DROUGHT TOLERANT GENE IDENTIFICATION BY RICE GENOME COMPARISON AND GENE CHARACTERIZATION IN ARABIDOPSIS PLANT MODEL

Miss Chutarat Punchkhon

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biotechnology
Common Course
FACULTY OF SCIENCE
Chulalongkorn University
Academic Year 2020

การระบุยีนทนแล้งโดยการเปรียบเทียบจีโนมข้าวและลักษณะสมบัติของยีนในพืชต้นแบบอะราบิดอปซิส

น.ส.จุฑารัตน์ ปัญจขันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ ไม่สังกัดภาควิชา/เทียบเท่า
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2563

Thesis Title	DROUGHT TOLERANT GENE IDENTIFICATION BY RICE GENOME COMPARISON AND GENE CHARACTERIZATION IN ARABIDOPSIS PLANT MODEL
By	Miss Chutarat Punchkhon
Field of Study	Biotechnology
Thesis Advisor	Professor SUPACHITRA CHADCHAWAN, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the FACULTY OF SCIENCE
(Professor POLKIT SANGVANICH, Ph.D.)

DISSERTATION COMMITTEE

..... Chairman
(Associate Professor PATTAMAWADEE YANATATSANEEJIT, Ph.D.)

..... Thesis Advisor
(Professor SUPACHITRA CHADCHAWAN, Ph.D.)

..... Examiner
(Associate Professor SEHANAT PRASONGSUK, Ph.D.)

..... Examiner
(Assistant Professor JUTHAMAS CHAIWANON, Ph.D.)

..... External Examiner
(Jonaliza L. Siangliw, Ph.D.)

จุฑารัตน์ ปัญจขันธ์ : การระบุยีนทนแล้งโดยการเปรียบเทียบจีโนมข้าวและลักษณะสมบัติของยีนในพืชต้นแบบอะราบิดอปซิส. (DROUGHT TOLERANT GENE IDENTIFICATION BY RICE GENOME COMPARISON AND GENE CHARACTERIZATION IN ARABIDOPSIS PLANT MODEL) อ.ที่ปรึกษาหลัก : ศ. ดร.ศุภจิตรา ชัชวาลย์

ความเครียดจากภาวะทางกายภาพโดยเฉพาะอย่างยิ่งความแล้งและความเค็ม ส่งผลต่อการเจริญเติบโตและผลิตผลของพืช ข้าวขาวดอกมะลิ 105 ข้าวขาวดอกมะลิ 105 เป็นข้าวที่ต้องเผชิญกับภาวะแล้งและภาวะเค็ม เนื่องจากเป็นข้าวที่ปลูกบนพื้นที่แห้งแล้งและดินเค็มทางภาคตะวันออกเฉียงเหนือของประเทศไทย เนื่องจากเป็นพื้นที่ที่การชลประทานไม่ทั่วถึง ข้าวสายพันธุ์ CSSL104 เป็นข้าวที่ได้รับการแทนที่บนบางส่วนของโครโมโซม (CSSLs) ที่ถูกพัฒนาขึ้นเพื่อให้ทนทานต่อสภาวะแล้ง โดยการแทนที่ชิ้นส่วน DT-QTL จากข้าวสายพันธุ์ DH103 ค่าการสังเคราะห์ด้วยแสงของสายพันธุ์ CSSL104 และสายพันธุ์พ่อแม่ (ขาวดอกมะลิ 105 และ DH103) ถูกศึกษาเมื่อปลูกในภาวะปกติและภาวะแล้ง ค่าการสังเคราะห์ด้วยแสงของทุกสายพันธุ์มีค่าเท่ากันในภาวะปกติ ในภาวะแล้งค่าการสังเคราะห์ด้วยแสงของทุกสายพันธุ์ลดลง แต่ CSSL104 และ DH103 มีค่าการสังเคราะห์ด้วยแสงสูงกว่าข้าวขาวดอกมะลิ 105 อย่างมีนัยสำคัญภายใต้ภาวะแล้ง อ้างอิงจากการเปรียบเทียบสนิปส์ระหว่าง CSSL104 กับข้าวขาวดอกมะลิ 105 และการศึกษาเครือข่ายการแสดงของร่วมของยีน พบว่ามี 9 ยีนที่เกี่ยวข้องกับการสังเคราะห์ด้วยแสง และ 6 ยีนจากทั้งหมดถูกนำมาศึกษาในสายพันธุ์อะราบิดอปซิสดัดแปลงพันธุ์ที่มีการกลายพันธุ์ที่มีลักษณะเป็น homologous ของยีนที่ถูกทำนายไว้ อะราบิดอปซิสดัดแปลงพันธุ์ที่มีการกลายพันธุ์ใน *LOC_Os01g72950*, *LOC_Os07g37550*, *LOC_Os07g38300* และ *LOC_Os10g10170* สามารถเจริญเติบโตได้ดีกว่าสายพันธุ์ปกติภายใต้ภาวะแล้ง และการแสดงออกของยีน *LOC_Os07g37550* และ *LOC_Os08g41460* ในข้าวขาวดอกมะลิสูงกว่า CSSL104 และ DH103 ในภาวะแล้ง ซึ่งสนับสนุนผลการศึกษาค่าความสามารถในการสังเคราะห์แสงที่ว่า ข้าวขาวดอกมะลิ 105 มีความสามารถในการทนแล้งต่ำกว่า CSSL104 และ DH103 โดยยีนที่กล่าวไปข้างต้นอาจเกี่ยวข้องกับความสามารถในการทนแล้งของข้าวสายพันธุ์ CSSL104

ยีนทนแล้งที่ถูกทำนายไว้จาก CSSL104 คือ *Ndh-O Lhcb3 Rrf Pgrl5-like LOC_Os09g39390 Mrl1* และ *LOC_Os01g68450* ที่ถูกทำนายว่าเป็นยีนทนเค็มจาก CSSL16 ถูกนำมาประเมินความเกี่ยวข้องกับการทนเค็มในสายพันธุ์อะราบิดอปซิสดัดแปลงพันธุ์ที่มีการกลายพันธุ์ที่มีลักษณะเป็น homologous ของยีนที่ถูกทำนายข้างต้น *At1g65230* เป็น orthologous ของ *LOC_Os01g68450* ในข้าว และอะราบิดอปซิสดัดแปลงพันธุ์ *at1g65230* มีการถูกยับยั้งการเจริญเติบโตสูงที่สุดภายใต้ภาวะเค็มเมื่อเปรียบเทียบกับสายพันธุ์อะราบิดอปซิสดัดแปลงพันธุ์อื่นๆ สายพันธุ์ revertant และ ectopic expression แสดงค่าการสังเคราะห์ด้วยแสงและปริมาณรงควัตุสูงภายใต้ภาวะเค็ม ผลเหล่านี้แสดงให้เห็นว่ายีน *LOC_Os01g68450* มีการทำงานเกี่ยวกับการรักษาปริมาณรงควัตุภายใต้ภาวะแล้งและภาวะเค็ม ซึ่งนำไปสู่การปรับปรุงการสังเคราะห์ด้วยแสงเมื่อได้รับภาวะแล้งและภาวะเค็ม

สาขาวิชา	เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต .....
ปีการศึกษา	2563	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5872806523 : MAJOR BIOTECHNOLOGY
KEYWORD: CSSLs, KDML105, Drought stress, Salt stress

Chutarat Punchkhon : DROUGHT TOLERANT GENE IDENTIFICATION BY RICE GENOME COMPARISON AND GENE CHARACTERIZATION IN ARABIDOPSIS PLANT MODEL. Advisor: Prof. SUPACHITRA CHADCHAWAN, Ph.D.

Abiotic stresses, especially drought and salt stresses can affect plant growth and productivity. 'Kao Dawk Mali 105' ('KDML105') rice is one of the crops which has to face both drought and salt stresses as it is grown in rain-fed saline soil in the northeastern region of Thailand, where the irrigation is limited. CSSL104, a chromosome substitution line with 'KDML105' genetic background, was developed in order to improve drought tolerant ability of 'KDML105' rice by transferring the drought tolerant (DT)-QTL region from double haploid line, DH103. Photosynthesis parameters of CSSL104 and its parental lines, 'KDML105' and DH103, were investigated, when they were grown in normal and drought-stress conditions. Net photosynthesis rates of all lines were similar in normal condition. In drought stress, net photosynthesis rate of all lines was declined, but CSSL104 and DH103 rice had the significant higher photosynthetic rate than 'KDML105' rice under drought-stressed condition. Based on the SNPs comparison between CSSL104 and 'KDML105' rice and gene co-expression network analysis, nine genes were involved in photosynthesis and six genes from those genes were used to study in Arabidopsis mutant lines containing the mutation in the homologous genes of the predicted ones. The mutant lines containing the mutation in *LOC_Os01g72950*, *LOC_Os07g37550*, *LOC_Os07g38300*, and *LOC_Os10g10170* showed better growth than wild type (WT) under drought stress. The expression of *LOC_Os07g37550* and *LOC_Os08g41460* in 'KDML105' was higher than the expression in CSSL104 and DH103 under drought stress. It supported the result of net photosynthesis that 'KDML105' was more susceptible to drought stress than CSSL104 and DH103. Therefore, it was proposed that these genes were involved in drought tolerant mechanism in CSSL104.

The predicted drought tolerance genes from CSSL104, *Ndh-O*, *Lhcb3*, *Rrf*, *Pgrl5-like*, *LOC_Os09g39390*, *Mrl1*, together with *LOC_Os01g68450*, the predicted salt tolerance gene from CSSL16 were evaluated for salt tolerance involvement by using the Arabidopsis mutant lines containing the mutation in the homologous genes of the predicted ones. *At1g65230* is orthologous to *LOC_Os01g68450* gene in rice and *at1g65230 Arabidopsis* mutant performed the highest growth inhibition under salt stress condition when compared to other mutant lines. The revertant and ectopic expression lines with *LOC_Os01g68450* gene show the higher growth parameters than the *at1g65230* mutant under drought and salt stress. Moreover, the ectopic expression lines showed high net photosynthesis and pigments content under salt stress. These suggested that *LOC_Os01g68450* gene has a function to maintain pigments contents under drought and salt stress leading to the photosynthesis adaptation during drought and salt stress.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my special thanks of gratitude to my dissertation advisors, Prof. Supachitra Chadchawan, for giving me the opportunity to do this research and always providing me the valuable guidance and kindly supporting in all of my PhD life i.e. scholarship, research funding, publications and this dissertation. I would like to express my sincere to Assoc. Prof. Dr. Teerapong Buaboocha, Assist. Prof. Dr. Kitiporn Plaimas for their guidance and suggesting. I would like to thank my dissertation committee, Assoc. Prof. Dr. Pattamawadee Yanatatsaneejit, Assoc. Prof. Dr. Sehanat Prasongsuk, Assist. Prof. Dr. Juthamas Chaiwanon and Dr. Jonaliza L. Siangliw for their help and suggestion.

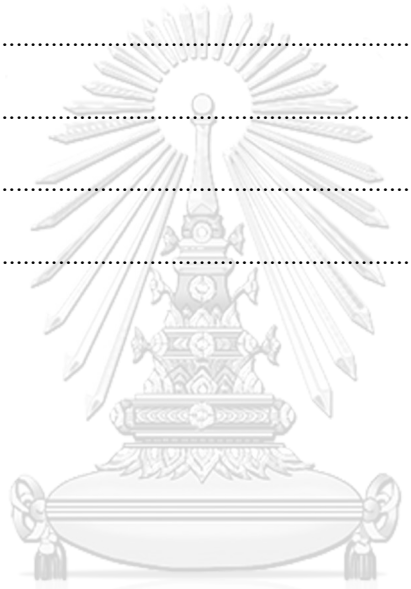
This dissertation was supported by The 100th Anniversary of Chulalongkorn University Doctoral Scholarship and 90th Anniversary of Chulalongkorn University Scholarship. And I would like to thank Innovative Plant Biotechnology and Precision Agriculture Research Team (APBT), BIOTEC for providing CSSL seeds for this research.

This dissertation could not be completed without the kind support of the members of Center of Excellence in Environmental and Plant Physiology to help me to take care of the plants and share the useful techniques in laboratory.

Finally, I would like to say thank you to my family, especially my parents who always give me their understanding and support throughout my study.

Chutarat Punchkhon

TABLE OF CONTENTS



LIST OF TABLE

LIST OF FIGURES

CONTENTS OF DISSERTATION

The contents of this dissertation start with the introduction which explains about rationales, objectives, scope of research and expected benefits of the dissertation. Then, it is followed by chapter II and chapter III, the publications of this dissertation which are separated into two parts:

1. Drought-tolerance gene identification using genome comparison and co-expression network analysis of chromosome substitution lines in rice
2. Role of *LOC_Os01g68450* in salt tolerance is mediated *via* the maintenance of the light-harvesting complex

The identification of drought tolerance gene in CSSL104, chromosome segment substitution line with DT-QTLs on chromosome 8 with 'KDML105' genetic background, is presented in the first publication. Then, the *LOC_Os01g68450*, the salt tolerance gene predicted from CSSL16, was studied with Arabidopsis mutant line, containing the mutation in the *LOC_Os01g68450* ortholog, *At1g65230* gene. The revertants with *LOC_Os01g68450* expression in the *at1g65230* mutant background and the ectopic expression lines with *LOC_Os01g68450* gene in WT were investigated for the stress response phenotypes.

Lastly, this dissertation shows the additional results, which include:

1. Gene expression of drought stress genes predicted by gene co-expression network analysis
2. Green area of Arabidopsis WT, *at1g65230* mutant and ectopic expression lines under drought stress conditions.

The first additional results show expression of *Ndh-O*, *Lhcb3*, *Rrf*, *Pgrl1* and *Mrl1* genes, the drought tolerant genes predicted in the first publication. The second

part of additional results presented the study of *LOC_Os01g68450* gene function in Arabidopsis under drought stress.

CHAPTER I

RATIONALES

Drought is an abiotic stress, affecting plant growth and productivity. Drought in term of plant physiology means the imbalance of water uptake from root and water loss from plant. When water potential in the soil is lower than water potential in roots, the roots cannot absorb water from the soil and lead to water deficiency. Moreover, drought can occur by transpiration and evaporation in the air. Global warming triggers drought stress. The drought area increases about 2 percent of farmland every year. It decreases productivity of crop yields by 1-2 percent per year during 1950 - 2009. Nowadays, the drought affects 5% reduction of global crop yields every year, and the reduction percentage is increasing every year (Lesk and Anderson, 2021). The severity of drought stress to plants is up to the duration of plant exposure to drought and the response of the plants after recovery (Salehi-Lisar and Bakhshayeshan-Agdam, 2016).

Drought stress can change morphological, physiological, biochemical and molecular mechanisms of plants. It reduces growth, development and yield quality, especially crop plants. Cell division, cell elongation and cell differentiation are the most important steps in plant growth and establishment. Drought stress inhibits seed germination, seedling growth, length of hypocotyl, shoot dry weight and root dry weight (Fahad et al., 2017; Hussain et al., 2013). When drought stress is developed, it causes the dehydration in plant cells, leading to the loss of turgor pressure. The cell expansion and cell elongation are inhibited and result in the limit of plant growth. Leaf size and number of stomata are decreased under drought stress. Cell wall thickening, leaf senescence and root to shoot ratios are increased during drought stress (Farooq et al., 2009; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). There are many research about the reduction of plants growth and yields under drought stress

including wheat (*Triticum aestivum* L.) (Dhanda et al., 2004; Saqib et al., 2013), barley (*Hordeum vulgare* L.) (Blum, 1989; Pham et al., 2019), alfafa (*Medicago sativa* L.) (Mouradi et al., 2016) and rice (*Oryza sativa* L.) (Swapna and Shylaraj, 2017). Plants protect themselves via osmotic adjustment by accumulation of osmolytes such as proline, glycine betaine and sugars to maintain osmotic potential and turgor pressure (Verbruggen and Hermans, 2008)

Photosynthesis is very important mechanism of plants. It is very sensitive to drought stress. When plant exposed to drought stress, stomatal closure is the initial response of plant to stress to prevent water loss from leaf cell by transpiration and evaporation (Marchin et al., 2020; Mukarram et al., 2021). Stomatal closure decreases carbon dioxide absorption and leads to carbon starvation and photosynthesis rate reduction (Sharma et al., 2020). Moreover, the decrease of leaf expansion, leaf area and stomatal conductance can limit CO_2 uptake (Cornic and Massacci, 1996). Under drought stress, activity of Calvin cycle is decreased and results in the decline of $NADP^+$ regeneration (Hajiboland, 2014). Low concentration of carbon affects change of electron transfer chain at photosystem I. The excess electrons transfer to oxygen instead of $NADP^+$ and generate reactive oxygen species (ROS) such as singlet oxygen, superoxide radical, hydrogen peroxide and hydroxyl radical (Reddy et al., 2004). The ROS cause oxidative stress which is the main factor that damages plant macromolecules (Munir, 2018). ROS scavenging enzymes, such as superoxide dismutase, glutathione peroxidase and glutathione reductase, are activated to prevent oxidative damage (Wang et al., 2019). Chlorophyll *a* content and chlorophyll *b* content are reduced by oxidative stress and result in the decrease of light absorption and maximum photosynthesis rate (Anjum et al., 2003; Mafakheri et al., 2010).

Linear electron flow (LEF) is the normal pathway to transfer electrons from water to $NADP^+$ which can produce ATP and NADPH. But cyclic electron flow (CEF) transfers electrons from ferredoxin (Fd) back to plastoquinone (PQ) and produces

only ATP (Suorsa, 2015). Drought stimulates CEF as the mechanism to minimize the ROS and protect photosystems under carbon dioxide limitation (Golding and Johnson, 2003; Johnson, 2011).

Drought affects molecular response in plant cells. Plants have complex mechanisms of gene regulation to response to drought stress (Singh et al., 2020). Plants respond to drought stress differently *via* expression pattern of genes and proteins. There are many researches about expression profiling in many various plant species under drought stress such as rice (*Oryza sativa* L.) (Degenkolbe et al., 2009), soybean (*Glycine max* (L.) Merr.) (Fan et al., 2013), sugarcane (*Saccharum* sp.) (Devi et al., 2019) and sorghum (*Sorghum bicolor* L. Moench) (Abou-Elwafa and Shehzad, 2018)

Rice is the first genome sequenced cereal crop plant, meaning that rice has many genomic resources on public databases. The expression profiling study showed that 413 genes were up-regulated and 249 genes were down-regulated under drought stress. The numbers of gene expression were more significant regulated in sensitive cultivars than tolerant cultivars (Degenkolbe et al., 2009). The genes involved in photosynthesis and water use were more downregulated in the tolerant cultivars than the sensitive ones, while genes involved in degradation processed were induced in the sensitive cultivars more than the tolerant cultivars (Ding et al., 2013).

In Thailand, rice is the major exported product, which is exported about more than five million tons per year. ‘Khao Dawk Mali 105 (KDML105)’ rice is a special rice variety, because of the scent and texture of rice seeds. ‘KDML105’ rice is normally grown in the rain-fed farms in the northeastern of Thailand where the irrigation is limited (Siangliw et al., 2007). Therefore, ‘KDML105’ rice must face drought stress which leads to the reduction of growth and yield.

Chromosome segment substitution lines (CSSLs) were developed (Kanjoo et al., 2011). CT9993 rice with good rooting system and IR62266 rice with high osmotic adjustment ability, were used to generate double haploid lines. The double haploid lines were selected by evaluation of yield, yield components and morpho-physiological characters under drought stress condition with DT-QTLs controlling traits. DH103 and DH212, which carried CT9993 alleles on chromosome 1, 3, 4 and 9 and IR62266 alleles on chromosome 8, were selected to be donors in CSSL development. 'KDML105' rice was used as a recipient. The selected lines with the putative drought tolerant regions on chromosomes 1, 3, 4, 8 or 9 were backcrossed with 'KDML105' rice for 5 generations to generate the putative drought tolerant CSSL with 'KDML105' rice genetic background. The CSSLs with the putative drought tolerant genes from chromosome 8 obtained from DH103 (Siangliw et al., 2007) were used in this study.

CSSL104 was selected for this research according to the drought tolerant phenotypes, such as higher relative water content, higher chlorophyll fluorescence (F_v/F_m) and lower leaf drying score under drought stress condition at 50% field capacity, when compared to 'KDML105' rice (Punchkhon et al., 2015). This implied that the putative region from DH103 carried the drought tolerant genes to CSSL104.

To investigate the mechanisms affected by the introgression of DT-QTL from DH103, the physiological responses to drought stress of CSSL104 was evaluated in comparison with 'KDML105' rice and DH103 donor line. The drought tolerant gene was predicted by genomic sequencing, followed by co-expression network analysis. The putative drought tolerant gene was validated using Arabidopsis mutant line(s) containing the knock-out or knock-down mutation in the homologous gene of the predicted rice gene.

OBJECTIVES

1. To study the photosynthesis rate of CSSL104 compared to parental lines, 'KDML105' and DH103 under drought stress
2. To compare the genome sequence of drought tolerant CSSL rice line (CSSL104) with background genome
3. To identify and characterize drought and salt tolerant gene in rice by using homologous gene in the Arabidopsis plant model

SCOPE OF RESEARCH

CSSL14 rice, a chromosome segment substitution line containing segment of chromosome 8 from DH103 line with genetic background of 'KDML105' rice and 'KDML105' were used as the materials for high-throughput genome sequencing. The whole genome sequence comparison between CSSL14 and 'KDML105' rice was performed to locate the different regions in the genome. Co-expression network analysis of the genes located in the regions from DH103 was performed to predict the important genes involve in drought tolerance. Moreover, the predicted genes from CSSL104 and *LOC_Os01g68450*, the predicted gene responsible for salt tolerance from CSSL16 (Chutimanukul et al., 2018), were investigated under drought- and salt- stress conditions using the Arabidopsis mutant lines.

The complementation with the rice gene expression in the selected Arabidopsis mutant line was performed to verify the equivalence of the gene function in both species. Phenotyping of the transgenic plants with knocked-out mutation or putative gene over-expression under normal and drought conditions was used to evaluate the role of the selected gene.

EXPECTED BENEFITS

This research can identify and characterize the important gene involve in abiotic stress tolerance mechanism in CSSL rice including drought stress and salt stress, which can be used to screen drought and salt tolerance regulated in the genome for improving abiotic stress tolerance rice. It may benefit for breeders to generate and select drought and salt tolerance rice in the future.

CHAPTER II

RESEARCH ARTICLES

Drought-tolerance gene identification using genome comparison and co-expression network analysis of chromosome substitution lines in rice

Chutarat Punchkhon [1,2], Kitiporn Plaimas [3,4] , Teerapong Buaboocha [4,5], Jonaliza L. Siangliw [6], Theerayut Toojinda [6], Luca Comai [7], Nuria De Diego [8] , Lukáš Spíchal [8] and Supachitra Chadchawan [2,5,*]

[1] Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10300, Thailand; chutarat_tom@hotmail.com

[2] Center of Excellence in Environment and Plant Physiology, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10300, Thailand

[3] Department of Mathematics and Computer Science, Faculty of Science, Chulalongkorn University, Bangkok 10300, Thailand; kitiporn.p@chula.ac.th

[4] Omics Science and Bioinformatics Center, Faculty of Science, Chulalongkorn University, Bangkok 10300, Thailand; Teerapong.b@chula.ac.th

[5] Molecular Crop Research Unit, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10300, Thailand

[6] National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Phahonyothin Rd. Khlong Nueng, Khlong Luang, Pathumthani 12120, Thailand; jsiangliw@gmail.com (J.L.S.); theerayut@biotec.or.th (T.T.)

[7] Genome Center and Department of Plant Biology, UC Davis Genome Center, UC Davis, Davis, CA 95616, USA; lcomai@ucdavis.edu

[8] Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 27, CZ-783 71 Olomouc, Czech Republic; nuria.de@upol.cz (N.D.D.); lukas.spichal@upol.cz (L.S.)

* Correspondence: Supachitra.c@chula.ac.th or s_chadchawan@hotmail.com

Abstract: Drought stress limits plant growth and productivity. It triggers many responses by inducing changes in plant morphology and physiology. KDML105 rice is a key rice variety in Thailand and is normally grown in the northeastern part of the country. The chromosome segment substitution lines (CSSLs) were developed by transferring putative drought tolerance loci (QTLs) on chromosome 1, 3, 4, 8, or 9 into the KDML105 rice genome. CSSL104 is a drought-tolerant line with higher net photosynthesis and leaf water potential than KDML105 rice. The analysis of CSSL104 gene regulation identified the loci associated with these traits via gene co-expression network analysis. Most of the predicted genes are involved in the photosynthesis process. These genes are also conserved in *Arabidopsis thaliana*. Seven genes encoding chloroplast proteins were selected for further analysis through characterization of *Arabidopsis* tagged mutants. The response of these mutants to drought stress was analyzed daily for seven days after treatment by scoring green tissue areas via the PlantScreen™ XYZ system. Mutation of these genes affected green areas of the plant and stability index under drought stress, suggesting their involvement in drought tolerance.

Keywords: CSSLs; drought stress; ‘KDML105’ rice; co-expression network

1. Introduction

Rice (Oryza sativa L.) is one of the important cereal crops of the world [1]. In Thailand, rice is the major agricultural export, especially Khao Dawk Mali 105 (KDML105) rice. The cooked kernels of KDML105 rice have a highly prized scent and texture [2]. KDML105 rice is normally grown in the northeast of Thailand, based on rain with limited irrigation [3]. Therefore, it is always affected by drought stress, leading to the reduction in growth and yield.

Drought stress affects plant morphology, physiology, and molecular mechanisms. Upon drought stress, cell turgor pressure is decreased due to low water potential in cells. This causes a decrease in the relative water content, leaf water potential, stomatal conductance, and transpiration rate [4]. Cell expansion and elongation are inhibited, resulting in the reduction of plant height, leaf area, growth, and yield [5]. Photosynthesis is one of the important physiological mechanisms affected by drought stress. The decrease in leaf expansion, leaf area, and stomatal conductance limits CO_2 uptake [6]. The photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids) can also be damaged by drought stress, resulting in their degradation and decreased light absorption and maximum photosynthetic rate [7]. During drought stress, phosphoenolpyruvate carboxylase, nicotinamide adenine dinucleotide phosphate-malic enzyme, Rubisco, fructose-1,6-bisphosphatase, and pyruvate orthophosphate dikinase activities are decreased, which can reduce the photosynthetic and electron transport rate [8]. The physiological responses to drought tolerance include osmotic adjustment, osmoprotection, antioxidation, scavenging defense, and photorespiration [9,10].

Kanjoo et al. [11] developed chromosome segment substitution lines (CSSLs) in the background of variety KDML105. CT9993, a variety with a good rooting system, and IR62266, a variety with high osmotic adjustment ability, were hybridized, and their F1 was used to generate double haploid lines. The double haploid lines were evaluated for yield, yield components, and morpho-physiological characters under

drought-stress conditions, defining drought-tolerant quantitative trait loci (DT-QTLs) on chromosomes 1, 3, 4, 8 and 9. The doubled haploid line DH212 carries CT9993 alleles on in all chromosomes, while DH103 has IR62266 alleles on chromosome 8. These lines were selected as donor lines for CSSL development. Repeated crossing to KDML105 resulted in CSSLs with the putative drought-tolerant genes from chromosome 8, donated by DH103, and the CSSLs with the DT-QTL from chromosome, 1, 3, 4 and 9, donated by DH212 [3].

CSSL104 is a drought-tolerant KDML105 CSSL carrying the chromosome 8 introgression from inbred DH103 [11]. Compared to KDML105, CSSL104 had higher relative water content, higher chlorophyll fluorescence (Fv/Fm), and lower leaf drying score under 50% field capacity drought-stress conditions [12]. This implied that the region introgressed from DH103 carried the putative drought-tolerant genes.

To investigate the mechanisms affected by the introgression of DH103 genes, the physiological responses to drought stress of CSSL104 were evaluated relative to KDML105 and the DH103 donor line. Then, drought-tolerance genes were predicted based on genomic sequence comparison and co-expression network analysis. Finally, the putative drought-tolerance genes were validated using the corresponding *Arabidopsis* mutants. This study will be beneficial to the future development of drought-tolerant rice.

