fig. 1). One domain, known as the cyanobacterial aminoacyl-tRNA synthetase appended domain (CAAD), was found spanning the transmembrane region from amino acid position Ala_{70} to Gly_{154}, and the presence of signal peptide, as predicted by PrediSi, was found at the N-terminal of the polypeptides. Further investigation at the 1kb upstream of the transcription start site (TSS) showed the presence of several hypothetical abiotic stress-related motifs, as shown in Table 1. This however needs to be further validified through experiments.

Phylogenetic tree of CURT1 protein from photosynthetic organisms

Phylogenetic relationships of OsCURT1A and protein members from the CURT1 family were analysed by the Neighbour-Joining method (Fig. 2). Subfamily classification of each protein was assigned in accordance to Armbruster et al. (2013). A total of 31 amino acid sequences were retrieved from the NCBI non-redundant protein sequence database for the phylogenetic analysis. A sequence of aminoacyl-tRNA synthetase from *Streptococcus agalactiae* was used as the out-group. The evolutionary tree was grouped into six major clusters, which were made up of four clusters from plants (CURT1A, CURT1B, CURT1C and CURT1D) and two clusters from microbes (algae and cyanobacteria). The OsCURT1A protein was found to be assigned into the cluster of CURT1A, under the clade of monocot, as expected. Despite of high functional and sequential conservation, the CURT1A proteins from monocots and dicots surprisingly showed a clear separation into two different clades, implying that the inheritance of CURT1A genes in plants is most likely done through vertical gene transfer.

Down regulated expression of OsCURT1A under various abiotic stresses

To observe the expression pattern of OsCURT1A in respond to various stresses in rice, an qRT-PCR analysis was performed. As shown in figure 3, the *OsCURT1A* transcript is significantly down-regulated in two-week old rice seedlings treated with 20% PEG-8000. This result of down-regulated expression was also similar to the 200 mM NaCl treatment. These results might be an indicator that the OsCURT1A play a role during stress.

Phenotypic analysis of OsCURT1A overexpression line in Arabidopsis seedling

Due to the low transformation efficiency in the *Indica* variety, we over-expressed *OsCURT1A* in *Arabidopsis* to determine the gene's function in plants. To generate a transgenic *Arabidopsis* plant expressing the *OsCURT1A*, the gene's open reading frame (ORF) was integrated between the 35S promoter and NOS terminator region in the binary vector pB2GW7. The *Arabidopsis* ecotype Columbia (Col-0) plant was transformed using the 35S::*OsCURT1A* construct according to the floral dipping method (Clough and Bent, 1998). Among 20 lines of positive transformants, one homozygous line was selected for further characterisation (*35::OsCURT1A-5)*. However, we did not observe any phenotypic differences between the wild type, transgenic, and mutant seedlings (Fig. 4).

Effect of OsCURT1A on Arabidopsis root development

In order to relate the gene effect towards biomass production during stress, we measure the Root development. Two growth parameters which were Root Length (RL) and Root Fresh Weight (RFW) were evaluated. Based on our analysis on RL, we observed that there is no significant difference in RL for both independent transgenic lines of *35S::OsCURT1A* compared to the *atcurt1a* mutant and WT in the control media (½ MS) (Fig. 5a). Whereas, under the PEG treated experiment, we found that the RL of the homozygous transgenic lines of *35S::OsCURT1A* were slightly shorter compared to WT. However, in the *atcurt1a* mutant, the RL was significantly reduced. Under the NaCl treatment, we found that only the *atcurt1a* mutant showed a significantly reduced RL (Fig.5a). Meanwhile, for RFW, only the *atcurt1a* mutant showed a significant difference under the NaCl treatment (Fig.5b). This result indicates that the NaCl has a negative effect on the root growth of *atcurt1a* compared to the PEG treatment.

