EXPRESSION OF PREDICTED SALT TOLERANT GENE IN RICE *Oryza sativa* L. AND CHARACTERIZATION OF *OsBTBZ1* GENE

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การแสดงออกของยีนที่ถูกทำนายว่าเป็นยีนทนเค็มในข้าว *Oryza sativa* L. และลักษณะสมบัติของยีน *OsBTBZ1*

นายทรีโอโน บากัส ซาปุโตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	EXPRESSION OF PREDICTED SALT TOLERANT GENE IN RICE *Oryza sativa* L. AND CHARACTERIZATION OF *OsBTBZ1* GENE
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ทรีโอโน บากัส ซาปุโตร : การแสดงออกของยีนที่ถูกทำนายว่าเป็นยีนทนเค็มในข้าว *Oryza sativa* L. และลักษณะสมบัติของยีน *OsBTBZ1*. (EXPRESSION OF PREDICTED SALT TOLERANT GENE IN RICE *Oryza sativa* L. AND CHARACTERIZATION OF *OsBTBZ1* GENE) อ.ที่ปรึกษาหลัก : ศุภจิตรา ชัชวาลย์, อ.ที่ปรึกษาร่วม : ธีรพงษ์ บัวบูชา

ข้าว (*Oryza sativa* L.) เป็นแหล่งอาหารหลักของประชากรทั่วโลก โดยเฉพาะอย่างยิ่งในภูมิภาคเอเชียที่การผลิตข้าวมีบทบาทสำคัญในการเสริมสร้างความมั่นคงทางอาหาร ความเค็มจึงถือเป็นข้อจำกัดสำคัญต่อการปลูกพืชเช่นข้าว เนื่องจากความแล็มส่งผลให้การเจริญเติบโต และผลผลิตโดยรวมลดลง ความก้าวหน้าทางทรานสคริปโทมิกส์ที่นำมาใช้ทางการเกษตรช่วยทำให้เกิดความเข้าใจทางชีววิทยาระดับโมเลกุลของพืช ซึ่งมีส่วนช่วยในการพัฒนาระบบการเกษตรอย่างยั่งยืนเนื่องจากทำให้มีการปรับตัวได้อย่างรวดเร็วและมีประสิทธิภาพ การศึกษาด้วยวิธีการทรานสคริปโทมิกส์ด้วยการใช้ข้าวสองสายพันธุ์ / พันธุ์ ที่มีความใกล้ชิดทางพันธุกรรม แต่มีความสามารถในการทนเค็มที่แตกต่างกันคือ CSSL16 และ KDML105 ร่วมกับพหุสัณฐานนิวคลีโอไทด์เดี่ยวมาใช้ในการระบุยีนทนเค็ม 9 ยีน โดยพบว่ายีน *LOC_Os01g64870*, *OsBTBZ1*, *OsERD4*, *LOC_01g73110* และ *OsSub34* มีการแสดงออกของยีนที่เพิ่มขึ้นทั้งในระยะเริ่มต้น (0 – 48 ชั่วโมงหลังจากได้รับความเครียด) และระยะปลาย (0 – 9วัน หลังจากได้รับความเครียด)

งานวิจัยนี้เลือกศึกษายีน *OsBTBZ1* เพื่อยืนยันความเกี่ยวข้องกับการทนเค็มผ่านการสร้างสายพันธุ์ revertant คือ สายพันธุ์ REV1 และ REV2 ใน *Arabidopsis* สายพันธุ์กลายคือ *Atbt3* นอกจากนี้ยังได้สร้างสายพันธุ์ที่มีการแสดงออกของ *OsBTBZ1* เพิ่มขึ้นที่มีพันธุกรรมพื้นฐานเป็นสายพันธุ์พื้นฐาน ได้แก่ สายพันธุ์ overexpressed คือ OE1 และ OE2 เพื่อศึกษาผลของการแสดงออกของ *OsBTBZ1* ที่มีต่อการทนเค็ม การศึกษาฟีโนไทป์ภายใต้ความเครียดจากภาวะเค็มที่มีการให้โซเดียมคลอไรด์เข้มข้น150 มิลลิโมลาร์ หรือ ABA ความเข้มข้น 1 ไมโครโมลาร์ หรือแมนนิทอลความเข้มข้น 150 มิลลิโมลาร์ แล้วศึกษาอัตราการงอกของเมล็ด ความยาวราก น้ำหนักสด ปริมาณคลอโรฟิลล์ *เอ* คลอโรฟิลล์ *บี* และแคโรทีนอยด์ เพื่อบ่งถึงความสามารถในการทนเค็ม ภายใต้ภาวะเค็มหรือการได้รับ ABA พบว่า สายพันธุ์กลาย *Atbt3* มีพารามิเตอร์ต่าง ๆ ข้างต้นลดลง ซึ่งค่าของพารามิเตอร์เหล่านั้นปรับขึ้นได้เมื่อมีการแสดงออกของยีน *OsBTBZ1* นอกจากนี้ การได้รับแมนนิทอลทำให้น้ำหนัก ความยาวราก และสารสีที่ใช้ในการสังเคราะห์ด้วยแสงของสายพันธุ์ต่าง ๆ ที่ทำการศึกษาลดลงใกล้เคียงกัน การแสดงออกของยีน *OsBTBZ1* ทั้งใน WT และในสายพันธุ์กลาย *Atbt3* ทำให้มีความทนเค็มสูงขึ้นทั้งในภาวะเครียดจากความเค็มและในภาวะเครียดจาก ABA ซึ่งจากข้อมูลเหล่านี้แสดงให้เห็นว่ายีน *OsBTBZ1* มีบทบาทในการตอบสนองต่อภาวะเครียดจากความเค็มมากกว่าความเครียดออสโทติกจากการเปลี่ยนแปลงที่เกิดจากการแสดงออกของยีน *OsBTBZ1* ตอบสนองต่อการให้ ABA ด้วย จึงชี้ให้เห็นว่าการทำงานของยีน *OsBTBZ1* นี้เป็นกลไกที่ขึ้นกับวิถีการตอบสนองผ่าน ABA ด้วย

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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Triono Bagus Saputro : EXPRESSION OF PREDICTED SALT TOLERANT GENE IN RICE *Oryza sativa* L. AND CHARACTERIZATION OF *OsBTBZ1* GENE. Advisor: Prof. Dr. SUPACHITRA CHADCHAWAN Co-advisor: Assoc. Prof. Dr. TEERAPONG BUABOOCHA

Rice (*Oryza sativa* L.) holds significant importance as a primary food source globally. In Asia, the production of rice plays a crucial role in enhancing food security. Salinity poses a substantial constraint on plants like rice, leading to a reduction in their growth and overall productivity. The advancement of transcriptomics in agriculture has provided a powerful tool for understanding the molecular processes in crops, contributing to the development of more resilient, productive, and sustainable agricultural systems. The transcriptomic approach in rice using two rice lines with close genetic relationships, but different salt tolerance ability, CSSL16 and KDML105 combined with single nucleotide polymorphism (SNP) gives the new insight in identifying the salt-tolerant genes and produces 9 candidates genes. *LOC_Os01g64870*, *OsBTBZ1*, *OsERD4*, *LOC_01g73110*, and *OsSub34* consistently showing the increment trend of gene expression in both early (0 – 48 h after stress) and late (0 – 9 d after stress) response during salt stress.

OsBTBZ1 was chosen to validate its involvement in salt tolerance through the creation of revertant lines, REV1 and REV2, in the *Atbt3 Arabidopsis* mutant. Additionally, overexpressed lines, OE1 and OE2, were generated in the wildtype lines to investigate the impact of elevated OsBTBZ1 expression on salt tolerance. The phenotyping under salt stress (150 mM NaCl), ABA 1 µM, and mannitol 150 mM were conducted. The germination, root length, fresh weight, Chl *a*, Chl *b* and carotenoid contents were chosen to describe the function of *OsBTBZ1*. Under salt and ABA treatment, the *Atbt3* mutant exhibits the highest reduction in all examined parameters, which were counteracted by *OsBTBZ1* expression. In addition, the exposure in mannitol resulted a comparable decrease in weight, root length, and photosynthetic pigment content across all tested lines. Furthermore, the expression of *OsBTBZ1* in both the WT and *Atbt3* mutant backgrounds demonstrated enhanced tolerance to abiotic stress, specifically under salt and ABA stress condition. Based on these data, *OsBTBZ1* is more responsible for the tolerance in salt stress rather than osmotic stress. The observed restoration of phenotypes in the mutant line upon introducing *OsBTBZ1* expression also occurred under ABA treatment, pointing to the involvement of the ABA-dependent pathway in *OsBTBZ1* function.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2023	Advisor's Signature .....
		Co-advisor's Signature .....

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The dissertation begins with an introductory section that outlines the background by providing insights into the underlying reasons for the research, its specific goals, the scope of the study, and the expected outcomes. Following the introduction, the dissertation is divided into two main chapters, Chapter II and Chapter III, which represent the core research publications.

Three transcriptome datasets in seedling stage, second leaves of booting stage and flag leaves of booting stages were used to figure out the dynamics of gene expression during high salt condition. Three different analyses were employed to obtain the responsible gene namely gene co-expression network (GCN), two-state co-expression with clustering coefficient (CC), and weighted gene co-expression network (WGCN). The *LOC_Os01g61010 (OsNodulin), LOC_Os01g64870, LOC_Os01g66890* (*OsBTBZ1*), *LOC_Os01g67370, LOC_Os01g72210* (*OsERD*), *LOC_Os01g71190 (OsPSB28), LOC_Os01g73110, LOC_Os04g03050* (*OsSub34*), and *LOC_Os06g46799* (*OsPeroxidase*) were the chosen genes to be further validated using qRT-PCR. The result of first objective was detailed in the paper entitled **"Combining Genome and Gene Co-expression Network Analyses for the Identification of Genes Potentially Regulating Salt Tolerance in Rice"** presented in chapter II.

The second paper entitled **"*OsBTBZ1* Confers Salt Stress Tolerance in *Arabidopsis thaliana*"** was the continuation of the first paper by expanding the searching of genes containing the BTB domain. Four BTB genes, *OsBTBZ1*, *OsBTBZ2*, *OsBTBN3*, and *OsBTBN7*, showed differential expression under salt stress. Furthermore, OsBTBZ1 and OsBTBZ2 contained the BTB and Transcription Adaptor putative zinc finger (TAZ zF or zF-TAZ) domains that relate to plant growth and stress response, whereas OsBTBN3 and OsBTBN7 contain the non-phototropic hypocotyl3 (NPH3) domain that mostly responds to light. OsBTBZ1 gene was found to be closely related to salt-responsive

protein in protein-protein interaction result. Then, it was further examined its function through complementation test in *Arabidopsis* model plant.

CHAPTER I

RATIONALES

Rice, scientifically known as *Oryza sativa* L., plays a crucial role as a primary food source on a global scale. In Asia, rice production has been a principal concern to be improved to fulfill food security. Salinity is identified as a major obstacle for plant growth, leading to decreased growth and productivity in various plant species, including rice (Flowers, 2004). Furthermore, excessive salt levels have detrimental effects on grain yield, panicle length, spikelet number per panicle, seed weight per panicle, and the weight of 1000 grains (Khatun & Flowers, 1995; Zeng, 2000). Salinity in soils can arise naturally or be induced by human activities through continuous irrigation with low-quality groundwater (Shahid et al., 2018). The main characteristic of saline soil is its high concentration of soluble salts with Ca^{+2}, Mg^{+2}, Na^{+}, K^{+} as cations and SO_4^{-2}, Cl^{-}, HCO_3^{-}, CO_3^{-2}, and NO_3^{-} as anions, with electrical conductivity (EC) ≥ 4 dSm^{-1} (Fahad et al., 2019). The high concentration of salt can affect plant physiology, ion toxicity on plant cells, changes in plant growth, and decrement of photosynthetic rate (Roychoudhury et al., 2011). Salt tolerance is an intricate mechanism evolved by plants to withstand the harmful impacts of salt stress. The ability of plants to endure high salt levels is contingent on the alteration in gene expression, where a numerous of genes are induced to generate specific proteins and metabolites.

The chromosome segment substitution line 16 (CSSL16) of rice is considered a salt-tolerant line. 511 differentially expressed sequence (DEseq) genes at the seedling stage, 520 DEseq genes in the secondary leaves, and 584 DEseq genes in the flag leaves at the booting stage were found when the transcriptome data of the CSSL16 line under normal and salt stress conditions were compared. A comparison of the transcriptomic data of the CSSL16 line under normal and salt stress conditions revealed 511 differentially expressed sequence (DEseq) genes at the seedling stage, 520 DEseq genes in the secondary leaves, and 584 DEseq genes in the flag leaves at the booting stage. Moreover, 92 genes were identified based on gene co-expression network

(GCN), two-state co-expression with clustering coefficient (CC), and weighted gene co-expression network (WGCN) analysis. Out of 92 genes, only 9 genes show the single nucleotide polymorphism (SNPs) between 'KDML105' and CSSL16. The expression study was be performed to validate all those nine genes. *LOC_Os01g61010 (OsNodulin), LOC_Os01g64870, LOC_Os01g66890* (*OsBTBZ1*), *LOC_Os01g67370, LOC_Os01g72210* (*OsERD*), *LOC_Os01g71190 (OsPSB28), LOC_Os01g73110, LOC_Os04g03050* (*OsSub34*), and *LOC_Os06g46799* (*OsPeroxidase*). Furthermore, *LOC_Os01g66890* (*OsBTBZ1*) and *LOC_Os01g72210* (*OsERD*) appear in all three analysis methods.

The Bric-a-Brac, Tramtrack, and Broad Complex BTB domain with TAZ zinc finger and Calmodulin-binding domains (*OsBTBZ1*) gene will be further characterized since it contains an important domain, a BTB domain. BTB was reported to participate in plant responses to abiotic stresses, ubiquitination, and development. In addition, based on subcellular prediction, only *LOC_Os01g66890* (*OsBTBZ1*) resides in the nucleus and possibly regulates many downstream genes related to salt stress. *CaBPM4* (*Capsicum annuum* BTB-POZ and MATH domain protein) from pepper was shown to be up-regulated in salt stress exposure (He et al., 2019). *Arabidopsis thaliana Stress-Induced BTB protein 1* (*AtSIBP1*) was demonstrated to be a positive regulator for salinity responses in *Arabidopsis* (Wan et al., 2019). MdBT2 of apple responding the nitrate by its interaction with MdCIbHLH1 transcription factor and ubiquitinates this protein *via* the ubiquitin/26S proteasome pathway (Zhang et al., 2020). (Robert et al., 2009) reported BT1, BT2, BT3, BT4, BT5 performed crucial roles in gametophyte development of *Arabidopsis*. Furthermore, the combination of BTB with TAZ (Transcription Adaptors Zinc finger) is a feature that is present only in plants (Gingerich et al., 2007). SlBTB18 in tomato contains the TAZ domain and shows a dramatic increment in cold, salt stress, and oxidative stress (Li et al., 2018). Genes containing the BTB TAZ domain might exhibit a diverse role and part in various mechanisms in responding to salt stress.

To delve deeper into the involvement of BTB proteins in salt stress, an analysis of three transcriptomic datasets was conducted. Four BTB genes, *OsBTBZ1*, *OsBTBZ2*, *OsBTBN3*, and *OsBTBN7*, showed differential expression under salt stress. Significantly, *OsBTBZ1* was specifically differentially expressed during the seedling stage, while the other genes showed differential expression during the booting stage. The STRING database indicated that OsBTBZ1 had stronger associations than other abiotic stress-related proteins compared to other BTB genes. Notably, *OsBTBZ1* exhibited its highest expression levels in the sheaths of young leaves. Further supporting the idea of its involvement in transcriptional regulation, the OsBTBZ1-GFP fusion protein was localized within the nucleus. The *Arabidopsis* mutant line *bt3* displayed sensitivity to NaCl and abscisic acid (ABA) but showed no susceptibility to mannitol.

The germination and growth of the mutant lines were adversely affected by NaCl and abscisic acid (ABA). Additionally, introducing the ectopic expression of *OsBTBZ1* reinstated normal phenotypes in the *bt3* mutant line and boosted the growth of wild-type *Arabidopsis* in stress conditions. These results indicate that *OsBTBZ1* is a salt-tolerant gene, operating within ABA-dependent pathways.

OBJECTIVES

There are two main objectives in this research :

1. To validate gene expression of the predicted salt tolerant genes obtained from transcriptome analysis
2. To characterize of *OsBTBZ1* gene by using *Arabidopsis* as the heterologous system

SCOPE OF RESEARCH

In this research, the gene expression study of the predicted salt tolerant genes was determined to validate the result of transcriptomic data. Then, the expression of *BTBZ1* gene was conducted by quantitative PCR technique to determine the tissue-specific expression pattern. The second is a complementation study in *bt3* mutant, a null *BT3* allele in *Arabidopsis*. Revertant and ectopic expression lines in wild type (WT) *Arabidopsis* were generated using transgenic technology mediated by *Agrobacterium tumefaciens*. The phenotyping study of the transgenic lines with *OsBTBZ1* expression were performed to have a deep understanding of the gene function in salt-stress conditions. Several physiological parameters such as shoot and root length, fresh weight, dry weight, photosynthetic pigment contents were determined in wild type, *bt3* mutant, revertant, and ectopic expression lines.