2. Materials and methods

2.1. Plant materials

We used CSSLs with the genetic background of KDML105 rice and containing a putative drought-tolerance segment of chromosome 8 from DH103 between markers RM5353 and RM3480 [11]. These are CSSL97, CSSL104, CSSL106, CSSL107, and parental lines (KDML105 and DH103). They were used to study drought-stress responses. All rice seeds were provided by the Innovative Plant Biotechnology and

Precision Agriculture Research Team (APBT) at the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand.

2.2. Evaluation of physiological responses at vegetative stage under drought-stress conditions

2.2.1. Rice growth condition

KDML105 rice, DH103, and CSSL seeds were incubated at 60 ℃ for 48 h before planting. The seeds were then germinated by soaking in distilled water for seven days in plastic cups. Rice seedlings were transferred to a plastic tray and continuously grown in WP nutrient solution [13]. Twenty-eight days after germination, rice plants were drought-stressed for three days by the addition of 10% polyethylene glycol 6000 (PEG6000). This condition was previously shown to cause drought stress in rice [14,15]. In order to induce the stronger drought-stress condition, after treatment with WP nutrient solution with 10% PEG6000 for three days, the solution was then changed to WP nutrient solution with 15% PEG. Plants grown in WP nutrient solution without PEG6000 were used as controls. A complete randomized design (CRD) with four replicates was used for physiological evaluation in each parameter.

2.2.2. Net photosynthesis rate and leaf water status detection

The net photosynthesis rate (Pn) of twenty-eight-day-old rice plants was determined with a LI-6400 XT portable photosynthesis system (LI-COR, Lincoln, NE, USA). The measurement was taken at the middle part of the youngest fully expanded leaves between 9 am and 2 pm, with the following conditions: the molar flow of air per unit leaf area was 500 mmol l^{-1} m^{-2} s^{-1}, the photosynthetically active radiation (PAR) at the leaf surface was 1200 μmol m^{-2} s^{-1}, the leaf temperature

ranged from 30.0 to 37.0 °C, with a CO_2 concentration of 380.0 mol mol^{-1}. The leaf water potential (LWP) was measured in the youngest fully expanded leaves using plant water status console model 3005 (Soilmoisture, Goleta, CA, USA).

2.3. Identification of drought-tolerance gene

2.3.1. Whole genome sequencing

The aboveground parts of KDML105, DH103, and CSSL104 rice plants were collected at fourteen days after germination. Rice genomic DNA was extracted using a Genomic DNA Mini Kit, 'Plant' (Geneaid, New Taipei City, Taiwan). Genomic DNA libraries were prepared for sequencing by using an Illumina genome analyzer (Illumina, San Diego, CA, USA) with Illumina HiSeq3000's protocol. For genome analyses, the sequence reads were classified into specific categories using the pipeline developed by Missirian et al. [16]. The rice genomic sequence from the Rice Genome Annotation Project database [17] was used as a reference genome [18] to map the sequence reads. The raw reads were submitted to GenBank at the NCBI under BioProject no. PRJNA659381. Bioinformatic tools were used to compare the genome of CSSL104 with the KDML105 rice genome to identify loci containing different single nucleotide polymorphisms (SNPs). These loci may contribute to the drought-tolerance phenotypes of CSSL104. The genome comparison first started by discarding all SNPs shared by both CSSL104 and KDML105. The remaining differential SNPs were counted within a sliding window of 5000 background nucleotides. To visualize the chromosome plots with the marks of different SNPs' loci, the window region containing more than 100 SNPs in CSSL104 with different nucleotides from KDML105 was marked as a blue line on the chromosome plots. The analysis of the locations of SNPs in the candidate genes was analyzed.

2.3.2. Gene co-expression network analysis

The rice loci containing the different SNPs were used for a gene co-expression network analysis. To predict the important loci involved in drought tolerance, a rice oligonucleotide array database was used with abiotic stress-induced gene expression data with a correlation coefficient cut-off 0.95 [19]. The predicted loci were searched for gene ontology and expression patterns from the rice expression database [20].

2.4. Identification of drought-tolerance gene function in *Arabidopsis*

2.4.1. *Arabidopsis* homologous gene

The best candidate genes were used to search for the homologous gene in *Arabidopsis* from the Rice Genome Annotation Project database [17] and The *Arabidopsis* Information Resource [19]. *Arabidopsis* mutant lines with T-DNA insertion in the selected gene were ordered from the *Arabidopsis* Biological Resource Center (ABRC). The *Arabidopsis* seeds were screened for homozygous mutant lines via specific primers, LP and RP, to the gene of interest; the LB primer was used for a specific T-DNA region.

2.4.2. *Arabidopsis* growth condition

Four days after germination, *Arabidopsis thaliana* ecotype Col-0 seeds and seven mutant lines [21] including at1g74880, at5g54270, at3g63190, at4g11960, at4g22890, at2g27680, and at4g34830 were sowed and transferred to 48-well plates, containing Murashige and Skoog (MS) agar media for normal conditions, MS agar media supplemented with 75 mM mannitol for mild drought stress, or MS agar media supplemented with 150 mM mannitol for severe drought stress. The plants were then grown in a growth chamber at 22 ℃/20 ℃, 16/8 h light/dark cycle, 120 μmol photons of PAR m^{-2} s^{-1}, and 60% humidity. RGB imaging was used to collect the green

area of plants twice a day via a PlantScreen™ XYZ system (Photon Systems Instruments, Drásov, Czech Republic) [22].

2.5. Statistical analysis

Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were used for data analysis by SPSS Statistics program version 22 (IBM, Armonk, NY, USA). The images from the PlantScreen™ XYZ system were analyzed using MATLAB (R2015; MathWorks, Inc., Natick, MA, USA), and the data were analyzed by independent t-tests using SPSS.

3. Results

3.1. Evaluation of physiological responses of CSSLs of KDML105 under drought-stress conditions

Selected CSSLs, namely CSSL97, CSSL104, CSSL106, and CSSL107, were evaluated for drought tolerance by growing the seedlings in soil with 100% or 50% field capacity. In normal growth conditions (100% field capacity), all of the lines were similar (Figure 1A,C), but they differed under 50% field capacity (Figure 1B,D). CSSL104 displayed the most drought-tolerant phenotype, with the lowest leaf dying score and the highest photosystem II (PSII) efficiency (Fv/Fm). This was similar to the performance of DH103 (the drought-tolerant parental line). The highest leaf-death score was detected in CSSL106, while CSSL97 had the lowest PSII efficiency under drought stress. These data suggest that CSSL104 is the most drought-tolerant line, while CSSL97 and CSSL106 are the most susceptible. Therefore, CSSL104 was selected for further characterization.

CSSL104 and its parental lines, KDML105 and DH103 rice, were grown in nutrient solutions corresponding to normal growth conditions and in a solution

supplemented with 15% PEG for the drought-stress treatment, which caused about a 50–180% reduction in leaf water potential (Table 1). After nine days of drought stress, we measured the highest reduction of leaf water potential for all lines. Under these conditions, CSSL104 had the lowest leaf water potential at -7.75 MPa, which was about threefold lower than the LWP of the plants grown in normal conditions. The parental lines, KDML105 and DH103, had about a twofold reduction in LWP. Drought stress reduced all parameters of photosynthesis, including net photosynthesis rate, stomatal conductance, transpiration rate, intercellular CO_2 concentration, ϕPSII, and electron transport rate in all lines. However, after six days under drought stress, CSSL104 had a significantly higher photosynthetic rate than the KDML105 parent. In addition, CSSL104 had a greater tendency than KDML105 toward higher values for all photosynthetic parameters after nine days of drought treatment (Table 1).

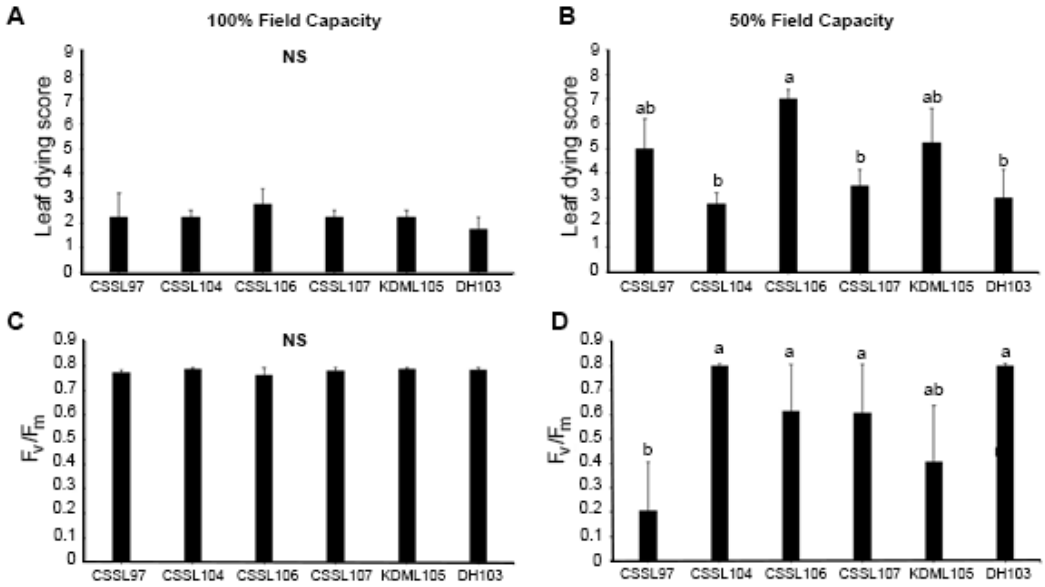


Figure 1. Response to drought stress in chromosome segment substitution lines (CSSLs) and parents. CSSLs, CSSL97, CSSL104, CSSL106, and CSSL107, and their parental lines KDML105 and DH103, were compared for leaf death (leaf dying score) and photosystem II efficiency (Fv/Fm) under (**A**,**C**) normal (100% field capacity) and (**B**,**D**) drought-stress (50% field capacity) conditions. The mean ± standard error (SE) was derived from four replicates. Means with a different lowercase letter above them are significantly different ($p < 0.05$). NS demonstrates no significant difference among lines.

Table 1. Photosynthetic performance of CSSL104 and parent lines. Net photosynthesis rate, transpiration rate, stomatal conductance, ϕPSII, electron transport rate, intercellular CO_2 concentration, F_v'/F_m', relative chlorophyll content (determined by portable chlorophyll meter SPAD-501) and leaf water potential of rice at vegetative stage in normal and drought stress conditions. ANOVA and Duncan's Multiple Range Test (DMRT) were used for statistical analysis. The data show mean ± SE. Different superscript letters show the significant difference among lines at *p* value ≤ 0.05.

Condition	Normal (0% PEG)				Drought stress (15% PEG)			
Timing (days after stress)	0	3	6	9	0	3	6	9
Net photosynthesis rate ($\mu mol.m^{-2}.s^{-1}$)								
KDML105	19.44 ± 4.46	14.50 ± 0.81	17.85 ± 1.85	20.17 ± 0.88	19.44 ± 4.46	10.30 ± 1.74	5.51 ± 0.42^{b}	8.64 ± 1.11
DH103	14.47 ± 2.31	17.45 ± 1.21	18.92 ± 1.53	21.35 ± 2.02	14.47 ± 2.31	10.67 ± 0.38	10.20 ± 1.23^{a}	12.35 ± 0.59
CSSL104	15.12 ± 1.28	16.74 ± 1.10	18.47 ± 1.61	15.76 ± 0.87	15.12 ± 1.28	11.31 ± 1.88	10.31 ± 1.33^{a}	9.38 ± 1.79
Transpiration rate ($mmol.m^{-2}.s^{-1}$)								
KDML105	5.26 ± 0.56	4.86 ± 0.44	0.23 ± 0.01	5.10 ± 0.39^{b}	5.26 ± 0.56	2.42 ± 0.14	0.18 ± 0.02	1.87 ± 0.26^{b}
DH103	4.99 ±0.48	5.87 ± 0.12	0.26 ± 0.02	6.77 ± 0.71^{a}	4.99 ±0.48	2.65 ± 0.24	0.17 ± 0.02	3.33 ± 0.10^{a}
CSSL104	4.54 ± 0.28	4.99 ± 0.34	0.25 ± 0.01	4.48 ± 0.27^{b}	4.54 ± 0.28	2.57 ± 0.32	0.17 ± 0.02	2.16 ± 0.34^{b}
Stomatal conductance ($mol.m^{-2}.s^{-1}$)								
KDML105	0.35 ± 0.09	0.32 ± 0.05	0.32 ± 0.06^{b}	0.33 ± 0.04^{b}	0.35 ± 0.09	0.12 ± 0.01	0.08 ± 0.01	0.09 ± 0.01^{b}
DH103	0.43 ± 0.05	0.44 ± 0.02	0.44 ± 0.03^{a}	0.53 ± 0.07^{a}	0.43 ± 0.05	0.12 ± 0.01	0.19 ± 0.04	0.17 ± 0.01^{a}
CSSL104	0.36 ± 0.03	0.33 ± 0.06	0.33 ± 0.06^{b}	0.28 ± 0.02^{b}	0.36 ± 0.03	0.15 ± 0.02	0.11 ± 0.02	0.10 ± 0.02^{b}

Condition	Normal (0% PEG)				Drought stress (15% PEG)			
Timing (days after stress)	0	3	6	9	0	3	6	9
ϕPS2								
KDML105	0.25 ± 0.00	0.23 ± 0.01	0.23 ± 0.01	0.23 ± 0.01^{b}	0.25 ± 0.00	0.20 ± 0.01	0.18 ± 0.02	0.15 ± 0.01
DH103	0.22 ± 0.02	0.25 ± 0.02	0.26 ± 0.02	0.29 ± 0.01^{a}	0.22 ± 0.02	0.21 ± 0.03	0.17 ± 0.02	0.18 ± 0.02
CSSL104	0.24 ± 0.01	0.21 ± 0.01	0.25 ± 0.01	0.22 ± 0.00^{b}	0.24 ± 0.01	0.21 ± 0.01	0.17 ± 0.02	0.17 ± 0.01
Electron transport rate								
KDML105	163.02 ± 2.37	149.08 ± 9.83	153.51 ± 4.96	152.38 ± 3.91^{b}	163.02 ± 2.37	130.73 ± 4.36	117.25 ± 10.71	100.65 ± 8.13
DH103	143.72 ± 10.16	163.29 ± 11.08	168.02 ± 11.52	188.68 ± 4.99^{a}	143.72 ± 10.16	139.94 ± 16.65	110.58 ± 11.43	118.43 ± 10.95
CSSL104	155.86 ± 6.09	141.86 ± 7.08	161.10 ± 6.45	144.28 ± 2.79^{b}	155.86 ± 6.09	133.31 ± 11.19	113.12 ± 12.52	112.18 ± 8.52
Intercellular CO_2 concentration (μmol.mol^{-1})								
KDML105	327.27 ± 5.37	305.04 ± 11.48	282.47 ± 14.96^{b}	282.98 ± 10.67	327.27 ± 5.37	255.39 ± 18.30	269.74 ± 18.42	215.42 ± 21.55^{a}
DH103	330.97 ± 5.82	318.58 ± 3.45	312.79 ± 8.84^{a}	313.36 ± 8.12	330.97 ± 5.82	260.64 ± 19.39	286.68 ± 18.62	265.87 ± 8.18^{b}
CSSL104	317.29 ± 2.12	294.48 ± 11.18	281.92 ± 13.68^{b}	289.50 ± 9.46	317.29 ± 2.12	241.03 ± 14.35	227.89 ± 18.71	237.02 ± 13.81^{ab}
F_v'/F_m'								
KDML105	0.54 ± 0.01^{b}	0.53 ± 0.01	0.50 ± 0.01	0.50 ± 0.02	0.54 ± 0.01^{b}	0.50 ± 0.01	0.48 ± 0.01	0.45 ± 0.02
DH103	0.63 ± 0.02^{a}	0.60 ± 0.03	0.55 ± 0.02	0.52 ± 0.01	0.63 ± 0.02^{a}	0.53 ± 0.04	0.53 ± 0.08	0.47 ± 0.03
CSSL104	0.53 ± 0.01^{b}	0.54 ± 0.01	0.53 ± 0.03	0.52 ± 0.01	0.53 ± 0.01^{b}	0.52 ± 0.03	0.48 ± 0.01	0.43 ± 0.02
SPAD								
KDML105	35.93 ± 0.41	36.63 ± 0.95	36.40 ± 0.81	38.30 ± 1.05	35.93 ± 0.41	37.58 ± 0.40	37.10 ± 0.57	34.20 ± 1.02
DH103	34.43 ± 0.43	35.78 ± 0.21	36.55 ± 0.34	38.15 ± 0.95	34.43 ± 0.43	33.25 ± 1.12	32.58 ± 2.18	34.38 ± 0.97
CSSL104	34.90 ± 0.65	35.70 ± 0.39	38.65 ± 1.70	39.00 ± 0.38	34.90 ± 0.65	35.93 ± 1.06	36.25 ± 0.71	34.48 ± 1.21

Condition	Normal (0% PEG)				Drought stress (15% PEG)			
Timing (days after stress)	0	3	6	9	0	3	6	9
Leaf water potential (MPa)								
KDML105	-1.20 ± 0.14	-2.70 ± 0.77	-5.15 ± 1.08	-2.90 ± 0.26	-1.60 ± 0.08	-4.05 ± 0.48	-9.00 ± 1.00	-6.85 ± 0.43
DH103	-1.35 ± 0.10	-2.60 ± 0.54	-3.90 ± 0.33	-3.25 ± 0.10	-2.05 ± 0.32	-4.60 ± 0.50	-7.00 ± 0.38	-6.95 ± 1.03
CSSL104	-1.20 ± 0.14	-1.95 ± 0.15	-4.20 ± 1.29	-2.70 ± 0.13	-1.65 ± 0.05	-4.08 ± 0.31	-7.10 ± 1.04	-7.75 ± 1.50

3.2. Whole genome sequence comparison between CSSL104 and KDML105 and co-expression network analysis revealed that major hub genes have a role in photosynthesis

We compared whole genome sequences of CSSL104 and its drought susceptible parental line KDML105 to define the genes responsible for drought tolerance in CSSL104. A total of 101,950 SNPs located on 3440 genes were detected. The regions with a high density of SNPs were on chromosomes 1, 8, 9, and 11 (Figure 2).

The eighteen rice genes reported here have homologs in *Arabidopsis* for which tagged mutants are available (Table 2). Nine of them (*CPFTSY*, *NDH-O*, *SOQ1*, *LHCB3*, *RRF*, *PGRL1B*, *HCF244*, *NAD(P)-linked oxidoreductase*, and *MRL1*) were annotated to be involved in the photosynthesis process [24–32]. Moreover, the homolog of *LOC_Os11g43600* is *CPRF1*, an *Arabidopsis* gene required for chloroplast development [33]. These findings suggest that these rice genes are involved in photosynthesis adaptation during drought stress.

Therefore, we obtained seven homozygous, T-DNA tagged *Arabidopsis* mutant lines corresponding to *ndhO* (*at1g74880*), *lhcb3* (*at5g54270*), *rrf* (*at3g63190*), *pgrl1b* (*at4g11960*), *pgrl1a* (*at4g22890*), *at2g27680*, and *mrl1* (*at4g34830*). These lines were drought stressed by growing them in MS medium supplemented with 0 mM, 75 mM, or 150 mM mannitol. Their growth response was assessed by measuring the green pixel area per plant and compared to the wild type (WT).

Under normal conditions, all mutant lines displayed a significantly lower number of green pixels than WT, suggesting lower growth than WT (Figure 4A). Both drought-stress treatments decreased growth in all lines, with the 150mMcausing the more severe reduction. At 75mMmannitol, *pgrl1b* had a significantly lower number of green pixels than WT, while *pgrl1a* showed similar growth to WT. Other mutant lines showed better growth than WT (Figure 4B). Under the severe drought-stress

conditions induced with 150 mM mannitol, the growth of *lhcb3*, *at2g27680*, *mrl1*, and WT were similar. Mutants *pgrl1b* and *pgrl1a* had a significantly lower growth than WT, while *rrf* had a lower growth at the beginning of the treatment but displayed better growth than WT after 5 days of the treatment. However, similar growth between WT and *rrf* was found after seven days of drought stress. Among the mutant lines, *ndhO* was the only mutant line that had significantly better growth than WT under severe drought stress (Figure 4C).

The stability indexes of Arabidopsis mutants and WT were calculated to compare drought tolerance after six days of drought stress. After the intermediate drought stress (75 mM mannitol), all mutant lines except *pgrl1a* displayed significantly higher stability than WT, suggesting the contribution of *NDH-O*, *LHCB3*, *RRF*, *PGRL1b*, *at2g27680*, and *MRL1* to drought-tolerance adaptation (Figure 5A). Under severe drought (150 mM mannitol), significantly higher stability than WTs was detected for the *ndh-o*, *rrf*, *pgrl1b*, *at2g27680*, and *mrl1* mutants (Figure 5B). The *rrf* mutant line displayed the highest stability under both intermediate and severe drought-stress conditions.

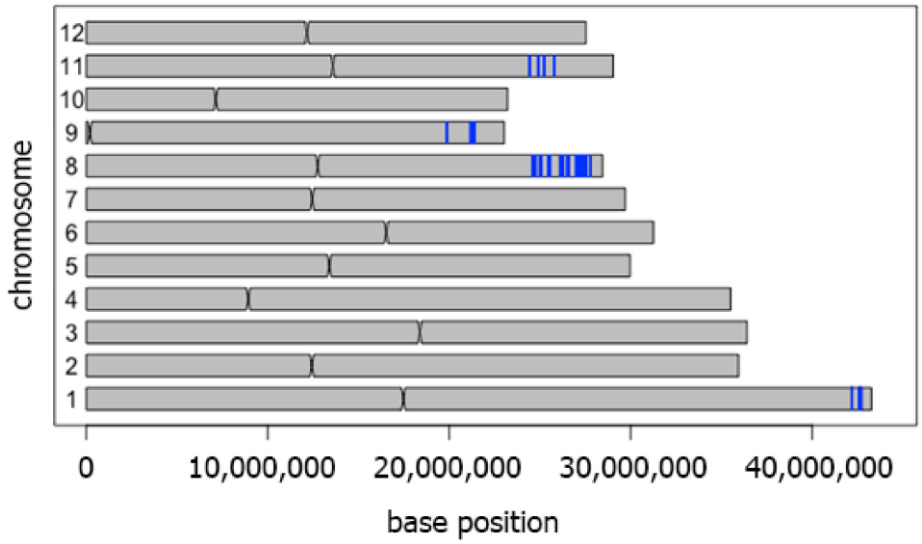


Figure 2. Genetic regions introgressed into the KDML105 genome. Single nucleotide polymorphisms (SNPs) between CSSL104 and KDML105 rice. The blue lines show 100 SNPs within 5000 background nucleotides. All loci containing SNPs were subjected to a co-expression network analysis. The results, shown in Figure 3A, revealed 18 major nodes with a high connection to other genes. The gene ontology of these 18 genes is listed in Table 2. The map position of these genes is shown in Figure 3B. Based on quantitative trait loci (QTL) data from the Qtaro database [23], six loci are located in QTL regions for drought-stress tolerance on chromosomes 1, 3, and 8 (Figure 3B). The high-density SNP on chromosome 1 was consistent with the location of the drought-tolerant (DT)-QTL, which is flanked by markers RZ14 and R117. In this QTL, co-expression network analysis identified two genes, *LOC_Os01g72800* and *LOC_Os01g72950*, as the major nodes. Chromosome 3 did not display high-density SNPs. On this chromosome, *LOC_Os03g02590* and *LOC_Os03g03910* were located in two drought-tolerance QTLs mapped between markers RM7332, RM545, and RG104, RZ329. Another node gene, *LOC_Os03g52460*, is located between markers C136 and R1618, corresponding to another drought-tolerance QTL. Chromosome 8 displays several major nodes: LOC_Os08g16570 is located between markers RM72 and RM331,

while LOC_Os08g41040 and LOC_Os08g41460 are located between RM5353 and RM 3480. This region on chromosome 8 also displayed high-density SNPs between CSSL104 and KDML105 (Figures 2 and 3B).

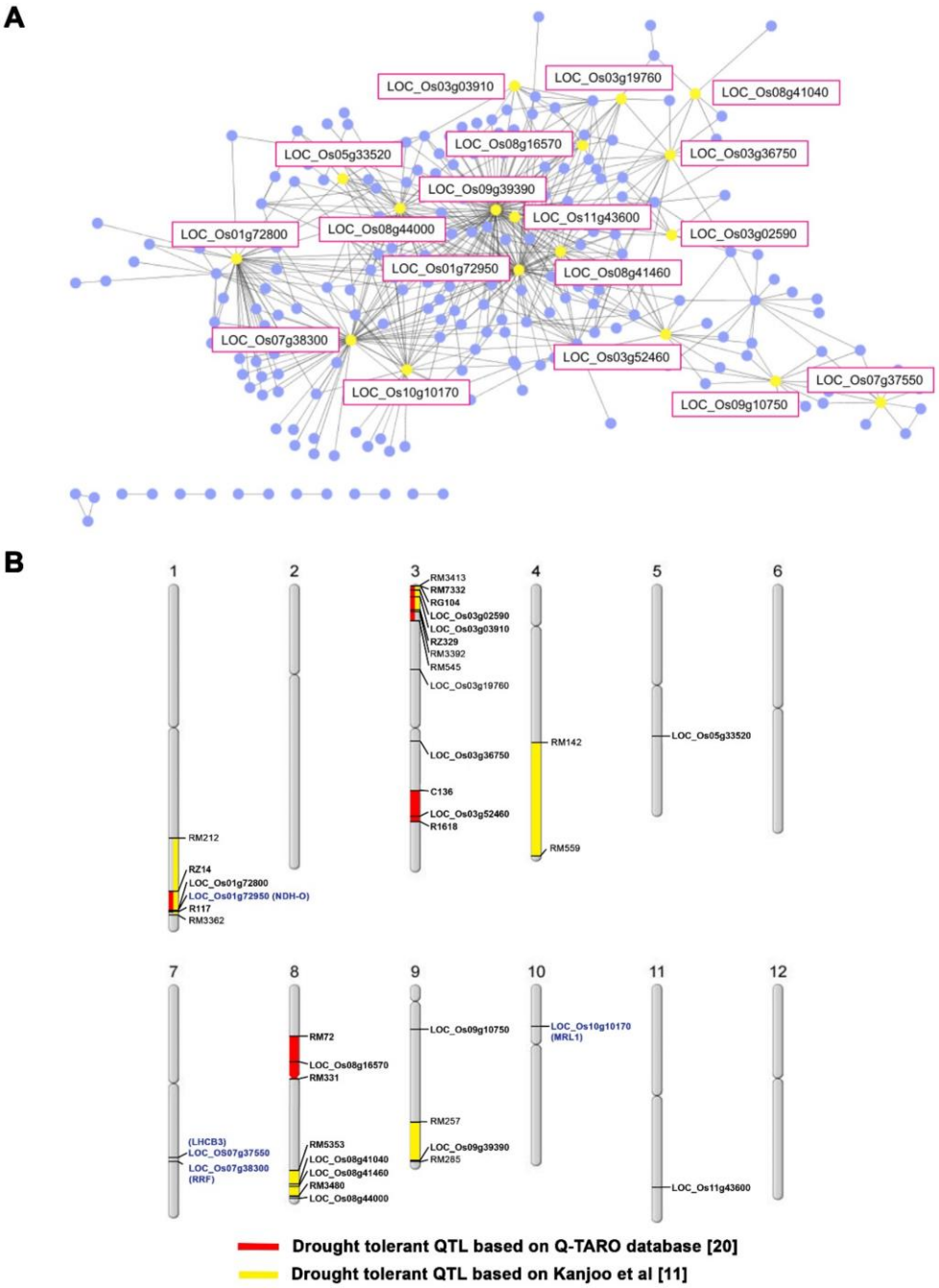


Figure 3. Candidate genes for drought tolerance. Gene co-expression network was analyzed by using the Rice Oligonucleotide Array Database [19], showing the major node genes with yellow dots (**A**), while DT-QTLs from the Q-TARO database [23] and Kanjoo et al. [11] are shown in red and yellow boxes on the chromosome, respectively. (**B**) Loci written in blue letters indicate the proposed drought-tolerance loci based on this study.