Table 1. Analysis of ~1 kB upstream of *OsCURT1A* (promoter region) where several abiotic stress-related and other motifs are enriched**.**

Family name	Function	Position	Sequence	Enrichment score
ARF	Auxin-regulated gene expression of primary response genes	-828	gAGACA	1
C2H2	DNA and RNA binding, protein-protein interaction especially specific protein which requires contact with ZF domain	-348	tAGTGTa	1
		-229	aAGTGTg	1
		2	cAGTGTg	1
TCP	Abiotic stress, chaperoning, tiller development	-223	GGACC	1
		-59	GGTCC	1
WRKY	Biotic and abiotic stresses, development	-568	ttgTTGACta	1
Zf-HD	Abiotic stress	-405	tatGATTAtg	1
NAC/NAM	Stress response, growth and development	-567	tgTTGACta	1
		-517	tTGCGTactg	1
		-9	gataGCGTGa	0.99
ERF	Biotic and abiotic stresses, development	-39	gggtgCACCG	0.98
bZIP	pathogen defence, light and stress signalling, seed maturation, flower development	-125	cctgcCACGTgtcagc	1
		-89	gCCACGtgg	1
		-969	gGACAGcaca	0.97
		-940	tGACAGttca	0.97
		-843	gctCGTCAtg	0.97
		-827	aGACAGcagt	0.97
SBP	Development	-519	cattgCGTACtggatg	0.95
		-350	tatagtGTACAgtatg	0.95
		-324	gaaaaaGTACAcccag	0.95
MYB/ MYB-related	Stress response, development	-997	caagGAATCt	1
		-506	atgGAATCat	1
		-442	TAACAaa	1
		-34	caCCGTTa	0.95
		-448	aggagaTAACAaaac	0.87
		-555	atTGTTA	0.86
E2F	Key components of cyclin D/ retinoblastoma/ E2F pathway	-188	ccGGGAAt	1
		-738	ctGCCAAt	0.94
		-687	caGGCAAt	0.94
HD-ZIP	Abiotic stress, development	-508	ggatggaATCATtccagaa	0.9
MADS	Flower development	-647	gctgaacaaaataAGAAAaga	0.89
bHLH	Development	-124	ctgcCACGTg	1
		-91	ctgcCACGTg	1
		-720	GCAAGtgc	0.88
LFY	Flower development	-735	CCAATgc	0.86

Fig 1. Multiple sequence alignment of OsCURT1A and its CURT1 homologs. Residues responsible for the transmembrane domains are indicated in boxes. The signal peptide region is shown in blue, whereas the cleavage site of the signal peptide is indicated in red. The secondary structure elements for α-helix and β-strand are shown in green and orange, respectively. The red line above the sequences depicts the region of the predicted CAAD domain. The sequence conservation symbols are indicated as follows: "*" for identical residues in all aligned sequences; ":" for conserved substitutions of aligned sequences; "." for semi-conserved substitutions in aligned sequences.