EXPECTED BENEFITS

This research aims to obtain conclusive data of the pivotal role of the *OsBTBZ1* gene in regulating plant defense mechanisms under salt stress. Elucidating the functions of the gene contributes to salt tolerance will enable the breeder or plant scientist to optimize the potential development of salt-resistant rice varieties in the years to come.

CHAPTER II

RESEARCH ARTICLES

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Abstract:

Salinity stress tolerance is a complex polygenic trait involving multi-molecular pathways. This study aims to demonstrate an effective transcriptomic approach for identifying genes regulating salt tolerance in rice. The chromosome segment substitution lines (CSSLs) of 'Khao Dawk Mali 105 (KDML105)' rice containing various regions of DH212 between markers RM1003 and RM3362 displayed differential salt tolerance at the booting stage. CSSL16 and its nearly isogenic parent, KDML105, were used for transcriptome analysis. Differentially expressed genes in the leaves of seedlings, flag leaves, and second leaves of CSSL16 and KDML105 under normal and salt stress conditions were subjected to analyses based on gene co-expression network (GCN), on two-state co-expression with clustering coefficient (CC), and on weighted gene co-expression network (WGCN). GCN identified 57 genes, while 30 and 59 genes were identified using CC and WGCN, respectively. With the three methods, some of the identified genes overlapped, bringing the maximum number of predicted salt tolerance genes to 92. Among the 92 genes, nine genes, *OsNodulin*, *OsBTBZ1*, *OsPSB28*, *OsERD*, *OsSub34*, *peroxidase precursor* genes, and three expressed protein genes, displayed SNPs between CSSL16 and KDML105. The nine genes were differentially expressed in CSSL16 and KDML105 under normal and salt stress conditions. *OsBTBZ1* and *OsERD* were identified by the three methods. These results suggest that the transcriptomic approach described here effectively identified the genes regulating salt tolerance in rice and support the identification of appropriate QTL for salt tolerance improvement.

Keywords : transcriptome analysis, gene co-expression network, salt-tolerant genes, rice, clustering co-efficient

1. **Introduction**

Salinity is a major environmental stressor that affects rice production worldwide. Salt stress decreases crop yield and limits agricultural productivity (Munns, 2002), particularly in non-irrigated farmlands by triggering two primary effects on plants, osmotic stress, and ion toxicity (Boyer, 1982). In most rice cultivars, the seedling and early booting stages are the most sensitive to salt stress (Lafitte et al., 2007). High concentrations of sodium ions are toxic to most plants (Dionisio-Sese & Tobita, 2000). A combination of ion toxicity and osmotic stress inhibits growth and affects plant development or cause cell death (Hasegawa et al., 2000; Zhu, 2002). Moreover, these factors affect enzyme activities, which lead to a reduction in photosynthetic rate, metabolism, growth, and development; additionally, pollen germination may also be affected, lowering fertility. These effects contribute to the lower yield of crops exposed to salt stress (Abdullah, 2001).

Salt tolerance is a polygenic trait, and although several genes regulating salt tolerance have been identified, there are still some genes regulating salt tolerance in different rice varieties that are yet to be identified. Thai jasmine rice or ‘Khao Dawk Mali 105’ (‘KDML105’) rice is one of the most popular Thai rice cultivars among consumers. The high quality KDML105 grains are produced in rain-fed farms in the northeastern part of Thailand, and the farmlands are characterized by high soil salinity (2–16 $dS.m^{-1}$). Drought tolerant line was developed by generating chromosome substitution lines (CSSLs) in the KDML105 rice genetic background (Kanjoo et al., 2012). The introgressions in these CSSLs contain drought-tolerant quantitative trait loci (QTL) on chromosome 1 and were engineered via marker-assisted breeding by crossing KDML105 to a drought-tolerant donor, DH212. CSSL16, a CSSL from this population, exhibited salt tolerance when compared to other CSSLs and KDML105 at the vegetative and seedling stage (Chutimanukul, 2018b).

RNA-seq has been widely used to investigate transcriptomes under biotic and abiotic stress conditions in several plants ((Garg et al., 2014; Song

et al., 2014). High-throughput information can be analyzed to understand plant responses at the transcriptional level using various methods. The gene co-expression network (GCN) is a simplified method used in investigating the biological functions of genes under different conditions using the node degree or hub centrality. GCN analysis was applied to identify the gene modules that regulate drought tolerance (Sircar & Parekh, 2015), salt tolerance (Chutimanukul, 2018b), and osmotic stress tolerance (Nounjan et al., 2018). However, this type of network is an undirect graph, which contains nodes corresponding to genes and edges representing neighborhood relations (Lee et al., 2004; Stuart, 2003). Recently, the analysis of complex data is being carried out using high-performance computing systems. Consequently, the clustering coefficient method was developed to identify genes in plants or animals exposed to different environments (Zhang & Horvath, 2005).

In the analysis of network topological features, the node degree is one of the most generally used analytical techniques to identify the connection between the number of hub genes and neighboring nodes in the network. The consideration of the important genes can refer to the high number of neighboring nodes. The local density of the connection, referred to as the clustering coefficient (CC), is the measurement of the local density that quantifies the network's tendency of the connections (Ravasz et al., 2002; Watts & Strogatz, 1998). Furthermore, CC was developed from a simple binary network to a weighted network to fulfill the prediction constant degree of any real-world network (Humphries & Gurney, 2008). There have been reports of CC in GCN datasets from yeast and cancer microarrays (Zhang & Horvath, 2005). Moreover, the data analysis of degree on weighted gene co-expression network (WGCN) can be used to construct the signed gene co-expression network to define transcriptional modules (Horvath, 2011). This technique can identify the hub genes in plants or animals subjected to different conditions and the genes responsible for human diseases (Horvath, 2011; Mukund & Subramaniam, 2015; Riquelme Medina & Lubovac-Pilav, 2016).

To perform the expression network analysis for the identification of genes regulating salt tolerance in rice, we used the expression datasets from a

single pair of rice lines with similar genetic backgrounds, but different levels of salt tolerance. Therefore, we selected the CSSL population because the lines share a similar genetic background but possess different levels of salt tolerance. To create an expression network, transcriptome datasets of the selected lines at seedling and booting stages were used to identify the major (hub) genes responsible for salt tolerance, as these two stages are the most susceptible to salt stress in rice.

In this study, we compared various CSSLs with different size segments of the putative abiotic stress tolerance genomic region to validate the salt tolerance of CSSL16 at the booting stage. The transcriptome data from leaves at the seedling stage, second leaf, and flag leaf at the booting stage of CSSL16 were analyzed using GCN, CC, and WGCN to predict the major genes responsible for salt tolerance. The expression of some predicted genes was investigated in both salt-tolerant and susceptible lines.

2. Materials and Methods

2.1 Plant materials

Rice (*Oryza sativa* L.) seeds of CSSL lines (CSSL10, CSSL14, and CSSL16) with 'KDML105' rice genetic background, and their parents (DH212 and KDML105) were obtained from the Rice Gene Discovery Unit (RGDU), National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. CSSL16 contained the full segment of the putative salt tolerance region between RM1003–RM3362 (Chutimanukul et al., 2018b), while CSSL10 contained the segment between RM1003–RM6827, and CSSL14 contained the segment between RM3468–RM3362 (Figure 2.1). The three CSSL lines, CSSL10, CSSL14, and CSSL16, were compared with KDML105 and DH212 for salt stress responses. Then the best CSSL candidate for salt tolerance was selected for transcriptomic analysis.

2.1 Determination of the photosynthetic rate and yield components of the lines at booting stage

2.2.1 Plant growth condition

CSSL10, CSSL14, CSSL16, and their parental lines, 'KDML105'and DH212 were grown in plastic pots containing soil. We supplied the necessary

nutrients by applying Bangsai nutrient solution (1:100) to the soil. At the booting stage, 75 mM NaCl was added to the nutrient solution of the treatment groups, but not to the control group. The addition of NaCl increased the soil EC to 8 $dS.m^{-1}$, thus inducing salt stress. The experiment was performed in randomized complete block design with four replicates. Three plants per replicate were used for collecting the data. Analysis of variance was performed, and means were compared with Duncan's multiple range test.

2.2.2 Measurement of Physiological Parameters

After 6 days of salt-stress at the booting stage, standard physiological responses, such as net photosynthetic rate (*Pn*), stomatal conductance (gs), internal CO_2 concentration (*Ci*), transpiration rate (E), F_v/F_m, and performance index (*Pi*), were evaluated. In parallel, every 3 days from day 0 to 9 during salt-stress treatment, we classified rice responses using the standard evaluation system (SES) of rice (IRRI, 1996). After 9 days of salt stress, the saline solution was washed out to reduce soil salinity to 2 $dS.m^{-1}$. Plants were then grown until seed harvest and yield components were determined.

At day 6 of salt stress, we measured gas exchange parameters in the middle portion of the flag leaves using a portable photosynthesis system (LI-6400 XT; LI-COR, Lincoln, NE). We used three plants per group as a replicate. The leaves were examined under the following conditions: 500 mmol m^{-2} s^{-1} air flow per unit leaf area, 1,200 mol m^{-2} s^{-1} photosynthetically active radiation (PAR) at leaf surface, leaf temperature ranged from 31.0 to 35.0◦C, and a CO_2 concentration of 380 mol mol^{-1}. F_v/F_m and P_i were measured according to the recommended procedures of FMS 2 (Hansatech, King's Lynn, UK). Leaves were dark-adapted for 40 min using dark-adapted leaf clips before measurement.

2.2.3 Experimental design and statistical analysis

The study was laid out in a completely randomized design (CRD), with four replicates per treatment group (samples from three plants in a group constituted a replicate). Data of physiological parameters were subjected to analysis of variance (ANOVA) and significant means were compared using

Duncan's multiple range tests (DMRT) by using SPSS version 21 (IBM Corp, Armonk, USA). Values were considered statistically significant at $p < 0.05$.

2.3 Identification of the putative salt tolerant genes via transcriptome analysis

*2.3.1 RNA **e**xtraction and **s**equencing*

To identify the genes regulating salt tolerance in rice, we focused on transcriptome analysis of CSSL16, which had the highest salt tolerance in the seedling and booting stages. Three replicates were used for each condition (CSSL16 grown under normal condition and under salt stress (75 mM NaCl treatment), respectively). Leaf tissues were collected at the seedling and booting stages. We harvested leaf samples from 21 days old seedlings after 0 and 2 days of salt stress, while flag leaves and second leaves were harvested at the booting stage on days0 and 3. Leaves from the seedlings, flag leaves, and second leaves of untreated plants were used as the control. Three biological replications were conducted for this experiment. Total RNA was extracted from the leave samples using plant RNA purification reagent (Invitrogen, USA), and contaminated genomic DNA was removed with DNaseI (Invitrogen). cDNA libraries were constructed using the KAPA Stranded RNA-Seq Library Preparation Kit from Illumina R© (Kapa Biosystem, USA). All short reads with a size of ~300 bp were selected and connected with adaptors. Thereafter, all fragments were enriched by PCR for 12 cycles. The cDNA libraries were sequenced using Illumina Next-Generation sequencing (Illumina, USA).

For transcriptome analysis, all short-sequence reads were classified into the right category and QC was performed using a pipeline created by Missirian et al. (2011). The transcriptome sequences were uploaded to the NCBI database with BioProject ID, PRJNA507040. The sequence reads were aligned and mapped to the rice genome database (Ouyang et al., 2007) using Bowtie2 (Langmead & Salzberg, 2012). The DESeq program (version1.24.0) was used to identify differentially expressed genes (Anders & Huber, 2010). Genes with p-value < 0.01 were identified as differentially expressed genes.

2.4 Identification of marker genes by GCN and CC analysis

The read count of the RNA-Seq was analyzed and normalized using the DESeq package in software R (Anders & Huber, 2010). We constructed the gene co-expression network of the rice lines under normal and salt stress conditions at the growth stages (Suratanee et al., 2018), and these constructs were combined as whole-state networks. The expression levels of whole-state networks were mixed. The edges in the network were recognized by calculating and selecting gene pairs with highly correlated ($r \geq 0.9$) levels of expression. Node degree is the number of edges connected to a node in a network, and clustering coefficient is a measure of the proportion of true connections and the number of all possible connections among neighbors of a gene node. The nodes represent the investigated genes, and the edges represent the significant co-expression level of any of the gene pairs. GCN identifies genes by using the degree or hub centrality. The clustering coefficient (CC) is a common measure of the true proportion of the link between the gene nodes and neighbors. The original clustering coefficient (small-world network) (Watts & Strogatz, 1998) is as follows:

$$C(i) = \frac{\sum_j \sum_{q \neq j} (a_{(ij)} a_{(iq)} a_{(jq)})}{k_i(k_i - 1)} \quad (1)$$

C(i) varies from 0 to 1. a_{ij} is a binary value from the connection between node *i* and node *j*. The degree of node *i* is k_i. If all neighbors of *i* are themselves connected to another, CC equals 1, and if the neighbors of *i* do not connect to each other, CC equals 0. Based on a real-world network, their nodes are mostly connected with some level of strength connections or weights. Moreover, the clustering coefficient for a weighted graph was constructed from the total weights of the neighbors (Onnela et al., 2005).

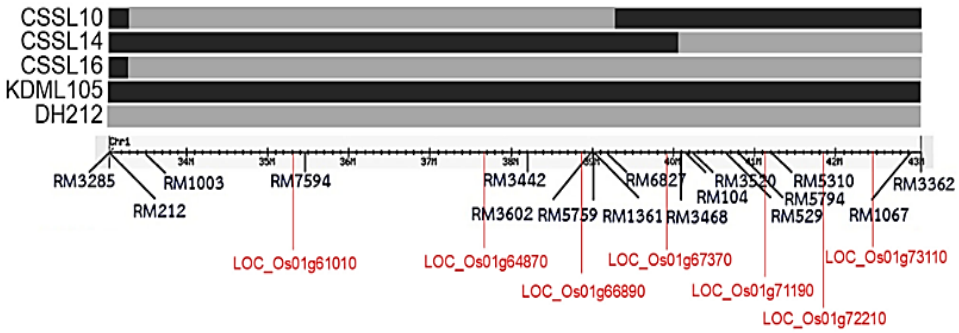

Figure 2.1 The chromosomal segment substitution line of CSSL10, CSSL14, and CSSL16 with regions between RM1003 and RM3362 markers on chromosome 1. Some genes with putative functions, Nodulin (LOC_Os01g61010), BTBZ1 (LOC_Os01g66890), PSB28 (LOC_Os01g71190), and ERD (LOC_Os01g72210), are included.

2.5 Identification of marker genes by weighted co-expression network (WGCN)

For WGCN, the connection of the network has its own values as a binary network of 0 or 1. Therefore, a weighted degree is the sum of all edges connecting the given node and neighbors. A weighted graph of the clustering coefficient is obtained by taking the geometric mean of the total weights of its neighbors (Onnela et al., 2005). Moreover, these connection weights can be positive or negative. While 0 represents no connection with neighbors, 1 represents the highest connection with all neighbors. The formula for using the real weights in the network is as follows:

$$C_{\text{realweight}}(i) = \frac{\sum_j \sum_{q \neq j} \left| w_{(ij)} w_{(iq)} w_{(jq)} \right|^{\frac{1}{3}}}{k_i(k_i - 1)} \quad (2)$$

The weight of the edge connecting nodes i and j is w_{ij}. The connection weights can be categorized as positive or negative. The value of $C_{realweight}$ (i) is distributed in the range [0, 1], where 0 means that there were no neighbors to connect to each other, and 1 means that there were high connections with neighbors. This formula was used to calculate the clustering coefficient for the real weights in the network, while the original formula was performed using a cut-off for the weight estimation into a binary class.

To clarify the analysis of GCN, CC, and WGCN, Figure 2.2 shows an example of a gene co-expression network in the form of a binary network (Figure 2.2A) and in the form of a weighted network (Figure 2.2B). Gene identification by GCN analysis involves calculating the degree for each gene in the binary network in Figure 2.2A. Then, the highly connected nodes are recognized as marker genes. Therefore, G_1 with degree of 4, G_2 with degree of 6, and G_3 with degree of 4 have more connections than the other genes and are identified as important markers. On the other hand, gene identification of CC explores the possibility of connections among the neighbors of a certain node. There are no connections among the neighbors of G1 and among the neighbors of G_3, while there is one connection among the neighbors of G2. Therefore, the CC values of G_1 and G_3 are zero while the CC value of G_2 is 1/15 since 15 is the total number of all possible connections among the six neighbors.