Table 2. Rice gene candidates for drought tolerance, their *Arabidopsis* homologs, and the inferred function of the genes in rice.

Rice locus ID	Arabidopsis locus ID	Gene description	Mutant stock	Homozygous	involve in photosynthesis
LOC_Os01g72800	AT2G45770	Chloroplast SRP receptor homolog, alpha subunit CPFTSY. Required for LHCP integration into isolated thylakoids.	SALK_070410C	✓	✓
LOC_Os01g72950	AT1G74880	NDH-O, encoding subunit NDH-O of NAD(P)H:plastoquinone dehydrogenase complex (Ndh complex) present in the thylakoid membrane of chloroplasts. This subunit is thought to be required for Ndh complex assembly.	SALK_097351C	✓	✓
LOC_Os03g02590	AT1G01820	PEROXIN11C, member of the peroxin11 (PEX11) gene family, integral to peroxisome membrane, controls peroxisome proliferation	SALK_057358C	✓	
LOC_Os03g03910	AT4G35090	CAT2	SALK_076998	✓	
LOC_Os03g19760	AT1G56500	SOQ1 (Suppressor of quenching 1) prevents the formation of a slowly reversible form of antenna quenching, thereby maintaining the efficiency of light harvesting.	SALK_097577		✓
LOC_Os03g36750	AT3G48420	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein	SALK_025204	✓	
LOC_Os03g52460	AT5G19220	APL1, the large subunit of ADP-glucose pyrophosphorylase which catalyzes the first and rate-limiting step in starch biosynthesis.	CS478981	✓	
LOC_Os05g33520	AT2G48070	RPH1 is a chloroplast protein RPH1 (resistance to Phytophthora 1) involved in immune response to *Phytophthora brassicae*	SALK_102558C	✓	
LOC_Os07g37550	AT5G54270	LHCB3 is a component of the main light harvesting chlorophyll a/b-protein complex of Photosystem II	SALK_020314C	✓	✓

		(LHC II)			
LOC_Os07g38300	AT3G63190	RRF, encoding a chloroplast ribosome recycling factor homologue.	SALK_015954C	✓	✓
LOC_Os08g16570	AT1G16080	Nuclear protein	SALK_007790C	✓	
LOC_Os08g41040	AT4G31115	DUF1997 family protein	SALK_010690C	✓	
LOC_Os08g41460	AT4G11960	PGRL1B - a transmembrane protein present in thylakoids. Plants lacking PGRL1 show perturbation of cyclic electron flow.	SALK_059238C	✓	✓
	AT4G22890		SALK_133856C	✓	✓
LOC_Os08g44000	AT4G35250	HCF244 is a member of the atypical short-chain dehydrogenase/reductase superfamily, a modified group, required for the biogenesis of photosystem II (PSII), especially for the synthesis of the reaction center proteins (e.g. D1)	SALK_058477		✓
LOC_Os09g10750	AT2G42220	Rhodanese/cell cycle control phosphatase superfamily protein	SALK_045769	✓	
LOC_Os09g39390	AT2G27680	NAD(P)-linked oxidoreductase superfamily protein	SALK_073120C	✓	✓
LOC_Os10g10170	AT4G34830	MRL1 (a conserved pentatricopeptide repeat protein) required for stabilization of *rbcL* mRNA	SALK_060806C	✓	✓
LOC_Os11g43600	AT3G62910	Chloroplast ribosome release factor 1, CPRF1, encoding a plastid-localized ribosome release factor 1 that is essential in chloroplast development	SALK_117765C		

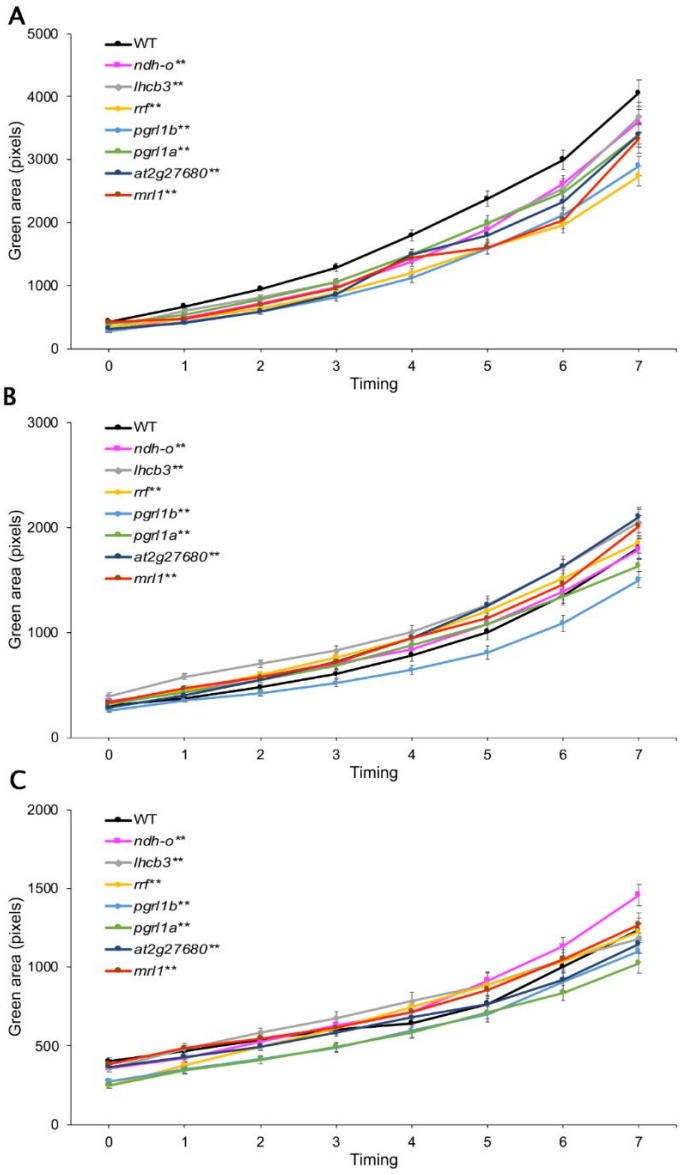


Figure 4. Growth response of seven mutant *Arabidopsis* lines to drought stress. Comparison of growth (green pixels per plant) of wild type (WT) and the T-DNA insertion mutant lines *ndhO* (*at1g74880*), *lhcb3* (*at5g54270*), *rrf* (*at3g63190*), *pgrl1b* (*at4g11960*), *pgrl1a* (*at4g22890*), *at2g27680*, and *mrl1*(*at4g34830*) grown in (**A**) normal Murashige and Skoog (MS) medium, (**B**) under intermediate drought stress (MS medium supplemented with 75 mM mannitol), and (**C**) under severe drought stress (MS medium supplemented with 150 mM mannitol). Statistical analysis was by t-test. * and ** above the name of the mutant line represent significant difference ($p < 0.05$) and highly significant difference ($p < 0.01$) between WT and mutant, respectively.

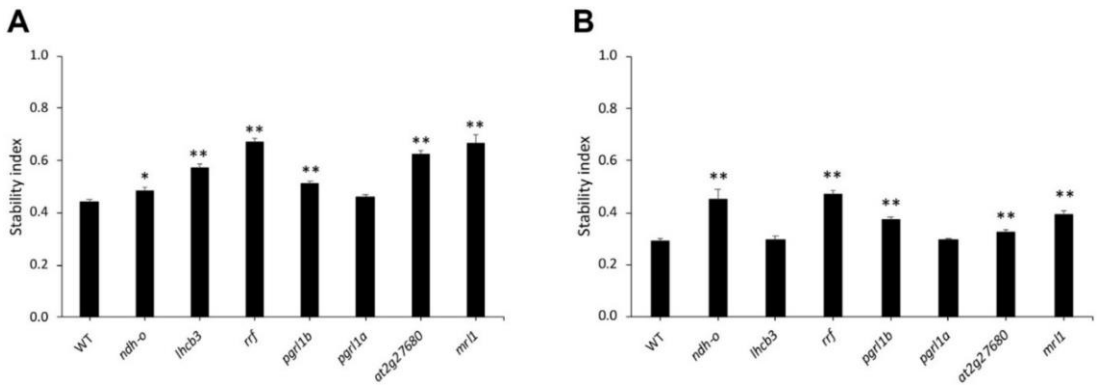


Figure 5. Stability during drought stress. The stability index, which is the ratio between the values from stressed plants and normal growth plants, is shown in (**A**) 75 mM mannitol and (**B**) 150 mM. This figure represents the mean ± SE of WT and mutant lines. The * shows a significant difference at *p* values ≤ 0.05 and ** indicates *p* value ≤ 0.01.

4. Discussion

We investigated the effect of a drought-stress QTL introgressed into the elite rice line KDML105. Using leaf water potential under drought stress, we found that introgression line CSSL104 manifested drought tolerance similar to that of the parent line DH103. Both tended to have a better ability to maintain water status in the first fully expanded leaves. After six days of drought stress, both lines had about a 22% higher leaf water potential than the KDML105 parent. Drought stress limits water uptake from the rice root and reduces water availability in the cells, which is critical for survival under drought stress. Water depletion can compromise photosynthesis and cell growth [34], and three main maintenance mechanisms are used by plants to set water loss: leaf rolling, stomatal closure, and osmoregulation [35,36]. Evidence for the drought tolerance of CSSL104 was also provided by the lower leaf-death score and higher F_v/F_m displayed by CSSL104 compared to KDML105 (Figure 1).

Drought stress resulted in decreased stomatal conductance in all lines (KDML105, DH103, and CSSL104; Table 1). This was a water-preservation mechanism

that also resulted in a decline in the net photosynthesis rate. After three days of drought stress, the photosynthesis parameters, net photosynthesis rate, stomatal conductance, transpiration rate, ϕPSII, electron transport rate, intercellular CO_2 concentration, and F_v'/F_m', were similar among all lines. After six days under drought stress, the net photosynthesis rate of DH103 and CSSL104 were about twofold higher than the net photosynthesis rate of KDML105. In comparison with normal plants, KDML105 rice had a nearly 70% reduction in photosynthesis rate, while DH103 and CSSL104 had only a 46 and 44% reduction, respectively. Interestingly, the ϕPSII and electron transport rate of all lines were similar, while the stomatal conductance of KDML105 was 58% lower than DH103. These findings suggest that stomatal closure could be one of the major factors contributing to the decline in the net photosynthesis rate of KDML105. Although the stomatal conductance of CSSL104 was lower than DH103 by 42%, CSSL104 could maintain a net photosynthesis rate (Table 1). These indicated that this CSSL is better adapted than its drought-tolerant parental line. It is possible that a KDML105 locus contributed to maintenance of the photosynthesis process through an epistatic interaction with the introgressed DH103 region.

Using whole genome sequence comparison and co-expression network analysis, we characterized the molecular fingerprint of the introgression. The first detected DNA segments introgressed, while the second identified genes connected with the drought response. During this stress, 18 genes were highly co-expressed with other genes (Figure 3A and Table 2). Nine of them (*CPFTSY*, *NDH-O*, *SOQ1*, *LHCB3*, *RRF*, *PGRL1*, *HCF244*, *NAD(P)-linked oxidoreductase*, and *MRL1*) are involved in the photosynthesis process, and *CPRF1* is essential for chloroplast development. These findings indicate that the drought-adaptation QTL affects photosynthetic genes whose modulation maintains the net photosynthesis rate of CSSL104.

The function of the identified genes is as follows. CPFTSY (chloroplast FtsY, i.e., chloroplast signal-recognition particle) is required for light-harvesting chlorophyll

a/b-binding protein (LHCP) integration into thylakoids [25], and NDH-O is the subunit required for the NADH dehydrogenase-like (NDH) complex assembly that functions in cyclic electron flow [25,37]. SOQ1 is required to maintain light harvesting efficiency especially during nonphotochemical quenching (NPQ) recovery [26]. Light-harvesting chlorophyll (LHC) functions as a light receptor to capture light energy and deliver it to photosystems. The *Lhcb3* gene product regulates the rate of state transition by changing the excitation energy transfer and charge separation [38]. RRF is a ribosome recycling factor in chloroplast [39]. *RRF* is required to maintain photosystem II efficiency (F_v/F_m) and proper stacking of the internal membranes of chloroplast. Loss of these functions led to a lower growth rate for the rrf mutant compared to WT [28], which is consistent with the phenotype documented in this study (Figure 4A). *PGRL1A* (*AT4G22890*) and *PGRL1B* (*AT4G11960*) are paralogous genes whose products switch linear electron flow to cyclic electron flow. PGRL1 is the elusive ferredoxin-plastoquinone reductase (FQR) [29].

During drought stress, plants shift the electron transfer route from linear to cyclic to balance the energy flow from light reaction to Calvin cycle and photorespiration [37,40,41]; this change is due to CO_2 limitations caused by stomatal closure. It was recently shown that tomato with co-silencing of the *PGR5/PGRL1A* gene was more susceptible to cold stress [42]. This is consistent with our finding that the *pgrl1* mutant had a significantly lower growth rate than WT in both intermediate and severe drought stress (Figure 5B,C). HCF244 is required for the biogenesis of photosystem II (PSII), and specifically for the synthesis of the reaction center proteins [30–32]. The NAD(P)-linked oxidoreductase gene is involved in redox reactions, but the details are still unclear. *MRL1* is the only gene in this study whose product is involved in the Calvin cycle. It is involved either in the processing or in the stabilization of the large subunit (LS) of *RuBisCO* transcripts [43].

Not surprisingly, impairment of these photosynthesis genes significantly reduced growth under normal conditions (Figure 4A). Moreover, we could not obtain

homozygous lines of the *soq1*, *hcf244*, and *cprf1* mutation, probably because the homozygous mutants are lethal. However, under drought-stress conditions, some of the mutants in the genes involved in the light reaction process (*ndhO* (*at1g74880*), *lhcb3* (a*t5g54270*), *rrf* (*at3g63190*), and *at2g27680* mutants) displayed significantly higher growth than WT. These responses suggested that the decrease in light energy harvest during drought stress could prevent damage to chloroplasts and prolong survival of photosynthetic tissues. The *mrl1* mutant was expected to lack of the ability to stabilize the *rbcL* mRNA, which could result in a decline in Calvin cycle activity. The higher stability index of the mrl1 mutant line under intermediate and severe drought stress indicated that a slower rate of the Calvin cycle may help plants cope with drought stress.

Based on the growth phenotype under drought stress, the *ndhO*, *lhcb3*, *rrf*, and *mrl1* mutants showed a higher growth rate than WT (Figure 4B,C). The homologs in rice of these genes are *LOC_Os01g72950*, *LOC_Os07g37550*, *LOC_Os07g38300*, and *LOC_Os10g10170*, respectively. Therefore, we would like to propose that these genes contribute to drought-tolerance regulation in rice by mediating adaptation in the photosynthesis process. *LOC_Os01g72950* is located in the previously reported drought-tolerance QTL between RZ14 and R117 [21], while the other three genes are not. Collectively, our results indicate that the combination of SNP analysis with co-expression network analysis is a suitable method for drought-tolerance gene prediction. This approach will help future exploration to identify the candidate genes for abiotic stress tolerance.

5. Conclusions

The KDML105 chromosome substitution line CSSL104 displayed a drought-tolerance phenotype based on photosynthetic maintenance ability. Identification of SNPs between KDML105 and the tolerant CSSL, together with co-expression network

analysis, predicted 18 candidate drought-tolerance genes—ten of which were involved in photosynthesis or chloroplast development. Seven of them were selected for the characterization by using Arabidopsis mutant lines for the homologous genes. Four out of seven mutants showed a higher growth rate than WT under drought stress. Therefore, *LOC_Os01g72950*, *LOC_Os07g37550*, *LOC_Os07g38300*, and *LOC_Os10g10170* are proposed to be the drought-tolerance genes in CSSL104 rice.

Author Contributions: Conceptualization, S.C. and L.C.; methodology, K.P., T.P., L.C., N.D.D., and L.S.; software, K.P., T.P., N.D.D., and L.S.; validation, C.P.; formal analysis, C.P.; investigation, C.P.; resources, J.L.S. and T.T.; data curation, C.P. and T.B.; writing—original draft preparation, C.P.; writing—review and editing, S.C., T.B., and L.C.; visualization, C.P., S.C.; supervision, S.C., N.D.D., and L.S.; project administration, S.C.; funding acquisition, S.C. All authors have read and agreed to the published version of the manuscript.

Funding: C.P. was supported by the 100th Anniversary of Chulalongkorn University Doctoral Scholarship, and this study was supported by the 90th Anniversary of Chulalongkorn University, Rachadapisek Sompote Fund, grant no. GCUGR1125614034D. The collaboration between CEEPP and CRH was supported by the graduate school and the Rachadapisek Sompote Fund, Chulalongkorn University, Thailand. L.S. and N.D.D. acknowledge the ERDF project entitled “Development of Pre-Applied Research in Nanotechnology and Biotechnology” (No. CZ.02.1.01/0.0/0.0/17_048/0007323).

Acknowledgments: We thank Noppakhun Khunpolwattana for rice DNA extraction and Panita Chutimanukul and Thammaporn Kojonna for their kind help.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Saikumar, S.; Gouda, P.K.; Saiharini, A.; Varma, C.M.K.; Vineesha, G.; Padmavathi, G.; Shenoy, V.V. Major QTL for enhancing rice grain yield under lowland reproductive drought stress identify using *O. sativa/O. glaberrima* introgression line. *Field Crops Res.* 2014, 163, 119–131.

2. Tulyathan, V.; Leeharatanaluk, B. Change in quality of rice (*Oryza sativa* L.) cv. Khao Dawk Mali 105 during storage. *J. Food Biochem*. 2007, 31, 415–425.

3. Siangliw, J.; Jongdee, B.; Pantuwan, G.; Toojinda, T. Developing KDML105 backcross introgression lines using marker-assisted selection for QTLs associated with drought tolerance in rice. *Sci. Asia* 2007, 33, 207–214.

4. Siddique, M.R.B.; Hamid, A.; Islam, M.S. Drought stress effects on water relations of wheat. *Bot. Bull.Acad. Sin.* 2001, 41, 35–39.

5. Hussain, M.; Malik, M.A.; Farooq, M.; Ashraf, M.Y.; Cheema, M.A. Improving drought tolerance by exogenous application of glycine-betaine and salicylic acid in sunflower. *J. Agron. Crop Sci*. 2008, 194, 193.

6. Cornic, G.; Massacci, A. Leaf photosynthesis under drought. In *Photosynthesis and the Environment*; Baker, N.R., Ed.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1996; pp. 347–366.

7. Anjum, F.; Yaseen, M.; Rasul, E.; Wahid, A.; Anjum, S. Water stress in barley (*Hordeum valgare* L.). I. effect on chemical composition and chlorophyll content. Pak. *J. Agron. Sci*. 2003, 40, 45–49.

8. Du, Y.C.; Kawamitsu, Y.; Nose, A.; Hiyane, S.; Murayama, S.; Wasano, K.; Uchida, Y. Effect on water stress on carbon exchange rate and activities of photosynthetic enzymes in leaves of sugarcane (*Saccharum* sp.). *Aust. J. Plant Physiol*. 1996, 23, 719–726.

9. Farooq, M.;Wahid, A.; Kobayashi, N.; Fujita, D.; Basra, S.M.A. Plant drought stress: Effects, mechanisms and management. *Agron. Sustain. Dev*. 2009, 29, 185–212.

10. Zargar, S.M.; Gupta, N.; Nazir, M.; Mahajan, R.; Malik, F.A.; Sofi, N.R.; Shikari, A.B.; Salgotra, R.K. Impact of drought on photosynthesis: Molecular perspective. *Plant Gene* 2017, 11, 154–159.

11. Kanjoo, V.; Punyawaew, K.; Siangliw, J.L.; Jearakongman, S.; Vanavichit, A.; Toojinda, T. Evaluation of agronomic traits in chromosome segment substitution lines of KDML105 containing drought tolerance QTL under drought stress. *Rice Sci*. 2012, 19, 117–124.

12. Punchkhon, C.; Kasetranunt, W.; Kositsup, B.; Siengliw, J.; Chadchawan, S. Evaluation of drought tolerance ability at seedling stage of CSSL rice populations with drought tolerant genes on chromosome 8. In Proceedings of the 9th Botanical Conference of Thailand, Bangkok, Thailand, 3–5 June 2015; pp. 61–71.

13. Vajrabhaya, M.; Vajrabhaya, T. Somaclonal variation of salt tolerance in rice. In *Biotechnology Agriculture Forestry*; Bajaj, Y.P.S., Ed.; Springer: Berlin/Heidelberg, Germany, 1991; pp. 368–382.

14. Pongprayoon, W.; Roytrakul, S.; Pichayangkura, R.; Chadchawan, S. The role of hydrogen peroxide in chitosan-induced resistance to osmotic stress in rice (*Oryza sativa* L.). *Plant Growth Regul.* 2013, 70, 159–173.

15. Chintakovid, N.; Maipoka, M.; Phaonakrop, N.; Mickelbart, M.V.; Roytrakul, S.; Chadchawan, S. Proteomic analysis of drought-responsive proteins in rice reveals photosynthesis-related adaptations to drought stress. *Acta Physiol. Plant*. 2017, 39, 240.

16. Missirian, V.; Comai, L.; Filkov, V. Statistical mutation calling from sequenced overlapping DNA pools in TILLING experiments. *BMC Bioinf.* 2011, 12, 287.

17. Ouyang, S.; Zhu, L.; Hamilton, J.; Lin, H.; Campbell, M.; Childs, K.; Thibaud-Nissen, F.; Malek, R.L.; Lee, Y.; Zheng, L.; et al. The TIGR rice genome annotation resource: Improvements and new features. *Nucleic Acids Res*. 2007, 35, 883–887.

18. Kawahara, Y.; de la Bastide, M.; Hamilton, J.P.; Kanamori, H.; McCombie, W.R.; Ouyang, S.; Schwartz, D.C.; Tanaka, T.; Zhou, S.; Childs, K.L.; et al. Improvement of the *Oryza sativa* Nipponbare reference genome using next generation sequence and optical map data. *Rice* 2013, 6, 4.

19. Cao, P.; Jung, K.; Choi, D.; Hwang, D.; Zhu, J.; Ronald, P. The rice oligonucleotide array database: An atlas of rice gene expression. *Rice* 2012, 5, 17.

20. Xia, L.; Zou, D.; Sang, J.; Xu, X.J.; Yin, H.Y.; Li, M.W.; Wu, S.Y.; Hu, S.N.; Hao, L.L.; Zhang, Z. Rice Expression Database (RED): An integrated RNA-Seq-derived gene expression database for rice. *J. Genet. Genom.* 2017, 44, 235–241.

21. Lamesch, P.; Berardini, T.Z.; Li, D.; Swarbreck, D.; Wilks, C.; Sasidharan, R.; Muller, R.; Dreher, K.; Alexander, D.L.; Garcia-Hernandez, M.; et al. The arabidopsis information resource (TAIR): Improved gene annotation and new tools. *Nucleic Acids Res.* 2012, 40, D1202–D1210.

22. De Diego, N.; Fürst, T.; Humplik, J.; Ugena, L.; Podlešáková, K.; Spíchal, L. An automated method for high-throughput screening of Arabidopsis rosette growth in multi-well plates and its validation stress condition. *Front. Plan Sci*. 2017, 8, 1702.

23. Yonemaru, J.; Yamamoto, T.; Fukuoka, S.; Uga, Y.; Hori, K.; Yano, M. Q-TARO:QTL Annotation rice online database. *Rice* 2010, 3, 194.

24. Tu, C.J.; Schuenemann, D.; Ho man, N.E. Chloroplast FtsY, chloroplast signal recognition particle, and GTP are required to reconstitute the soluble phase of light-harvesting chlorophyll protein transport into thylakoid membranes. *J. Biochem. Chem*. 1999, 274, 27219–27224.

25. Rumeau, D.; Bécuwe-Linka, N.; Beyly, A.; Louwagie, M.; Garin, J.; Peltier, G. New subunit NDH-M, -N, -O, encoded by nuclear genes, are essential for plastid Ndh complex functioning in higher plants. *Plant Cell* 2005, 17, 219–232.

26. Brooks, M.D.; Sylak-Glassman, E.J.; Fleming, G.R.; Niyogi, K.K. A thioredoxin-like/ -propeller protein maintains the effciency of light harvesting in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 2013, 110, E2733–E2740.

27. Adamiec, M., Gibasiewicz, K., Luciński, R., Giera, W., Chełminiak, P., Szewczyk, S., Sipińska, W., Grondelle, R. and Jackowski, G. Excitation energy transfer and charge separation are affected in Arabidopsis thaliana mutants lacking light-harvesting chlorophyll a/b binding protein Lhcb3. *J Photochem & Photobiology, B: Biology* **2015**, *153*: 423–428.

28. Guan, Z.; Wang, W.; Yu, X.; Lin, W.; Maio, Y. Comparative proteomic analysis of coregulation of CIPK14 and WHIRLY1/3 mediated pale yellowing of leaves in *Arabidopsis*. *Int. J. Mol. Sci*. 2018, 19, 2331.

29. Hertle, A.P.; Blunder, T.; Wunder, T.; Pesaresi, P.; Pribil, M.; Armbruster, U.; Leister, D. PGRL1 is the elusive ferredoxin-plastoquinone reductase in photosynthetic cyclic electron flow. *Mol. Cell* 2013, 49, 511–523.

30. Hey, D.; Grimm, B. ONE-HELIX PROTEIN2 (OHP2) is required for the stability of OHP1 and assembly factor HCF244 and is functionally linked to PSII biogenesis. *Plant Physiol.* 2018, 177, 1453–1472.

31. Li, Y.; Liu, B.; Zhang, J.; Kong, F.; Zhang, L.; Meng, H.; Li,W.; Rochaix, J.D.; Li, D.; Peng, L. OHP1, OHP2, and HCF244 form a transient functional complex with the photosystem II reaction center. *Plant Physiol*. 2019, 179, 195–208.

32. Chotewutmontri, P.;Williams-Carrier, R.; Barkan, A. Exploring the link between photosystem II assembly and translation of the chloroplast *psbA* mRNA. *Plants* 2020, 9, 152.

33. Motohashi, R.; Yamazaki, T.; Myouga, F.; Ito, T.; Ito, K.; Satou, M.; Kobayashi, M.; Nagata, N.; Yoshida, S.; Nagashima, A.; et al. Chloroplast ribosome release factor 1 (AtcpRF1) is essential for chloroplast development. *Plant Mol. Biol*. 2007, 646, 481–497.

34. Tezara,W.; Mitchell, V.J.; Driscoll, S.D.; Lawlor, D.W.Water stress inhibits plant photosynthesis by decreaseing coupling factor and ATP. *Nature* 1999, 401, 914–917.

35. Teulat, B.; Monneveux, P.; Wery, J.; Borries, C.; Souyris, I.; Charrier, A.; This, D. Relationships between relative water content and growth parameters under water stress in barley: A QTL study. *New Phytol*. 1997, 137, 99–107.

36. González, L.; González-Vilar, M. Determination of relative water content. In *Handbook of Plant Ecophysiology Techniques*; Springer: Berlin/Heidelberg, Germany, 2001; pp. 207–212.

37. Nikkanen, L.; Toivola, J.; Trotta, A.; Diaz, M.G.; Tikkanen, M.; Aro, E.; Rintamäki, E. Regulation of cyclic electron flow by chloroplast NADPH-dependent thioredoxin system. *Plant Direct* 2018, 2, e00093.