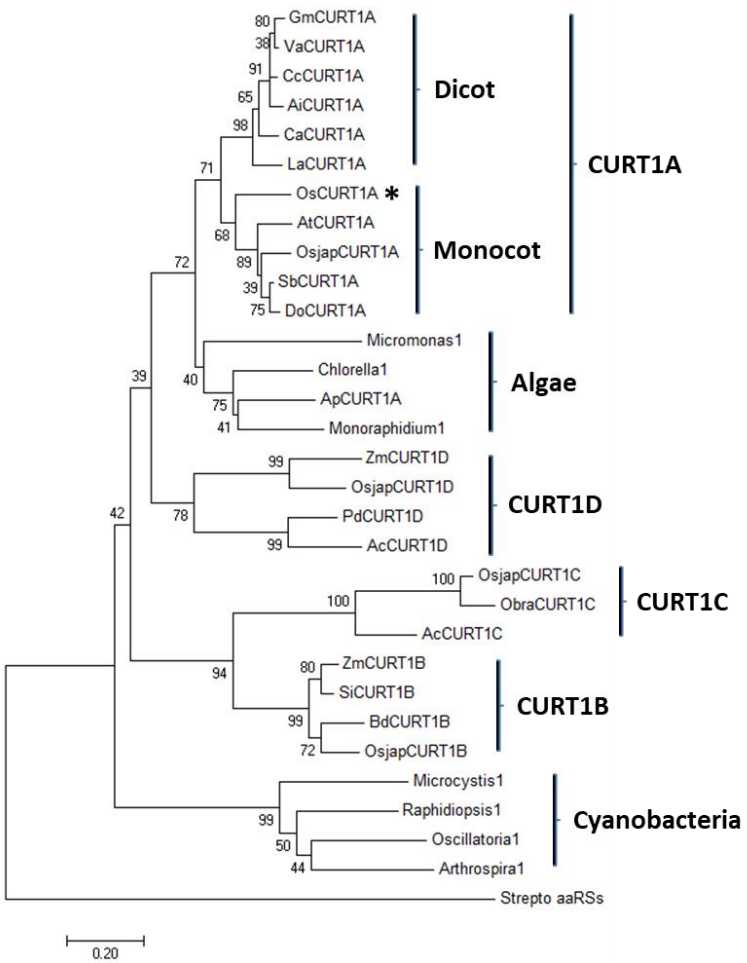

Fig 2. Phylogenetic analysis of the CURT1 protein family. The phylogenetic tree of CURT1 proteins was constructed using the Neighbour-Joining method. The evolutionary tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

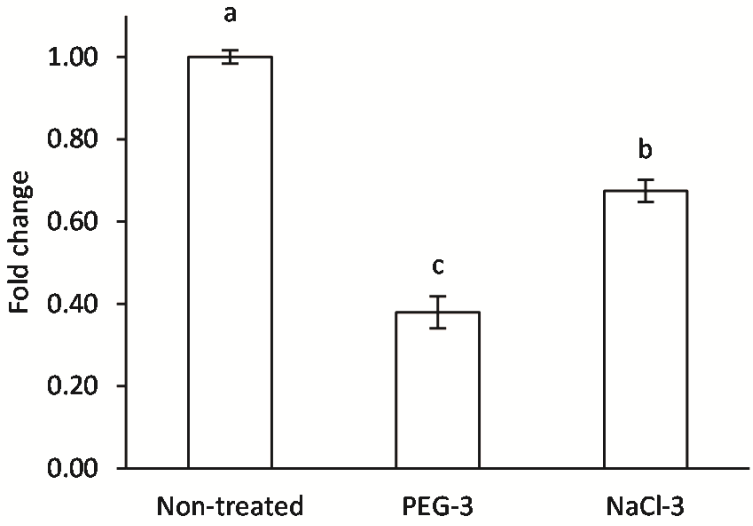

Fig 3. The qRT-PCR analyses found that *OsCURT1A* expression is significantly downregulated in leaves of two-week old Kuku Belang, treated hydroponically with 20 % PEG-8000 (PEG-3) and 200 mM NaCl (NaCl-3) for 3hr compared to the control. The expression value was expressed as a mean fold change ± SD (n=3). Values with different letters indicate significant difference ($p < 0.05$).

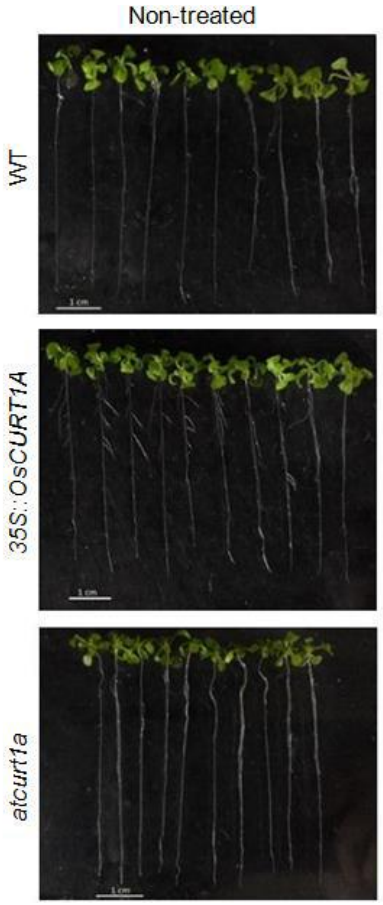

Fig 4. Phenotypic analysis showed that there is no morphological different between the wild type, *35S::OsCURT1A* overexpression, and the *atcurt1a* mutant of 14-day seedlings under normal conditions.