Gene identification by WGCN involves the direct calculation of a weighted degree, that is the sum of all edge weights for a certain node in Figure 2.2B. With the use of weighted network, there are more edges with known strength as more information needs to be considered. Thus, the weighted degree of G_1 is 4.6, the weighted degree of G_2 is 5, and the weighted degree of G_3 is 4.3. Comparing with the degree values above, the weighted network indicates that G_1 is more important than G_3 while they have the same level of importance in the binary network.

2.6 Validation of the salt tolerant candidate genes by gene expression analysis

To validate regulation of salt-tolerance candidate genes by qRT-PCR, CSSL16, which had the highest salt tolerance at the seedling and booting stages, was compared with KDML105. The seeds of CSSL16 and KDML105 were soaked in water to induce germination. After 7 days, the seedlings were transplanted to nutrient solution (Udomchalothorn et al., 2014) with three replicates (three seedlings per replicate). Subsequently, after 7 days, the seedlings were transferred to nutrient solution without NaCl (control) and nutrient solution containing 75 mM NaCl (treatment

group). Seedlings were harvested after salt stress treatment for 0, 3, 6, 12, 24, and 48 h for the early response and for the late response, seedlings were harvested on days 0, 3, and 6 of treatment.

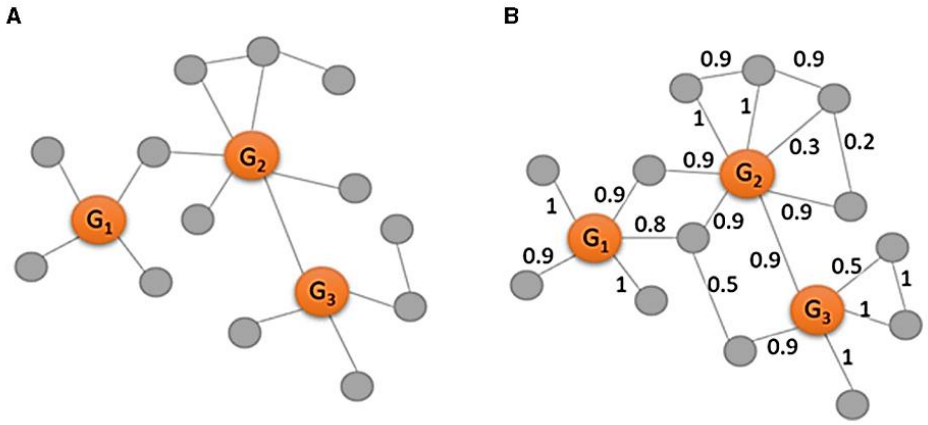

Figure 2.2 Examples of a binary gene co-expression network (A) and a weighted gene co-expression network (B) consisting of three observed genes (in orange) and 13 genes (in gray). The edges in the network (A) are recognized by calculating and selecting gene pairs with highly correlated ($r \geq 0.9$), while the edges in the network (B) are weighted by the absolute values of the correlation.

2.7 Gene expression analysis

Total RNA was extracted from the shoots of seedlings from the control and treatment groups using GENEzol GZR100 (Geneaid Biotech, Taiwan). The RNA was treated with DNase I (Thermo Scientific, USA) and converted into cDNA. cDNA synthesis was performed using an Accupower RT premix (Bioneer Inc., Alameda, USA). The synthesized cDNA was used as template for the PCR. qRT-PCR was conducted using Luna Universal qPCR master mix M3003L (New England Biolabs Inc., USA).

Quantitative RT-PCR reactions were conducted on three technical replicates for each sample. No template (NTC) was used as a negative control, and EF-1α primers (Chutimanukul et al., 2018b) were used as an internal control to standardize

the equal template in the reaction. Gene sequences were obtained from the rice genome database (Ouyang et al., 2007) and then submitted to Primer3

to generate specific primers for the nine selected genes (Table 1). Relative gene expression was determined by qRT-PCR. The PCR conditions were as follows: an initial denaturation step at 95◦ C for 60 s, followed by 35 cycles of denaturation at 95◦ C for 15 s, annealing steps with the temperature shown in Table 1 for 30 s, and continued with an extension step at 75◦ C for 30 s. The melt curve and plate read were set at 60–94◦ C with increasing temperature at the rate of 5◦ C per 5 s. Average cycle threshold (Cq) values of all genes were normalized to the level of EF-1α reference genes in the same sample and then used to measure relative gene expression by following the 11Ct method as described by Pfaffl (2001). The gene expression analysis was interpreted based on the relative expression levels, and SPSS software was used for the analysis of variance ($p < 0.05$).

2.8 Analysis of *Arabidopsis* mutant lines for salt stress responses

The selected mutant seeds were ordered from *Arabidopsis* Biological Resource Center (ABRC). The homozygous mutant lines were screened according to SALK T-DNA primer design. The homozygous mutant lines used in this experiment were bt3, psb28, AT5G45310, sbt3.3, sbt3.4, and per3 mutants. Col-0wild type (WT) was used as a control. The evaluation of salt stress response was performed with a complete randomized design with three replicates. Each replicate contained 20 seedlings. Mutant lines and WT seeds were sterilized and germinated for 7 days after stratification at 4◦C for 48 h. Then, 7-day-old seedlings were transferred to the freshly prepared MS medium with or without 100 mM NaCl addition. After 7-day incubation under light intensity of 35 $mmol.m^{-2}.s^{-1}$, 16/8 light/dark cycle at 22 ◦C, dry weight was measured with 15 plants per treatment. Photosynthetic pigment contents were determined from 5 plants per treatment according to (Wellburn, 1994). The absorbance at A470, A646.8, and A663.2 were measured to determine Chlorophyll *a*, chlorophyll *b* and carotenoid contents by using the following equations:

Chlorophyll *a* (Chl *a*) content = $12.25A_{663.2} - 2.79A_{646.8}$ (3)

Chlorophyll *b* (Chl *b*) content = $21.5A_{646.8} - 5.1A_{663.2}$ (4)

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

2.9 Putative promoter analysis

The putative promoter region (2 kb upstream from coding region) of *OsBTBZ1* gene of KDML105 and CSSL16 was retrieved from PRJNA659381. Sequence alignment was performed by using Needle tool via EMBOSS. Cis-elements were searched against PLACE database (Higo et al., 1999).

Table 2.1 Quantitative RT-PCR rice primers.

Name/annotation		Sequence 5'à3'	Product size	Position	Annealing temperature
LOC_Os01g61010 (Nodulin)	FW	CCGCGAAAAGTGGCTACTCCA	101 bp	1,179–1,282	60.0°C
	RV	AAAGAAGTCCCGCTGGTTGAG			
LOC_Os01g64870	FW	CGAGCAGTTTGCCAGGTTGAAT	183 bp	974–1,156	61.5°C
	RV	AGCCTTTGGAATGCAAGCTCCT			
LOC_Os01g66890(BTBZ1)	FW	TTCCTGCCTGCAAGGGCATC	172 bp	1,108–1,280	61.5°C
	RV	TCCTTGAAATGCCTACAGAGGGG			
LOC_Os01g67370	FW	GGCGGATTTACCGAACATATTTGA	173 bp	260–432	60.5°C
	RV	TGTCAGCCAGGAAGGTTGGA			
LOC_Os01g72210 (ERD)	FW	GGTTCTAACAAGCTTTGGGTGC	141 bp	562–703	61.5°C
	RV	TTGGTCAGGCCGTTTCCTGT			
LOC_Os01g71190 (PSB28)	FW	GATGCCCCGCAGGTTCGTC	170 bp	218–387	60.0°C
	RV	GGTGCCCTGGATGAACTGGA			
LOC_Os01g73110	*FW*	CCGATGGTGATGGTTGGCTG	180 bp	160–339	61.0°C
	RV	CCGATCCAGCTTGCGCTCT			
LOC_Os04g03050 (Sub34)	FW	TGTGGTTATCACCTTGGGCG	124 bp	1,164–1,287	61.0°C
	RV	ATTGTCGGCATTGCAGTCGT			
LOC_Os06g46799 (Peroxidase)	FW	CCTCTCCTCCTTCCAGAGCAA	97 bp	629–725	61.0°C
	RV	GCTGAACGAGTTGCAGTGCG			
EF1α	FW	ATGGTTGTGGAGACCTTC	127 bp	1,326–1,435	60.0°C
	RV	TCACCTTGGCACCGGTTG			

3. Result

3.1 CSSL16 sustained photosynthetic responses under salt stress at booting stage.

The physiological study showed that the net photosynthesis rate (P_n) of the flag leaves of the rice lines under normal grown condition was not significantly different (Figure 2.3A). However, the P_n of the second leaves of the lines were significantly different, with the second leaves of 'KDML105' recording the highest P_n values, while the second leaves of CSSL14 grown under normal conditions had the lowest P_n values.

Salt stress caused a decrease in the P_n of the flag leaf and second leaf of the lines (Figure 2.3B). The flag leaves of CSSL10, CSSL16, and DH212 had significantly higher P_n than those of 'KDML105' and CSSL14, while the second leaves of CSSL10 had similar P_n values to those of 'KDML105' and DH212. A similar response was also found in stomatal conductance (Figures 2.3 C, D). The C_i levels of rice grown under normal conditions were not

significantly different; contrarily, the C_i levels of both flag leaves and second leaves of rice lines grown under salt stress were significantly different with the second leaves of CSSL16 recording the highest C_i level (Figures 2.3 E, F). The transpiration rate of these plants was consistent with their g_s (Figures 2.3 G, H).

Salt stress did not affect the PSII efficiency (F_v/F_m) of the flag leaves (Figures 2.4A, B). Additionally, the P_i's of the flag leaves were not significantly different under normal growth condition; contrarily, salt stress significantly affected the P_i's of the flag leaves, with CSSL14 recording the highest P_i, while 'KDML105' recorded the lowest. The second leaves of CSSL16 recorded the highest P_i both under normal growth condition and under salt stress, while the second leaves of 'KDML105' had the lowest P_i both under normal growth condition and under salt stress. Overall, the P_i's of the second leaves of the rice lines were significantly different both under normal growth conditions and under salt stress (Figures 2.4C, D). During the first 6 days and after 9 days under salt stress conditions (Figure 2.5), CSSL16 and DH212 had significantly lower SES than the other lines.

3.2 CSSL16 had higher yield components than that did 'KDML105' and other CSSLs

After exposing the rice seedlings to salt stress at 8 $dS.m^{-1}$ for 9 days, soil salinity was reduced to 2 $dS.m^{-1}$ and the plants were grown under this condition until grain harvest. The yield components of the different lines were determined after harvest (Table 2.2). Results showed that rice lines with KDML105 genetic background recorded higher tiller numbers per plant than the corresponding, introgression-free line DH212. Salt stress decreased tiller numbers per plant, panicle numbers per plant, panicle length, total seed number, and number of filled grains per plant. Moreover, shoot fresh weight, dry weight, and height were affected by salt stress (Table 2.2). CSSL16 had the highest tiller numbers per plant, panicle number per plant, total seed number per panicle, filled grain, and seed number per plant, compared to the other lines. Based on gas exchange parameters, PSII efficiency and yield

component, CSSL16 was the most tolerant line under high salt stress at the booting stage. This suggested that the presence of the whole QTL region was required to achieve the best tolerance, implicating the action of two or more genes Therefore, CSSL16 was chosen for transcriptome analysis.

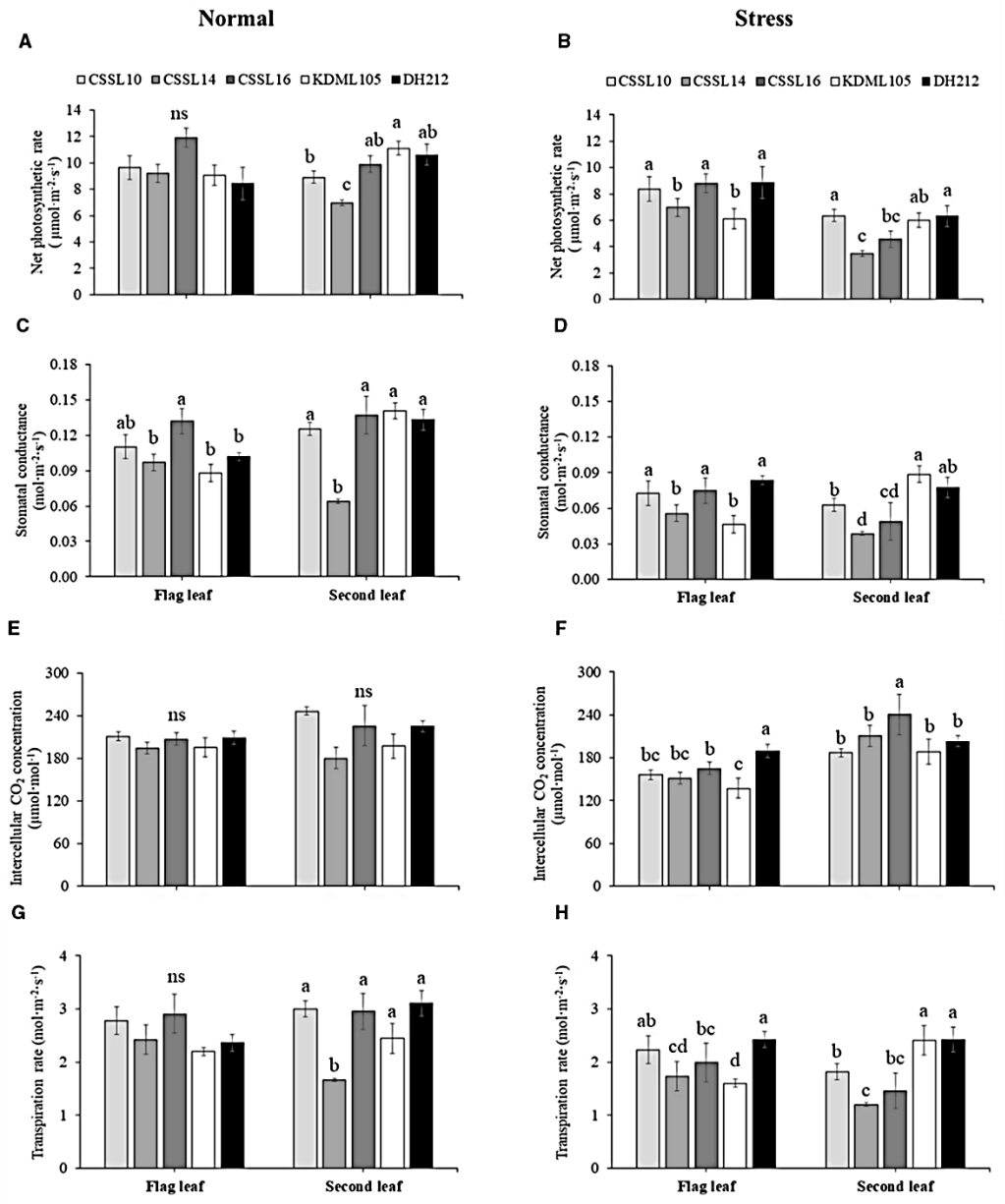

Figure 2.3 Gas exchange parameters, net photosynthesis rate [P_n, (A,B)], stomatal conductance [g_s , (C, D)], internal CO_2 concentration [C_i, (E,F)], and transpiration rate (E, G, H) of flag leaves and second leaves of CSSL10, CSSL14, CSSL16, 'KDML105' and DH212 under normal and salt stress conditions. Values are represented as mean ± SE (n = 4). Different letters

above bars indicate significant difference between lines at $p < 0.05$. "ns" indicates no significant difference.

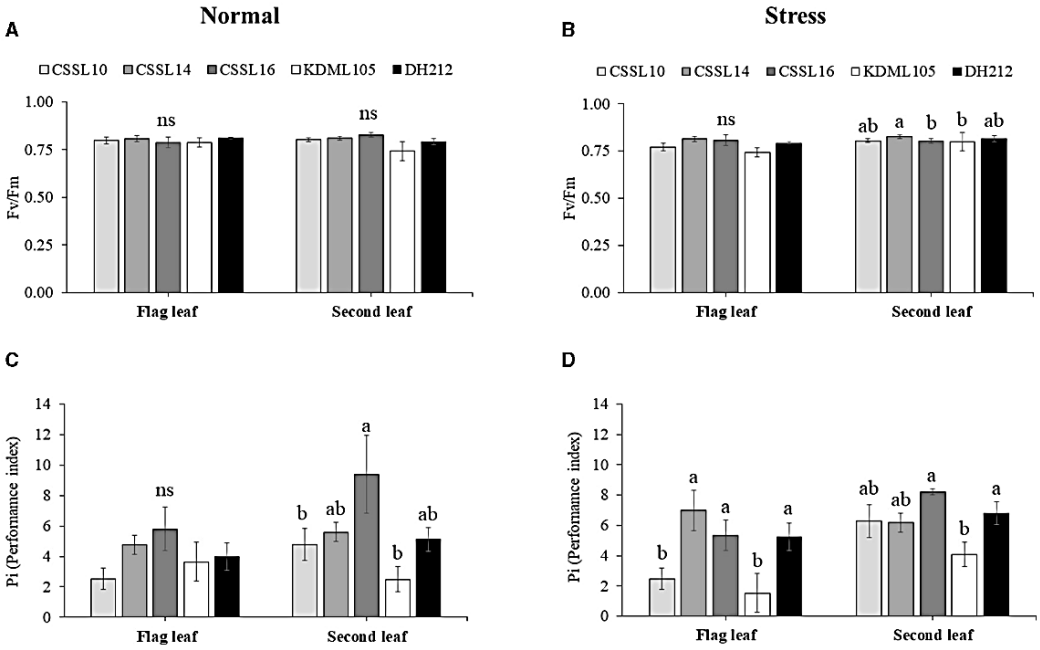

Figure 2.4 Maximum PSII efficiency (F_v /F_m) (A, B) and Performance index (P_i) (C,D) of flag leaves and second leaves in CSSL10, CSSL14, CSSL16, 'KDML105' and DH212 under normal and salt stress conditions. Values are represented as mean ± SE (*n = 4*). Different letters above bars indicate significant difference between lines at $p < 0.05$. "ns" indicates no significant difference.