38. Damkjaer, J.T.; Kereïche, S.; Johnson, M.; Kovacs, L.; Kiss, A.Z.; Boekema, E.J.; Ruban, A.V.; Horton, P.; Jansson, S. The photosystem II light-harvesting protein Lhcb3 affects the macrostructure of photosystem II and the rate of state transitions in *Arabidopsis*. *Plant Cell* 2009, 21, 3245–3256.

39. Wang, L.; Ouyang, M.; Li, Q.; Zou, M.; Gou, J.; Ma, J.; Lu, C.; Zhang, L. The *Arabidopsis* chloroplast ribosome recycling factor is essential for embryogenesis and chloroplast biogenesis. *Plant Mol. Biol*. 2010, 74, 47–59.

40. DalCorso, G.; Pesaresi, P.; Masiero, S.; Aseeva, E.; Schünemann, D.; Finazzi, G.; Joliot, P.; Barbato, R.; Leister, D. A complex containing PGRL1 and PGRL5 is involved in the switch between linear and cyclic electron flow in *Arabidopsis*. *Cell* 2008, 132, 273–285.

41. Suorsa, M.; Rossi, F.; Tadini, L.; Labs, M.; Colombo, M.; Jahns, P.; Kater, M.M.; Leister, D.; Finazzi, G.; Aro, E.; et al. PGRL5-PGRL1-dependent cyclic electron transport modulates linear electron transport rate in *Arabidopsis thaliana*. *Mol. Plant* 2016, 9, 271–288.

42. Wang, F.; Yan, J.; Ahammed, G.J.;Wang, X.; Bu, X.; Xiang, H.; Li, Y.; Lu, J.; Liu, Y.; Qi, H.; et al. PGR5/PGRL1 and NDH mediate far-red light-induced photoprotection in response to chilling stress in tomato. *Front. Plant Sci*. 2020, 11, 669.

43. Johnson, X.; Wostrikoff, K.; Finazzi, G.; Kuras, R.; Schwarz, C.; Bujaldon, S.; Nickelsen, J.; Stern, D.B.; Wollman, F.; Vallon, O. MRL1, a conserved pentatricopeptide repeat protein, is required for stabilization of rbcL mRNA in *Chlamydomas* and *Arabidopsis*. *Plant Cell* 2010, 22, 234–248.

CHAPTER III

RESEARCH ARTICLES

Role of *LOC_Os01g68450* in Salt Tolerance is Mediated via the Maintenance of the Light-Harvesting Complex

Chutarat Punchkhon[1,2], Panita Chutimanukul[3], Aleel K. Grennan[4], Nuria De Diego[5], Lukáš Spíchal[5] and Supachitra Chadchawan[2,6]

[1] Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand; chutarat_tom@hotmail.com

[2] Center of Excellence in Environment and Plant Physiology, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

[3] National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Phahonyothin Rd. Khlong Nueng, Khlong Luang, Pathumthani, 12120, Thailand; panita.chu@bitec.or.th

[4] Biology Department, Worcester State University, Worcester, MA, 01602, USA; agrennan@worcester.edu

[5] Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 27, CZ-783 71 Olomouc, Czech Republic; nuria.de@upol.cz (N.D.D.); lukas.spichal@upol.cz (L.S.)

[6] Omics Science and Bioinformatics Center, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

* Correspondence: Supachitra.c@chula.ac.th or s_chadchawan@hotmail.com

Abstract

Salt stress affects plant growth and productivity. In this study, we determined the roles of eight genes involved in photosynthesis, predicted by using gene co-expression network analysis, under salt-stress conditions using Arabidopsis knockout mutants. Green area of the leaves was minimum in the at1g65230 mutant line. Rice LOC_Os01g68450, a homolog of At1g65230, was ectopically expressed in the at1g65230 mutant line to generate revertant lines. Salt stress increased the content of photosynthetic pigments in the revertant line than in the mutant line. Under salt stress, the net photosynthetic rate of the revertant line was higher than that of the wildtype (WT) and mutant lines, whereas the stomatal conductance and transpiration rate of the revertant line were lower than those of the WT plants. The excitation capture efficiency, operating efficiency of photosystem II, and electron transport rate decreased in the WT and mutant line after five days of exposure to salt stress, but they were maintained in the revertant line. Moreover, the revertant line accumulated more photosynthetic pigments. These findings suggest the important role of the gene product of LOC_Os01g68450 in the maintenance of the light-harvesting complex and adaptation of photosynthesis under salt stress.

Keywords: LOC_Os01g68450; at1g65230 mutant line; light-harvesting complex; photosynthetic pigment; salt stress; excitation capture efficiency; PhiPSII; electron transport rate; stomatal conductance

1. Introduction

Salt stress is one of the most common abiotic stresses that affects plant growth and productivity. In 2019, approximately 1.125 MH of land exhibited highly saline conditions, and the area is likely to increase by 1%–2% each year [1]. Salt stress affects plants *via* osmotic stress, inhibition of nutrient absorption, ion toxicity, metabolism imbalance, and oxidative stress, which affect biological processes, including growth, photosynthesis, protein synthesis, and protein and phospholipid metabolism. Under salt-stress conditions, plants close their stomata to prevent water loss from the leaves, resulting in decreased carbon assimilation. Moreover, salt stress induces the production of reactive oxygen species (ROS), which results in thylakoid disruption and chlorophyll degradation. Moreover, the plant responses induced under salt stress suppress photosynthesis [2,3], thereby reducing growth and yield of crops.

Salt and drought stress are abiotic stresses, which promote water deficit in plants. These stresses have similar effects on plants during the early stages of stress. Prolonged salt stress induces hyperionic and hyperosmotic stresses in plants. In rice, drought-tolerant quantitative trait loci in the chromosome segment substitution line (CSSL) 'KDML105' were co-localized with the markers of salt tolerance [4]. Recently, based on the comparison between the genomes of 'CSSL104' and 'KDML105' and co-expression network analysis, ten genes, namely *CPFTSY*, *NDH-O*, *SOQ1*, *LHCB3*, *RRF*, *PGRL1B*, *HCF244*, *NAD(P)-linked oxidoreductase*, *LOC_Os09g39390*, and *MRL1*, have been identified to function in the adaptation of photosynthesis during drought stress. The analysis of seven T-DNA-tagged Arabidopsis mutant lines, corresponding to seven of the ten genes, namely *ndhO* (*at1g74880*), *lhcb3* (*at5g54270*), *rrf* (*at3g63190*), *pgrl1b* (*at4g11960*), *pgrl1a* (*at4g22890*), *at2g27680*, and *mrl1* (*at4g34830*), revealed that all the genes, except *pgrl1a*, contributed to drought tolerance [5]. Furthermore, salt tolerance has been investigated in rice CSSLs. It was reported that CSSL16 exhibited salt tolerance at the seedling and tillering stages, with several photosynthesis-related genes predicted to play roles in salt tolerance, including *OsPsbS1*, which encodes the chlorophyll *a*- and *b*-binding protein in the photosystem II, and *OsNDH-O*, which is involved in the adaptation of photosynthesis during drought stress in CSSL104 [5–7].

Among the predicted genes involved in salt tolerance from CSSL16, *LOC_Os01g68450* has not been characterized [6]. Therefore, in this study, we aimed to determine the roles of the photosynthesis-related genes predicted to be involved in drought tolerance, namely, *NDH-O*, *LHCB3*, *RRF*, *PGRL1A*, *PGRL1B,* *LOC_Os09g39390*, and *MRL1*, using the T-DNA-tagged Arabidopsis mutant lines corresponding to these genes. Comparison between the Arabidopsis mutant line *at1g65230*, corresponding to *LOC_Os01g68450*, and the uncharacterized gene from CSSL16, predicted to be involved in drought tolerance, under salt stress revealed that *at1g65230* exhibited the most susceptible phenotype. Thus, the phenotype of the *at1g65230* revertant lines, with the ectopic expression of *LOC_Os01g68450*, was investigated to determine the role of *LOC_Os01g68450* under salt stress.

2. Results

2.1 Mutation in At1g65230 Strongly Inhibited Plant Growth Under Salt Stress

Arabidopsis mutant lines with T-DNA insertions at the orthologous rice genes were used to determine the total green area of the leaves cultured under control and salt-stress conditions.

Under control conditions, the values of the total green areas in the leaves of all the Arabidopsis mutant lines were significantly lower than that of the wildtype (WT) (Figure 1A), whereas under salt stress, the mutant lines exhibited higher values of the green areas in the leaves. For the *ndho*, *lhbc3*, and *mrl1* mutants, the values of the green areas of the leaves were similar to those of the WT under salt stress, whereas the values of the green areas in the leaves of the remaining mutants were significantly lower than that of the WT. Moreover, the value of the green area in the leaves of the *at2g27680* mutant was significantly lower than that of the WT, but after five days of exposure to salt stress, its value was similar to that of the WT. The *at1g65230* mutant exhibited the lowest green area in the leaves (Figure 1B), lowest growth ratio (Figure 1C), and maximum decrease in the leaf green area percentage (Figure 1D) compared to the WT under salt stress, suggesting the role of *At1g65230* in

adapting to salt stress. Therefore, the *at1g65230* mutant was selected for further characterization.

2.2 Ectopic Expression of LOC_Os01g68450 Reverted the Susceptibility of the at1g65230 Mutant to Salt Stress

To investigate the reversal of susceptibility of the *at1g65230* mutant to salt stress by ectopic expression of *LOC_Os01g68450*, the ectopic expression construct was transformed into the *at1g65230* mutant. T2 homozygous expression lines were used to determine the phenotype under the control and salt-stress conditions. Both the revertant lines grew better than the *at1g65230* mutant under control conditions. The revertant line rev-1 exhibited green area development similar to that of the WT (Figure 2A), whereas under salt stress, both the revertant lines exhibited significantly higher green areas in the leaves than the mutant line. However, after five days of exposure to salt stress, the revertant lines exhibited significantly lower values of the green area in the leaves than the WT (Figure 2B). Therefore, the revertant line was selected for further characterization of the photosynthetic parameters.

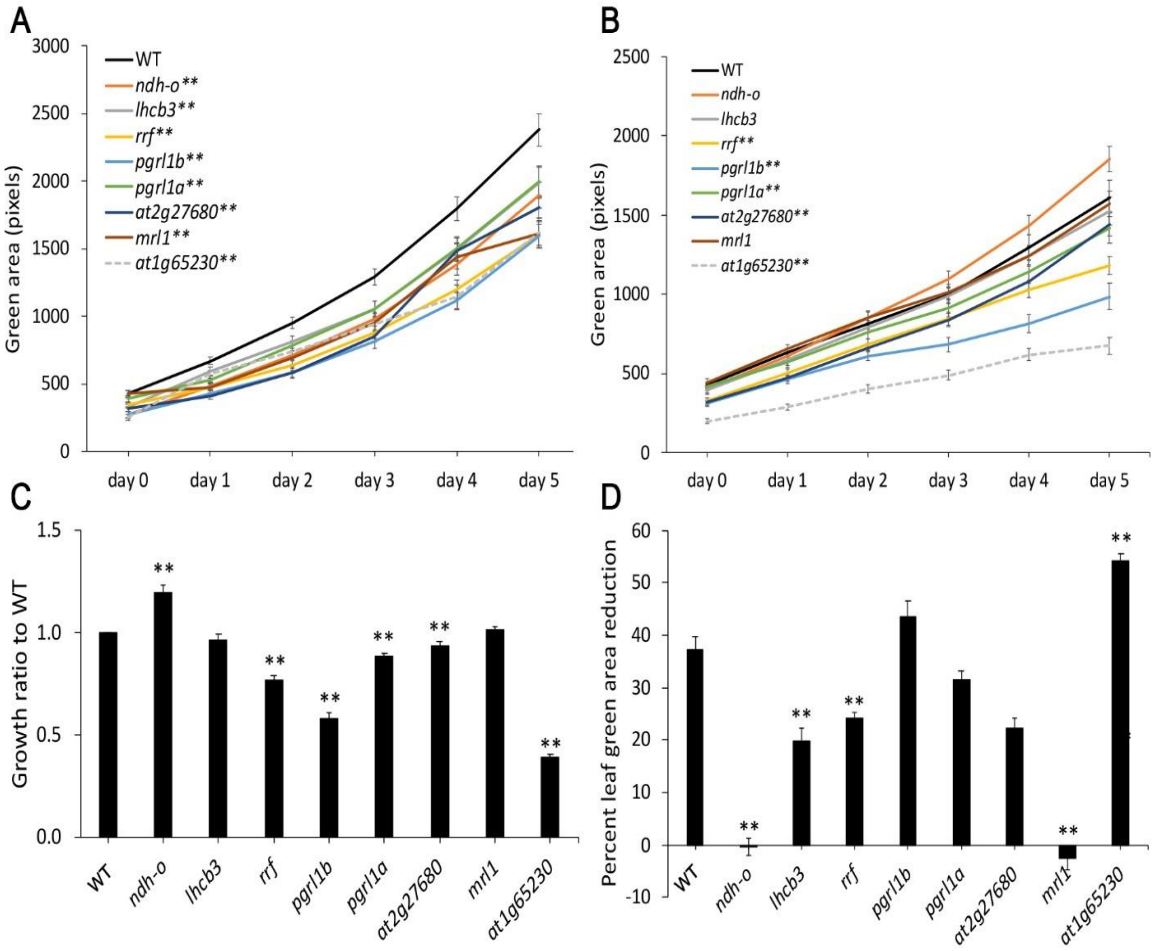


Figure 1. Total green area of the leaves in the Arabidopsis wildtype (WT) and mutant lines under the control conditions **(A)** and 75-mM NaCl stress **(B)**. Relative growth rates of the Arabidopsis WT and mutant lines after five-day-exposure to the control conditions **(C)** and 75 mM NaCl **(D)**.

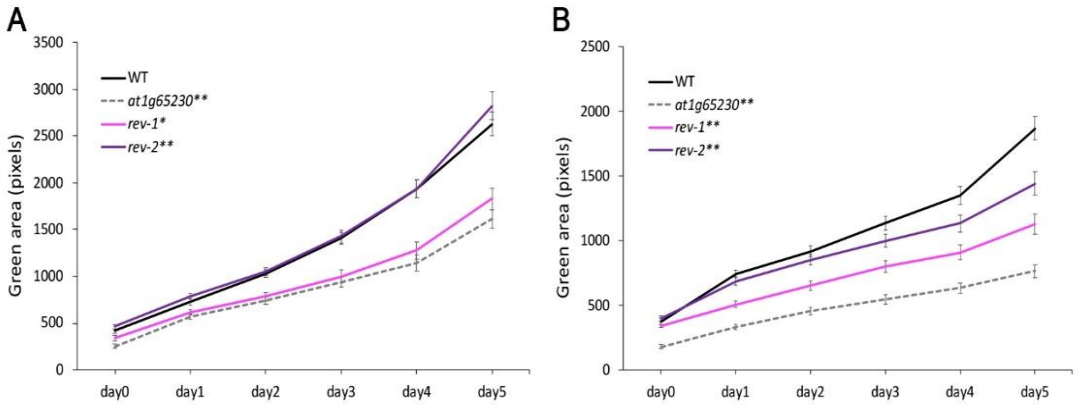


Figure 2 Total green area of the leaves in the Arabidopsis wildtype (WT) and revertant lines under the control conditions **(A)** and 75-mM NaCl stress **(B)**.

Under control conditions, the net photosynthetic rates (P_n) of the mutant, revertant lines, and WT were similar, whereas salt stress resulted in a significant decrease in the P_n of all the lines. However, the revertant line exhibited a significantly higher P_n than the WT and mutant line under salt stress (Figure 3A). This response was not affected by the increase in the stomatal conductance (g_s) under salt stress, because g_s of the revertant line was similar to that of the mutant line. Nonetheless, both the mutant and revertant lines exhibited lower g_s than the WT under salt stress (Figure 3B), which did not affect the intracellular CO_2 concentration (C_i) of the plants (Figure 3C). Owing to the decrease in g_s, the transpiration rates of the mutant and revertant lines were significantly lower than that of the WT (Figure 3D).

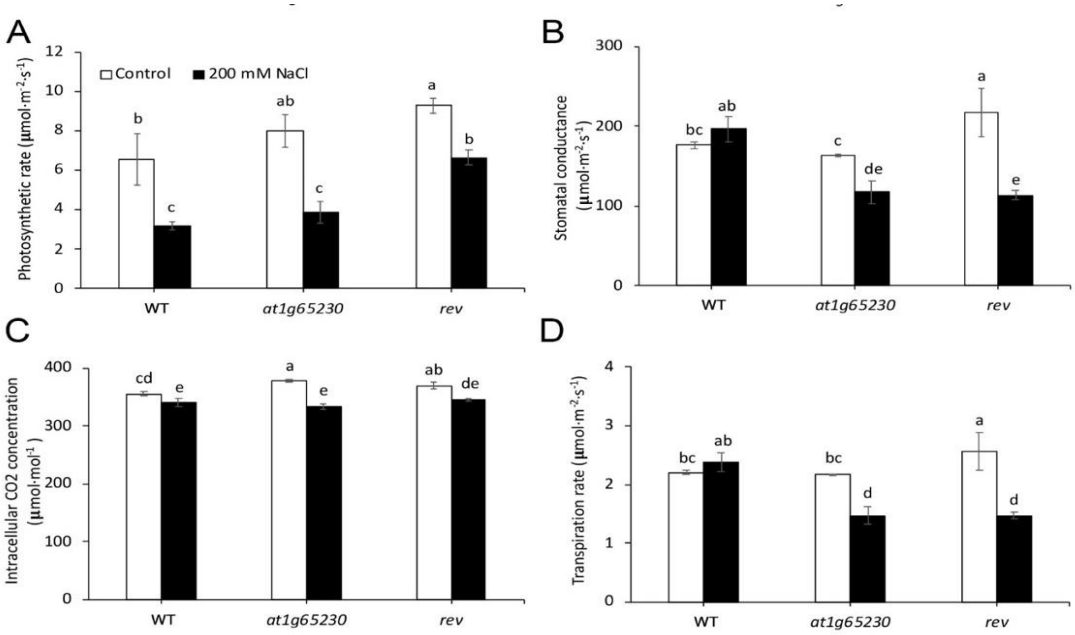


Figure 3 Photosynthetic parameters of the Arabidopsis wildtype (WT), *at1g65230* mutant, and revertant line (*rev*) cultured under control and salt-stress (200 mM NaCl) conditions. Photosynthetic rate (P_n) **(A)**, stomatal conductance (g_s) **(B)**, intracellular CO_2 concentration (C_i), and transpiration rate (E) of were determined after five days of treatments.

The P_n of the revertant line was higher than that of the WT and mutant line, but the g_s of the revertant line was lower, suggesting that the higher P_n of the revertant line did not result from the upregulation of the Calvin cycle. Therefore, the light-harvesting efficiency and content of photosynthetic pigments were estimated under the control and salt-stress conditions.

Under control conditions, the three lines exhibited similar excitation capture efficiency (Fv′/Fm′) and operating efficiency of photosystem II (ΦPSII) (Figure 4A and 4B). By contrast, the electron transport rate (ETR) of the revertant line was significantly higher than that of the WT and mutant line (Figure 4C). After five days of exposure to salt stress, the Fv′/Fm′ and ΦPSII of the WT and mutant line significantly decreased, whereas those of the revertant line were maintained (Figure

4A and 4B). However, salt stress did not affect the ETR in the WT and mutant line, whereas it induced a higher ETR in the revertant line (Figure 4C).

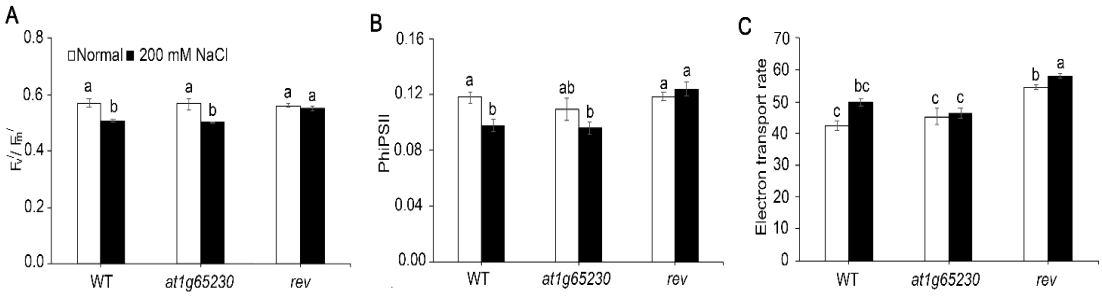


Figure 4 Efficiency of the light reaction in the Arabidopsis wildtype (WT), *at1g65230* mutant, and revertant line (*rev*) determined after five days of treatment under control and salt-stress (200 mM NaCl) conditions. Excitation capture efficiency **(A)**, operating efficiency of PSII **(B)**, and electron transport rate.

The chlorophyll *a* content in the mutant line was lower than in the WT (Figure 5A) under control conditions, whereas the chlorophyll *b* content was similar to that of the WT (Figure 5B). Therefore, the total chlorophyll content in the mutant line was lower than that in the WT (Figure 5C). However, the mutant line exhibited significantly lower chlorophyll *a/b* ratio than the WT (Figure 5 D). When *LOC_Os01g68450* was ectopically expressed in the revertant line, the chlorophyll *a* content significantly increased to a level similar to that in the WT under the control conditions (Figure 5A). Salt stress did not affect the chlorophyll *b* content in the revertant line (Figure 5B), leading to significantly higher total chlorophyll content and chlorophyll *a/b* ratio in the revertant line than the mutant line (Figure 5C and 5D).

After five days of exposure to salt stress, the content of chlorophyll *a* decreased in all the lines, whereas only the mutant line exhibited a significantly lower chlorophyll b content than the control plants. Furthermore, salt stress resulted in a significantly lower chlorophyll a content in the WT (Figure 5A) and significantly lower chlorophyll b content in the mutant line. Nevertheless, both the WT and revertant line maintained chlorophyll *b* content under salt stress (Figure 5B), resulting in a lower chlorophyll *a/b* ratio in the WT and significantly higher

chlorophyll a/b ratio in the mutant line after five days of exposure to salt stress. By contrast, no significant changes were observed in the chlorophyll a/b ratio of the revertant line after salt stress (Figure 5D).

The mutant line exhibited a lower carotenoid content than the WT and revertant line under control conditions. By contrast, salt stress decreased the carotenoid content in all the lines. However, a remarkably lower carotenoid content was observed only in the WT (Figure 5E).

The revertant line accumulated anthocyanins under control conditions, whereas salt stress decreased the anthocyanin content in all the lines. However, the anthocyanin content in the revertant line was dramatically higher than that in the WT and mutant line after salt stress (Figure 5F).

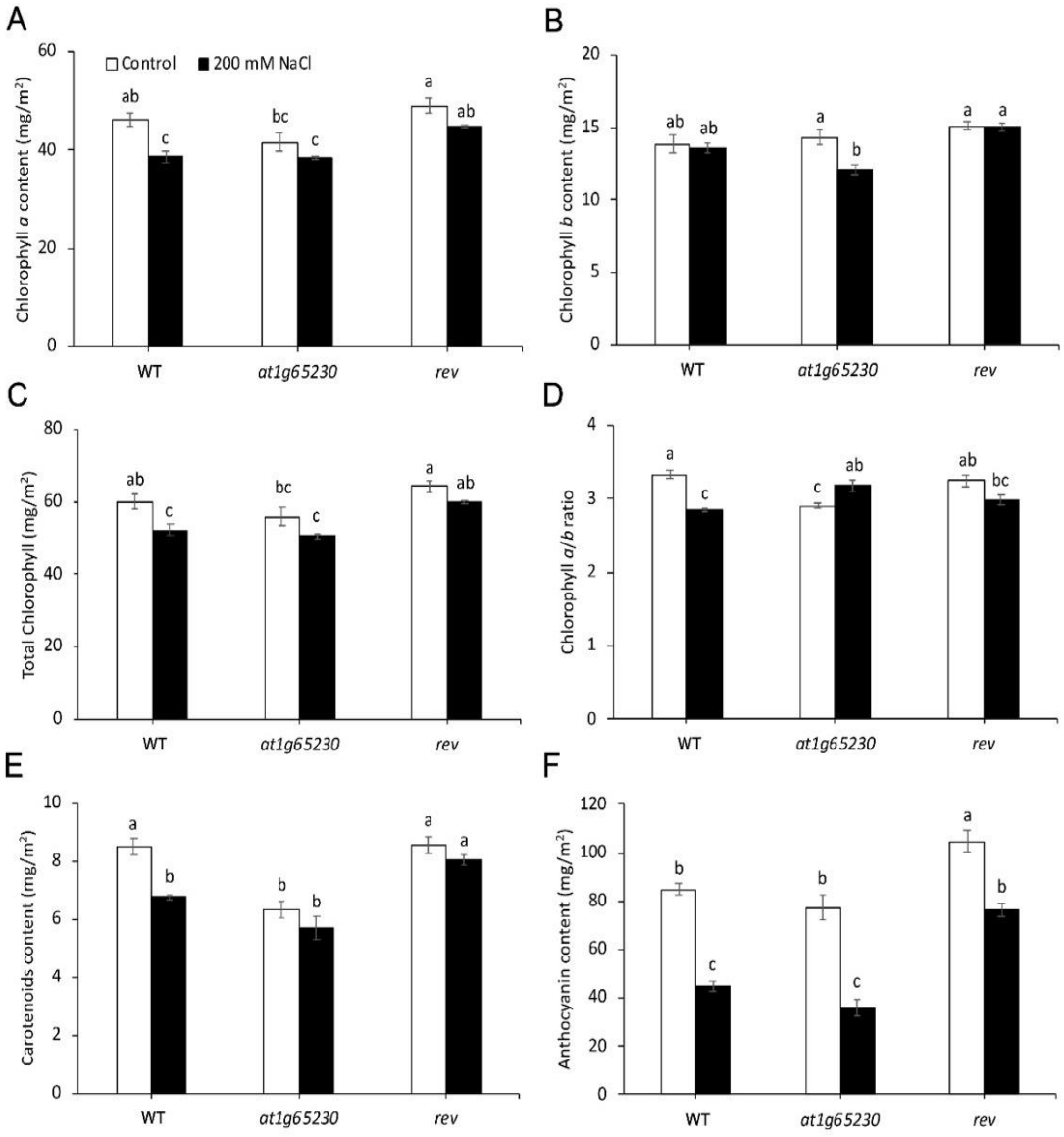


Figure 5 Pigment contents in the Arabidopsis wildtype (WT), *at1g65230* mutant, and revertant line (*rev*) determined after five days of culturing under control and salt-stress (200 mM NaCl) conditions: Chlorophyll a **(A)**, chlorophyll b **(B)**, total chlorophyll **(C)**, chlorophyll a/*b* ratio **(D)**, carotenoids **(E)**, and anthocyanin **(F)**.

3. Discussion

The growth responses of the selected mutant lines under salt stress were different from that exhibited under drought stress. Based on a previous study, *ndhO*, *lhcb3, rrf*, and *at2g27680* mutants exhibited higher growth rates than the WT under drought stress [5], because the impairment in the components of the light-harvesting complex prevented chloroplast damage. However, this response was not observed under salt stress (Figure 1), which can be attributed to the increased ion imbalance and toxicity. The knockout mutants of *RRF*, *PGRL1B*, *PGRL1A*, *At2g27680*, and *At1g65230* exhibited growth inhibition under both the control and salt-stress conditions, particularly the *at1g65230* mutant, which was highly susceptible to salt stress (Figure 1). This suggests an important role of *At1g65230* in salt tolerance.

Rice *LOC_Os01g68450* is the homolog of *At1g65230*, encoding the protein Q8L604. Amino acid sequence alignment revealed 67.1% similarity between the two homologs. Furthermore, the DUF2358 and transmembrane domains were conserved between the two homologs, suggesting similar functions of their protein products (Figure 6). Thus, to validate the role of *LOC_Os01g68450*, it was ectopically expressed in the *at1g65230* mutant and its phenotype reversal ability was determined under salt stress.