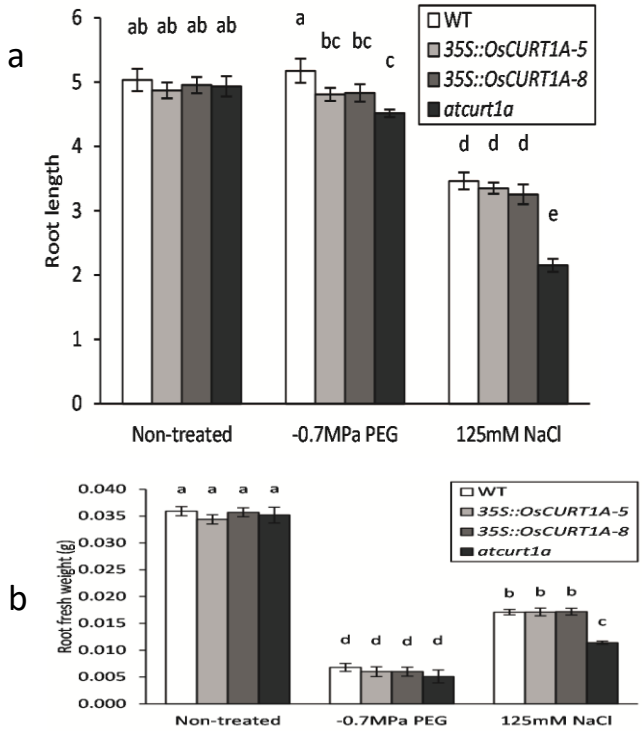

Fig 5. (a) Root length (RL) of WT, two independent lines of *35S::OsCURT1A* and *atcurt1a* grown on non-treated, PEG-infused (ψ_w -0.7 MPa), and NaCl-containing (125 mM) media. RL was stated as mean length (cm) ± SD (n=3). Values with different letters indicate significant difference ($p < 0.05$). (b) Root fresh weight (RFW) of WT, two independent lines of *35S::OsCURT1A* and *atcurt1a* grown on non-treated, PEG-infused (ψ_w -0.7 MPa), and NaCl-containing (125 mM) media. RFW was stated as mean fresh weight (g) ± SD (n=3). Values with different letters indicate significant difference ($p < 0.05$).

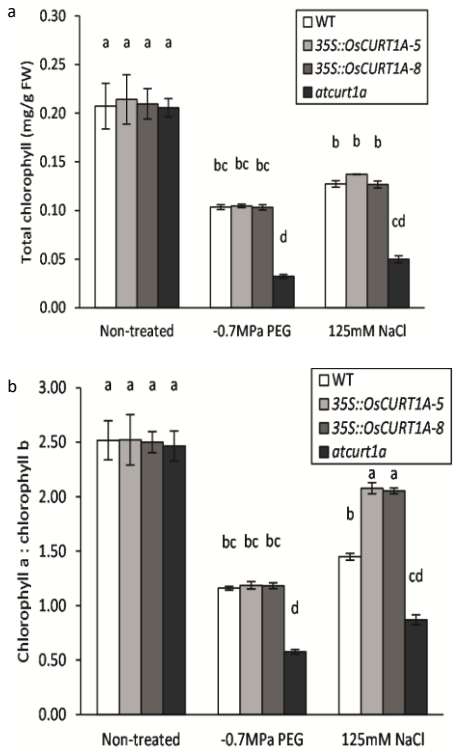

Fig 6. (a) Total chlorophyll content (chl_t) of WT, two independent lines of *35S::OsCURT1A* and *atcurt1a* grown on non-treated, PEG-infused (ψ_w -0.7 MPa), and NaCl-containing (125 mM) media. chl_t was stated as mean content (mg/g fresh weight) $\pm$ SD (n=3). Values with different letters indicate significant difference ($p < 0.05$). (b) Chlorophyll a to b ratio (chl a:b) of WT, two independent lines of *35S::OsCURT1A* and *atcurt1a* grown on non-treated, PEG-infused (ψ_w -0.7 MPa), and NaCl-containing (125 mM) media. Chl a:b was stated as mean ratio $\pm$ SD (n=3). Values with different letters indicate significant difference ($p < 0.05$).