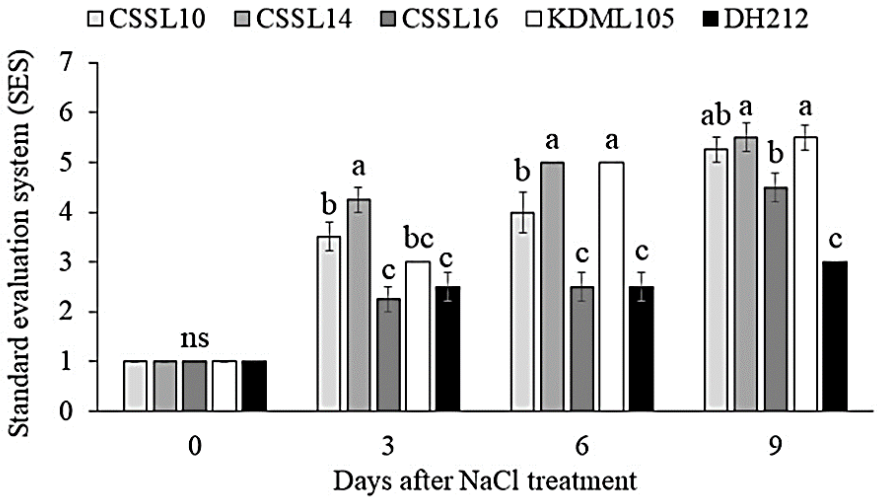

Figure 2.5 Standard evaluation system (SES) determined from the appearance of plants under salt stress condition for 0, 3, 6, and 9 days. Values are presented as mean ± SE (*n = 4*). Different letters above bars

indicate significant difference between lines at $p < 0.05$. “ns” indicates no significant difference.

3.3 Transcriptomics profile of CSSL16 rice at seedling and booting stages

To identify genes regulating salt tolerance in rice, we analyzed the transcriptome of three seedling leaves, and from the flag and second leaves of CSSL16 plants exposed to normal growth condition and salt stress, respectively. Gene expression was examined by RNA sequencing of the leaves of seedlings at 0 and 2 days of treatment. At the booting stage, RNA sequencing was performed from flag leaf and second leaf samples at 0 and 3 days of treatments. We identified 511 differentially expressed genes in the leaves of the seedling, while 520 and 584 differentially expressed genes were identified in the second leaf and flag leaf, respectively (Supplementary Files 1, 2). More than 50% of the differentially expressed genes were downregulated by salt stress at the seedling stage and in the flag leaves at the booting stage. Contrarily, <50% of the differentially expressed genes were downregulated by salt stress in the second leaf.

We used the Clue Go tool to screen gene ontology (GO) terms that were significantly enriched by the DEGs. The results showed that genes enriched in biological processes, such as response to inorganic substances, oxygen-containing compounds, alcohol, heat, and temperature stimulus were downregulated in the leaves of the seedlings, while the genes involved in cell wall biogenesis, cellular glucan metabolism, and glucan metabolism were upregulated (Supplementary Figure 1). We compared the transcriptomes of the second leaf before and after 3 days of salt stress. The GO enrichment analysis of the second leaf indicated a significant upregulation of genes regulating temperature and heat responses, and the sizes of cellular components and anatomical structures (Supplementary Figure 2), while genes enriched in cellular chemical homeostasis and chemical homeostasis were downregulated. When the plants were exposed to salt stress, the upregulated genes were enriched in response to heat and temperature stimulus (Supplementary Figure 3).

Table 2.2 Yield components of CSSL10, CSSL14, CSSL16, 'KDML105' and DH212 grown under normal or salt stress conditions (8 $dS.m^{-1}$) at booting stage for 9 days.

Yield components†	Condition	Rice lines					*F*-test
		CSSL10	CSSL14	CSSL16	KDML105	DH212	
Tiller number per plant	Normal	14.25 ± 0.85^{a}	15 ± 1.77^{a}	16.75 ± 0.63^{a}	13.25 ± 0.85^{a}	8.5 ± 1.19^{b}	*
	Salt stress	10.25 ± 0.85^{bc}	12 ± 0.71^{ab}	14 ± 1.08^{a}	12.25 ± 0.47^{ab}	7.75 ± 1.03^{c}	*
Panicle number per plant	Normal	9.25 ± 0.85^{b}	9.75 ± 0.85^{b}	14.5 ± 0.29^{a}	10.25 ± 0.95^{b}	8.5 ± 1.55^{b}	*
	Salt stress	7.75 ± 1.11	9.25 ± 0.95	11 ± 1.47	9.75 ± 1.11	7.5 ± 1.19	ns
Panicle length (cm)	Normal	27.78 ± 0.54^{a}	26.14 ± 0.33^{b}	24.98 ± 0.29^{c}	25.96 ± 0.17^{bc}	27.31 ± 0.21^{a}	*
	Salt stress	24.31 ± 0.17^{b}	24.12 ± 0.36^{b}	23.49 ± 0.26^{b}	21.46 ± 0.53^{c}	25.92 ± 0.26^{a}	*
Total seed per panicle	Normal	130.25 ± 3.94^{bc}	116.75 ± 4.31^{c}	152.25 ± 3.68^{a}	124.25 ± 5.78^{c}	143.75 ± 4.40^{ab}	*
	Salt stress	121.50 ± 4.13^{bc}	114.75 ± 1.75^{c}	142.75 ± 4.05^{a}	113.5 ± 3.10^{c}	134.75 ± 2.63^{ab}	*
Filled grains per plant	Normal	91.75 ± 0.48^{c}	94.5 ± 2.10^{c}	130.25 ± 2.25^{a}	109.75 ± 5.20^{b}	114.25 ± 4.85^{b}	*
	Salt stress	74.25 ± 2.50^{d}	75 ± 1.68^{d}	117 ± 3.03^{a}	90.25 ± 2.29^{c}	98.25 ± 2.87^{b}	*
100 Seeds weight (g)	Normal	1.89 ± 0.07^{b}	1.90 ± 0.10^{b}	1.92 ± 0.04^{b}	2.20 ± 0.10^{a}	2.08 ± 0.02^{ab}	*
	Salt stress	1.14 ± 0.15^{b}	1.67 ± 0.08^{a}	1.65 ± 0.07^{a}	1.85 ± 0.07^{a}	1.55 ± 0.16^{a}	*
Plant height (cm)	Normal	178.25 ± 2.62^{a}	171.75 ± 5.21^{ab}	156 ± 8.95^{b}	121.25 ± 3.35^{c}	164.75 ± 4.19^{ab}	*
	Salt stress	152.5 ± 2.75^{a}	160 ± 5.05^{a}	139 ± 2.68^{b}	109.5 ± 0.65^{c}	152.75 ± 2.56^{a}	*
Shoot fresh weight (g)	Normal	179.25 ± 8.01^{a}	147.5 ± 10.13^{ab}	157.7 ± 10.40^{ab}	116.22 ± 11.54^{b}	132.19 ± 22.07^{b}	*
	Salt stress	138.5 ± 7.053	118.25 ± 11.44	125.75 ± 12.30	86.5 ± 13.37	117.25 ± 16.12	ns
Shoot dry weight (g)	Normal	28.74 ± 2.19	28.67 ± 2.10	32.76 ± 0.95	24.56 ± 1.27	29.07 ± 2.27	ns
	Salt stress	24.49 ± 1.41^{bc}	27.39 ± 1.02^{ab}	29.01 ± 0.53^{a}	22.13 ± 0.88^{c}	27.94 ± 1.71^{ab}	*

† Values are represented as mean ± SE (n = 4). Different letters indicate significant difference between lines at $p < 0.05$. "ns" indicates no significant difference. * Significant difference at $p < 0.05$.

3.4 Combining the gene co-expression network analysis with SNP information can identify salt tolerant genes.

The co-expression networks under salinity and normal conditions were constructed by calculating the correlation of the expression levels of DEGs in the plants (leaves of the seedlings, flag leaves, and second leaves). Genes that were highly correlated ($r > 0.9$) under normal condition were used to construct the normal-state network. Similarly, genes that were highly correlated under salinity stress were used to construct the salinity-state network. We found 579 DEGs in the normal-state network and 573 DEGs in the salinity-state network. The results showed that the network created from expression data under normal conditions had higher number of nodes, edges, connection per node, and average degree than those of the network created from the expression data under the salt stress condition. The genes involved in salt tolerance were selected from genes with high connections per node under salt stress conditions and low connections per node under normal conditions. Fifty-seven

candidate genes (Supplementary File 2) were selected. Most of the selected genes were on chromosome 1. Four of them, *LOC_Os01g64870*, *LOC_Os01g66890*, *LOC_Os01g67370,* and *LOC_Os01g72210* were located in the salt/drought tolerant QTL reported by Kanjoo et al. (2012). LOC_Os01g72210 and LOC_Os01g67370 encoded unknown expressed proteins, while LOC_Os01g66890 was annotated as BTBZ1 and LOC_Os01g72210, was annotated as a protein part of the early response to dehydration (ERD) protein. Both *BTBZ1* and *ERD* displayed SNPs between CSSL16 and 'KDML105' in the promoter, 5'UTR, exons, introns, and 3'UTR.

We analyzed the distributions of the clustering coefficients for the binary network by comparing a dense local cluster between salt stress and normal conditions. The clustering coefficient analysis identified 30 genes involved in salt tolerance (Supplementary File 3). Four genes were located in the salt/drought tolerant QTL (Kanjoo et al., 2012), *LOC_Os01g61010*, *LOC_Os01g66890* (*BTBZ1*), *LOC_Os01g72210* (*ERD*), and *LOC_Os01g73110*. The CC analysis identified *BTBZ1* and *ERD*, which were also identified by GCN analysis. *LOC_Os01g61010* was annotated as encoding a Nodulin, while *LOC_Os01g73110* encoded an unknown expressed protein.

Furthermore, we identified 59 genes using weighted co-expression network analysis (Supplementary File 2). *LOC_Os01g64870*, *LOC_Os01g66890* (*BTBZ1*), *LOC_Os01g71190*, *LOC_Os01g72210* (*ERD*), and *LOC_Os04g03050* were located in the salt/drought QTL (Kanjoo et al., 2012; Kanjoo, 2011; Koyama et al., 2001). Moreover, three out of the five genes (*LOC_Os01g64870*, *BTBZ1*, and *ERD*) were identified by both the co-expression network and clustering coefficient analyses. The other three genes included *LOC_Os01g71190* (*PSB28*), which was annotated to encode the protein involved in photosystem II reaction center, while *LOC_Os04g03050* and *LOC_Os06g46799* encoded subtilisin (OsSub34) and peroxidase precursor, respectively.

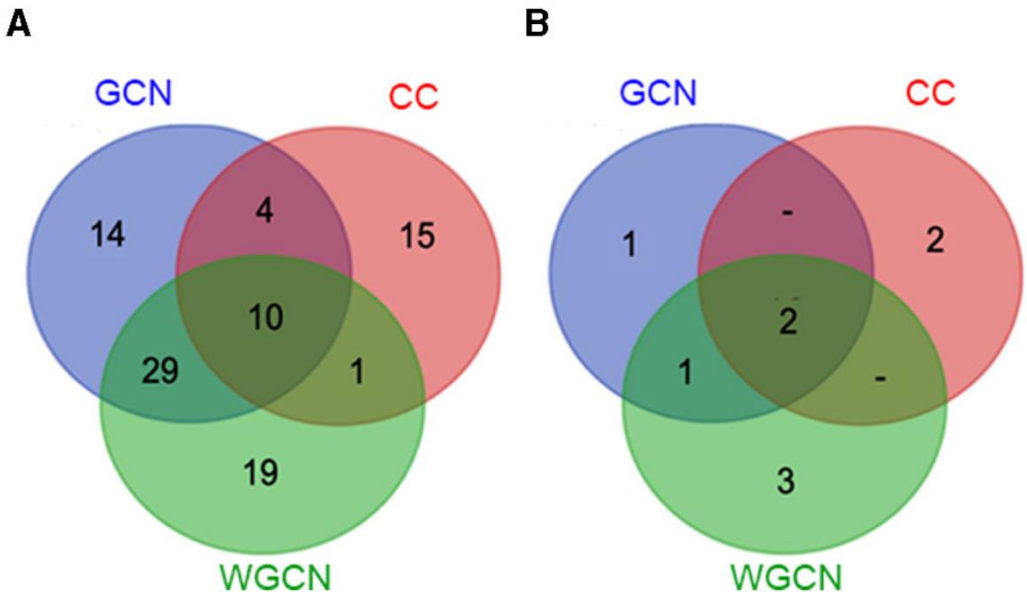

Figure 2.6 Venn diagram (A) showing the number of salt-responsive genes from co-expression network analysis (blue circle), Clustering coefficient analysis (red circle), weighted co-expression network analysis (green circle), and Venn diagram (B) showing number of salt-responsive genes containing the SNPs in each method analysis

Table 2.3. Salt-tolerant genes consistently predicted by GCN, CC, and WGCN

Locus	Annotation
LOC_Os01g66890 (*BTBZ1*)	BTBZ1—Bric-a-Brac, Tramtrack, and Broad Complex BTB domain with TAZ zinc finger and Calmodulin-binding domains, expressed
LOC_Os01g72210 (*ERD*)	Early-Responsive to Dehydration protein-related, putative, expressed
LOC_Os02g08100	AMP-binding domain containing protein, expressed
LOC_Os02g45950	cytochrome b_6f complex subunit, putative, expressed expressed protein
LOC_Os03g55720	Cytochrome b_6f complex subunit, putative, expressed
LOC_Os06g28630	Expressed protein
LOC_Os07g02540	HLS, putative, expressed
LOC_Os09g26880	Aldehyde dehydrogenase, putative, expressed
LOC_Os09g39910	ABC transporter, ATP-binding protein, putative, expressed
LOC_Os11g42500	Dirigent, putative, expressed

Figure 2.6A displays a Venn diagram of the genes identified using the three network analyses. The blue, red, and green circles included genes identified by GCN, CC, and WGCN, respectively (Figure 2.6A). In total, we

identified 92 genes using the three methods. Among the genes, 10 were identified by each of the three methods (Table 2.3). The co-expression network of 92 genes identified by GCN, CC, and WGCN is shown in Figure 2.7. The 10 genes, identified by these three techniques (GCN, CC, and WGCN), are displayed as red circles.

Using SNPs found in CSSL16 and 'KDML105', the number of genes identified by GCN, CC, and WGCN were 4, 4, and 6, respectively (Figure 2.6B). Together with the three methods of transcriptome analysis and SNP information of the salt tolerant and susceptible lines, we identified nine genes, which were responsible for salt tolerance in rice (Figure 2.6B and Table 2.4). Two out of these genes, which are *LOC_Os01g66890* (*BTBZ1*) and *LOC_Os01g72210* (*ERD*), contain SNPs between CSSL16 and 'KDML105' rice. In addition, these two genes are connected to each other in the network (Figure 2.7). We hypothesize that the nine genes were responsible for the salt tolerance of CSSL16 compared with KDML105 rice.