Salt stress suppresses photosynthesis in various plant species, including rice (*Oryza sativa* L.) [8], chick-pea (*Cicer arietinum* L.) [9], rocket (*Eruca sativa* L.) [10], and maize (*Zea mays* L.) [11]. Two independent revertant lines, with the ectopic expression of *LOC_Os01g68450*, revealed significantly higher values of the green area in the leaves under salt stress, suggesting that *LOC_Os01g68450* increased salt tolerance in the *at1g65230* mutant (Figure 2). Moreover, the increased P_n and decreased *ETR* in the revertant lines (Figure 3) prolonged the survival ability under salt stress owing to the increase in water use efficiency (WUE). Plants have various mechanisms to regulate the process of gaseous exchange to adapt to drought and salt-stress conditions [12]. Treatment to increase WUE is one of the strategies to increase salt tolerance in plants [13, 14]. Furthermore, the overexpression of *AtHRDY* in rice ensured higher WUE than in the WT and increased drought and salt tolerance [15].

The P_n of the revertant line was approximately twice the P_n of the WT and mutant line (Figure 3A), but all of them exhibited similar C_i, resulting in the increased carboxylation efficiency of the revertant line under salt stress. The inoculation of salt-tolerant plant growth-promoting bacteria to tomato and rice plants increased plant growth rates under salt stress; the increase in the ΦPSII, ETR, and carboxylation efficiency were observed in the salt-tolerant phenotypes [16]. Similar observations were reported in the revertant line (Figure 4), indicating that the ectopic expression of *LOC_Os01g68450* in the *at1g65230* mutant increased salt tolerance by enhancing the efficiency of the light-harvesting complex under salt stress. This is the first report on the characterization of the role of a protein with DUF2358 domain in the enhancement of the efficiency of the light-harvesting complex under salt stress.

To further analyze the effects of salt stress on the components of the light-harvesting complex and effects of the ectopic expression of *LOC_Os01g68450*, the contents of the photosynthetic pigments and anthocyanins were determined. The *at1g65230* mutant exhibited lower chlorophyll *a* and carotenoid contents, without any effect on the chlorophyll *b* content. The revertant line exhibited chlorophyll and carotenoid contents similar to those of the WT (Figure 5). Moreover, under salt stress, the ectopic expression of *LOC_Os01g68450* helped plants to maintain chlorophyll *a, b*, and carotenoids, which increased the stability of the light-harvesting complex.

Furthermore, the revertant line accumulated more anthocyanin under salt stress (Figure 5F). The overexpression of the gene encoding leucoanthocyanidin dioxygenase from *Reaumuria trigyna* Maxim in Arabidopsis conferred tolerance of abiotic stress through anthocyanin accumulation [17]. Anthocyanins function as antioxidants under stress conditions [18], indirectly preventing chlorophyll degradation and maintaining the photosynthetic activity [19]. Carotenoids and anthocyanins also scavenge ROS, which are generated in the cell after stress [20, 21].

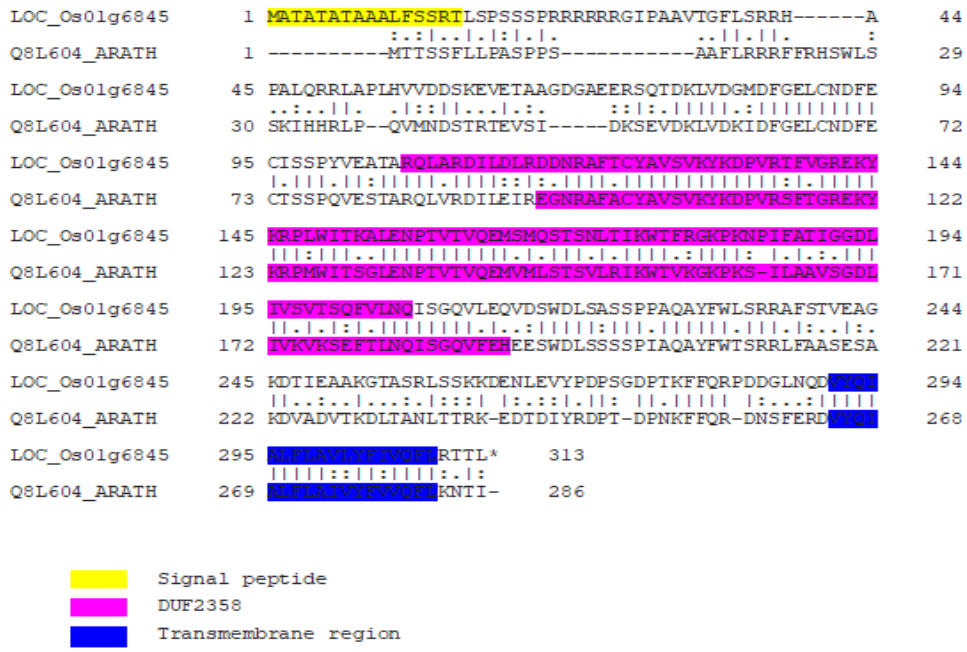


Figure 6 Amino acid sequence alignment of LOC_Os01g68450 and Q8L604, the protein product of *At1g65230*. Different motifs are demonstrated in yellow, pink, and blue. LOC_Os01g68450 sequence was obtained from the Rice Genome Annotation Project [22] and that of Q8L604 was obtained from The Arabidopsis Information Resource [23].

4. Materials and Methods

4.1 Plant Material

In this study, the Arabidopsis ecotype Columbia-0 and mutant lines with T-DNA insertions in the genes of interest were used. The mutant lines SALK_097351, SALK_020314, SALK_015954, SALK_059238, SALK_133856, SALK_073120, SALK_060806, and SALK_130615 had the T-DNA inserted in the genes *at1g74880*, *at5g54270*, *at3g63190*, *at4g11960*, *at4g22890*, *at2g27680*, *at4g34830*, and *at1g65230*, respectively. All Arabidopsis mutant lines were obtained from the Arabidopsis Biological Resources Center [24].

4.2 Ectopic Expression and Revertant Line Generation

The cDNA of the rice ortholog of *at1g65230*, *LOC_Os01g68450* (Accession no: AK068538), was cloned into the pJIM19 vector using the restriction enzymes *XbaI* and *XhoI* and T4 ligase. The plasmid was then introduced into *Agrobacterium tumefaciens* GV3101 using freeze-thaw transformation, and kanamycin, rifampicin, and gentamicin were used as the selectable markers. The Col-0 ecotype and *at1g65230* mutant line were transformed using the floral dipping method to generate the revertant lines [6, 7]. A completely randomized design (CRD) with 48 plants per line was used as the experimental design.

4.3 Estimation of the Green Area of the Leaves

The seeds of all the Arabidopsis lines were sterilized, placed on square plates, and kept undisturbed in the refrigerator for four days. The square plates were then placed in a growth chamber for germination at 22 ℃/20 ℃, 16/8 h light/dark cycle, 120 µmol photons of PAR $m^{-2} \cdot s^{-1}$, and 60% humidity. After four days of germination, the seedlings were transferred to 48-well plates, containing full Murashige and Skoog (MS) solid medium (control) or MS solid medium supplemented with 75 mM sodium chloride (salt stress) and grown under the same condition. The green area of the leaves were estimated everyday using a PlantScreen XYZ system (Photon Systems Instruments, Drásov, Czech Republic) [25].

4.4 Estimation of Physiological Parameters

4.4.1 Growth Conditions

The seeds of all the Arabidopsis lines were soaked in distilled water and kept in dark for two days. The seeds were then sown in the soil mixture containing peat moss, perlite, and vermiculite (3: 1: 1). Plants were grown at 22 °C, 180 µmol photons of PAR $m^{-2} \cdot s^{-1}$, and 16/8 h light/dark cycle. Twenty-eight-day-old plants were treated with distilled water (control) and 200 mM sodium chloride (salt stress). The experiment was designed using a CRD, with four replicates and four plants per replicate for each treatment.

4.4.2 Estimation of Photosynthetic Parameters

Photosynthetic parameters were estimated five days after treatment using the seventh leaf of all the Arabidopsis lines and a LI-6400XT portable photosynthesis system (LICOR, Lincoln, NE, USA) [26]. P_n, C_i, g_s, transpiration rate, Fv′/Fm′, ΦPSII, and ETR were estimated at 300 $mmol^{-1}$ of flow of air $m^{-2} \cdot s^{-1}$, 1000 mol photon of PAR $m^{-2} \cdot s^{-1}$, 23–24 °C, and 400 $mol \cdot mol^{-1}$ of CO_2 concentration.

4.4.3 Estimation of the Content of the Photosynthetic Pigments and Anthocyanins

Leaf disks (diameter: 7 mm) of all the Arabidopsis lines collected five days after treatment were soaked in 80% acetone and maintained at 14 °C for 72 h to extract chlorophylls and carotenoids. A spectrophotometer was used to measure the absorbance of the leaf extracts at A_{470}, $A_{646.8}$, and $A_{663.2}$. The contents of the photosynthetic pigments were calculated using the following formulas [27]:

$$\text{Chlorophyll a (Chl } a\text{) content} = 12.25A_{663.2} - 2.79A_{646.8} \quad (1)$$

$$\text{Chlorophyll b (Chl } b\text{) content} = 21.5A_{646.8} - 5.1A_{663.2} \quad (2)$$

$$\text{Total chlorophyll content} = \text{Chl a} + \text{Chl b} \quad (3)$$

$$\text{Chlorophyll a/b ratio} = \text{Chl } a \text{ / Chl } b \quad (4)$$

$$\text{Total carotenoids} = (100A_{470} - 1.82\text{Chl } a - 85.02\text{chl } b) / 198 \quad (5)$$

Anthocyanin extraction was performed using the leaf disks soaked in 1% HCl in methanol and kept at 14 °C overnight in microcentrifuge tubes. After overnight-soaking, 200 µL of milli-Q water and 500 µL of chloroform were added to each sample and centrifuged at 16,000 x g for 3 min. The supernatant was discarded, 400 µL of 60% aqueous solution of 1% HCl in methanol was added to the pellet, and absorbance was measured at A_{530} and A_{675} using a microplate reader (Synergy™ HTX Multi-Mode Microplate Reader, BioTex, USA). The anthocyanin content in the leaves was calculated using the following formula [28].

$$\text{Anthocyanin content} = (A_{657} - A_{530}) / \text{area } (m^2) \quad (6)$$

4.5 Statistical Analyses

The images of the green areas of the leaves were analyzed using MATLAB (R2015; MathWorks Inc., Natick, MA, USA). Independent *t*-tests were performed using SPSS version 22 (IBM, Armonk, NY, USA). Analysis of variance and Duncan's multiple range test were used to analyze the photosynthetic parameters and pigment contents.

5. Conclusions

Based on this study, *LOC_Os01g68450* confers salt tolerance to plants by maintaining higher contents of the photosynthetic pigments, including chlorophyll *a* and carotenoids, and anthocyanins, which protect the light-harvesting complex and increase the photosynthetic ability of the plants under salt stress. It is located in salt tolerance QTL previously reported by Kanjoo et al. [4]. Therefore, it suggested that this salt tolerance QTL supports the photosynthesis adaptation under salt stress condition can be a target region for rice breeding program.

Author Contributions:

Funding: LS and NDD acknowledge the ERDF project titled, "Development of Pre-Applied Research in Nanotechnology and Biotechnology" (No. CZ.02.1.01/0.0/0.0/17_048/0007323).

Acknowledgments: JP was supported by the 100th Anniversary of Chulalongkorn University Doctoral Scholarship and the 90th Anniversary of Rachadapisek Sompote Fund, Chulalongkorn University. PC was supported by C2F post-doctoral scholarship.

Conflicts of Interest:

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Hossain, M.S. Present Scenario of Global Salt Affected Soil, its Management and Importance of Salinity Research. Int. Res. J. Biol. Sci. **2019**, *1*, 1–3.
2. Li, W.; Li, Q. Effect of Environment Salt Stress on Plant and the Molecular Mechanism of Salt Stress Tolerance. Int. J. Environ. Sci. Nat. Resour. **2017**, *7*, 81–86.
3. Isayenkov, S.V.; Maathuis, F.J.M. Plant Salinity Stress: Many Unanswered Questions Remain. Front. Plant Sci. **2019**, 10, 80.
4. Kanjoo, V.; Jearakongman, S.; Punyawaew, K.; Siangliw, J.L.; Siangliw, M.; Vanavichit; A.; Toojinda, T. Co-Location of Quantitative Trait Loci for Drought and Salinity Tolerance in Rice. Thai J. Genet. **2011**, *4*, 126–138.
5. Punchkhon, C.; Plaimas, K.; Buaboocha, T.; Siangliw, J.L.; Toojinda, T.; Comai, L.; De Diego, N.; Spíchal, L.; Chadchawan, S. Drought-tolerance gene identification using genome comparison and co-expression network analysis of chromosome substitution lines in rice. Genes. **2020**, *11*, 1197.
6. Chutimanukul, P.; Kositsup, B.; Plaimas, K.; Buaboocha, T.; Siangliw, M.; Toojinda, T.; Comai, L.; Chadchawan, S. Photosynthetic Responses and Identification of Salt Tolerance Genes in a Chromosome Segment Substitution Line of 'Khao Dawk Mali 105' Rice. Environ. Exp. Bot. **2018**, *155*, 497–508.
7. Chutimanukul, P. Salt Tolerant Gene Identification in Rice Using Chromosome Substitution Lines with 'Khao Dawk Mali 105' Rice Genetic Background, Doctoral Dissertation, Chulalongkorn University, 30 June **2018**.
8. Moradi, F.; Ismail, A.M. Responses of Photosynthesis, Chlorophyll Fluorescence and ROS-Scavenging Systems to Salt Stress during Seedling and Reproductive Stages in Rice. Ann. Bot. **2007**, *99*, 1161–1173.
9. Soussi, M.; Ocana, A.; Lluch, C. Effects of Salt Stress on Growth, Photosynthesis and Nitrogen Fixation in Chick-Pea (Cicer arietinum L.). J. Exp. Bot. **1998**, *49*, 1329–1337.
10. Helena, H.; František, H.; Jaroslava, M.; Kamil, K. Effects of Salt Stress on Water Status, Photosynthesis and Chlorophyll Fluorescence of Rocket. Plant Soil Environ. **2017**, *63*, 362–367.

11. Jiang, C.; Zu, C.; Lu, D.; Zheng, Q.; Shen, J.; Wang, H.; Li, D. Effect of Exogenous Selenium Supply on Photosynthesis, Na+ Accumulation and Antioxidative Capacity of Maize (*Zea mays* L.) under Salinity Stress. Sci. Rep. **2017**, *7*, 42039.
12. Chaves, M.M.; Flexas, J.; Pinheiro, C. Photosynthesis under Drought and Salt Stress: Regulation Mechanisms from Whole Plant to Cell. Ann. Bot. **2009**, *103*, 551–560.
13. Hamani, A.K.M.; Li, S.; Chen, J.; Amin, A.S.; Wang, G.; Xiaojun, S.; Zain, M.; Gao, Y. Linking Exogenous Foliar Application of Glycine Betaine and Stomatal Characteristics with Salinity Stress Tolerance in Cotton (*Gossypium hirsutum* L.) Seedlings. B.M.C. Plant Biol. **2021**, *21*, 146.
14. González-Grande, P.; Suárez, N.; Marín, O. Effect of Salinity and Seed Salt Priming on the Physiology of Adult Plants of *Solanum lycopersicum* cv. 'Río Grande'. Braz. J. Bot. **2020**, *43*, 775–787.
15. Karaba, A.; Dixit, S.; Greco, R.; Aharoni, A.; Trijatmiko, K.R.; Marsch-Martinez, N.; Krishnan, A.; Nataraja, K.N.; Udayakumar, M.; Pereira, A. Improvement of Water Use Efficiency in Rice by Expression of HARDY, an Arabidopsis Drought and Salt Tolerance Gene. Proc. Natl. Acad. Sci. U. S. A. **2007**, *104,* 15270–15275.
16. Taj, Z.; Challabathula, D. Protection of Photosynthesis by Halotolerant Staphylococcus sciuri ET101 in Tomato (Lycoperiscon esculentum) and Rice (*Oryza sativa*) Plants during Salinity Stress: Possible Interplay between Carboxylation and Oxygenation in Stress Mitigation. Front. Microbiol. **2021**, *11*, 1–24.
17. Li, N.; Wang, X.; Ma, B.; Wu, Z.; Zheng, L.; Qi, Z.; Wang, Y. A Leucoanthocyanidin Dioxygenase Gene (RtLDOX2) from the Feral Forage Plant Reaumuria trigyna Promotes the Accumulation of Flavonoids and Improves Tolerance to Abiotic Stresses. J. Plant Res. **2021**, 1–8**;** doi:10.1007/s10265-021-01315-2.
18. Cebrián, G.; Iglesias-Moya, J.; García, A.; Martínez, J.; Romero, J.; Regalado, J.J.; Martínez, C.; Valenzuela, J.L.; Jamilena, M. Involvement of Ethylene Receptors in the Salt Tolerance Response of Cucurbita pepo. Hortic. Res. **2021**, *8*, 73.
19. Sharma, A.; Shahzad, B.; Rehman, A.; Bhardwaj, R.; Landi, M.; Zheng, B. Response of Phenylpropanoid Pathway and the Role of Polyphenols in Plants under Abiotic Stress. Molecules. **2019**, *24*, 2452.

20. Xu, Z.; Mahmood, K.; Rothstein, S.J. ROS Induces Anthocyanin Production via Late Biosynthetic Genes and Anthocyanin Deficiency Confers the Hypersensitivity to ROS-Generating Stresses in Arabidopsis. Plant Cell Physiol. **2017**, *58*, 1364–1377.
21. Shen, Y.; Li, J.; Gu, R.; Yue, L.; Wang, H.; Zhan, X.; Xing, B. Carotenoid and Superoxide Dismutase Are the Most Effective Antioxidants Participating in ROS Scavenging in Phenanthrene Accumulated Wheat Leaf. Chemosphere. **2018**, *197,* 513–525.
22. Ouyang, S.; Zhu, W.; Hamilton, J.; Lin, H.; Campbell, M.; Childs, K.; Thibaud-Nissen, F.; Malek, R.L.; Lee, Y.; Zheng, L.; Orvis, J.; Haas, B.; Wortman, J.; Buell, C.R. The TIGR Rice Genome Annotation Resource: Improvements and New Features. Nucleic Acids Res. **2007**, *35*, D883-D887.
23. Berardini, T.Z.; Reiser, L.; Li, D.; Mezheritsky, Y.; Muller, R.; Strait, E.; Huala, E. The Arabidopsis Information Resource: Making and Mining the 'Gold Standard' Annotated Reference Plant Genome. Genesis. **2015**, *53,* 474–485; DOI: 10.1002/dvg.22877.
24. Lamesch, P.; Berardini, T.Z.; Li, D.; Swarbreck, D.; Wilks, C.; Sasidharan, R.; Muller, R.; Dreher, K.; Alexander, D.L.; Garcia-Hernandez, M.; Karthikeyan, A.S.; Lee, C.H.; Nelson, W.D.; Ploetz, L.; Singh, S.; Wensel, A.; Huala, E. The Arabidopsis Information Resource (TAIR): Improved Gene Annotation and New Tools. Nucleic Acids Res. **2012**, *40*, D1202–D1210.
25. De Diego, N.; Fürst, T.; Humplík, J.F.; Ugena, L.; Podlešáková, K.; Spíchal, L. An Automated Method for High-Throughput Screening of Arabidopsis Rosette Growth in Multi-Well Plates and Its Validation in Stress Condition. Front. Plant Sci. **2017**, *8*, 1702.
26. Hu, X.; Tanaka, A.; Tanaka, R. Simple Extraction Methods That Prevent the Artifactual Conversion of Chlorophyll to Chlorophyllide during Pigment Isolation from Leaf Samples. Plant Methods. **2013**, *9*, 19.
27. Wellburn, A.R. The Spectral Determination of Chlorophylls a and b, as well as Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution. J. Plant Physiol. **1994**, *144*, 307–313.

28. Neff, M.M.; Chory, J. Genetic Interactions between Phytochrome A, Phytochrome B, and Cryptochrome 1 during Arabidopsis Development. Plant Physiol. **1998**, *118*, 27–35.

CHAPTER IV

ADDITIONAL RESULTS

Gene expression of drought stress genes predicted by gene co-expression network analysis

Introduction

Many genes are up-regulated and down-regulated by drought stress (Chamani Mohasses et al., 2020). *LOC_Os01g72950* (*Ndh-O*), *LOC_Os07g37550* (*Lhcb3*), *LOC_Os07g38300* (*Rrf*) and *LOC_Os10g10170* (*Mrl1*) were predicted to be drought tolerance related genes. Their roles in drought tolerance were investigated by using Arabidopsis *thaliana* mutant lines, *ndh-O*, *lhcb3*, *rrf*, and *mrl1* mutants. The previous results showed the higher green area of *ndh-O*, *lhcb3*, *rrf*, and *mrl1* mutants than wild-type under drought stress condition. Moreover, *pgrl1a* and *pgrl1b* mutant, which was mutated in the homologous genes of LOC_Os08g41460, show the lowest green area under drought stress condition. Therefore, the gene expression of these genes 'KDML105', DH103 and CSSL104 rice was monitored under drought stress condition.

Materials and methods

Plant materials and growing conditions

KDML105, DH103 and CSSL104 rice seeds were incubated in the 60°C oven for two days. Then, the hot-air treated seeds were soaked in distilled water for 7 days for germination. During the germination period, the distilled water was changed daily. Seven-day old seedlings were transferred into a half-strength WP solution (Vajrabhaya and Vajrabhaya, 1991). The WP solution was changed to full-strength WP solution when rice plants were at the age of 14 and 21 days old. When rice plants were 28 days old, the rice plants were separated into 2 groups, one was grown in the

freshly prepared WP solution as a control condition, and the other group was transferred to the freshly prepared WP solution supplemented with 15% of polyethylene glycol 6000 (PEG6000) for drought stress condition. The experiment was designed as Completely Randomize Design (CRD) with 4 replications.

RNA extraction and gene expression analysis by qPCR

The fresh leaves of KDML105, DH103 and CSSL104 rice were collected on day 0, 3, 6 and after treatment and then, the tissues were frozen directly in the liquid nitrogen. The samples were grinded by using mortar and pestle. GENEzol^{TM} reagent (Geneaid, Taiwan) was used for RNA extraction. The total RNA concentration was measured by NanoDrop spectrophotometers (Thermo Fisher Sciencitific, USA). The RNA samples were treated with DNase I (Thermo Fisher scientific, USA) to remove DNA. Then, the RNA was reverted to DNA by using AccuPower RT Premix (Bioneer, Korea) and keep in -80 °C freezer.

The quantitative RT-PCR were conducted using Luna qPCR master mix (NEB, USA) with 3 technical replications for each sample. The primers for qPCR were used following Table 1.

Table 1 Primer and product size for RT-PCR of *NdhO, Lhcb3, Rrf, Pgrl5-like and Mrl1*

Gene name	RT-PCR primer (5'→3')		product size
NdhO	FW	TCTCTCGCAGGCAGTGAAGC	199
	Rv	TCGTGCCATCCAGAACCAGC	
Lhcb3	FW	GTCGACTTCAAGGAGCCCGT	197
	Rv	CCCATGAGGACGACCTGGAA	
Rrf	FW	TACTGTGAGGACAGGGCGGG	140
	Rv	GTGTAACCCCCAGATTTGCAGC	
Pgrl5-like	FW	TGTGGCTGGCAACCCCATTA	179
	Rv	TCAGAGCTAAAACTGCTGCTGGA	
Mrl1	FW	GCTGTTCCGGAAGCTGTTCGT	191
	Rv	TTCCACACTTGGCACAAGTCGATA	

The condition of PCR is followed initial denaturation 95°C for 60 seconds. then, followed by 40 cycles of denaturation 95°C for 15 seconds, extension 61.5°C for 30 seconds and 95°C for 5 seconds. The melt curve is at 60-94°C with temperature increasing 5°C per 5 seconds.

The transcription levels were normalized by using EF1α (Bevitori et al., 2014). The relative expression levels was calculated by using the $\Delta\Delta$Ct method (Pfaffl, 2001).

Statistical analysis

The data were analyzed using Analysis of Variance (ANOVA) and the means were compared with Duncan's Multiple Range Test (DMRT) by SPSS Statistics program version 22 (IBM, Armonk, NY, USA).

Results

Expression of *LOC_Os01g72950* (*NDH-O*) gene

The significant difference of *LOC_Os01g72950* gene expression was first detected after 6 days of the treatment. The gene expression tended to decline, except the expression in DH103, which could be maintained in control condition. This resulted in the significant higher expression of *LOC_Os01g72950* gene in normal-grown DH103. However, after 9 days, no significant difference of *LOC_Os01g72950* gene expression could be detected (Figure 1).

Expression of *LOC_Os07g37550* (*LHCB3)* gene

The relative expression of *LOC_Os07g37550* gene in 'KDML105', DH103, and CSSL104 was significantly different on day 0. The highest expression was detected in CSSL104, while 'KDML105' had the lowest level of expression. After nine days of treatment, in normal grown condition, all lines show the similar level of expression, but drought stress induced the *LOC_Os07g37550* gene expression in 'KDML105' and DH103, but no significant changes in gene expression level were detected in CSSL104 (Figure 2).

Expression of *LOC_Os07g38300* (ribosome recycling factor, *RRF*) gene

Under the experimental condition, all lines exhibited the similar level of gene expression, and drought stress did not affect the expression of *LOC_Os07g38300* gene in leaf tissues.

Expression of *LOC_Os08g41460* (*PGR5-like*) gene

At the start of the experiment (day 0), CSSL104 showed the highest relative expression compared to other lines. After 3 days of treatment, *LOC_Os08g41460* gene in KDML105 was increased in normal grown condition after 3 days of transplanting. However, drought stress caused the decrease of *LOC_Os08g41460* gene expression in DH103 and CSSL104 after 3 days of the treatment. After transplanting in normal grown condition, *LOC_Os08g41460* gene expression in all lines were decreased. Contrastingly, the expression in drought stress for 9 days tended to cause the higher expression of *LOC_Os08g41460* gene in all lines tested (Figure 4).

Expression of *LOC_Os10g10170* gene

There was no significant difference of *LOC_Os10g10170* gene expression in all lines and conditions tested in this experiment (Figure 5).