Chlorophyll a:b content analysis of transgenic plants

Two photosynthesis parameters, which are total chlorophyll content (chl_t) and chlorophyll a to b ratio (chl a/b) were determined (Fig. 6a & 6b). No significant differences in chl_t were observed in two-week old homozygous transgenic lines of *35S::OsCURT1A* and *atcurt1a,* compared to WT plants when grown on the control media (½ MS). However, under abiotic stress treatments, only the *atcurt1a* mutant showed a significant decline in chl. (Fig 6a). No significant changes in chl_t were observed for *35S::OsCURT1A* overexpressing lines under the control and treated conditions. Based on the observation of the mutant, we suggest that the *atcurt1a* mutant might have a role in maintaining the total chlorophyll content, especially when under stress. Besides looking at the total chlorophyll content, we also carried out an experiment to observe the ratio of chl a/b. Under control conditions (½ MS), no changes in the chl a/b ratio were observed in homozygous transgenic lines of *35S::OsCURT1A and atcurt1a as* compared to WT. Despite that, in homozygous overexpression lines, the ratio of chl a/b was dramatically increased under 125 mM NaCl. On the other hand, the *atcurt1a* mutant showed a significantly reduced chl a/b under 125 mM NaCl and PEG (ψ_w -0.7 MPa) (Fig. 6b). Our results described above suggested that, overexpressing *OsCURT1A* can increase the steady state properties of PSII, and this might contribute to the abiotic stress tolerance.

Discussion

In a search for a drought-related gene in the rice transcriptomic RNA-seq data, we identified that the *OsCURT1A* transcript is differentially regulated in PEG-treated Kuku Belang. This is the first report of the O*. sativa* CURT1A protein. Although the CURT1 protein was identified a decade ago, only recently was the CURT1 protein characterised in *A. thaliana* (Armbruster et al., 2013) and *Synechocystis* sp. PCC6803 (Heinz et al., 2016). It was proposed that the CURT1 protein is involved in maintaining thylakoid membrane architecture, as well as the biogenesis of PSII centres. Additionally, Heinz et al (2016) suggested that CURT1 plays an important role during osmotic stress.

In silico analysis showed that *OsCURT1A* has a conserved CAAD domain which contains two transmembrane helices, like other eukaryotic CURT1 proteins. The deletion study of the CAAD domain suggested that its function is required to anchor aminoacyl-tRNA synthetases (aaRSs) to the membrane, especially under nitrogen-limiting conditions (Olmedo et al., 2011). The CAAD domain has been extensively studied *in silico* by Luque (2014), who suggested that the CAAD domain might be involved in protein sorting and localisation in thylakoid membranes. OsCURT1A is the nuclear encoded and this is strongly supported by the

presence of transit peptide at the N-terminal. This suggests that the OsCURT1A protein is synthesized in the cytoplasm and translocated into the thylakoid membrane. Phylogenetic clustering showed that CURT1A algae are well separated from the higher plants clade. This suggests that the differences may result from sequential or structural features acquired or lost during evolution, which led to some degree of functional divergence.

The promoter analysis sequence of 1kb upstream from the TSS showed that there were several abiotic stress-related motifs present in this region. For example, the presence of the C2H2-type transcription factor, known to play critical roles in DNA or RNA binding and protein-protein interaction during dehydration, salinity, and cold stresses, was identified (Flexas et al., 2004). Besides that, the presence of NAC/NAM and MYB motifs in this region strengthen our hypothesis that this gene is involved in the stress response (Abe et al., 2003). The presence of several drought-related cis-elements in this region support our findings that this gene may play a role under stress conditions. However, nothing was known to date about gene regulation at the cellular level responds to stress. In our rice RT-qPCR analysis of *OsCURT1A*, we found that the expression level of *OsCURT1A* was significantly downregulated under osmotic stress exerted by PEG and NaCl. This change in expression suggests that *OsCURT1A* may very likely to play a role in the osmotic stress response mechanism.

To further examine the functional roles of *OsCURT1A,* we produced transgenic 35S:*OsCURT1A* as well as a T-DNA mutant obtained from Nottingham Arabidopsis Stock Centre. In this study we determined the roles of this gene in root development. Roots are primary responding organs in plants, especially during the stress acclimatization process, through avoidance and tolerance mechanisms (Harb et al., 2010). A perceptible decrease in cellular growth of roots can be observed even under mild water deficit, and even more severely under moderate and severe water deficit. The decrease in the cellular growth of the roots during water deficit stress is due to lowered cell turgor (Van Der Weele et al., 2000). From our physiological analysis on the *atcurt1a* mutant, we observed that NaCl has more negative effects on the RL compared to PEG stress. We observed that impairment in root elongation by NaCl stress is paralleled to the decrease in RFW. This may be caused by the K+ toxicity (Munns, 2002; Verslues et al., 2006; Zhu, 2002). The effects of PEG are regulated by osmotic adjustments, and the main mechanism is through avoidance and tolerance, while NaCl is regulated by ion homeostasis which somehow caused toxicity in the cell in excessive amounts.