3.5 Significantly different expression levels of the candidate genes in CSSL16 after salt-stress treatment.

To examine the salt-tolerance candidate gene expression, we used qRT-PCR to study the expression response to salt stress of the nine genes in Table 4. After growing rice seedlings for 14 days, 75 mM NaCl was added to the nutrient solution. We compared their expression in CSSL16, the salt-tolerant genotype, and in its salt-susceptible parent, 'KDML105'. The comparison was performed in two sets of experiments to investigate the early (0, 3, 6, 12, 24, and 48 h after stress) and late (0, 3, and 6 days after stress) responses. After 6 days of salt stress, morphology of the plants is displayed in Figures 2.8 A, B. For early stress responses, *OsNodulin* expression did not vary much during this period of salt stress (Figure 2.9 A), while *LOC_Os01g64870* expression in the salt-treated CSSL16 after 12 h of salt treatment was increased to more than 7-fold higher than treated KDML105 (Figure 2.9B). The expression levels of *OsBTBZ1* (Figure 2.9 C), *LOC_01g67370* (Figure 2.9 D), and *OsPeroxidase* (Figure 2.9 I) in the salt-

treated CSSL16 were also significantly higher than those of the salt-treated KDML105 after 12 h of the treatment, while the expression levels of *OsERD* (Figure 2.9 E), *LOC_01g73110* (Figure 2.9 G), and *OsSub34* (Figure 2.9 H) in CSSL16 was dramatically higher than KDML105 after 6 h of salt stress. It is worth mentioning that the expression of *OsBTBZ1*, *OsERD*, *OsSub34*, and *LOC_01g73110* was induced more than 15-fold by salt stress in the early response. The expression level of *OsPSB28* (Figure 2.9F) was higher in CSSL16 after 6 and 48 h of stress, but the level of expression was fluctuating and did not show much difference during this early response.

For the late response, the expression of *Nodulin* (Figure 2.10 A), *LOC_Os01g64870* (Figure 2.10B), *BTBZ1* (Figure 2.10C), *LOC_Os0167370* (Figure 2.10D), and *PSB28* (Figure 2.10 F), increased significantly in CSSL16, but decreased in KDML105 at 3 days of exposure to salt stress. However, the expression of *ERD* (Figure 2.10E) and *LOC_Os01g73110* (Figure 2.10G) increased in both CSSL16 and KDML105 at 3 days of salt stress. After 6 days of salt stress, the expression of *Nodulin*, *LOC_Os01g64870*, and *BTBZ1* was still higher in CSSL16 compared with that of KDML105, but the expression of *LOC_Os01g73110* decreased, while the expression of *ERD* increased. After 6 days of salt stress, the expression of *ERD* increased by more than 4.5 and 4 fold in CSSL16 and KDML105, respectively. The expression of *OsSub34* was reduced by salt stress in both lines, however, this decrease was more pronounced in CSSL16 than that in 'KDML105' (Figure 2.10H). Peroxidase increased after 6 days of salt stress in both lines (Figure 2.10I). The results suggest that the nine candidate genes may be involved in salt tolerance in rice.

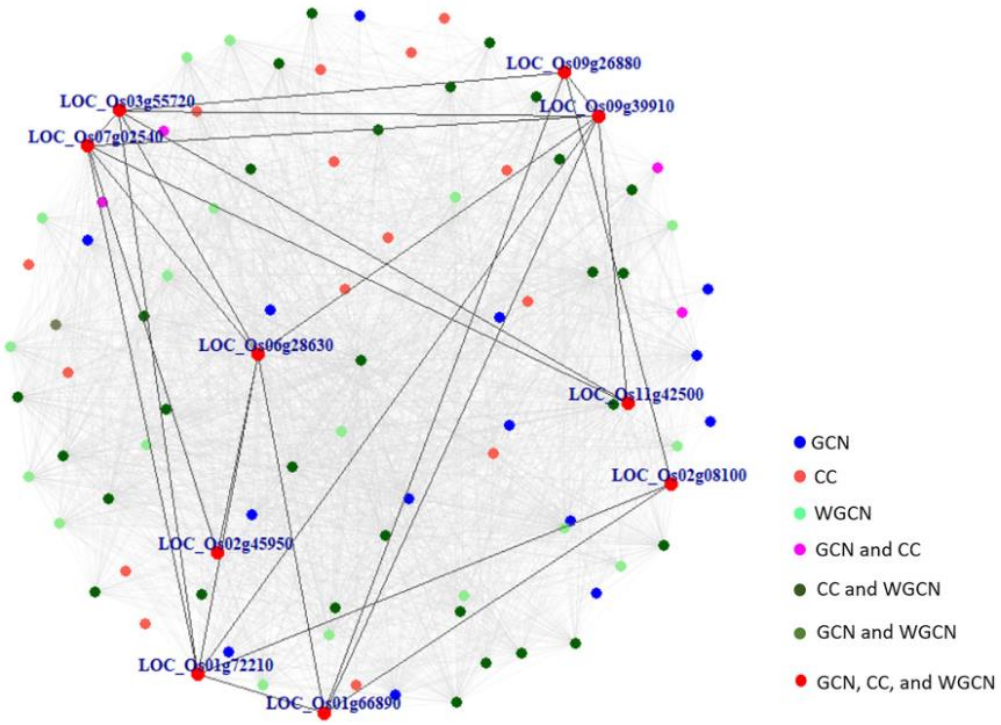

Figure 2.7 The co-expression network of 92 marker genes identified by GCN, CC, and WGCN. Ten genes in red were detected by all methods and the connections among them were shown in darked lines. All gray lines represent the connections among these 92 marker genes.

3.6 The predicted genes have the potentials to function in salt tolerance

In order to investigate the potential of these predicted genes for functioning in salt tolerance, *Arabidopsis* mutant lines containing T-DNA insertion in the genes orthologous to the predicted rice genes, were analyzed for their salt responsive phenotypes. Due to the dramatically higher induction at early response of *OsBTBZ1*, *OsSub34*, and *LOC_01g73110*, the *Arabidopsis* mutants of their orthologous genes (Table 2.4), namely *bt3*, *sbt3.3*, *sbt3.4*, and *at5g45310* mutants, were analyzed. Although *OsERD4* displayed high level during early induction, the *erd4* mutant was not included in this analysis because no homozygous insertion lines could be obtained. Finally, the *psb28* and *per3* mutants were included in this experiment and Col-0 wild type (WT) was used as a control.

Under normal growth condition, *sbt3.4*, *psb28*, and *per3* mutants showed significantly higher dry weights than WT, while the *bt3* mutant had

significantly lower dry weight. The photosynthetic pigment contents were also different among these lines. The *at5g45310* mutant displayed a similar phenotype to the WT, and so did *sbt3.3*, except that *sbt3.3* had higher Chl a content than the WT (Table 2.5).

Salt stress caused dry weight reduction in the WT, but it had decreased effects on the *sbt3.3*, *sbt3.4*, *at5g45310*, *per3*, and *psb28* mutants. A negative effect of salt stress on dry weight was detected in the *bt3* mutant, with more than 60% reduction in dry weight. Salt stress conditions caused the reduction of photosynthetic pigments content in all lines, especially the *bt3* mutant, whose photosynthetic pigments content was decreased more than 65%. Interestingly, the carotenoid content in *sbt3.3* and *per3* mutants was dramatically decreased by salt stress (more than 80% reduction), but both mutants displayed better Chl *b* maintenance than the WT (Table 2.5). These changes in salt stress responses in these mutant lines, when compared to WT, suggest a role for these genes in salt stress adaptation in *Arabidopsis* and reinforce the hypothesis of functions of these gene families in other plant species, including rice.

Because the *bt3* mutant displayed the highest growth inhibition and photosynthesis pigment reduction and the *OsBTBZ1* gene was highly-induced under salt stress, we focused on its promoter. We compared putative regulatory sequences 2 kb base pairs upstream from the coding region of *OsBTBZ1* in the KDML105 and CSSL16 accessions analyzing it for putative regulatory cis-elements (Supplementary Figure 4).

Three ABA responsive elements (ABREs) are located within 250 base pairs upstream of the gene. Moreover, four MYC binding sites, which represent water-stress responsive elements, are located within this region, and two out of four overlapped with the ABREs. Beyond this region, −251 to −2,000 bp, 12 more MYC binding sites are found. The MYB transcription factor was also reported for water stress and salt stress regulation (Ponce et al., 2021). Five MYB binding sites are located in the putative regulatory sequence of *OsBTBZ1* gene.

Two elements that are found only in the putative regulatory region of CSSL16's *OsBTBZ1* gene, but not in 'KDML105's are an endosperm-specific element (AAAG) and GAGA-binding site. The insertion and base substitution in KDML105 eliminate the two elements found in CSSL16. This polymorphism may contribute to the difference in *OsBTBZ1* gene expression level in these two rice lines.

Table 2.4. Putative salt tolerance genes predicted by GCN, CC, and WGCN containing SNPs between CSSL16 and 'KDML105' rice

Locus	Annotation	Types of network analysis	Orthologous gene in Arabidopsis
LOC_Os01g61010 (*Nodulin*)	Nodulin, putative, expressed	CC	-
LOC_Os01g64870	expressed protein	GCN, WGCN	*AT1G71240*
LOC_Os01g66890 (*BTBZ1*)	BTBZ1—Bric-a-Brac, Tramtrack, and Broad Complex BTB domain with TAZ zinc finger and Calmodulin-binding domains, expressed	GCN, CC, WGCN	*AT1G05690* (*BT3*)
LOC_Os01g67370	Expressed protein	GCN	*AT3G59300*
LOC_Os01g71190	Photosystem II reaction center PSB28 protein, chloroplast precursor, putative, expressed	WGCN	*AT4G28660* (*PSB28*)
LOC_Os01g72210 (*ERD*)	Early-responsive to dehydration protein-related, putative, expressed	GCN, CC, WGCN	*AT3G54510* (*ERD4*)
LOC_Os01g73110	Expressed protein	CC	*AT5G45310*
LOC_Os04g03050	OsSub34—Putative Subtilisin homolog, expressed	WGCN	*AT1G32940* (*SBT3.5*) *AT1G32950* (*SBT3.4*) *AT1G32960* (*SBT3.3*) *AT4G10510* *AT4G10540* (*SBT3.8*) *AT4G10550*
LOC_Os06g46799	Peroxidase precursor, putative, expressed	WGCN	*AT1G05260* (*PER3*)

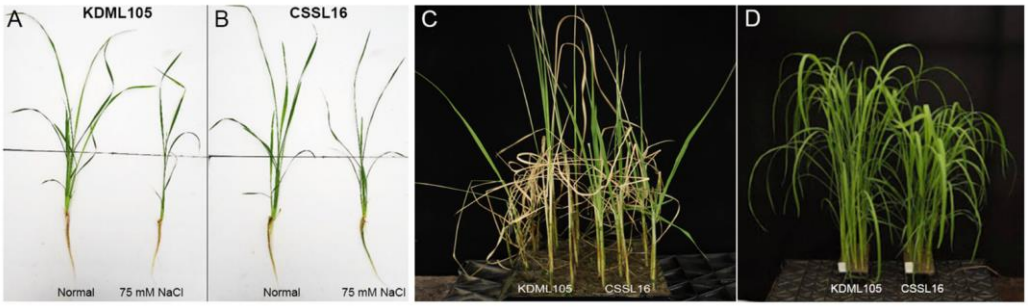

Figure 2.8 Fourteen day-old KDML105 and CSSL16 seedlings after growing in nutrient solution in nomral condition or supplemented with 75 mM NaCl for 6 days (A,B) and the seedlings that were soil-grown and treated with 75 mM for 12 days (C) or grown in normal condition (D).

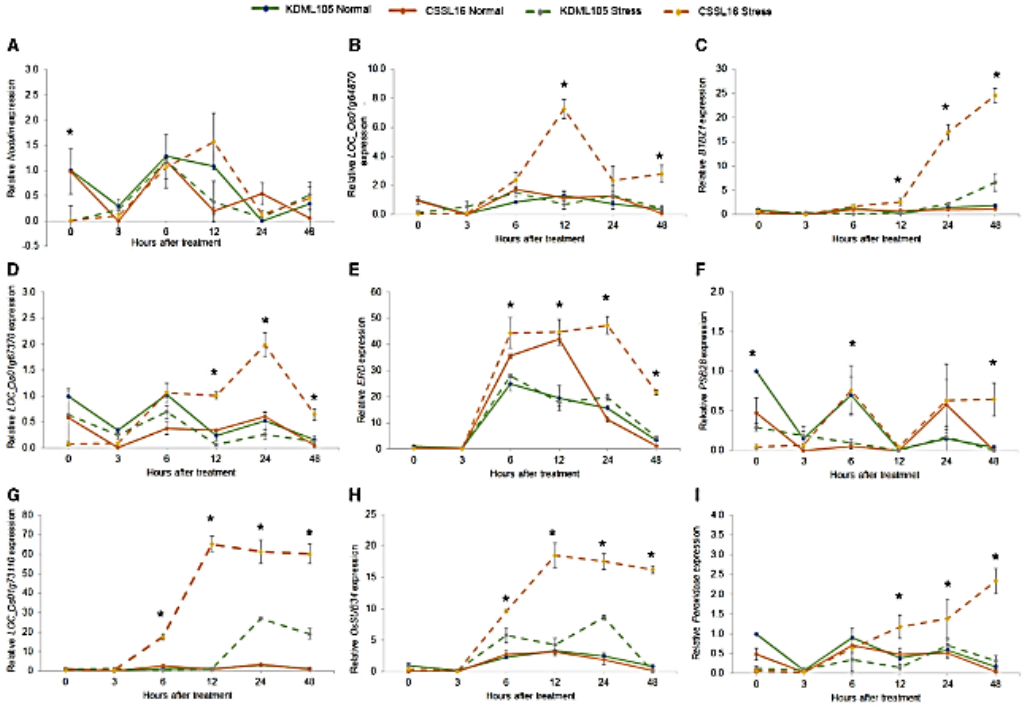

Figure 2.9 Gene expression analysis of nine candidate genes, *Nodulin* (A), *Os01g64870* (B), *BTBZ1* (C), *Os01g67370* (D), *ERD* (E), *PSBS28* (F), *Os01g73110* (G), *Sub34* (H), and *Peroxidase* (I) in CSSL16 and KDML105 under normal and salt stress conditions after 0, 3, 6, 9, 12, 24, and 48 h of salt stress. *indicates the significant difference among mean of the gene expression at $p < 0.05$.

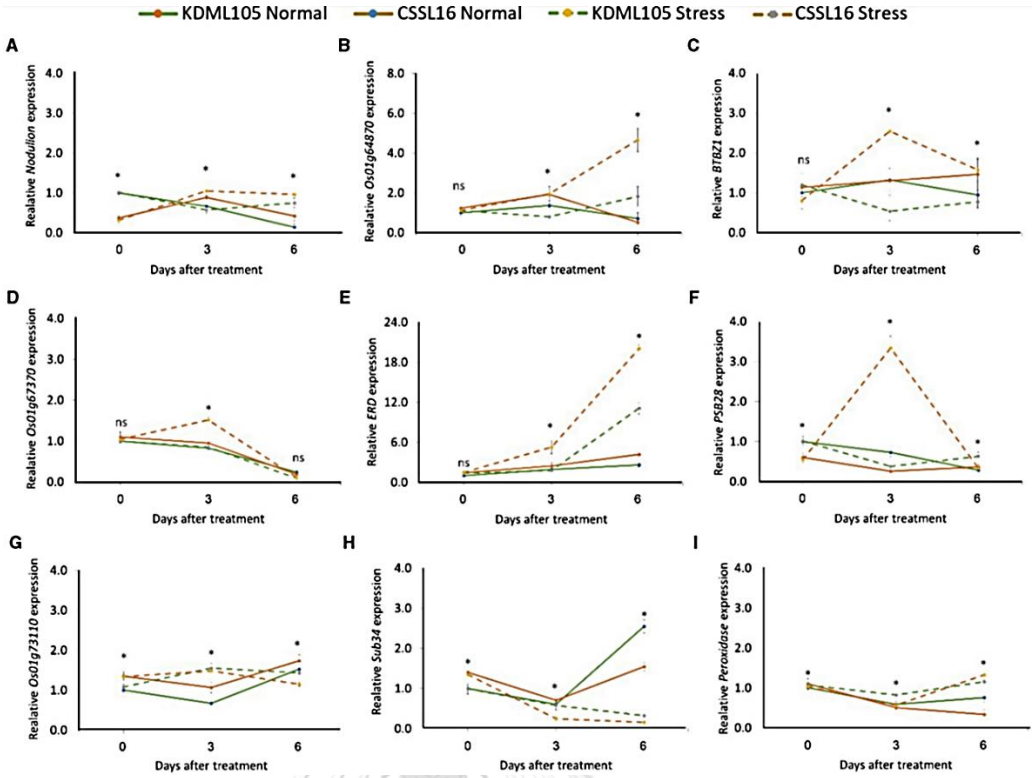

Figure 2.10 Gene expression analysis of nine candidate genes, *Nodulin* (A), *Os01g64870* (B), *BTBZ1* (C), *Os01g67370* (D), *ERD* (E), *PSBS28* (F), *Os01g73110* (G), *Sub34* (H), and *Peroxidase* (I) in CSSL16 and KDML105 under normal and salt stress conditions at day 0, 3, and 6 of salt stress. *indicates the significant difference among mean of the gene expression at p< 0.05.