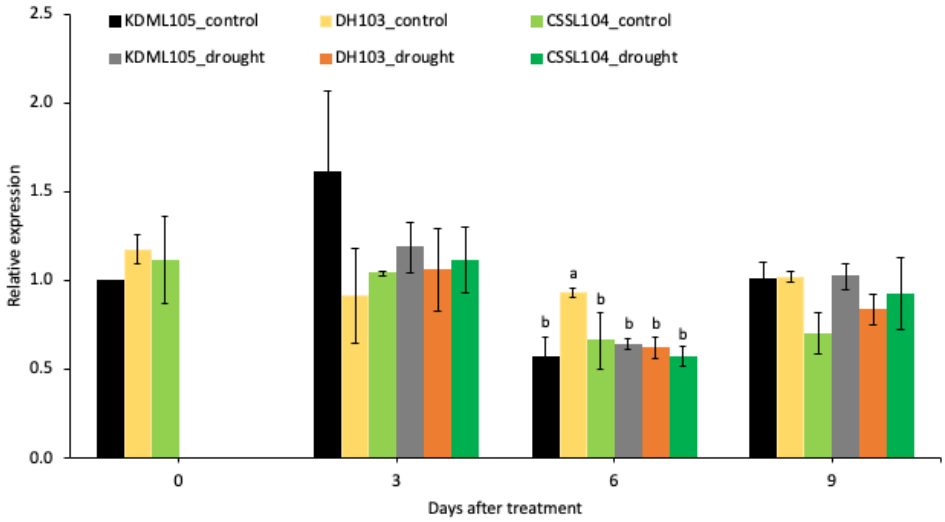


Figure 1 Relative expression of *LOC_Os01g72950* gene in KDML105, DH103 and CSSL104 under control and drought stress (15% PEG) at 0, 3, 6 and 9 days after treatment

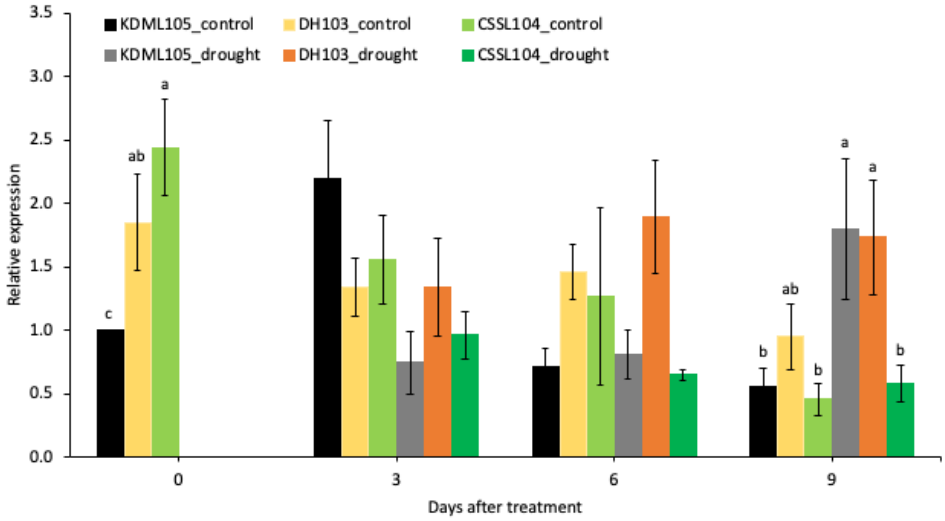


Figure 2 Relative expression of *LOC_Os07g37550* gene in KDML105, DH103 and CSSL104 under control and drought stress (15% PEG) at 0, 3, 6 and 9 days after treatment

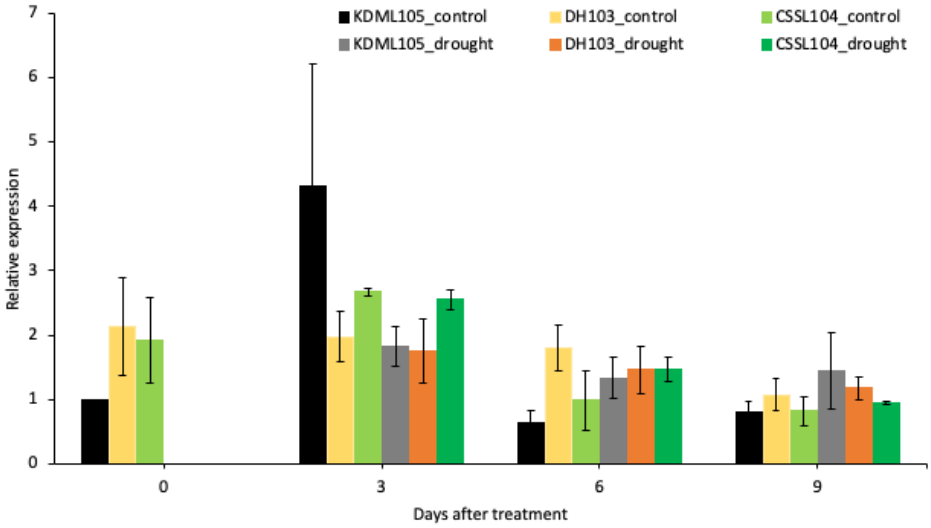


Figure 3 Relative expression of *LOC_Os07g38300* gene in KDML105, DH103 and CSSL104 under control and drought stress (15% PEG) at 0, 3, 6 and 9 days after treatment

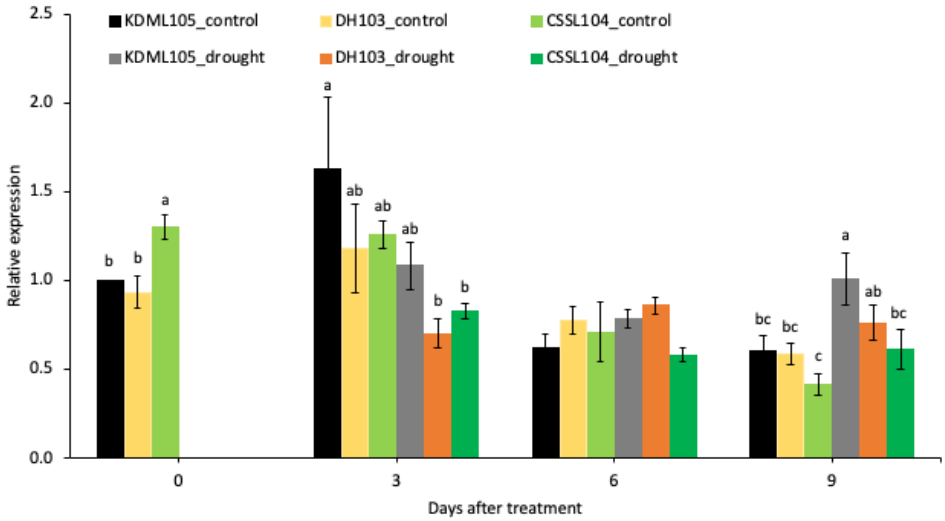


Figure 4 Relative expression of *LOC_Os08g41460* gene in KDML105, DH103 and CSSL104 under control and drought stress (15% PEG) at 0, 3, 6 and 9 days after treatment

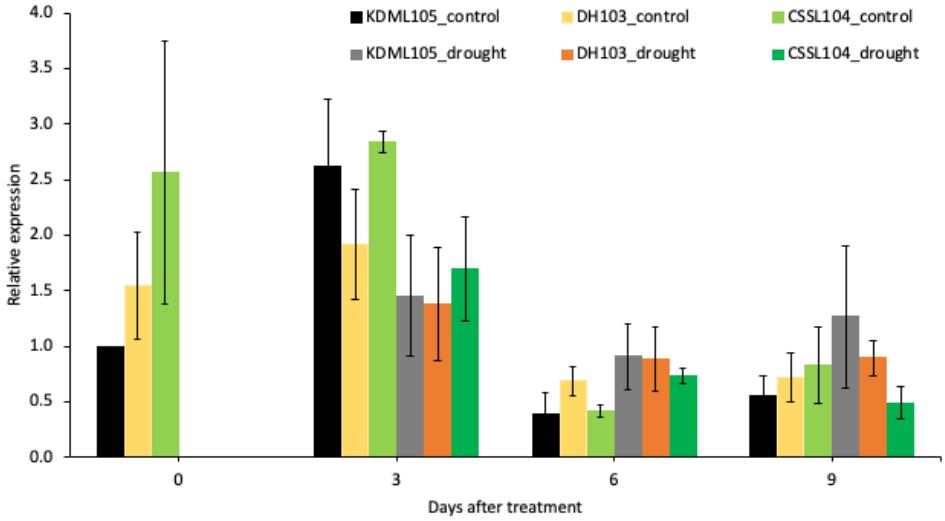


Figure 5 Relative expression of *LOC_Os10g10170* gene in KDML105, DH103 and CSSL104 under control and drought stress (15% PEG) at 0, 3, 6 and 9 days after treatment

Discussion

LOC_Os01g72950 is the gene encoded for chloroplast NADH dehydrogenase (NDH) subunit O. NDH-O is a subunit in the stroma-exposed subcomplex A (SubA), which is one of five subcomplexes of NDH complex. NDH complex interacts with photosystem I (PSI) to form NDH-PSI supercomplex (Peng et al., 2009), which is required to stabilize NDH (Peng and Shikanai, 2011). NDH complex is responsible for Fd oxidization, electron transfer, and complex stabilization (Fan et al., 2015). NDH-O involves in triggering cyclic electron transfer for ATP supply in chloroplast under stress condition such as heat and drought stress (Shikanai, 2016; Sirpiö et al., 2009). However, in the drought condition in this study, the expression of *LOC_Os01g72950* gene in all lines and condition was similar, suggesting no regulation at transcriptional level of this gene due to drought stress

LOC_Os07g37550, encoding LHCB3, which functions in the main light harvesting complex chlorophyll a/b binding protein in photosystem II. LHCB1, LHCB2 and LHCB3 capture light energy in antenna complex (Damkjær et al., 2009; Xu et al., 2012). The disruption of *LHCB3* gene was shown to affect responsiveness of stomatal movement to ABA, resulted in the decrease of drought tolerance (Xu et al., 2012). Based on this study, the induction of *LHCB3* gene by drought stress was detected in 'KDML105' after 9 days of treatment, suggesting that *LHCB3* may play a role to cope with drought stress in rice. However, the induction of *LHCB3* gene was not detected in CSSL104 after 9 days of drought stress. This suggested that CSSL104 had other mechanisms to cope with drought stress, which was not directly involving *LHCB3* transcriptional regulations.

LOC_Os07g38300 is expressed protein in rice, and *AT3G63190* is a homologous gene in Arabidopsis. *At3g63190* gene encodes ribosome recycling factor (RRF), whose functions are splicing and splitting ribosomes to subunit for recycling the ribosome to next translation. The *hlf-108* Arabidopsis mutant with T-DNA insertion in *At3g63190* decreased *RRF* gene expression and resulted in the decrease of ribosome splicing efficiency (Wang et al., 2010). In this experiment, the expression of *LOC_Os07g38300* was unchanged by drought stress, suggesting that drought stress did not affect the regulation of RRF gene expression at transcriptional level and the efficiency of ribosome recycling in all rice lines might be not affected in this stress condition.

LOC_Os08g41460 encodes the PGR5-like protein, which functions in photosystem and involves in photosynthetic electron flow. *At4g11960* and *At4g22890*, encoding *Pgrl1b* and *Pgrl1a*, respectively, are the homologous genes of *LOC_Os0841460*. The expression of *Pgrl1* was induced by drought stress in *Arabidopsis* (Lehtimäki et al., 2010; Suorsa, 2015). This gene product triggers cyclic electron flow which is important mechanism to help plant survive under stress condition (Huang et al., 2012). The relative expression of *LOC_Os08g41460* gene in

KDML105 under drought stress was higher than that in control condition after nine days of drought treatment. Moreover, the expression level of this gene in drought-stressed DH103 and CSSL104 had the tendency to be higher than normal-grown ones (Figure 4). This supports the role of *LOC_Os0841460* that is similar to *AtPgrl1*, involving the enhancement of cyclic electron transfer during drought stress.

LOC_Os10g10170 is conserved pentatricopeptide repeat protein, MRL1. This gene function by interact with *rbcL* for stabilization by acting at 5' untranslated region of *rbcL* to prevent degradation. *Atmrl1* mutant showed slightly decrease of RuBisCO content (Johnson et al., 2010). In this study, the expression of *LOC_Os10g10170* was not affected under drought stress, suggesting no direct effect of drought stress on the regulation of *LOC_Os10g10170* gene at transcriptional level.

Overall, KDML105 showed the induction of *LHCB3* and *PGR5-like* gene expression under drought stress condition, while the expression of these genes in DH103 and CSSL104 was lower. This implies that DH103 and CSSL104 may have other mechanisms to protect the photosynthesis and prevent from drought stress, while LHCB3 and PGR5-like may be the important factors for drought stress adaptation in 'KDML105' rice.

Conclusion

Based on the gene expression study in 'KDML105', DH103 and CSSL104, it was suggested that *LHCB3* and *PGR5-like* genes may play an important role in drought stress response in 'KDML105', while in the other two lines, DH103 and CSSL104, the induction of these two genes was not dramatically different from the expression in normal-grown condition. Therefore, these two rice lines may have other mechanisms to cope with drought stress.

Green area of Arabidopsis wild-type, *at1g65230* mutant and ectopic expression lines under drought stress conditions.

Introduction

Drought and salt stress are abiotic stress. It can limit growth, yield and productivity of plants. Both salt and drought stress can cause water deficit stress to plants. Osmotic adjustment can be induced by salt and drought stress. Moreover, reactive oxygen species are generated by salt and drought stress. But the difference of salt and drought stresses is ion toxicity that causes metabolism imbalance and premature senescence in plants (Chaves et al., 2008; Isayenkov and Maathuis, 2019) . *LOC_Os01g68450* gene was identified as interesting node gene via co-expression network analysis under salt stress in CSSLs which have the putative drought tolerant chromosome segment on chromosome 1 (Chutimanukul et al., 2018). The aim of this study is to investigate the impact of *LOC_Os01g68450* gene expression in *at1G65230 Arabidopsis* mutant, revertant by *LOC_Os01g68450* gene expression and ectopic expression lines in WT Arabidopsis under drought stress condition.

Materials and methods

Plant materials

The *Arabidopsis thaliana* ecotype Columbia-0 (wild type), mutant lines with T-DNA insertion in the AT1G65230 gene (SALK_130615), *LOC_Os01g68450* ectopic gene expression and revertant lines were used in this study. The Arabidopsis mutant line was ordered from The Arabidopsis Biological Resources Center (ARBC) (Lamesch et al., 2012).

Expression vector construction

The *LOC_Os01g68450* gene was amplified from AK068538 cDNA in pFLC1 vector using PCR and primer pairs as described below.

Forward primer	5’ TCTAGATACCAATGGCCACCGCCACCGCCA 3’
Reverse primer	5’GACAGGCTCGAGGGAAATAGATACACACAC 3’

The *LOC_Os01g68450* gene was cloned into binary vector, pJIM19, via *xbaI* and *xhoI* restriction sites to generated pJIM19_LOC_Os01g68450. The pJIM19_LOC_Os01g68450 plasmids were transferred into *Agrobacterium tumefaciens* GV3101 by freeze-thaw method (Jyothishwaran et al., 2007). The kanamycin, rifampicin and gentamycin were used as selectable markers to select for the right clones. Plant transformation was performed to transfer the gene cassette to WT and *at1g65230* mutant by floral dipping method (Zhang et al., 2006) in order to generate ectopic expression of *LOC_Os01g68450* and revertant lines, respectively. The ectopic expression of *LOC_Os01g68450* and revertant lines were grow on MS agar media supplemented with kanamycin to screened T_1, T_2 and T_3 generation. The homozygous transgenic lines were selected by monitoring of segregation analysis on MS medium supplemented with 50 mg/L kanamycin and PCR analysis to detect the existing of the inserted gene.

Drought stress treatment and growing conditions

Arabidopsis thaliana ecotype Columbia-0, *at1g65230* mutant lines, revertant lines, and transgenic Arabidopsis with ectopic expression of *LOC_Os01g68450* gene were sterile and germinated on filter paper covering over Murashige and Skoog (MS) agar media for four days. Then, the seedlings were transferred to 48-well plates filled

with MS agar media for control condition, MS agar media supplemented with 75 mM mannitol or 150 mM mannitol for mild and severe drought stress conditions, respectively. Seedlings were grown in growth chamber with the condition of 22°C/20°C, 16/8 h light/dark cycle, 120 μmol photons of PAR m^{-2} s^{-1}, and 60% humidity. The green area were collected by RGB imaging using PlantScreen™ XYZ system (Photon Systems Instruments, Drásov, Czech Republic) (De Diego et al., 2017). Each plate contained 48 plants / line.

Statistical analysis

The images from the PlantScreen™ XYZ system were analyzed using MATLAB (R2015; MathWorks, Inc., Natick, MA, USA). Mean of green area were analyzed by independent sample t-test using SPSS statistics program version 22 (IBM, Armonk, NY, USA) to compared with WT.

Results

The mutation in *At1g65230* gene resulted in growth inhibition in normal grown condition, but conferred less effect of drought stress on plant growth

When comparing the green area of WT and *at1g65230* mutant of normally grown plants, the mutation in *At1g65230* gene resulted in growth inhibition, according to the significantly less green area in the mutant line (Figure 6). In mild drought stress (75 mM mannitol), the significant lower green area of the mutant line was also detected (Figure 7), but under severe drought stress (150 mM mannitol) condition, wild-type and *at1g65230* mutant plant show no significant different green area between lines (Figure 8). If we compare the green area reduction percentage, the green area reduction percentage of the mutant was less than WT.

Ectopic expression of *LOC_Os01g68450* gene in WT Arabidopsis helped maintenance of green area under severe drought condition.

In normal grown condition, at the beginning of the experiment, green area of WT and ectopic expression of *LOC_Os01g68450* was similar, but after 7 days, ectopic expression lines had the significant higher level of green area than WT (Figure 9). At 75 mM mannitol, *ect-1* and WT showed the similar average green area, while the green area of *ect-2* leaves were significantly lower than wild-type during day 2- 6 after treatment. However, seven days of treatment, the green area of *ect-2* become similar to wild-type after 7 days of mild drought stress (Figure 10). In severe drought (150 mM mannitol) condition, the leaf green area of *ect-1* and *ect-2* was higher than WT for the whole experiment (Figure 11). This suggested the role of *LOC_Os01g68450* gene in drought tolerance.

The expression of *LOC_Os01g68450* gene rescued growth inhibition effect due to mutation in *At1g65230* gene and confer drought tolerance in mild stress.

When compared green area of *at1g65230* mutant line with revertant lines with ectopic expression of *LOC_Os01g68450*, the *rev-1* and *rev-2* showed significantly better than the mutant line under control condition (Figure 12). Under mild drought stress, the *rev-1* line showed significantly more green area than the mutant line, but after 7 days of treatment, the level of green area of *rev-1* was similar to the mutant line. This was contrast to *rev-2*, whose green area was similar to the mutant line at the beginning of the experiment, but it showed significantly more green area than mutant line after three days of treatment (Figure 13). In severe drought stress (150 mM mannitol) condition, the *rev-1* line had significantly higher green area when compared to the mutant line, while *rev-2* line showed less of green area than wild-type (Figure 14).

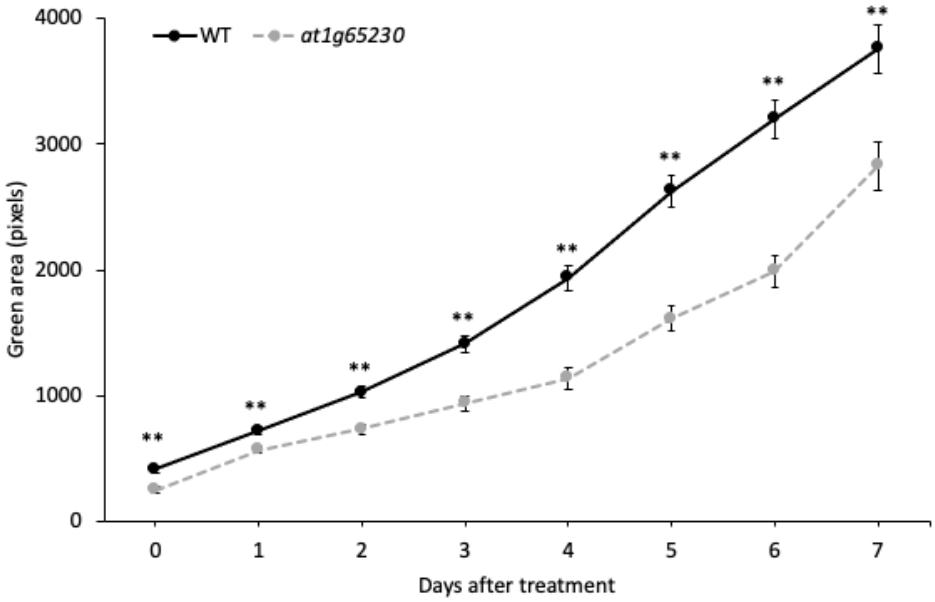


Figure 6 Green area (pixels) of Arabidopsis wild-type and *at1g65230* mutant plants under control condition

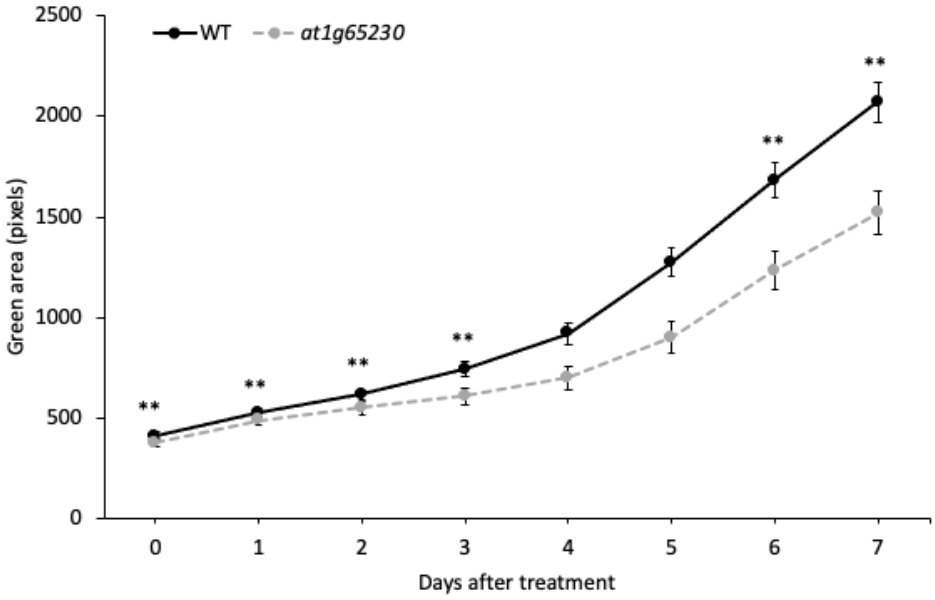


Figure 7 Green area (pixels) of Arabidopsis wild-type and *at1g65230* mutant plants under mild drought stress condition (75 mM mannitol)

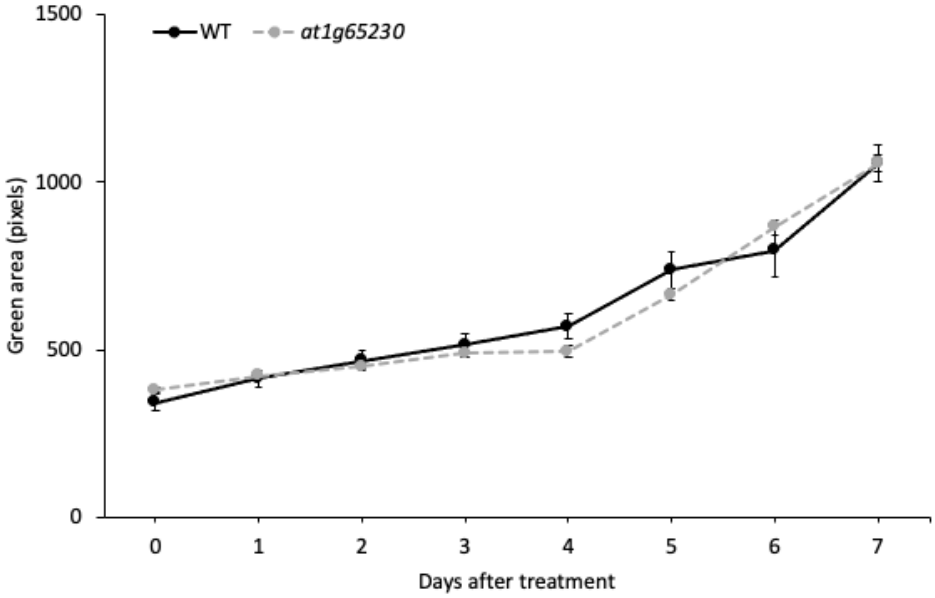


Figure 8 Green area (pixels) of Arabidopsis wild-type and *at1g65230* mutant plants under severe drought stress condition (150 mM mannitol)

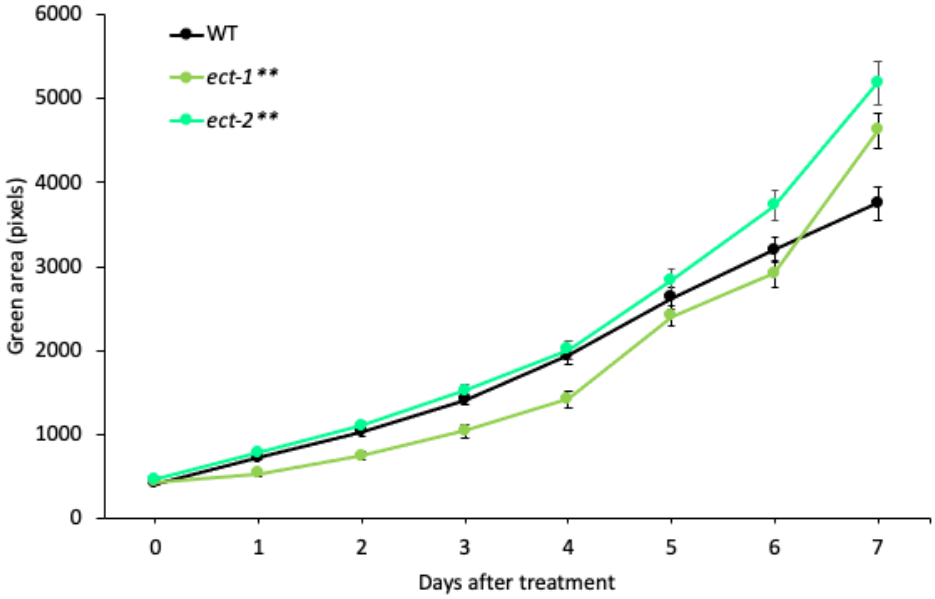


Figure 9 Green area (pixels) of Arabidopsis wild-type and ectopic expression of *LOC_Os01g68450* (*ect-1* and *ect-2*) lines under control condition

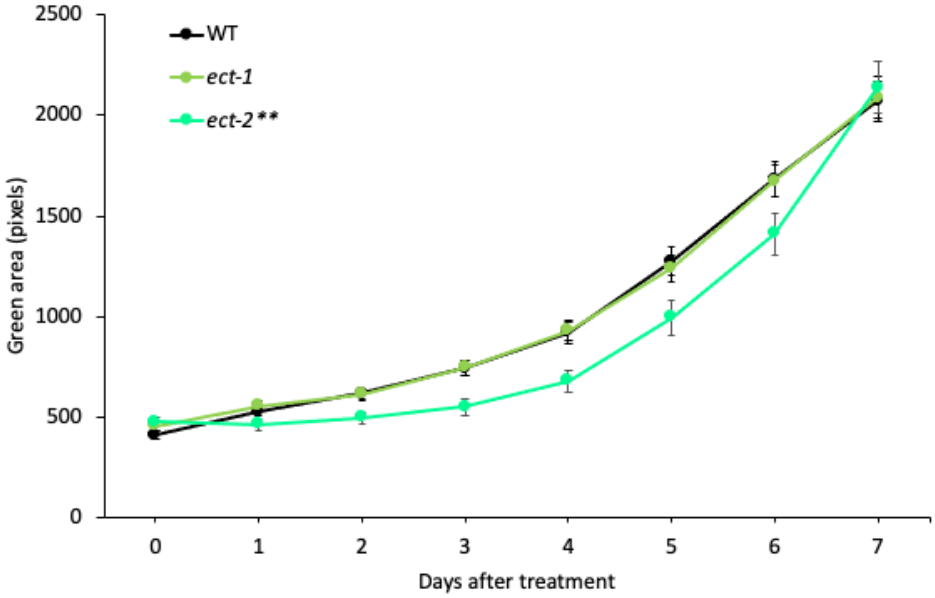


Figure 10 Green area (pixels) of Arabidopsis wild-type and ectopic expression of *LOC_Os01g68450* (*ect-1* and *ect-2*) lines under mild drought stress condition (75 mM mannitol)