Severe abiotic stress may affect the chlorophyll content by damaging the photosynthetic apparatus (Picorel et al., 2017; Pinheiro et al., 2008). Photosynthesis is a sophisticated and crucial function in plant productivity; therefore, the ability of plants to adapt to the fluctuations in their environments is related to the plasticity of their photosynthesis. The fastest way to access the plant's stress status is via measuring the chlorophyll content. It is thought that a lower chlorophyll content indicates a decrease in photosynthesis pigment production, which might be due to the impairment of chlorophyll biosynthesis and/or an increment of chlorophyll degradation. This ultimately causes a decrease in the efficiency rate of photosynthesis. A decrease in the total chlorophyll (chl_t) signifies a lower capacity for harvesting light (Mafakheri et al., 2010). From our results, we observed that the *atcurt1a* mutant showed a significant decline in chl_t, thus this might likely indicate that the loss of functioning mutation of *atcurt1a* might affect the capacity for light harvesting, compared to WT, in response to stress. According to our findings, the presence of chlorophyll b was much higher under both stresses in the mutant line. This result supports the claim that chlorophyll b should increase to ensure the light harvesting complex (LHC) is stabilised during stress. This is to ensure the efficient capture of photons and increase the electron transport rate (Biswal et al., 2012). The difference in the amount of chlorophyll a:b ratio, in response to difference stresses, suggest that they are likely to be triggered by different mechanisms.

Materials and Methods

Plant material and growth condition

Kuku Belang seeds were obtained from the Malaysian Agricultural Research and Development Institute (MARDI). Germinated seeds were grown in three miniature hydroponic systems containing half-strength (½) Yoshida solution (Yoshida et al., 1976), placed in a controlled room at a temperature of 26ºC day/22ºC night, with a relative humidity of 70-75 %, and were illuminated for 12 hr using fluorescent light at 165 $\mu molm^{-2}s^{-1}$. Nutrient solutions were changed every week. After 14 days, one of the miniature hydroponic systems was filled with ½ Yoshida solution, containing 20 % PEG-8000 to mimic drought stress, and another was filled with ½ Yoshida solution containing 200 mM of NaCl to mimic salt stress. *OsCURT1A* accession number in rice cv. Indica (EAZ00433.1). Gabi-Kat (GK) mutants *atcurt1a* accession number (AT4G01150)

Bioinformatic analysis

Information regarding *OsCURT1A* and its products were obtained from various databases, such as Ensemble Plants (http://plants.ensembl.org/index.html), Uniprot (http://www.uniprot.org/), and RiceDB (http://ricedb.plantenergy.uwa.edu.au/). The orthologue of *OsCURT1A* was searched for using the EggNOG v4.5 (http://eggnogdb.embl.de/) and OrthoDB (http://www.orthodb.org/). A promoter analysis was performed using the PLANTPan (http://plantpan2.itps.ncku.edu.tw/) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) databases. The physico-chemical characteristics were predicted using ExPASy-ProtParam (http://web.expasy.org/protparam/). A multiple sequence alignment was conducted using ClustalW version 1.83 (http://www.genome.jp/tools-bin/clustalw). The secondary structure elements of each sequence were predicted using Psipred's online server (Buchan et al. 2013). The prediction of a transmembrane domain of the protein sequence was conducted with the MINNOU server (Cao et al. 2006). The presence of a signal peptide sequence and their cleavage site in CURT1 proteins was predicted with PrediSi software tool (Menne et al. 2000).

Construction of phylogenetic tree of CURT1 proteins

Phylogenetic analysis of CURT1 proteins was conducted using a molecular evolutionary genetics analysis integrated tool, MEGA 7 (Kumar et al., 2008). All homologous amino acid sequences of *OsCURT1A* were retrieved from the NCBI non-redundant sequence database (a complete list of accession numbers are given in the supplementary information section) and aligned using ClustalW (default settings, gap extension: 1, protein weight matrix: BLOSUM). Phylogenetic reconstruction was conducted using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in 1000 replicates of the bootstrap test are provided next to each branch (Felsenstein 1985). The evolutionary distance was calculated with the Poisson correction method (Zucherkandl & Pauling 1965).