4. Discussion

In the present study, the results of the gas exchange parameters and yield components indicated that CSSL16 was more resistant to salt stress than KDML105 at the booting stage, as it recorded higher *Pn* and yield components than KDML105 (Table 2.2). This was consistent with the previous reports in rice at the seedling and vegetative stages examined under salt tolerance (Chutimanukul et al., 2018a; Chutimanukul, 2018b). Salt-tolerant rice varieties can maintain their photosynthetic ability after a short period of salt stress (Moradi & Ismail, 2007), however, shoot biomass may decrease (Bhowmik, 2009; Krishnamurthy et al., 2009). In the present study, we documented higher stomatal conductance in CSSL16 than in 'KDML105', which may have

contributed to the higher net photosynthetic rate observed in CSSL16 (Figures 1B, D). Robinson (1988) reported that stomatal conductance and transpiration rate adaptation were the most important mechanisms for salt tolerance. Although the *Pn* of the second leaves of CSSL16 was lower than the *Pn* of the second leaves of KDML105, the tiller number per plant and filled grain number of CSSL16 were higher than those of KDML105 after salt stress. These results suggest that photosynthetic activity in the flag leaves contributed more to grain filling than that of the second leaves. However, salt stress during the booting stage did affect the overall yield of the rice lines (Table 2.2).

Studies in various plant species have shown that salt stress results in a decrease in Fv /Fm (Huang et al., 2014; Martins et al., 2020; Sun et al., 2021). A reduction of Fv /Fm can be used as an indicator of photo-inhibition in stressed plants (Hichem, 2009). In the present study, the Fv /Fm values of the flag leaves were unaffected by salt stress at the booting stage. (Lisa et al., 2011) reported an increase in the expression of photosynthesis-related genes in salt tolerant rice cultivars. In the present study, photosynthesis was sustained in the CSSL16 at the vegetative stage under salt stress and this may be due to the higher expression of the *PsbS1* gene encoding the chlorophyll binding protein in photosystem II (Chutimanukul, 2018b). Contrarily, the 'KDML105' rice had the lowest *Pn*, suggesting that it was the most susceptible compared with the other lines. *Pi* refers to the quantum efficiency of primary photochemistry, the concentration of reaction centers, and excitation energy conversion in electron transport (Melis, 1999; Strasser, 2000). At the booting stage, CSSL14 and CSSL16 had higher *Pi* values under salt stress (Figure 2.3F), indicating that they were able to maintain the quantum efficiency of primary photochemistry. A comparison of the three methods of transcriptomic analysis showed that WGCN identified the highest number of salt tolerance candidate genes, while CC identified the lowest number of candidate genes. Among the 92 genes identified by the three methods, nine genes contained SNPs in CSSL16 and KDML105. The expression level of the nine genes was different in CSSL16 and KDML105, consistent with the notion that they may

be involved in regulating salt tolerance. Moreover, seven of the genes were located in the salt tolerance QTL (Kanjoo et al., 2012) as in Figure 2.1.

The expression analysis of these nine genes within 48 h (Figure 2.8) showed much higher induction in *OsBTBZ1*, *OsERD*, *LOC_Os01g73110*, and *OsSUB34* genes, when compared to the expression at later stages (Figure 2.9), suggesting that these four genes may function in the early response to salt stress. Therefore, we have tried to investigate the roles of these genes in salt stress tolerance by using the *Arabidopsis* mutant with T-DNA insertion in these orthologous genes. Unfortunately, we cannot obtain homozygous of *Arabidopsis* mutant with T-DNA insertion in *ERD4* at this moment. We also investigate the *Arabidopsis* mutant with T-DNA insertion in *PSB28* and *Per3* gene. The decrease in photosynthetic pigments and changes in dry weight response in the mutant lines support the role of the genes in salt tolerance.

Some of the nine genes were reported to be involved in stress responses. *LOC_Os01g61010* (*Nodulin*) encodes a member of a family of highly conserved proteins involved in regulating membrane transporters. Nodulin contributed to water permeability under osmotic stress in soybean (Wallace et al., 2006). Moreover, Nodulin stimulated phosphorylation to regulate the process of cellular transport during osmotic adaptation in soybean exposed to salt or drought stress (Guenther et al., 2003). *LOC_Os01g73110* has not been characterized. However, the function of *LOC_Os01g73110* was investigated using the AraNet and RGAP database identified its homolog in *Arabidopsis* as AT5G45310, whose product is involved in the biosynthesis of abscisic acid (ABA) (Sircar & Parekh, 2015). *LOC_Os01g67370 Arabidopsis* ortholog, *AT3G59300*, encodes a pentatricopeptide-repeat (PPR) superfamily protein. Some PPR proteins in *Arabidopsis* have been associated with abiotic stress responses, including oxidative stress and ABA responses (Liu et al., 2016). PSB28 was found to be associated with photosystem II reaction center and water splitting in light-dependent reactions Suorsa and Aro (2007) reported the molecular function of PSB28. The PSB28 rice mutant identified from the T-DNA insertion population exhibited a pale green plant (Jung et al., 2008). The expression of *PSB28* was reduced under water stress and heat

stress in tomato seedlings (Zhang et al., 2018) and Populus tomentosa (Ren et al., 2019), respectively. Moreover, Kosmala et al. (2009) found that expression of the *PSB28* gene responded to cold stress in Festuca pratensis. These results indicated that PSII and PSI were suppressed under stress conditions. Consequently, the accumulation of PSB28 might enhanced the electron transport rate and photochemical efficiency.

OsSub34 encodes a subtilisin protein associated with serine peptidase. Subtilisin contributes to plant responses under biotic and abiotic stress, organ abscission, senescence, and programmed cell death (Schaller et al., 2018). In rice, *LOC_Os06g46799* encodes a peroxidase precursor that is highly responsive to various abiotic stress stimuli and plays an important role in the regulation of reactive oxygen species (ROS) by converting H_2O_2 to water (Hiraga et al., 2001). Hiraga et al. (2001) identified a group of genes that encodes redox regulation-related proteins, including ascorbate peroxidase, peroxidase precursor, glutathione synthetase, and glutathione S-transferase, in rice exposed to drought stress. Moreover, Chutimanukul (2019) reported that CSSL16 had higher peroxidase activity than that did KDML105 under salt stress at the seedling stage, which supports the role of *LOC_Os06g46799* in the present study.

Table 2.5. Dry weight per plant, chlorophyll a, chlorophyll b and carotenoid contents of 14 day-old Col-0 wild type, *bt3*, *sbt3.3*, *sbt3.4*, *at5g45310*, *psb28*, and *per3* mutants grown in MS medium or MS medium supplemented with 100 mM NaCl for 7 days

	Line	Dry weight* (mg/pl)	Chlorophyll *a** (μg·mg^{-1} FW)	Chlorophyll *b** (μg·mg^{-1} FW)	Carotenoid* (μg·mg^{-1} FW)
Normal	Col-0	0.471 ± 0.062^{de}	0.482 ± 0.039^{b}	0.179 ± 0.028^{ab}	0.149 ± 0.001^{a}
	bt3	0.379 ± 0.023^{f}	0.378 ± 0.022^{d}	0.203 ± 0.003^{a}	0.138 ± 0.006^{bc}
	sbt3.3	0.496 ± 0.020^{d}	0.525 ± 0.028^{a}	0.189 ± 0.036^{ab}	0.177 ± 0.003^{a}
	sbt3.4	0.667 ± 0.089^{b}	0.418 ± 0.011^{c}	0.166 ± 0.004^{b}	0.133 ± 0.007^{bc}
	at5g45310	0.467 ± 0.039^{de}	0.480 ± 0.006^{b}	0.164 ± 0.005^{b}	0.161 ± 0.001^{ab}
	psb28	0.604 ± 0.072^{c}	0.512 ± 0.016^{ab}	0.180 ± 0.002^{ab}	0.117 ± 0.005^{cd}
	per3	0.704 ± 0.098^{ab}	0.354 ± 0.033^{d}	0.105 ± 0.005^{c}	0.102 ± 0.016^{d}
Salt stress	Col-0	0.416 ± 0.023^{ef}	0.222 ± 0.009^{ef}	0.068 ± 0.007^{d}	0.070 ± 0.008^{e}
	bt3	0.147 ± 0.016^{g}	0.077 ± 0.013^{h}	0.031 ± 0.02^{c}	0.044 ± 0.021^{ef}
	sbt3.3	0.518 ± 0.015^{d}	0.213 ± 0.036^{ef}	0.116 ± 0.05^{c}	0.030 ± 0.046^{gh}
	sbt3.4	0.758 ± 0.02^{a}	0.231 ± 0.010^{ef}	0.107 ± 0.008^{c}	0.038 ± 0.005^{gh}
	at5g45310	0.469 ± 0.015^{de}	0.199 ± 0.033^{f}	0.088 ± 0.004^{cd}	0.058 ± 0.015^{ef}
	psb28	0.667 ± 0.095^{b}	0.243 ± 0.012^{e}	0.099 ± 0.011^{cd}	0.045 ± 0.008^{ef}
	per3	0.713 ± 0.04^{ab}	0.162 ± 0.017^{g}	0.120 ± 0.007^{c}	0.015 ± 0.004^{h}

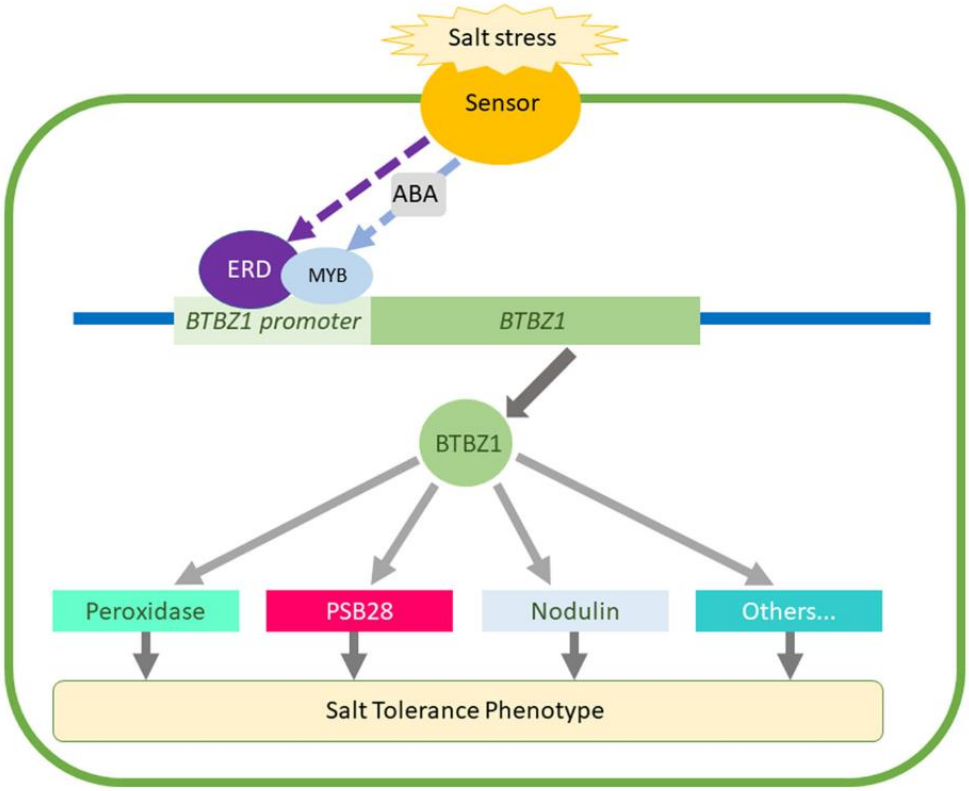

Figure 2.11. Hypothetical model for the function of the predicted genes obtained from the combining of genome and gene co-expression network analysis.

BTBZ1 and *ERD* are proposed to be the genes with the highest correlation with salt tolerance in the rice lines, as both were predicted by three methods of gene co-expression network analysis. Additionally, *BTBZ1* and *ERD* contained SNPs in CSSL16 and KDML105 and both genes were located in the salt/drought QTL previously identified by (Kanjoo, 2011). Consistent with a joint requirement for both genes for optimal stress tolerance, CSSL10 and CSSL14 carry, respectively, either the *BTBZ1* or the *ERD* allele of DH212 and neither displays the full tolerance phenotype of CSSL14. BTBZ1 belongs to the BricA-Brac/ Tramtrack/ Broad Complex (BTB) protein superfamily (subfamily C1) and contains a TAZ zinc finger and calmodulin binding domain. The homologous gene in *Arabidopsis*, *AtBT*3, encodes a nuclear CaM-binding protein. The expression of *AtBTs* can be triggered by stress stimuli (Du & Poovaiah, 2004). BTB-ZF proteins are known as the POK, POZ, and Krüppel zinc finger proteins (Deweindt, 1995). Moreover, Stogios et

al. (2005) reported that the BTB domain is a protein - protein interaction motif that is involved in cellular functions, including transcriptional regulation, cytoskeleton dynamics, ion channels, and targeting proteins for ubiquitination. Moreover, BTB-ZF genes constitute a supergene family encoding proteins that are thought to be transcription factors. Additionally, the analysis of protein-protein interactions from the Predicted Rice Interactome network (PRIN) indicated that the BTBZ1 protein interacted with a cullin protein (LOC_Os02g51180), which may be involved in the degradation of the target protein through the ubiquitin/proteasome pathway (Figueroa et al., 2005). Several reports have described the important role of BTB proteins in developmental programs, defense, and abiotic stress responses (Prasad et al., 2010; Weber & Hellmann, 2009). Nutrient, stress, and hormone responses were regulated by AtBT2 in *Arabidopsis* (Mandadi et al., 2009). However, an ortholog of the *BTBZ* gene in *Arabidopsis* (*AT1G05690*) was involved in plant development (Robert et al., 2009).

ERD was associated with early response to dehydration, which could be rapidly induced during drought stress and other abiotic stresses. *ERD* is a member of a large gene family, whose protein products are associated with triphosphate (ATP) dependent proteases, heat shock proteins (HSPs), membrane proteins, proline, sugar senescence-related genes, chloroplasts, biosynthesis, protein transporters, dehydrogenase, and ubiquitin extension proteins (Kiyosue, 1994; Simpson et al., 2003; Taji et al., 1999). Borah et al. (2017) reported that “Dhagaddeshi rice,” a drought-tolerant cultivar, had higher expression levels of *ERD1* and responded faster than the susceptible cultivar (IR20) to drought stress. Moreover, Liu et al. (2009) found that the *ERD4* gene played a key role in the adaptation of maize to the early stages of stress and enhanced the plant’s tolerance to abiotic stress conditions. In transgenic tobacco, the overexpression of *ERD15* increased the efficiency of PSII (Fv /Fm) through the protection of cellular membranes (Ziaf et al., 2011). Additionally, transgenic *Arabidopsis* plants overexpressing the *BjERD4* gene from *Brassica juncea* displayed increased tolerance to salt stress and drought, while the *Bjedr4* knockdown lines were susceptible to salt and

drought stress (Rai et al., 2017). Therefore, *ERD* may contribute to salt tolerance in rice.

The *bt3 Arabidopsis* mutant showed the highest reduction in growth and photosynthesis pigment content, while in rice, more than 20-fold induction of *OsBTBZ1* gene was detected after 48 h of salt stress treatment. This is consistent with the cis-regulatory elements found in the putative *OsBTBZ1* promoter (Supplementary Figure 4), which include 3 ABREs, 5 MYB binding sites, and 16 MYC binding sites. Many MYB proteins regulate salt tolerance through regulation of the ABA signaling pathway (Wang et al., 2021). Both MYB and MYC proteins function as the transcriptional activators in ABA signaling in *Arabidopsis* (Abe et al., 2003). Together with this literature information our finding support an *OsBTBZ1* contribution to salt tolerance phenotype of CSSL16. The upstream region of *OsBTBZ1* consists of multiple *ERD* binding sites. We identified *OsERD* as one of the key genes because it was highly induced prior to *OsBTBZ1* (45-fold induction) in CSSL16, while it was up-regulated only 25-fold in KDML105. Therefore, the interaction between *OsBTBZ1* and *OsERD* and their involvement in salt tolerance in rice should be further characterized.

Due to insertion and base substitution in the putative promoter region of *OsBTBZ1* in KDML105, GAGA binding site was detected only in CSSL16. In *Arabidopsis*, bHLH34 binds to GAGA element and is involved in ABA and salinity response (Min et al., 2017). Moreover, rice Trithorax factor ULTRAPETALA 1 (OsULT1) was found to bind the promoter region of the *OsDREB1b* gene during transcriptional activation. The binding of OsULT1 to GAGAG elements decreases trimethylation of lysine 27 on histone H3 (H3K27me3), which antagonizes the transcriptional repression effect of H3K27me3, favoring transcriptional activation of the gene (Roy et al., 2019). A similar phenomenon may occur in the regulation of *OsBTBZ1* leading to the higher expression in CSSL16 than KDML105.

Considering all our findings, we hypothesize that the predicted key regulatory genes in the network reported here coordinate a response that makes the rice plants more tolerant to salt stress. The earlier and higher

expression of *LOC_Os01g64870*, *OsBTBZ1*, *LOC_Os01g67370*, *OsERD*, *LOC_Os01g73110*, *OsSUB34*, and *OsPeroxidase* in CSSL16 leads to higher salt tolerance when compared to KDML105. Further investigations should be performed to validate this hypothesis in the future. Based on the *ERD* binding site in *OsBTBZ1* putative promoter, we hypothesize that the *ERD* protein regulates *OsBTBZ1* gene expression and regulates other genes such as *PSB28*, and *Peroxidase*. The proposed model for this hypothesis is shown in Figure 2.11.