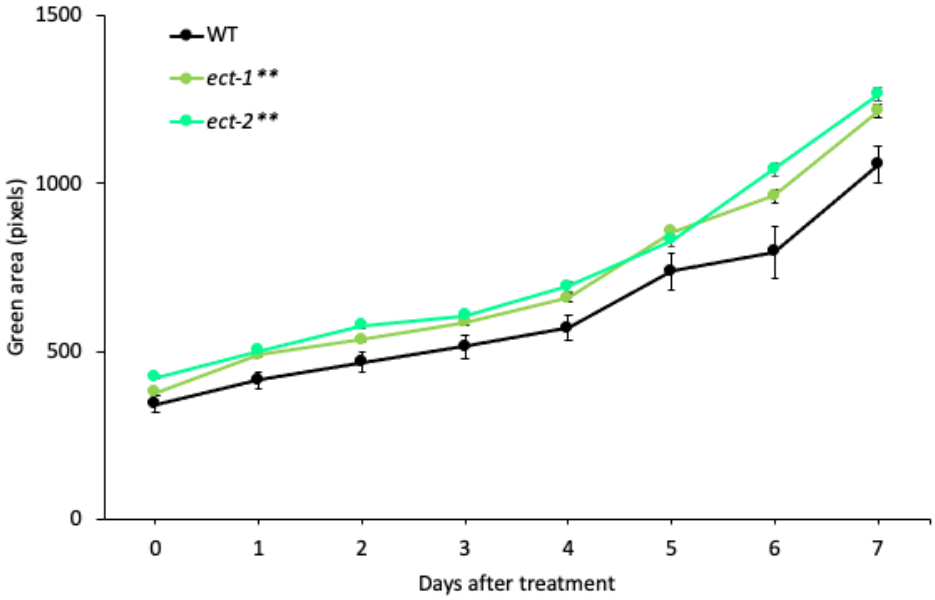


Figure 11 Green area (pixels) of Arabidopsis wild-type and ectopic expression of *LOC_Os01g68450* (*ect-1* and *ect-2*) lines under severe drought stress condition (150 mM mannitol)

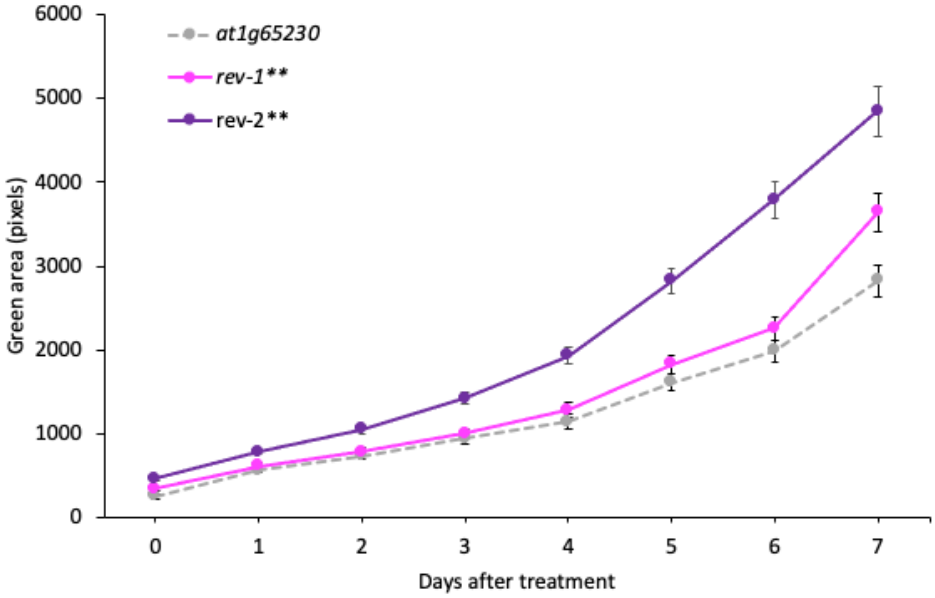


Figure 12 Green area (pixels) of Arabidopsis *at1g65230* mutant line and revertant lines with expression of *LOC_Os01g68450* gene (*rev-1* and *rev-2*) under control condition

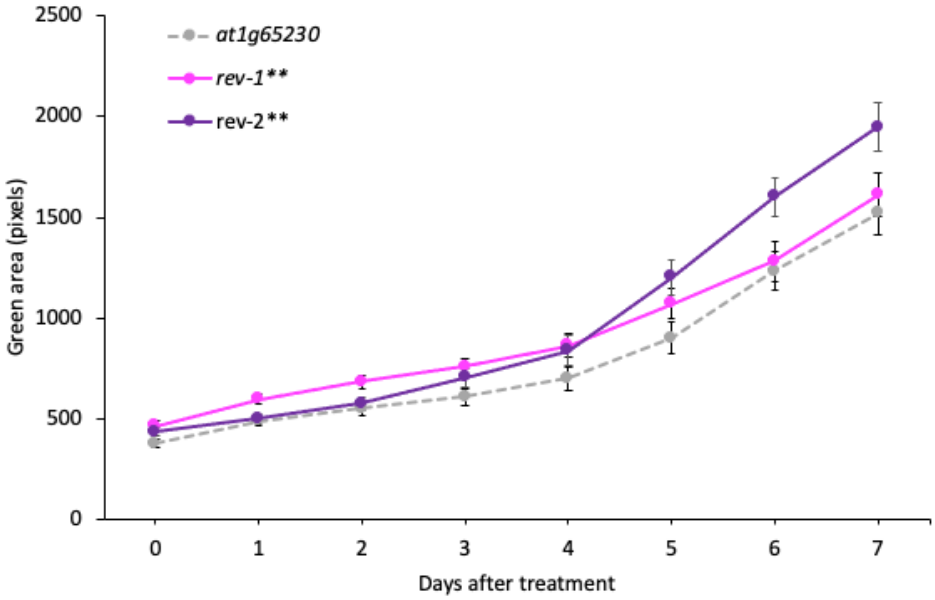


Figure 13 Green area (pixels) of Arabidopsis *at1g65230* mutant line and revertant lines with expression of *LOC_Os01g68450* gene (*rev-1* and *rev-2*) under mild drought stress condition (75 mM mannitol)

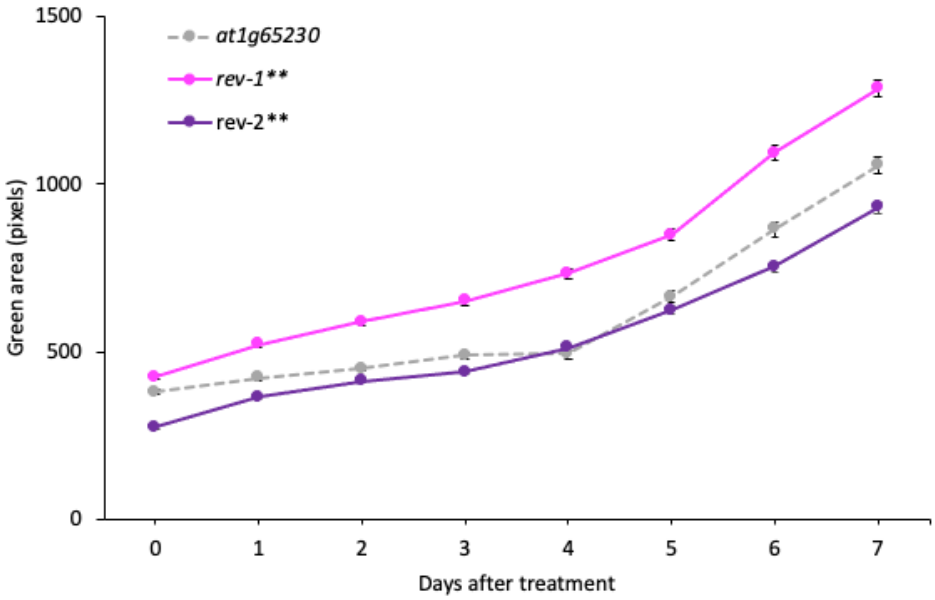


Figure 14 Green area (pixels) of Arabidopsis *at1g65230* mutant line and revertant lines with expression of *LOC_Os01g68450* gene (*rev-1* and *rev-2*) under severe drought stress condition (150 mM mannitol)

Discussion

The mutation in *AT1G65230* gene resulted in the decrease of green area, when compared to wild-type in normal-grown and drought stress condition. These results are similar to wild-type and *at1g65230* mutant in the experiment of salt stress (Chapter III). The green area in both lines show less decrease when grown under drought stress than salt stress. which means drought and salt affected plant growth, but salt stress is more severe than drought stress.

The green area of ectopic lines with expression of *LOC_Os01g68450* gene was higher than the green area of WT, especially under severe drought stress (150 mM mannitol). It can suggest that *LOC_Os01g68450* can help Arabidopsis plants to maintain growth rate under drought stress condition. The *rev-2*, revertant lines with expression of *LOC_Os01g68450* gene, show better growth rate under control and mild drought stress (75 mM mannitol). These results were the same as the rev-2 when grown under mild salt stress condition (75 mM NaCl) (Chapter III). It confirms

that *LOC_Os01g68450* gene can function instead of *AT1G65230* under drought and salt conditions. In contrast, *rev-1* showed less green area under control and mild drought stress condition. But this line showed higher green area than *at1g65230* mutant under severe drought stress. The growth of *rev-1* was better than *rev-2* under severe drought stress probably because of the high synthesis and accumulation of compounds in the cell. When plant synthesize unusual compound, it can cause negative effect on plant growth and development (Zargar et al., 2017). The differences in phenotypic responses under abiotic stress of different transgenic lines may be due to the position effects of the transgene.

LOC_Os01g68450 may function in drought stress in the similar response to salt stress. According to the increasing of the photosynthetic pigments and ability to maintain photosynthesis activity in the revertant and ectopic expression lines (Chapter III), leading to the better adaptation in abiotic stress, the function of *LOC_Os01g68450* gene in the maintenance of the light-harvesting complex stability can be proposed. *LOC_Os01g68450* could help plants to maintain pigment content, especially anthocyanin to reduce ROS in the cell and prevent photosynthesis reduction under drought stress (Sperdouli and Moustakas, 2012).

Conclusion

Based on the drought stress response of the transgenic lines with *LOC_Os01g68450* expression, may be proposed that *LOC_Os01g68450* functions in drought tolerance mechanism to maintain green area during drought stress.

CHAPTER V

CONCLUSION

The photosynthesis rate of CSSL104 is higher than 'KDML105' but similar to DH103 which is drought tolerant line. CSSL104 suggested to be drought tolerance line.

When compared the genome sequences of CSSL104 with 'KDML105' via whole genome sequencing and SNPs data found 101,950 SNPs located on 3440 genes separated on every chromosome. The dense SNPs located on chromosomes 1, 8 ,9 and 11. These SNPs data were analyzed by gene co-expression network analysis and found 18 gene nodes but 9 nodes are genes involve in photosynthesis including *LOC_Os01g72800* (*Cpftsy*), *LOC_Os01g72950* (*Ndh-o*), *LOC_Os03g19760* (*Soq1*), *LOC_Os07g37550* (*Lhcb3*), *LOC_Os07g38300* (*Rrf*), *LOC_Os08g41460* (*Pgrl5-like*), *LOC_Os08g44000* (*Hcf244*), *LOC_Os09g39390*, *LOC_Os10g10170* (*Mrl1*). These genes may have potential to be drought tolerance genes

Eight homozygous Arabidopsis mutant line with T-DNA insertion in the gene, which is ortholog with predicted rice gene, were used to study including *ndhO* (*at1g74880*), *lhcb3* (*at5g54270*), *rrf* (*at3g63190*), *pgrl1b* (*at4g11960*), *pgrl1a* (*at4g22890*), *at2g27680*, *mrl1*(*at4g34830*) and *at1g65230*, the orthologous gene with *LOC_Os01g68450* predicted from CSSL16. The results of green area show that the *ndhO, lhcb3*, *rrf* and *mrl1* mutant lines have higher green area than WT under drought stress and *at2g27680* mutant line show the lowest green area under salt stress condition. These genes may have potential in drought and salt tolerance in Arabidopsis and rice.

at1g65230 mutant, ectopic expression of *LOC_Os01g68450* and revertant lines were used to characterize the gene function of *LOC_Os01g68450*. The revertant lines

show high photosynthesis rate, Excitation capture efficiency (F_v'/F_m'), operating efficiency of PSII, electron transport rate, chlorophyll a content, carotenoids content and anthocyanin content under salt stress condition. The growth rate of *at1g65230* mutant and revertant lines that grow under salt stress are similar to these lines when grown under drought stress. The revertant lines can grow better than mutant lines under drought and salt stress. It means *LOC_Os01g68450* gene can function instead of *AT1G65230*. Which conclude that *LOC_Os01g68450* gene is function in light-harvesting complex stabilization by maintaining pigment content including chlorophyll a, carotenoids and anthocyanin under drought and salt stress.

Moreover, the high expression of *LOC_Os07g37550* and *LOC_Os08g41460* genes in 'KDML105' compared to DH103 and CSSL104 under drought stress condition can confirm the drought susceptibility of 'KDML105', which have more susceptible to drought than CSSL104 and DH103.

REFERENCES

Abou-Elwafa, S. F. and Shehzad, T. 2018. Genetic identification and expression profiling of drought responsive genes in sorghum. *Environmental and Experimental Botany* 155: 12-20.

Anjum, F., Yaseen, M., Rasool, E., Wahid, A. and Anjum, S. 2003. Water stress in barley (*Hordeum vulgare* L.). II. Effect on chemical composition and chlorophyll contents. *Pakistan J. Agric. Sci* 40.

Bevitori, R., Oliveira, M., Grossi-de-Sá, M., Lanna, A. and Silveira, R. 2014. Selection of optimized candidate reference genes for qRT-PCR normalization in rice (*Oryza sativa* L.) during Magnaporthe oryzae infection and drought. *Genetics and molecular research : GMR* 13: 9795-9805.

Blum, A. 1989. Osmotic Adjustment and Growth of Barley Genotypes under Drought Stress. *Crop Science* 29: 230-233.

Chamani Mohasses, F., Solouki, M., Ghareyazie, B., Fahmideh, L. and Mohsenpour, M. 2020. Correlation between gene expression levels under drought stress and synonymous codon usage in rice plant by in-silico study. *PLOS ONE* 15: e0237334.

Chaves, M. M., Flexas, J. and Pinheiro, C. 2008. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany* 103: 551-560.

Chutimanukul, P., Kositsup, B., Plaimas, K., Buaboocha, T., Siangliw, M., Toojinda, T., et al. 2018. Photosynthetic responses and identification of salt tolerance genes in a chromosome segment substitution line of 'Khao dawk Mali 105' rice. *Environmental and Experimental Botany* 155: 497-508.

Cornic, G. and Massacci, A. 1996. Leaf Photosynthesis Under Drought Stress. In N. R. Baker (Ed.), *Photosynthesis and the Environment* pp. 347-366. Natherlands: Kluwer Academic Publishers.

Damkjær, J. T., KereïChe, S., Johnson, M. P., Kovacs, L., Kiss, A. Z., Boekema, E. J., et al. 2009. The Photosystem II Light-Harvesting Protein *Lhcb3* Affects the Macrostructure of Photosystem II and the Rate of State Transitions in Arabidopsis. *The Plant Cell* 21: 3245-3256.

De Diego, N., Fürst, T., Humplík, J. F., Ugena, L., Podlešáková, K. and Spíchal, L. 2017. An Automated Method for High-Throughput Screening of Arabidopsis Rosette Growth in Multi-Well Plates and Its Validation in Stress Conditions. *Frontiers in Plant Science* 8.

Degenkolbe, T., Do, P. T., Zuther, E., Repsilber, D., Walther, D., Hincha, D. K., et al. 2009. Expression profiling of rice cultivars differing in their tolerance to long-term drought stress. *Plant Molecular Biology* 69: 133-153.

Devi, K., Prathima, P. T., Gomathi, R., Manimekalai, R., Lakshmi, K. and Selvi, A. 2019. Gene Expression Profiling in Sugarcane Genotypes During Drought Stress and Rehydration. *Sugar Tech* 21: 717-733.

Dhanda, S. S., Sethi, G. S. and Behl, R. K. 2004. Indices of Drought Tolerance in Wheat Genotypes at Early Stages of Plant Growth. *Journal of Agronomy and Crop Science* 190: 6-12.

Ding, X., Li, X. and Xiong, L. 2013. Insight into Differential Responses of Upland and Paddy Rice to Drought Stress by Comparative Expression Profiling Analysis. *International Journal of Molecular Sciences* 14: 5214-5238.

Fahad, S., Bajwa, A. A., Nazir, U., Anjum, S. A., Farooq, A., Zohaib, A., et al. 2017. Crop Production under Drought and Heat Stress: Plant Responses and Management Options. *Frontiers in Plant Science* 8.

Fan, X., Zhang, J., Li, W. and Peng, L. 2015. The *NdhV* subunit is required to stabilize the chloroplast NADH dehydrogenase-like complex in Arabidopsis. *The Plant Journal* 82: 221-231.

Fan, X. D., Wang, J. Q., Yang, N., Dong, Y. Y., Liu, L., Wang, F. W., et al. 2013. Gene expression profiling of soybean leaves and roots under salt, saline–alkali and drought stress by high-throughput Illumina sequencing. *Gene* 512: 392-402.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D. and Basra, S. M. A. 2009. Plant drought stress: effects, mechanisms and management. *Agronomy for Sustainable*

Development 29: 185-212.

Golding, A. J. and Johnson, G. N. 2003. Down-regulation of linear and activation of cyclic electron transport during drought. *Planta* 218: 107-114.

Hajiboland, R. 2014. Chapter 1 - Reactive Oxygen Species and Photosynthesis. In P. Ahmad (Ed.), *Oxidative Damage to Plants* pp. 1-63. San Diego: Academic Press.

Huang, W., Yang, S. J., Zhang, S. B., Zhang, J. L. and Cao, K. F. 2012. Cyclic electron flow plays an important role in photoprotection for the resurrection plant Paraboea rufescens under drought stress. *Planta* 235: 819-828.

Hussain, S., Farooq, M., Wahid, M. and Wahid, A. 2013. Seed Priming with Putrescine Improves the Drought Resistance of Maize Hybrids. *International Journal of Agriculture and Biology* 15: 1560-8530.

Isayenkov, S. V. and Maathuis, F. J. M. 2019. Plant Salinity Stress: Many Unanswered Questions Remain. *Frontiers in Plant Science* 10.

Johnson, G. N. 2011. Physiology of PSI cyclic electron transport in higher plants. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1807: 384-389.

Johnson, X., Wostrikoff, K., Finazzi, G., Kuras, R., Schwarz, C., Bujaldon, S., et al. 2010. MRL1, a Conserved Pentatricopeptide Repeat Protein, Is Required for Stabilization of *rbcL* mRNA in Chlamydomonas and Arabidopsis *The Plant Cell* 22: 234-248.

Jyothishwaran, G., Kotresha, D., Selvaraj, T., Srideshikan, S. M., Rajvanshi, P. K. and Jayabaskaran, C. 2007. A modified freeze–thaw method for efficient transformation of *Agrobacterium tumefaciens*. *Current Science* 93: 770-772.

Kanjoo, V., Jearakongman, S., Punyawaew, K., Siangliw, J., Siangliw, M., Vanavichit, A., et al. 2011. Co-location of quantitative trait loci for drought and salinity tolerance in rice. *Thai Journal of Genetics* 4: 126-138.

Lamesch, P., Berardini, T. Z., Li, D., Swarbreck, D., Wilks, C., Sasidharan, R., et al. 2012. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. *Nucleic Acids Research* 40: D1202-D1210.

Lehtimäki, N., Lintala, M., Allahverdiyeva, Y., Aro, E. M. and Mulo, P. 2010. Drought stress-induced upregulation of components involved in ferredoxin-dependent cyclic electron transfer. *J Plant Physiol* 167: 1018-1022.

Lesk, C. and Anderson, W. 2021. Decadal variability modulates trends in concurrent heat and drought over global croplands. *Environmental Research Letters* 16: 055024.

Mafakheri, A., Siosemardeh, A., Bahramnejad, B., Struik, P. and Sohrabi, Y. 2010. Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. *Australian Journal of Crop Science* 4: 8.

Marchin, R. M., Ossola, A., Leishman, M. R. and Ellsworth, D. S. 2020. A Simple Method for Simulating Drought Effects on Plants. *Frontiers in Plant Science* 10.

Mouradi, M., Bouizgaren, A., Farissi, M., Latrach, L., Qaddoury, A. and Ghoulam, C. 2016. Seed osmopriming improves plant growth, nodulation, chlorophyll fluorescence and nutrient uptake in alfalfa (*Medicago sativa* L.) – rhizobia symbiosis under drought stress. *Scientia Horticulturae* 213: 232-242.

Mukarram, M., Choudhary, S., Kurjak, D., Petek, A. and Khan, M. M. A. 2021. Drought: Sensing, signalling, effects and tolerance in higher plants. *Physiologia Plantarum* 172: 1291-1300.

Munir, S. 2018. Role of Reactive Oxygen Species and Contribution of New Players in Defense Mechanism under Drought Stress in Rice. *International Journal of Agriculture and Biology* 20: 1339-1352.

Peng, L., Fukao, Y., Fujiwara, M., Takami, T. and Shikanai, T. 2009. Efficient Operation of NAD(P)H Dehydrogenase Requires Supercomplex Formation with Photosystem I via Minor LHCI in Arabidopsis. *The Plant Cell* 21: 3623-3640.

Peng, L. and Shikanai, T. 2011. Supercomplex formation with photosystem I is required for the stabilization of the chloroplast NADH dehydrogenase-like complex in Arabidopsis. *Plant Physiol* 155: 1629-1639.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* 29: e45-e45.

Pham, A., Maurer, A., Pillen, K., Brien, C., Dowling, K., Berger, B., et al. 2019. Genome-wide association of barley plant growth under drought stress using a nested association mapping population. *BMC Plant Biology* 19.

Punchkhon, C., Kasetranunt, W., Kositsup, B., Siengliw, J. and Chadchawan, S. (2015). *Evaluation of drought tolerance ability at seedling stage of CSSL rice*

populations with drought tolerant genes on chromosome 8. In Proceeding of the 9th Botanical Conference of Thailand, Ambassador hotel, Bangkok.

Reddy, A. R., Chaitanya, K. V. and Vivekanandan, M. 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *Journal of Plant Physiology* 161: 1189-1202.

Salehi-Lisar, S. Y. and Bakhshayeshan-Agdam, H. 2016. Drought Stress in Plants: Causes, Consequences, and Tolerance. In M. A. Hossain, S. H. Wani, S. Bhattacharjee, D. J. Burritt, & L.-S. P. Tran (Eds.), *Drought Stress Tolerance in Plants, Vol 1: Physiology and Biochemistry* pp. 1-16. Cham: Springer International Publishing.

Saqib, M., Javaid, A., Abbas, G. and Nasim, M. 2013. Salinity and drought interaction in wheat (*Triticum aestivum* L.) is affected by the genotype and plant growth stage. *Acta Physiologiae Plantarum* 35.

Sharma, A., Kumar, V., Shahzad, B., Ramakrishnan, M., Singh Sidhu, G. P., Bali, A. S., et al. 2020. Photosynthetic Response of Plants Under Different Abiotic Stresses: A Review. *Journal of Plant Growth Regulation* 39: 509-531.

Shikanai, T. 2016. Chloroplast NDH: A different enzyme with a structure similar to that of respiratory NADH dehydrogenase. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1857: 1015-1022.

Siangliw, J., Jongdee, B., Pantuwan, G. and Toojinda, T. 2007. Developing KDML105 backcross introgression lines using marker-assisted selection for QTLs associated with drought tolerance in rice. *ScienceAsia* 33: 207-214.

Singh, S., Kumar, A., Panda, D., Modi, M. K. and Sen, P. 2020. Identification and characterization of drought responsive miRNAs from a drought tolerant rice genotype of Assam. *Plant Gene* 21: 100213.

Sirpiö, S., Allahverdiyeva, Y., Holmström, M., Khrouchtchova, A., Haldrup, A., Battchikova, N., et al. 2009. Novel Nuclear-encoded Subunits of the Chloroplast NAD(P)H Dehydrogenase Complex. *Journal of Biological Chemistry* 284: 905-912.

Sperdouli, I. and Moustakas, M. 2012. Interaction of proline, sugars, and anthocyanins during photosynthetic acclimation of *Arabidopsis thaliana* to drought stress. *Journal of Plant Physiology* 169: 577-585.

Suorsa, M. 2015. Cyclic electron flow provides acclimatory plasticity for the

photosynthetic machinery under various environmental conditions and developmental stages. *Frontiers in Plant Science* 6: 800-800.

Swapna, S. and Shylaraj, K. S. 2017. Screening for Osmotic Stress Responses in Rice Varieties under Drought Condition. *Rice Science* 24: 253-263.

Vajrabhaya, M. and Vajrabhaya, T. 1991. Somaclonal Variation for Salt Tolerance in Rice. In Y. P. S. Bajaj (Ed.), *Rice* pp. 368-382. Berlin, Heidelberg: Springer Berlin Heidelberg.

Verbruggen, N. and Hermans, C. 2008. Proline accumulation in plants: a review. *Amino Acids* 35: 753-759.

Wang, L., Ouyang, M., Li, Q., Zou, M., Guo, J., Ma, J., et al. 2010. The Arabidopsis chloroplast ribosome recycling factor is essential for embryogenesis and chloroplast biogenesis. *Plant Mol Biol* 74: 47-59.

Wang, X., Mao, Z., Zhang, J., Hemat, M., Huang, M., Cai, J., et al. 2019. Osmolyte accumulation plays important roles in the drought priming induced tolerance to post-anthesis drought stress in winter wheat (*Triticum aestivum* L.). *Environmental and Experimental Botany* 166: 103804.

Xu, Y., Liu, R., Yan, L., Liu, Z., Jiang, S., Shen, Y., et al. 2012. Light-harvesting chlorophyll a/b-binding proteins are required for stomatal response to abscisic acid in Arabidopsis. *Journal of Experimental Botany* 63: 1095-1106.

Zargar, S. M., Gupta, N., Nazir, M., Mahajan, R., Malik, F. A., Sofi, N. R., et al. 2017. Impact of drought on photosynthesis: Molecular perspective. *Plant Gene* 11: 154-159.

Zhang, X., Henriques, R., Lin, S. S., Niu, Q. W. and Chua, N. H. 2006. Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nature Protocols* 1: 641-646.

APPENDIX

APPENDIX

Supplementary Data

1. Drought-tolerance gene identification using genome comparison and co-expression network analysis of chromosome substitution lines in rice

Table 1 means of leaf dying score and Fv/Fm under normal (100% field capacity) and drought stress (50% field capacity).

	Lines	100% field capacity	50% field capacity
Leaf dying score	CSSL97	2.25 ± 0.95	5.00 ± 1.22^{ab}
	CSSL104	2.25 ± 0.25	2.75 ± 0.48^{b}
	CSSL106	2.75 ± 0.63	7.00 ± 0.4^{a}
	CSSL107	2.25 ± 0.25	3.50 ± 0.65^{b}
	KDML105	2.25 ± 0.25	5.25 ± 1.38^{ab}
	DH103	1.75 ± 0.48	3.00 ± 1.15^{b}
F_v/F_m	CSSL97	0.77 ± 0.01	0.21 ± 0.20^{b}
	CSSL104	0.79 ± 0.01	0.80 ± 0.01^{a}
	CSSL106	0.77 ± 0.03	0.61 ± 0.19^{a}
	CSSL107	0.78 ± 0.01	0.61 ± 0.20^{a}
	KDML105	0.79 ± 0.01	0.41 ± 0.23^{ab}
	DH103	0.78 ± 0.01	0.80 ± 0.01^{a}

Data are shown as the mean ± SE. Means in a column with a different superscript lowercase letter are significantly different (p value < 0.05).

Table 2 Means of Arabidopsis green area (Pixels) under control condition. * and ** above the mean ± SE of the mutant line represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant.