Isolation of total RNA from Kuku Belang

Leaves from the control, PEG-, and NaCl-treated group were sampled 3 hr after treatment in a triplicate biological control. The total RNA was extracted using a TRIzol reagent (Life Technologies) and was purified using the ethanol precipitation method (Weaver et al., 2017). Genomic DNA was discarded using the TURBO DNase Kit (Ambion) and first-strand cDNA was synthesised using the First Strand cDNA Synthesis Kit (Maxima). Full length *OsCURT1A* was isolated from the cDNA library (150 ng) of PEG-treated seedlings by touchdown PCR, using the KOD Hot Start High Fidelity DNA Polymerase kit (Toyobo) with the addition of 5 % DMSO.PCR, and was carried out using 0.3 µM forward (F) and reverse (R) primers; F: (5'-ACCGAC ATGGCCGCAG-3'), R: (5'-GCTGCTTAATTACTCCGTTCCAGC-3').

Real-Time Quantitative PCR (RT-qPCR) analysis

Gene-specific primers for the RT-qPCR analysis of *OsCURT1A* were designed according to the full length of *OsCURT1A,* whereas gene specific primers for two reference genes which are *OsU6* (BGIOSGA030886) and *OsEeF* (BGIOSGA011974) were designed according to their CDS using QuantPrime. The standard and melt curves were generated for each primer prior to the gene quantification analysis. The primers which were used to amplify *OsCURT1A* are 5'-GTCGGCGCCATCAACTC-3' and 5'-CAACTCTTTCCTGCTCTCCTTG-3'. The primers used to amplify *OsU6* are 5'-TACAGATAAGATTAGCATGGCCCC-3' and 5'-GGACCATTTCTCGATTTGTACGTG-3'. The primers used to amplify *OsEeF* are 5'-TTTCACTCTTGGTGTGAAGCAGAT-3' and 5'-GACTTCCTTCACGATTTCATCG TAA-3'. 6 ng/µL of cDNA for each sample was used as a template. The RT-qPCR was carried out using an iQ SYBR Green Supermix kit (Bio-Rad) using the iQ5 BioRad Real-Time thermal cycler. The expression values of *OsCURT1A* were normalised against both reference genes and calculated according to Schmittgen and Livak's (2008) method. The statistical significance of expression values was analysed using Statistical Analysis Software v9.3 (SAS 2011). qRT-PCR analysis was done in three biological replicates and three technical replicates.

Molecular cloning of OsCURT1A

Molecular cloning was carried out using the Gateway Cloning Technology. The attB adaptor sequences were added to the 5' end of both forward and reverse primers: F: (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCCGCCGCA GCCAC-3') and R: (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACTCCGTTCCAG CGATC TTC-3'). Amplicons were cloned into pDONR221 using BP clonase. Intermediate clones were sent for sequencing prior to the final cloning into the pB2GW7 vector using LR clonase. The presence of *OsCURT1A* fragments in the recombinant plasmid was determined via restriction enzyme digestion, *SpeI* and *HindIII* (England BioLab).

Agrobacterium-mediated transformation and selection of homozygous transgenic A. thaliana (35S::OsCURT1A)

Agrobacterium tumefaciens harbouring the *35S:: OsCURT1A* constructs were transformed into Columbia-0 via the floral-dip method (Zhang et al., 2006). The bacterial culture was supplemented with 5 % sucrose and 0.05 % silwet-77 prior to inflorescence dipping. T1 seeds were stratified and germinated, as described by Zhang et al., 2006. Screening for transformants was carried out by spraying 120 mg/L Basta on the 10^{th}, 12^{th}, 15^{th} and the 17^{th} day after stratification. Basta-resistant plants were considered putative T1 transformants, and thus were labelled and left to grow in the growth chamber with a temperature of 22°C day/20°C night, relative humidity of 60 %, and 16 hr (long day)/8 hr (short day) photoperiods at 100-150 µmol $m^{-2}s^{-1}$. For the transformant verification, PCR was carried out on the genomic DNA. PCR was carried out using 150ng of the plant's genomic DNA, 35S forward (5'-TCCCACTATCCTTCGCAAGACCC-3') and reverse (5'-GCTGCTTAATTACTC CGTTCCAGC-3'). T2 lines, which showed a 3:1 segregation ratio after growing for two weeks in ½ MS agar containing 10 mg/l Basta, were regarded as homozygous transgenic lines, thus were left to grow inside the growth chamber until forming mature T3 seeds. Matured T3 seeds were then harvested and stored at 4°C under desiccation. Two independent homozygous transgenic lines, which are *35::OsCURT1A-5* and *35::OsCURT1A-8,* were selected for germination and physiological analysis.