According to the comparison of the predicted alleles from chromosome 1 of DH212 in CSSLs, 3 candidate alleles from DH212, *Nodulin* (*LOC_Os01g61010*), *LOC_Os01g64870*, and *BTBZ1* (*LOC_Os01g66890*) are located in CSSL10, while CSSL14 contains another 3 candidates from DH212, which are *PSB28* (*LOC_Os01g71190*), *ERD* (*LOC_Os01g72210*), and *LOC_Os01g73110*. The salt tolerance phenotype of CSSL16 was significantly higher than CSSL10 and CSSL14 in all stages, seedling (Chutimanukul et al., 2018a; Chutimanukul, 2019), vegetative (Chutimanukul, 2018b) and booting stages. Therefore, we explicitly propose that the whole QTL in this region is necessary for salt tolerance in rice.

5. Conclusion

In the present study, we demonstrate an effective transcriptomic approach for identifying genes regulating salt tolerance in rice using two rice lines with close genetic relationships, but different salt tolerance ability. Combining GCN, CC, and WGCN analyses with available SNP information, we identified nine genes involved in salt tolerance in rice. Under salt stress, the expression levels of the nine genes differed in the two rice lines. Moreover, most of the genes were involved in abiotic stress responses. Therefore, we can conclude that the combination of the three methodologies for transcriptome analysis, GCN, CC, and WGCN with SNP information is an effective approach for the identification of genes involved in abiotic stress tolerance and it can support the identification of appropriate QTL for salt tolerance improvement.

6. Supplementary Materials

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.704549/full#supplementary-material

CHAPTER III

RESEARCH ARTICLES

OsBTBZ1 Confers Salt Stress Tolerance in *Arabidopsis thaliana*

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Abstract

Rice (*Oryza sativa* L.), one of the most important commodities and a primary food source worldwide, can be affected by adverse environmental factors. The chromosome segment substitution line 16 (CSSL16) of rice is considered salt-tolerant. A comparison of the transcriptomic data of the CSSL16 line under normal and salt stress conditions revealed 511 differentially expressed sequence (DEseq) genes at the seedling stage, 520 DEseq genes in the secondary leaves, and 584 DEseq genes in the flag leaves at the booting stage. Four *BTB* genes, *OsBTBZ1*, *OsBTBZ2*, *OsBTBN3*, and *OsBTBN7*, were differentially expressed under salt stress. Interestingly, only *OsBTBZ1* was differentially expressed at the seedling stage, whereas the other genes were differentially expressed at the booting stage. Based on the STRING database, *OsBTBZ1* was more closely associated with other abiotic stress-related proteins than other *BTB* genes. The highest expression of *OsBTBZ1* was observed in the sheaths of young leaves. The OsBTBZ1-GFP fusion protein was localized to the nucleus, supporting the hypothesis of a transcriptionally regulatory role for this protein. The *bt3 Arabidopsis* mutant line exhibited susceptibility to NaCl and abscisic acid (ABA) but not to mannitol. NaCl and ABA decreased the germination rate and growth of the mutant lines. Moreover, the ectopic expression of *OsBTBZ1* rescued the phenotypes of the *bt3* mutant line and enhanced the growth of wild-type *Arabidopsis* under stress conditions. These results suggest that *OsBTBZ1* is a salt-tolerant gene functioning in ABA-dependent pathways.

Keywords : abiotic stress; abscisic acid; BTB domain; *BTBZ*; salt stress; tolerance

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important primary food resources worldwide. In Asia, rice production is a principal factor for improving food security. Salinity is a major limiting factor for plants such as rice, decreasing their growth and productivity (Flowers, 2004). Moreover, salt toxicity adversely affects the grain yield, panicle length, spikelet number per panicle, seed weight per panicle, and 1000-grain weight (Khatun & Flowers, 1995; Zeng & Shannon, 2000). Soil salinity can occur naturally or can be induced by human activities, such as constant irrigation with low-quality groundwater (Shahid et al., 2018). High salt concentrations can adversely affect plant physiology through ion toxicity in the plant cells, which reduces the photosynthesis rate and growth of plants (Roychoudhury et al., 2011).

Salt stress tolerance is a polygenic trait controlled by multiple genes in the rice genome. Various efforts have been implemented to determine the genes or genomic regions responsible for this trait. Molecular markers for salt-tolerant phenotypes have been identified by several research groups (Ammar, 2007; Bimpong et al., 2014; Ghomi et al., 2013; Hossain et al., 2014; Koyama et al., 2001; Lee, 2007; Lin et al., 2004; Mohammadi et al., 2013; Prasad, 2000; Qiu et al., 2015; Yao, 2005). After the development of the omics sciences, genomics, transcriptomics, and proteomic approaches have been used to identify the genes/proteins involved in salt tolerance, including their functions in ion transport regulation (Ullah et al., 2022). A greater understanding of the salt tolerance mechanisms initiated by other genes has also been elucidated. Recently, the negative regulator (Kojonna et al., 2022) and protein with a role in absorbed light energy dissipation (Punchkhon et al., 2022) were reported to have a role in salt tolerance.

A genome-wide association study (GWAS) was conducted on the salt-tolerance traits in rice at the germination (Duan et al., 2022; Yu et al., 2018), seedling (Batayeva et al., 2018; Kim & Kim, 2023; Kojonna et al., 2022; Xu et al., 2023), early vegetative (Nayyeripasand et al., 2021; Yadav et al., 2021), and flowering stages (Lekklar et al., 2019; Warraich et al., 2020). In response to various environmental stress conditions, plants alter their gene expression to

deal with the negative effects of environmental signals. Transcriptomics is a prominent method for identifying the genes that potentially regulate salt tolerance in rice (Wang et al., 2020). Transcriptomics analysis can be used in combination with genomic data to predict salt tolerance genes in rice. (Lv et al., 2022) performed a GWAS using 3.82 million SNPs associated with the standard evaluation score (SES) of visual salt injury and then combined them with the differentially expressed genes between cultivars 93-11 and PA64s under normal and salinity stress conditions to predict 30 candidate salt-tolerant genes.

The chromosome segment substitution line 16 (CSSL16) is a salt-tolerant line. Based on the DEseq data of CSSL16, along with the genomic comparison between CSSL16 and its original genomic background, 'Khao Dawk Mali 105 (KDML105)', Chutimanukul et al. (2021) analyzed the transcriptome data of 'KDML105' and CSSL16 rice, using a gene co-expression network (GCN), a weighted gene co-expression network (WGCN), and clustering analysis and predicted 92 candidate salt-tolerant genes. Then, this information was combined with a genomic comparison between CSSL16 and KDML105 and revealed nine candidate genes, seven of which were in the salt-tolerant QTL previously reported by (Kanjoo et al., 2012; Kanjoo, 2011). In this research, we report the validation of the *OsBTBZ1* gene, which is one of the nine candidate salt-tolerant genes predicted by the combined methods of transcriptomic analysis via GCN, WGCN, and CC and genomic comparison.

OsBTBZ1 (*LOC_Os01g66890*) was predicted as an important gene responsible for the salt tolerance characteristics of CSSL16 (Chutimanukul et al., 2021). It is a Bric-a-Brac, Tramtrack, and Broad Complex BTB domain with a TAZ zinc finger and Calmodulin-binding domains. BTB proteins have been studied for many crops and play various roles, mainly in plant growth and in responses to abiotic stimuli. For example, the expression of the *CsBT1* gene in cucumber plants notably decreased under salt stress (Zhou et al., 2020b), whereas the *CaBPM4* gene in pepper was induced after 8 h under salt and drought exposure and 12 h after exposure to cold stress (He et al., 2019). Moreover, the expression in *Arabidopsis thaliana* of

the *IbBT4* gene from sweet potato enhanced drought tolerance (Zhou et al., 2020a).

In this research, to understand the function of *OsBTBZ1* in salt stress conditions, the *OsBTB* gene family expression at both the seedling and booting stages was investigated. Phylogenetic analysis of the *OsBTB* gene family was performed. The cis-elements in the promoters of *OsBTB* genes induced by salt stress were compared to support the salt-responsive expression of the genes. Based on the amino acid sequence of the OsBTBZ1 protein, it is predicted to be a transcription factor and to regulate other genes of the salt-tolerant phenotype (Chutimanukul et al., 2021). This gene was reported to be involved in plant growth regulation; however, its function during salt exposure has not yet been characterized fully. *AtBT3* is the *OsBTBZ1* ortholog in *Arabidopsis*. The *Atbtbz1* (*Atbt3*) mutant, a null mutant of the *AtBT3* gene, is more susceptible to salt stress (Chutimanukul et al., 2021). In this study, the *OsBTBZ1* expression cassette was transferred to *Arabidopsis* wild-type (WT) plants for ectopic expression and to the *Atbt3* mutant for a complementation study. The homozygous T_3 plants were used to investigate the salt, ABA, and mannitol responses and clarify the function of the OsBTBZ1 protein in these abiotic stresses.

2. Materials and Methods

2.1. Transcriptome analysis

A transcriptome study was conducted during the seedling and booting stages of the CSSL16 line. It is a chromosome substitution salt-tolerant line with a 'KDML105' genetic background from BC_5F_4, originally taken from a cross between 'KDML105' and DH105, which was selected as an abiotic stress-tolerant double haploid line. After the cross, F_1 progeny was backcrossed to 'KDML105' for 5 generations and then, self-fertilized to create BC_5F_4. Marker-assisted selection was used to select those CSSLs with the abiotic stress-tolerant regions from DH105 (Chutimanukul et al., 2021; Kanjoo, 2011). Transcriptomic data were retrieved from the database of the National Center for Biotechnology Information under the BioProject IDs

PRJNA507040 and PRJNA659381 (Chutimanukul et al., 2021). Briefly, the CSSL16 line plants were grown under normal and salt stress conditions (75 mM of NaCl treatment). The total RNA from 21-day-old seedling leaves was extracted after 0 and 48 h of salt stress treatment, whereas the total RNA from the flag and second leaves at the booting stage was extracted at 0 h and 72 h after salt stress, respectively, using a plant RNA purification reagent (Invitrogen, USA). Genomic DNA was extracted using DNase I (Invitrogen, Waltham, MA, USA). A KAPA stranded RNA-Seq library preparation kit (Illumina, San Diego, CA, USA) was used to synthesize the cDNA libraries, which were sequenced using Illumina next-generation sequencing (Illumina, USA). The differentially expressed genes were identified using the DESeq tool, version 1.24.0 (Anders & Huber, 2010). Genes with significantly different expressions were considered those with a *p*-value of < 0.01. The PC, sequencing data matrix, and box plot showing the quality of the transcriptome data are shown in Supplementary Figures S3–S5.

2.2. Phylogenetic analysis and an In silico analysis of BTB proteins in *Oryza sativa*

The amino acid sequences from the Rice Genome Annotation Project database were subjected to a motif search (https://www.genome.jp/tools/motif/ accessed on 10 October 2022) to predict the protein motifs (Kanehisa et al., 2002). All BTB proteins were retrieved from the Phytozome database, available at https://phytozome.jgi.doe.gov/pz/portal.html (Goodstein et al., 2012), accessed on 25 October 2022. Orthologous BTB proteins from *A. thaliana* were retrieved from the TAIR database (https://www.arabidopsis.org/ accessed on 25 October 2022). In total, 209 proteins (182 from rice and 27 from *A. thaliana*) containing the BTB domain were used to construct a phylogenetic tree. All BTB protein sequences were aligned in MEGAX, while for the maximum likelihood, the Jones–Taylor–Thornton (JTT) method was employed for phylogenetic tree construction. The phylogenetic tree thus obtained from MEGAX was visualized using iTOL, which is available at https://itol.embl.de/ (Letunic & Bork, 2021) accessed on

3 November 2022. All the *BTB* genes obtained from the Phytozome database were subsequently subjected to the Oryzabase-integrated rice science database http://viewer.shigen. info/oryzavw/maptool/MapTool.do (Kurata & Yamazaki, 2006) (accessed on 28 October 2022).

2.3. Putative promoter analysis

A promoter analysis of *BTBZ1* was carried out on a sequence retrieved from the Phytozome database (https://phytozome-next.jgi.doe.gov/, accessed on 1 November 2022) (Ouyang et al., 2007), from 0 to −2000 bps, and entered into the New Plant cis-acting regulatory DNA elements (New PLACE) website (https://www.dna.affrc.go.jp/PLACE/?action=newplace, accessed on 7 November 2023). The positions of stress-related cis-regulatory elements were visualized using the TBtools software, and the functions of these elements were mainly obtained from the New PLACE database and the published literature (Higo et al., 1999).

2.4. Protein-protein interaction (PPI) based on the STRING database

The PPI was predicted via STRING (Kang et al., 2022). The LOC_Os01g66890 (OsBTBZ1), LOC_Os01g68020 (OsBTBZ2), LOC_Os02g38120 (OsBTBN3), and LOC_Os03g41350 (OsBTBN7) proteins have been named according to the Rice Genome Annotation project (http://rice.uga.edu, accessed on 20 November 2022). However, the STRING website recognizes the Rice Annotation Project Database (RAP-DB) ID. To facilitate this analysis, the locus numbers based on the Rice Genome Annotation Project were converted into RAP-DB IDs using the following tool (https://rapdb.dna.affrc.go.jp/tools/converter/run, accessed on 20 November 2022).

The RAP-DB id of four *BTB* genes are as follows: *Os01g0893400* (*OsBTBZ1*), *Os01g0908200* (*OsBTBZ2*), *Os02g05 94700* (*OsBTBN3*), and *Os03g0609800* (*OsBTBN7*). The interactome was produced using a full-string network based on automated text mining, high-throughput experiments, prior stored databases, and co-expression sources. False-positive and false-negative results were reduced using a high confidence

score (0.700). The other parameters were set to the default values. Enrichment detection was used to predict a network that covered all the mapped proteins and their interconnections.

2.5. Detection of *OsBTBZ1* gene expression

A quantitative real-time polymerase chain reaction (qRT-PCR) was used to investigate the expression profile of *OsBTBZ1* in various tissues of Nipponbare rice varieties under normal conditions. Various tissues were selected at specific time points, as follows. In 15-day-old seedlings, the leaf blade and leaf sheath of the first fully expanded leaf, the second-youngest leaf, and the oldest leaf, including the root tissues, were collected for the gene expression study. Then, in 30-day-old plants, only the leaf blade of a fully expanded leaf was collected. At the reproductive stage, the leaf blade and leaf sheath of the flag leaf, the panicle, and the spikelets were collected to investigate gene expression. Three biological replicates were used for each analysis. Total RNA from all tissues was extracted using the GENEzolTM reagent GZR100, following the manufacturer's protocol, and then treated with DNase I. cDNA synthesis was performed using the Accupower RT premix (Bioneer Inc., Alameda, USA) and using oligoDT(T)18 as a primer to produce 40 ng/μL cDNA. The synthesized cDNA was used as a template and qRT-PCR was conducted on a Luna Universal qPCR master mix M3003L (New England Biolabs Inc., Ipswich, MA, USA). The qRT-PCR conditions were: 95 °C for 60 s, followed by 39 cycles at 95 °C for 15 s, 61.5 °C for 30 s, and 95 °C for 5 s; furthermore, the melt curve and plate read were at 60–94 °C, along with an increase in temperature of 5 °C per 5 s. A qRT-PCR was conducted in triplicate for each sample. The negative control was performed without a template and those reactions containing OsEF-1α primers (Table 7) were used as the internal reference genes (Udomchalothorn et al., 2017). The coding sequence obtained from the rice genome annotation database (Ouyang et al., 2007) was used to design primers that were specific to *OsBTBZ1* (Table 7). The average cycle threshold (Cq) values of the gene were normalized with the level of the *OsEF-1α* reference gene in the same sample and were then used to measure the relative gene expression using the method described by (Pfaffl,

2001). Gene expression was analyzed using the relative expression levels. The statistical program SPSS was used to conduct an analysis of variance (ANOVA, $p < 0.05$) and the means were compared using Duncan's multiple range test.

Table 6. Primer sequence for qRT-PCR to detect gene expression.

Name	Sequence 5'□3'	T_m (ºC)
qPCR_OsBTBZ1_FW	TTCCTGCCTGCAAGGGCATC	63
qPCR_OsBTBZ1_REV	TCCTTGAAATGCCTACAGAGGG	60
qPCR_OsEF1α_FW	ATGGTTGTGGAGACCTTC	53
qPCR_OsEF1α_REV	TCACCTTGGCACCGGTTG	60

2.6. Generation of complementation and over-expression of *Arabidopsis*lines with the *OsBTBZ1* Gene

A full-length *OsBTBZ1* cDNA clone, J023077N08, obtained from the NARO DNA Bank was cloned into the *Escherichia coli* DH5α strain and cultured onto LB semisolid medium (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 7.5 g agar per liter of water) along with 100 μg/mL of ampicillin. Single colonies were selected and then cultured into the LB broth with ampicillin for 16 h at 37 °C and 200 rpm. The plasmid was extracted using the Presto mini plasmid kit according to the manufacturer's protocol (Geneaid, Taiwan), and the sequence of the inserted fragment was determined using the M13 (-20) forward primer to validate the correct sequence of the *OsBTBZ1* gene.