Lines	day 0	day 1	day 2	day 3
WT	426.94 ± 23.28	669.88 ± 26.86	948.38 ± 38.36	1287.31 ± 58.02
ndh-o	271.17 ± 21.26**	486.06 ± 24.27**	713.71 ± 30.80**	975.40 ± 48.06**
lhcb3	325.19 ± 27.62**	595.75 ± 31.93	809.33 ± 45.62*	1049.19 ± 64.90**
rrf	342.33 ± 17.84**	469.75 ± 23.00**	635.85 ± 36.46**	878.79 ± 46.14**
pgrl1b	270.81 ± 18.84**	428.04 ± 23.05**	580.33 ± 36.36**	808.96 ± 51.05**
pgrl1a	387.79 ± 21.27	531.54 ± 27.76**	782.46 ± 37.04**	1054.21 ± 58.76**
at2g27680	313.38 ± 16.57**	409.00 ± 24.15**	585.33 ± 36.31**	852.60 ± 47.99**
mrl1	422.27 ± 30.08	471.04 ± 35.34**	691.42 ± 48.60**	954.04 ± 63.71**

Table 2 (cont.) Means of Arabidopsis green area (Pixels) under control condition. * and ** above the mean ± SE of the mutant line represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant.

Lines	day 4	day 5	day 6	day 7
WT	1793.40 ± 87.90	2377.69 ± 115.97	2995.44 ± 153.43	4056.81 ± 213.05
ndh-o	1380.46 ± 73.67**	1893.90 ± 97.86**	2617.08 ± 131.23	3605.31 ± 185.24
lhcb3	1494.65 ± 92.82*	1989.83 ± 115.68*	2548.31 ± 154.35*	3675.98 ± 236.75
rrf	1197.19 ± 70.19**	1603.17 ± 96.80**	1962.73 ± 116.74**	2740.27 ± 150.65**
pgrl1b	1114.48 ± 64.66**	1592.33 ± 89.05**	2121.56 ± 125.09**	2903.69 ± 152.06**
pgrl1a	1497.98 ± 83.97*	1995.19 ± 110.89*	2476.48 ± 133.29*	3392.46 ± 189.74*
at2g27680	1485.98 ± 58.00**	1804.71 ± 83.60**	2326.46 ± 101.41**	3395.60 ± 150.77*
mrl1	1440.50 ± 92.11**	1611.65 ± 110.90**	2041.79 ± 146.05**	3334.42 ± 229.88*

Table 3 Means of Arabidopsis green area (Pixels) under mild drought stress condition (75 mM mannitol). * and ** above the mean ± SE of the mutant line represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant.

Lines	day 0	day 1	day 2	day 3
WT	305.17 ± 18.62	374.75 ± 22.61	477.19 ± 27.40	602.58 ± 40.28
ndh-o	340.94 ± 22.02	450.48 ± 22.02*	572.96 ± 28.92*	696.44 ± 36.38
lhcb3	393.27 ± 30.74*	578.75 ± 31.76**	701.15 ± 37.21**	826.46 ± 42.71**
rrf	314.52 ± 20.03	451.71 ± 23.11*	595.79 ± 31.60**	760.63 ± 38.93**
pgrl1b	254.83 ± 14.15*	349.52 ± 17.32	421.56 ± 23.69	513.17 ± 32.51
pgrl1a	320.17 ± 19.91	427.15 ± 22.28	547.48 ± 28.53	681.54 ± 40.55
at2g27680	281.83 ± 18.74	399.48 ± 18.14	546.29 ± 24.79	716.79 ± 32.14*
mrl1	328.98 ± 25.25	465.42 ± 25.16**	573.85 ± 25.31*	710.58 ± 37.10

Table 3 (cont.) Means of Arabidopsis green area (Pixels) under mild drought stress condition (75 mM mannitol). * and ** above the mean ± SE of the mutant line represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant.

Lines	day 4	day 5	day 6	day 7
WT	778.48 ± 53.81	1002.21 ± 73.41	1352.25 ± 76.42	1803.04 ± 95.71
ndh-o	835.04 ± 47.52	1079.13 ± 63.99	1388.48 ± 82.31	1790.83 ± 100.57
lhcb3	1003.96 ± 64.27**	1265.33 ± 84.20*	1631.21 ± 96.98*	2046.08 ± 124.58
rrf	946.92 ± 55.36*	1201.98 ± 70.02	1512.04 ± 79.98	1852.04 ± 99.53
pgrl1b	640.27 ± 44.13*	803.75 ± 62.38*	1083.79 ± 75.64*	1500.19 ± 81.59*
pgrl1a	872.52 ± 50.64	1077.38 ± 70.69	1345.60 ± 90.66	1636.29 ± 113.42
at2g27680	947.83 ± 45.18*	1254.54 ± 58.55**	1627.46 ± 72.17**	2101.90 ± 83.89*
mrl1	938.40 ± 46.90*	1135.17 ± 64.32	1458.96 ± 73.39	2014.02 ± 95.06

Table 4 Means of Arabidopsis green area (Pixels) under severe drought stress condition (150 mM mannitol). * and ** above the mean ± SE of the mutant line represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant.

Lines	day 0	day 1	day 2	day 3
WT	401.23 ± 21.00	463.79 ± 21.54	537.25 ± 23.82	600.90 ± 27.15
ndh-o	353.79 ± 20.03	422.17 ± 19.98	523.77 ± 22.34	628.92 ± 32.24
lhcb3	359.73 ± 24.06	480.52 ± 24.86	583.71 ± 28.31	675.52 ± 39.74
rrf	246.08 ± 18.21**	378.85 ± 14.49**	492.81 ± 20.05	609.00 ± 25.02
pgrl1b	273.69 ± 18.19**	349.27 ± 21.20**	413.65 ± 24.58**	487.85 ± 30.63**
pgrl1a	247.90 ± 17.92**	341.13 ± 19.51**	409.73 ± 22.13**	493.21 ± 26.84**
at2g27680	365.04 ± 20.02	428.27 ± 18.30	494.73 ± 20.81	583.02 ± 23.1
mrl1	384.83 ± 28.56	485.92 ± 26.76	546.25 ± 29.28	613.17 ± 37.35

Table 4 (cont.) Means of Arabidopsis green area (Pixels) under severe drought stress condition (150 mM mannitol). * and ** above the mean ± SE of the mutant line represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant.

Lines	day 4	day 5	day 6	day 7
WT	644.81 ± 43.78	766.46 ± 55.67	1001.33 ± 66.14	1234.31 ± 79.11
ndh-o	715.23 ± 46.92	916.42 ± 51.55	1130.90 ± 59.29	1458.69 ± 68.82
lhcb3	781.52 ± 54.50	888.85 ± 71.17	1050.81 ± 81.23	1181.27 ± 89.26
rrf	745.27 ± 31.82	886.54 ± 43.04	1036.44 ± 52.41	1224.92 ± 56.44
pgrl1b	594.38 ± 38.09	701.10 ± 49.39	904.44 ± 58.80	1101.17 ± 67.32
pgrl1a	582.85 ± 35.99	712.54 ± 42.05	836.71 ± 48.95*	1024.13 ± 61.77*
at2g27680	677.38 ± 31.67	763.90 ± 47.94	919.08 ± 64.21	1149.08 ± 66.16
mrl1	713.04 ± 44.77	856.52 ± 55.39	1051.31 ± 58.99	1269.19 ± 72.35

Table 5 stability during mild drought stress (75 mM mannitol). * and ** above the mean ± SE of the mutant line represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant.

lines	Stability index
WT	0.44 ± 0.01
ndh-o	0.48 ± 0.01*
lhcb3	0.57 ± 0.01**
rrf	0.67 ± 0.02**
pgrl1b	0.51 ± 0.01**
pgrl1a	0.46 ± 0.01
at2g27680	0.62 ± 0.02**
mrl1	0.66 ± 0.03**

Table 6 stability during severe drought stress (150 mM mannitol). ** above the mean ± SE of the mutant line represent highly significant difference (*p* value < 0.01) between WT and mutant.

lines	Stability index
WT	0.29 ± 0.01
ndh-o	0.45 ± 0.04**
lhcb3	0.30 ± 0.01
rrf	0.47 ± 0.02**
pgrl1b	0.37 ± 0.01**
pgrl1a	0.30 ± 0.01
at2g27680	0.33 ± 0.01**
mrl1	0.39 ± 0.01**

2. Role of LOC_Os01g68450 in Salt Tolerance is Mediated via the Maintenance of the Light-Harvesting Complex

Table 7 Growth ratio to WT after 5 days of mild salt stress (75 mM NaCl). ** above the mean ± SE of the mutant line represent highly significant difference (*p* value < 0.01) between WT and mutant.

Lines	Growth ratio to WT
WT	1.00 ± 0.00
ndh-o	1.20 ± 0.03**
lhcb3	0.96 ± 0.03
rrf	0.77 ± 0.03**
pgrl1b	0.58 ± 0.03**
pgrl1a	0.88 ± 0.02**
at2g27680	0.93 ± 0.02**
mrl1	1.01 ± 0.02
at1g65230	0.39 ± 0.01**

Table 8 Percent leaf green are reduction after 5 days of mild salt stress (75 mM NaCl). ** above the mean ± SE of the mutant line represent highly significant difference (*p* value < 0.01) between WT and mutant.

lines	Percent leaf green area reduction
WT	37.15 ± 2.61
ndh-o	-0.43 ± 1.59**
lhcb3	19.86 ± 2.42**
rrf	24.25 ± 1.00**
pgrl1b	43.51 ± 3.16
pgrl1a	31.42 ± 1.77
at2g27680	22.25 ± 1.87**
mrl1	-2.49 ± 2.18**
at1g65230	54.12 ± 1.45**

Table 9 Means of Arabidopsis green area under control condition. * and ** above the mean ± SE of the mutant line represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant. * and ** above the mean ± SE of the *rev* lines represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between mutant and *rev*.

Lines	day 0	day 1	day 2	day 3	day 4	day 5
WT	415.52 ± 25.23	724.73 ± 30.21	1029.25 ± 43.72	1411.17 ± 62.16	1937.54 ± 98.08	2626.73 ± 124.83
at1g65230	252.10 ± 25.11**	569.40 ± 28.07**	737.10 ± 40.31**	941.98 ± 58.52**	1141.69 ± 86.94**	1615.10 ± 95.24**
rev-1	340.54 ± 25.16*	617.85 ± 31.78	784.48 ± 47.29	999.33 ± 68.29	1279.94 ± 91.37	1829.54 ± 114.32
rev-2	465.90 ± 23.11**	787.52 ± 32.97**	1052.67 ± 44.43**	1426.33 ± 67.03**	1934.13 ± 93.20**	2823.56 ± 150.69**

Table 10 Means of Arabidopsis green area under mild salt stress condition (75 mM NaCl). ** above the mean ± SE of the mutant line represent highly significant difference (*p* value < 0.01) between WT and mutant. ** above the mean ± SE of the *rev* lines represent highly significant difference (*p* value < 0.01) between mutant and *rev*.

Lines	day 0	day 1	day 2	day 3	day 4	day 5
WT	376.35 ± 22.22	742.77 ± 30.42	917.46 ± 40.18	1136.44 ± 51.09	1351.58 ± 70.71	1868.77 ± 91.53
at1g65230	177.31 ± 16.49**	332.83 ± 21.97**	456.25 ± 29.25**	547.31 ± 34.46**	632.17 ± 37.80**	766.48 ± 48.94**
rev-1	340.75 ± 15.49**	509.04 ± 26.03**	653.94 ± 35.83**	800.42 ± 42.16**	910.33 ± 59.18**	1127.63 ± 77.53**
rev-2	400.56 ± 21.51**	686.75 ± 32.22**	853.42 ± 42.37**	1000.52 ± 46.69**	1133.58 ± 63.78**	1441.19 ± 91.52**

Table 11 Photosynthetic parameters and Efficiency of the light reaction of the Arabidopsis wildtype (WT), *at1g65230* mutant, and revertant line (*rev*) under control and salt-stress (200 mM NaCl) conditions. Photosynthetic rate (P_n), stomatal conductance (g_s), intracellular CO_2 concentration (C_i), transpiration rate (E), Excitation capture efficiency (F_v'/F_m'), operating efficiency of PSII (PhiPSII), and electron transport rate (ETR) of were determined after five days of treatments. Data are shown as the mean ± SE. Means in a column with a different superscript lowercase letter are significantly different (p value < 0.05).

Conditions	Control			Salt stress (200 mM NaCl)		
Lines	WT	*at1g65230*	*rev*	WT	*at1g65230*	*rev*
P_n	6.55 ± 1.31^{b}	7.97 ± 0.83^{ab}	9.27 ± 0.37^{a}	3.18 ± 0.20^{c}	3.86 ± 0.56^{c}	6.65 ± 0.36^{b}
g_s	176.00 ± 4.12^{bc}	163.40 ± 1.81^{c}	217.10 ± 29.99^{a}	196.20 ± 15.47^{ab}	117.60 ± 14.16^{c}	113.70 ± 5.35^{e}
C_i	355.54 ± 3.28^{cd}	377.38 ± 1.73^{a}	369.62 ± 6.71^{ab}	340.53 ± 6.28^{e}	333.13 ± 5.58^{e}	345.57 ± 2.57^{de}
E	2.20 ± 0.04^{bc}	2.17 ± 0.01^{bc}	2.56 ± 0.32^{a}	2.37 ± 0.16^{ab}	1.47 ± 0.16^{d}	1.47 ± 0.06^{d}
Fv'/Fm'	0.57 ± 0.01^{a}	0.57 ± 0.02^{a}	0.56 ± 0.01^{a}	0.51 ± 0.01^{b}	0.50 ± 0.00^{b}	0.55 ± 0.01^{a}
PhiPSII	0.12 ± 0.00^{a}	0.11 ± 0.01^{ab}	0.12 ± 0.00^{a}	0.10 ± 0.00^{b}	0.10 ± 0.00^{b}	0.12 ± 0.00^{a}
ETR	42.47 ± 1.62^{c}	45.31 ± 2.52^{c}	54.59 ± 0.77^{b}	49.81 ± 1.09^{bc}	46.29 ± 1.46^{c}	57.99 ± 0.62^{a}

Table 12 Pigment content of Arabidopsis wildtype (WT), *at1g65230* mutant, and revertant line (*rev*) determined after five days of treatment under control and salt-stress (200 mM NaCl) conditions including chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (total Chl), Chlorophyll *a/b* ratio (Chl *a/b* ratio), Carotenoids and Anthocyanin. Data are shown as the mean ± SE. Means in a column with a different superscript lowercase letter are significantly different (p value < 0.05).

Conditions	Control			Salt stress (200 mM NaCl)		
Lines	WT	*at1g65230*	*rev*	WT	*at1g65230*	*rev*
Chl *a*	46.09 ± 1.38^{ab}	41.56 ± 1.93^{bc}	49.05 ± 1.44^{a}	38.66 ± 1.08^{c}	38.33 ± 0.34^{c}	44.78 ± 0.42^{ab}
Chl *b*	13.86 ± 0.60^{ab}	14.30 ± 0.48^{a}	15.11 ± 0.27^{a}	13.56 ± 0.37^{ab}	12.09 ± 0.39^{b}	15.01 ± 0.24^{a}
Total Chl	59.95 ± 1.97^{ab}	55.86 ± 2.41^{bc}	64.16 ± 1.61^{a}	52.21 ± 1.44^{c}	50.42 ± 0.71^{c}	59.79 ± 0.38^{ab}
Chl *a/b* ratio	3.33 ± 0.06^{a}	2.90 ± 0.04^{c}	3.25 ± 0.08^{ab}	2.85 ± 0.01^{c}	3.18 ± 0.08^{c}	2.99 ± 0.06^{bc}
Carotenoids	8.51 ± 0.30^{a}	6.36 ± 0.29^{b}	8.56 ± 0.26^{a}	6.78 ± 0.08^{b}	5.70 ± 0.38^{b}	8.04 ± 0.18^{a}
Anthocyanin	84.84 ± 2.47^{b}	77.42 ± 5.16^{b}	104.81 ± 4.46^{a}	44.54 ± 2.07^{c}	35.88 ± 3.69^{c}	76.60 ± 2.82^{b}

3. Gene expression of drought stress genes predicted by gene co-expression network analysis

Table 13 Relative expression of *LOC_Os01g72950* under control and drought stress (15% PEG). Data are shown as the mean ± SE. Means in a column with a different superscript lowercase letter are significantly different (*p* value < 0.05).

conditions	Lines	days after treatment			
		day 0	day 3	day 6	day 9
Control	KDML105	1.00 ± 0.00	1.61 ± 0.45	0.57 ± 0.12^{b}	1.01 ± 0.09
	DH103	1.17 ± 0.08	0.91 ± 0.27	0.93 ± 0.03^{a}	1.02 ± 0.03
	CSSL104	1.12 ± 0.24	1.04 ± 0.01	0.66 ± 0.16^{b}	0.70 ± 0.11
drought stress	KDML105	-	1.19 ± 0.14	0.64 ± 0.03^{b}	1.02 ± 0.07
(15% PEG)	DH103	-	1.06 ± 0.23	0.62 ± 0.06^{b}	0.84 ± 0.09
	CSSL104	-	1.11 ± 0.19	0.57 ± 0.06^{b}	0.93 ± 0.20

Table 14 Relative expression of *LOC_Os07g37550* under control and drought stress (15% PEG). Data are shown as the mean ± SE. Means in a column with a different superscript lowercase letter are significantly different (*p* value < 0.05).

conditions	Lines	days after treatment			
		day 0	day 3	day 6	day 9
Control	KDML105	1.00 ± 0.00	2.19 ± 0.46	0.71 ± 0.14	0.55 ± 0.15^{b}
	DH103	1.85 ± 0.38	1.34 ± 0.23	1.47 ± 0.22	0.95 ± 0.26^{ab}
	CSSL104	2.44 ± 0.38	1.56 ± 0.35	1.26 ± 0.70	0.45 ± 0.13^{b}
drought stress (15% PEG)	KDML105	-	0.75 ± 0.24	0.81 ± 0.19	1.80 ± 0.56^{a}
	DH103	-	1.34 ± 0.39	1.89 ± 0.45	1.73 ± 0.45^{a}
	CSSL104	-	0.96 ± 0.19	0.65 ± 0.04	0.58 ± 0.15^{b}

Table 15 Relative expression of *LOC_os07g38300* under control and drought stress (15% PEG). Data are shown as the mean ± SE.

conditions	Lines	days after treatment			
		day 0	day 3	day 6	day 9
Control	KDML105	1.00 ± 0.00	4.30 ± 1.91	0.63 ± 0.20	0.81 ± 0.17
	DH103	2.12 ± 0.76	1.97 ± 0.39	1.80 ± 0.36	1.07 ± 0.25
	CSSL104	1.91 ± 0.66	2.67 ± 0.06	0.98 ± 0.47	0.82 ± 0.24
drought stress (15% PEG)	KDML105	-	1.83 ± 0.31	1.33 ± 0.32	1.45 ± 0.60
	DH103	-	1.75 ± 0.49	1.46 ± 0.37	1.17 ± 0.17
	CSSL104	-	2.55 ± 0.15	1.47 ± 0.18	0.94 ± 0.03

Table 16 Relative expression of *LOC_08g41460* under control and drought stress (15% PEG). Data are shown as the mean ± SE. Means in a column with a different superscript lowercase letter are significantly different (*p* value < 0.05).

condition	Lines	days after treatment			
		day 0	day 3	day 6	day 9
Control	KDML105	1.00 ± 0.00^{b}	1.63 ± 0.40^{a}	0.62 ± 0.08	0.61 ± 0.08^{bc}
	DH103	0.94 ± 0.09^{b}	1.18 ± 0.25^{ab}	0.78 ± 0.08	0.59 ± 0.06^{bc}
	CSSL104	1.30 ± 0.07^{a}	1.26 ± 0.08^{ab}	0.71 ± 0.17	0.42 ± 0.06^{c}
Drought stress (15% PEG)	KDML105		1.08 ± 0.13^{ab}	0.78 ± 0.05	1.01 ± 0.15^{a}
	DH103		0.70 ± 0.08^{b}	0.86 ± 0.05	0.76 ± 0.15^{ab}
	CSSL104		0.83 ± 0.04^{b}	0.58 ± 0.04	0.61 ± 0.11b^{c}

Table 17 Relative expression of *LOC_Os10g10170* under control and drought stress (15% PEG). Data are shown as the mean ± SE. Means in a column with a different superscript lowercase letter are significantly different (*p* value < 0.05).

condition	Lines	days after treatment			
		day 0	day 3	day 6	day 9
Control	KDML105	1.00 ± 0.00	2.62 ± 0.61	0.39 ± 0.18	0.55 ± 0.18
	DH103	1.54 ± 0.48	1.91 ± 0.50	0.69 ± 0.13	0.72 ± 0.22
	CSSL104	2.56 ± 1.19	2.83 ± 0.10	0.41 ± 0.05	0.83 ± 0.35
drought stress (15% PEG)	KDML105	-	1.45 ± 0.54	0.91 ± 0.30	1.26 ± 0.64
	DH103	-	1.38 ± 0.51	0.88 ± 0.29	0.89 ± 0.16
	CSSL104	-	1.69 ± 0.47	0.73 ± 0.07	0.49 ± 0.15

4. Green area of Arabidopsis wild-type, *at1g65230* mutant and ectopic expression lines under drought stress conditions.

Table 18 Green area (pixels) of Arabidopsis WT, *at1g65230* mutant, *ect* lines and *rev* lines under control condition. * and ** above the mean ± SE represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant, WT and *ect*, mutant and *rev*.

Lines	day 0	day 1	day 2	day 3
WT	415.52 ± 25.23	724.73 ± 30.21	1029.25 ± 43.72	1411.17 ± 62.16
at1g65230	252.10 ± 25.11**	569.40 ± 28.07**	737.10 ± 40.31**	941.98 ± 58.52**
ect-1	439.10 ± 25.48	536.17 ± 38.36**	747.77 ± 52.34**	1040.81 ± 73.65**
ect-2	469.10 ± 26.39*	785.52 ± 40.31	1098.73 ± 51.87	1520.04 ± 75.47
rev-1	340.54 ± 25.16**	617.85 ± 31.78**	784.48 ± 47.29*	999.33 ± 68.29
rev-2	465.90 ± 23.11**	787.52 ± 32.97**	1052.67 ± 44.43**	1426.33 ± 67.03**

Table 18 (cont.) Green area (pixels) of Arabidopsis WT, *at1g65230* mutant, *ect* lines and *rev* lines under control condition. * and ** above the mean ± SE represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant, WT and *ect*, mutant and *rev*.

Lines	day 4	day 5	day 6	day 7
WT	1937.54 ± 98.08	2626.73 ± 124.83	3198.00 ± 154.44	3756.58 ± 198.12
at1g65230	1141.69 ± 86.94**	1615.10 ± 95.24**	1988.90 ± 126.89**	2826.46 ± 192.39**
ect-1	1418.73 ± 97.64**	2408.15 ± 116.42	2919.79 ± 158.58	4619.06 ± 216.91**
ect-2	2008.48 ± 103.15	2841.35 ± 139.67	3728.75 ± 187.39**	5191.35 ± 261.91**
rev-1	1279.94 ± 91.37**	1829.54 ± 114.32**	2259.23 ± 139.89**	3641.88 ± 231.29**
rev-2	1934.13 ± 93.20**	2823.56 ± 150.69**	3792.83 ± 219.34**	4849.33 ± 292.32**

Table 19 Green area (pixels) of Arabidopsis WT, *at1g65230* mutant, *ect* lines and *rev* lines under mild drought condition (75 mM mannitol). * and ** above the mean ± SE represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant, WT and *ect*, mutant and *rev*.

Lines	day 0	day 1	day 2	day 3
WT	409.85 ± 22.97	526.13 ± 21.78	616.90 ± 26.16	742.27 ± 38.31
at1g65230	378.15 ± 19.30	488.04 ± 21.50	550.29 ± 31.98	609.06 ± 42.13*
ect-1	456.25 ± 22.51	556.19 ± 24.75	611.35 ± 31.73	745.83 ± 35.07
ect-2	477.13 ± 21.26**	463.02 ± 26.50**	498.10 ± 29.91**	550.85 ± 42.55**
rev-1	465.67 ± 21.74**	597.63 ± 26.68**	684.25 ± 31.56**	756.63 ± 45.26**
rev-2	435.81 ± 21.92**	501.06 ± 25.41	575.40 ± 32.86	705.10 ± 47.33**

Table 19 (cont.) Green area (pixels) of Arabidopsis WT, *at1g65230* mutant, *ect* lines and *rev* lines under mild drought condition (75 mM mannitol). * and ** above the mean ± SE represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant, WT and *ect*, mutant and *rev*.

Lines	day 4	day 5	day 6	day 7
WT	921.54 ± 52.85	1274.40 ± 71.43	1682.73 ± 85.37	2070.46 ± 100.21
at1g65230	700.29 ± 59.45**	901.25 ± 82.19**	1231.83 ± 96.65**	1520.81 ± 109.13**
ect-1	930.13 ± 49.23	1238.75 ± 62.91	1673.44 ± 78.52	2087.96 ± 102.68
ect-2	678.00 ± 56.50**	994.19 ± 86.84**	1411.08 ± 105.28**	2138.85 ± 128.62
rev-1	862.33 ± 58.05**	1072.25 ± 74.84**	1282.15 ± 99.60	1612.33 ± 106.54
rev-2	839.23 ± 71.62**	1201.85 ± 84.84**	1602.10 ± 96.00**	1945.44 ± 120.04**

Table 20 Green area (pixels) of Arabidopsis WT, *at1g65230* mutant, *ect* lines and *rev* lines under severe drought condition (150 mM mannitol). * and ** above the mean ± SE represent significant difference (*p* value < 0.05) and highly significant difference (*p* value < 0.01) between WT and mutant, WT and *ect*, mutant and *rev*.

Lines	day 0	day 1	day 2	day 3
WT	342.44 ± 24.39	415.00 ± 26.03	467.67 ± 30.89	515.69 ± 34.78
at1g65230	380.88 ± 5.95	422.79 ± 7.49	450.54 ± 8.70	488.88 ± 11.94
ect-1	377.27 ± 6.50	490.94 ± 7.27**	534.17 ± 7.86*	587.35 ± 9.72*
ect-2	423.13 ± 5.15**	503.13 ± 6.57**	578.40 ± 8.29**	607.06 ± 10.02*
rev-1	424.92 ± 7.23**	522.58 ± 7.63**	589.23 ± 9.94**	651.23 ± 12.33**
rev-2	275.19 ± 5.69**	366.31 ± 4.64**	412.77 ± 5.56**	439.42 ± 7.2**

Table 20 (cont.) Green area (pixels) of Arabidopsis WT, *at1g65230* mutant, *ect* lines and *rev* lines under severe drought condition (150 mM mannitol). * and ** above the mean ± SE represent significant difference (p value < 0.05) and highly significant difference (p value < 0.01) between WT and mutant, WT and *ect*, mutant and *rev*.

Lines	day 4	day 5	day 6	day 7
WT	570.46 ± 38.98	740.23 ± 54.77	795.50 ± 78.29	1055.77 ± 54.26
at1g65230	495.60 ± 16.44	665.73 ± 19.52	866.38 ± 22.95	1057.10 ± 25.16
ect-1	660.31 ± 11.24*	856.21 ± 13.75*	963.02 ± 20.04*	1215.46 ± 19.05**
ect-2	693.54 ± 12.79**	831.85 ± 16.80	1043.33 ± 19.32**	1266.29 ± 19.48**
rev-1	734.83 ± 15.54**	848.71 ± 18.28**	1092.85 ± 21.91**	1285.88 ± 25.00**
rev-2	511.54 ± 9.18	625.75 ± 10.59	753.65 ± 14.77**	930.96 ± 17.66**

VITA

NAME	Chutarat Punchkhon
DATE OF BIRTH	23 June 1991
PLACE OF BIRTH	Phayao province
INSTITUTIONS ATTENDED	2012 - Bachelor of Science (Botany), Chulalongkorn university 2014 - Master of Science (Botany), Chulalongkorn university
HOME ADDRESS	62 moo 4, Sri Toi, Mae Chai, Phayao
PUBLICATION	Punchkhon, C., Plaimas, K., Buaboocha, T., Siangliw, J.L., Toojinda, T., Comai, L., De Diego, N., Spíchal, L. and Chadchawan, S. 2020. Drought-Tolerance Gene Identification Using Genome Comparison and Co-Expression Network Analysis of Chromosome Substitution Lines in Rice. Genes 11(10):1197.