Validation of atcurt1a T-DNA knockout mutant

Seeds of the Gabi-Kat (GK) mutants *atcurt1a* (AT4G01150) (catalogue no. GK-805B04) were obtained from the Nottingham Arabidopsis Stock Centre (NASC), UK. Genomic DNA was extracted from each knockout mutant using the standard CTAB method. PCR was carried out using 150 ng of genomic DNA with the following primers: forward (5'-AAATCGAAATTTTAGCGATGTACC-3'), reverse (5'-TCAACAGTTTACCTTAGG AGGACA-3'), and LB (5'-TTATATATGGAGGAGGAGCCATTG-3'). Knockout lines, which produced a single band with a product size of ~800 bp, were identified as homozygous knockout mutants, and therefore were left to grow in the growth chamber until forming mature seeds, which were then harvested and stored at 4°C. One independent homozygous knockout mutant, which is *atcurt1a*-1, was selected for germination and physiological analysis.

Plant materials for stress tolerance analysis

Seeds*35::OsCURT1A*, *atcurt1a,* and Col-0 (WT) were used during physiological assays. All Arabidopsis lines were germinated on ½ MS (control), ½ MS infused with PEG (ψ_w -0.7 MPa), and ½ MS containing 125 mM NaCl. PEG-infused (ψ_w -0.7 MPa) media were prepared using 40 % PEG-8000 ((ψ_w -0.7MPa), as described by Van der Weele et al (2000). As for the preparation of the 125 mM NaCl media, NaCl was added to the ½ MS media prior to autoclaving. Root length was measured across 30 biological replicates and experiments were repeated independently three times. Root length was estimated using ImageJ version 1.48. Fresh root weight was measured using a measuring balance by averaging 30 biological replicates. The data obtained were analysed by ANOVA using SAS 9.3.

Chlorophyll content

20 mg of shoots from all Arabidopsis lines (Col-0, *atcurt1a, 35S::OsCURT1A*) that were grown on ½ MS, ½ MS infused with PEG (ψ_w -0.7 MPa), and ½ MS containing 125 mM NaCl were selected. All samples were then transferred into a microcentrifuge tube containing 1 mL DMSO and were incubated for 30 min at 84°C. After 30min, the absorbance value of DMSO at 645 nm and 663 nm was determined using a Nanodrop UV-Vis spectrophotometer. The chlorophyll content of the Col-0, *atcurt1a,* and *35S::OsCURT1A* seedlings were calculated using Arnon's formula (Arnon., 1949). The data obtained were analysed by ANOVA using SAS 9.3.

Conclusion

Our work demonstrates the *in silico* and physiological characterisation of the *OsCURT1A* gene. From phylogenetic analysis, it has been demonstrated that this gene is well separated at the phylum level between higher plants and algae, but is strongly related to its homologue from algae. Promoter analysis indicates that the OsCURT1A protein maybe involved in abiotic stress due to the presence of an abiotic cis-element embedded in the promoter region, and this gene is negatively regulated during stress. In our mutant, the chlorophyll b content is high in its response to stress, however we cannot conclude the function of OsCURT1A in maintaining the chlorophyll b. Since the OsCURT1A has different subfamilies (class B, C, D), a single mutation gives very subtle changes, and more mutants including other CURT proteins should be considered to understand the interplay of different CURT proteins.

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Author contributions

Zamri Zainal and Bernadette Toni conceived and designed experiments. Bernadette Toni and Cheng Seng Tan performed the experiments. Zamri Zainal, Bernadette Toni, Nurulhikma Md Isa and Cheng Seng Tan analysed the data. Zamri Zainal, Bernadette Toni, Nurulhikma Md Isa, Cheng Seng Tan and Ismanizan Ismail wrote the manuscript.

Disclosure of potential conflicts of interest

The authors declare no conflict of interest due to the manuscript publication

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