The expression vector was constructed using the Gateway system. The *OsBTBZ1* gene was added with the CACC adaptor in 5′ ends, inserted into the pENTR D-TOPO plasmid (Thermo Fisher Scientific, Waltham, MA, USA) as a donor vector, and then cloned to the TOP10 *E. coli* strain using the heat shock method. The cells were then plated on an LB medium, supplemented with kanamycin (50 μg/mL), and were subsequently incubated at 37 °C overnight. The correct sequence of the plasmid host was utilized in the LR clonase reaction to switch the *OsBTBZ1* gene from the donor vector pENTR to the pGWB512 and pGWB505 plasmids as the destination vectors. The

destination vector was transferred to the *E. coli* strain DH5α and cultured on LB medium, supplemented with 50 µg/mL spectinomycin. Plasmids with the correct sequence were transferred to *Agrobacterium tumefaciens* GV3101-competent cells using the cold-shock method. To identify colonies with the inserted fragment, a polymerase chain reaction (PCR) was performed using the *CaMV35S* forward primer and the reverse primer in the *OsBTBZ1* gene to identify the correct clone for plant transformation.

2.7. Transformation of *A. thaliana*

An *OsBTBZ1* cDNA in the pGWB512 construct was inserted into *Arabidopsis* plants using the floral dip transformation (Clough & Bent, 1998). The resulting T_1 plants were positive for the *OsBTBZ1* gene and were grown in soil to obtain T_2 seeds, which were acquired from each transgenic line and then germinated on Murashige and Skoog (MS) medium, supplemented with 25 mg/L hygromycin. The 3:1 segregation ratio of resistance: sensitivity to hygromycin was determined to identify transgenic lines with a single insertion. The selected lines were then grown in soil to obtain homozygous T_3 seeds, which were used for further characterization (Endo et al., 2018).

2.8. Subcellular localization in onion inner epidermal cells

The agroinfiltration of onion (*Allium cepa*) inner epidermal cells was performed to observe the subcellular localization of OsBTBZ1. The *Agrobacterium* GV3101 harboring the *CaMV35S::OsBTBZ1-GFP* construct in pGWB505 was cultured into 5 mL LB, supplemented with the appropriate antibiotics (50 µg/mL spectinomycin, 25 µg/mL rifampicin, and 50 µg/mL gentamicin) at 28 °C for 1 d. Later, 250 µL of the culture was inoculated in 25 mL LB, supplemented with 10 mM MES (pH 5.6, 100 µM acetosyringone, and antibiotics), and grown at 28 °C to obtain an optical density (OD) at 600 of 0.8. Subsequently, the culture was centrifuged at 5000 rpm for 5 min. The cell pellet was resuspended in MMA liquid medium (10 mM $MgCl_2$, 10 mM MES (pH 5.6), and 100 µM acetosyringone) to a final OD600 of 0.8–1.0 (Liu et al., 2003). The mixture was incubated at room

temperature (25–26 °C) for 3 h and then infiltrated using a 5 mL needleless syringe, with only 500 µL injected per spot in the onion epidermal cells (Xu et al., 2014). The onions were incubated in dim light or in dark conditions at 22 °C under high humidity for 48 h. The *Agrobacterium* GV3101, containing *CaMV35S::GFP*, was used as a control and was treated in the same manner. The onion epidermal cell layers were cut into 1 × 1 cm^2 squares, peeled, and transferred directly to glass slides. Subsequently, 40 µL of 1 µg/mL 4′,6-diamidino-2-phenylindole, or DAPI dye was added to the epidermal cell sections. The green fluorescence protein (GFP) signal was observed under a Zeiss microscope (ZEISS Axio 10, Göttingen, Germany) at an excitation wavelength of 488 nm.

2.9. Evaluation of the effect of *OsBTBZ1* gene expression in transgenic *Arabidopsis* lines

The WT *Arabidopsis*, *Atbt3* mutants, two homozygous complemented lines, REV1 and REV2, and two ectopic expression lines in the WT background, OE1 and OE2, were used for phenotyping. The experiment was performed using a completely randomized design with three replicates. The seeds were germinated by subjecting the seeds to the 0.5× MS medium as a control or 0.5× MS medium supplemented with 150 mM NaCl, 1 µM ABA, or 150 mM mannitol. Germinating seeds were recorded from 0–11 d to calculate the germination rate.

To elucidate the growth responses, 7-day-old *Arabidopsis* seedlings of each line were grown in 0.5× MS medium and were then subjected to four treatments: 0.5× MS medium as a control, 0.5× MS medium, supplemented with 150 mM NaCl, 0.5× MS medium supplemented with 1 µM ABA, and 0.5× MS medium supplemented with 150 mM mannitol, to investigate the impact on fresh weight, root length, and pigment contents. All parameters were measured 0–6 d after initiating the treatments. Fresh weight was calculated as the weight per plant, while the root length was calculated as the change in root length during the experimental period. The pigments, chlorophyll *a*, chlorophyll *b*, and total carotenoid contents, were determined according to the procedures described by Wellburn (1994). All experiments

were performed in at least three replicates. An ANOVA was performed using SPSS Statistical Software version 23 (IBM Corp., Armonk, USA), followed by Duncan's multiple-range test, to compare the means of each parameter.

3. Results

3.1. Only four BTB genes were expressed in CSSL16 under salt stress

In order to investigate whether all *OsBTB* genes can be induced by salt stress, 182 genes containing the BTB domains were retrieved from the Phytozome database (Ouyang et al., 2007), and the differentially expressed genes at seedlings and booting stages were explored. These two stages, the seedling and booting stages, of rice were selected for transcriptome analysis because they are susceptible to salt stress (Kojonna et al., 2022; Lekklar et al., 2019). Moreover, the flag leaves and second leaves of rice at the booting stage are important for grain-filling. Under salt stress, 511 genes were differentially expressed at the seedling stage, whereas 520 and 584 differentially expressed genes were found in the second and flag leaves, respectively.

Among them, only four BTB genes, *LOC_Os01g66890* (*OsBTBZ1*), *LOC_Os01g68020* (*OsBTBZ2*), *LOC_Os02g38120* (*OsBTBN3*), and *LOC_Os03g41350* (*OsBTBN7*), were differentially expressed in salt - stressed CSSL16, while OsBTBZ1 was the only *BTB* gene expressed at the seedling stage, and the other genes were expressed at the booting stage in both second and flag leaves (Figure 3.1A). Furthermore, OsBTBZ1 and OsBTBZ2 contained the BTB and Transcription Adaptor putative zinc finger (TAZ zF or zF-TAZ) domains, whereas OsBTBN3 and OsBTBN7 contained the non-phototropic hypocotyl3 (NPH3) domain (Figure 3.1B).

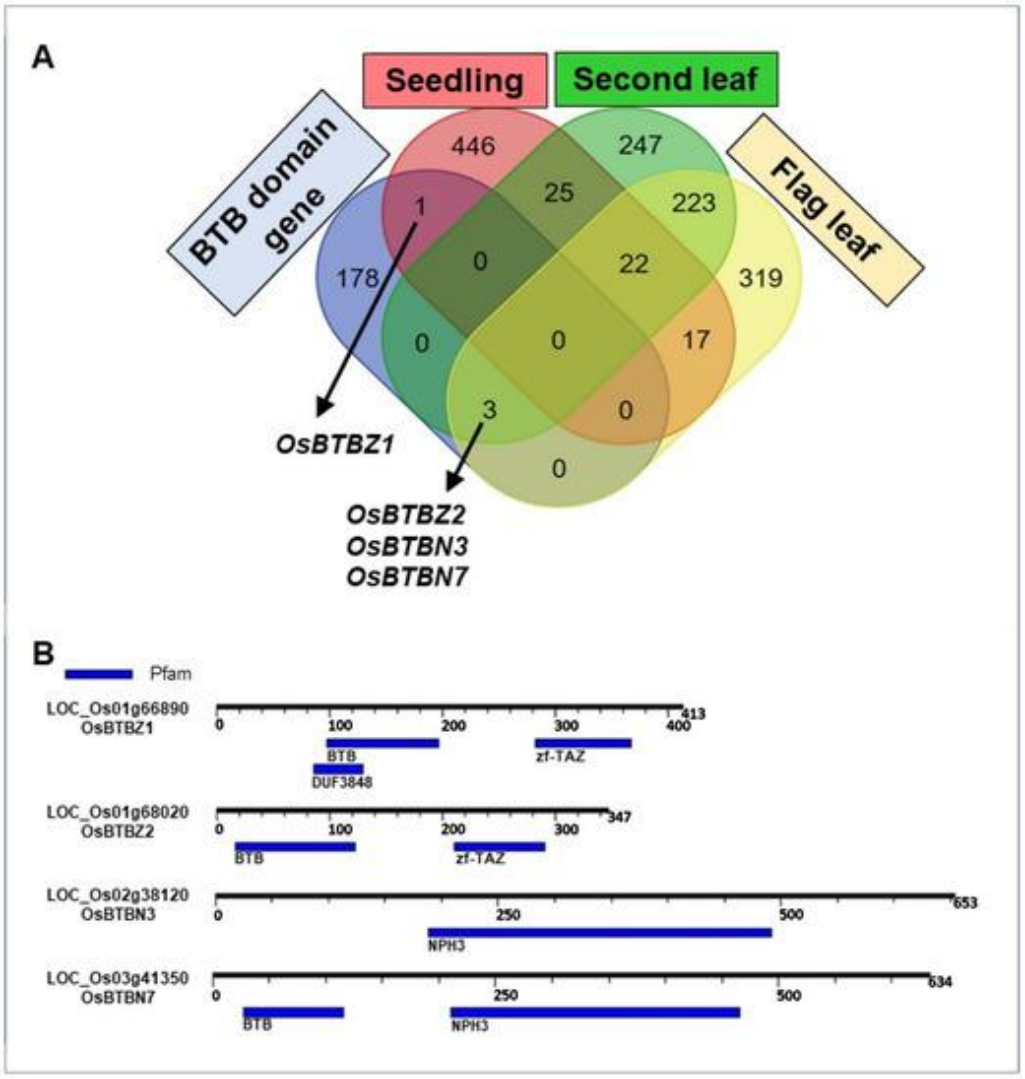


Figure 3.1 Venn diagram showing the intersection of DEseq data with the BTB protein in *O. sativa* (**A**). BTB, zf-TAZ, and NPH3 domains are present in the differentially expressed BTB genes under salt stress conditions, namely, *OsBTBZ1, OsBTBZ2, OsBTBN3, and OsBTBN7* (**B**).

Phylogenetic analysis of the genes containing the BTB domain in rice and *Arabidopsis* was conducted using the JTT model, which used a substitution model calculated from the nearest-neighbor proteins with more than 85% similarity. OsBTBZ1 and its ortholog in *Arabidopsis* AT1G05690 (AtBT3) belong to the same cluster. Both OsBTBZ1 and OsBTBZ2 are in the same cluster and contain the zf-TAZ domain. Moreover, OsBTBN3 and OsBTBN7, which contain an NPH3 domain, were clustered together (Figure

3.2A). The similar domains in OsBTBZs and OsBTBNs suggest a similar function for these proteins.

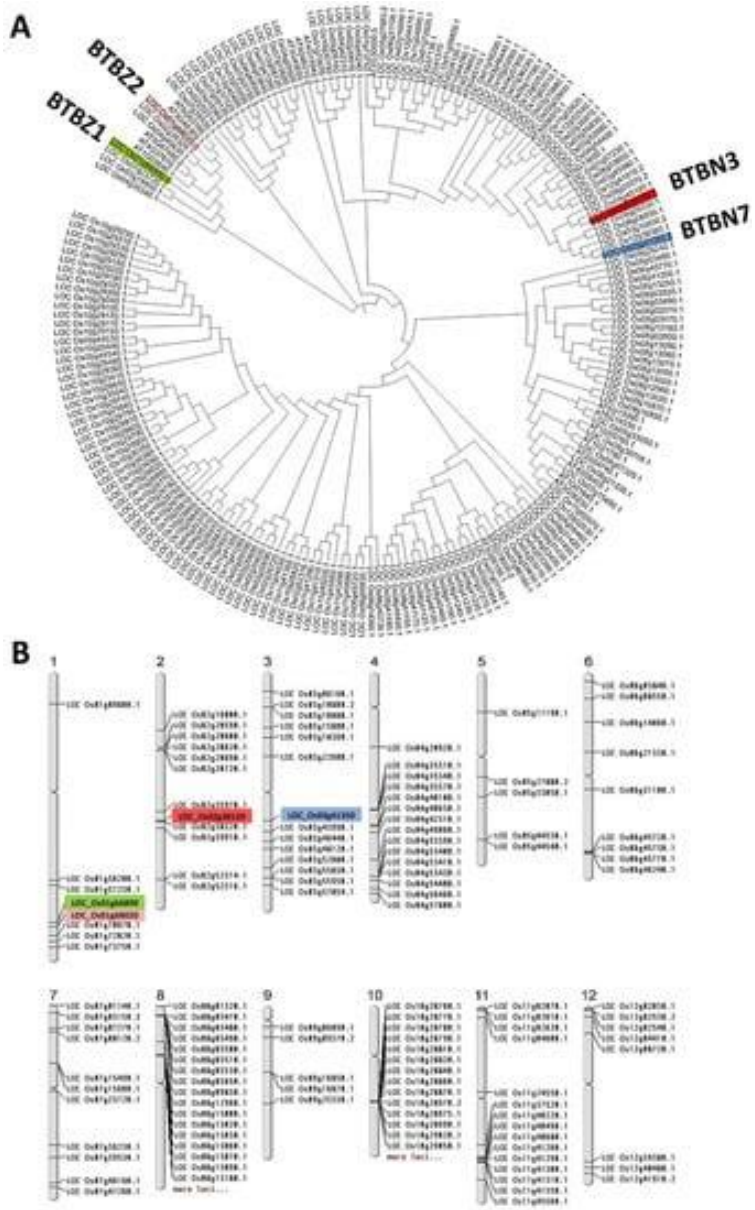


Figure 3.2 Phylogenetic tree and the position of BTB genes in the rice chromosome. (**A**) Maximum likelihood phylogenetic tree. The tree was constructed using the amino acid sequences of 27 BTB genes in Arabidopsis and 182 BTB genes in rice. LOC_Os01g66890 (BTBZ1) was marked, along with LOC_Os01g68020, LOC_Os02g38120, and LOC_Os03g41350. (**B**) The chromosomal location shows the distribution of the BTB gene family in the 12 rice chromosomes.

The salt-tolerant quantitative trait locus (QTL) identified on chromosome 1 and located between RM1003 and RM3362 contained four *BTB* genes: *OsBTBZ1*, *OsBTBZ2*, *OsBTBM1* (*LOC_Os01g70670*), and *OsBTBA3* (*LOC_Os01g72020*). *OsBTBN3* and *OsBTBN7* were located on chromosomes 2 and 3, respectively. Furthermore, chromosomes 8 and 10 contained dense clusters of *BTB* genes, with 34 and 46 *BTB* genes, respectively (Figure 3.2B).

3.2. *OsBTBZ1, OsBTBZ2, OsBTBN3,* and *OsBTBN7* promoters contain multiple cis-elements related to the water stress response

An investigation of the cis-element at the promoter regions of *OsBTBZ1*, *OsBTBZ2*, *OsBTBN3*, and *OsBTBN7* was performed to reveal the salt-responsive elements located in these *OsBTB* genes. Two thousand base pair sequences upstream of the *OsBTBZ1*, *OsBTBZ2*, *OsBTBN3*, and *OsBTBN7* transcription start sites were analyzed using New PLACE to identify the regulatory cis-elements located in the promoter regions of these genes (Figure 3.3). Table 3.1 summarizes the cis-elements related to abiotic stress. Multiple MYCCONSENSUSAT elements, which are water stress-responsive elements, were found in all the tested promoters. More than 10 of these elements were located on the *OsBTBZ1*, *OsBTBZ2*, and *OsBTBN3* promoters, which is consistent with the upregulation pattern under salt stress conditions. Moreover, other cis-elements related to dehydration stress, such as ACGTATERD1, DPBFCOREDCDC3, and MYBCORE, were found in all four promoters. Interestingly, ABA-responsive elements were found only in the + strand of *OsBTBZ1*. The GAGA-binding protein binding site (GAGAGMGSA1), which is specific to the CSSL16 allele, but not the KDML105 allele, was detected only in *OsBTBZ1* (+ strand) (Table 3.1 and Figure 3.3).

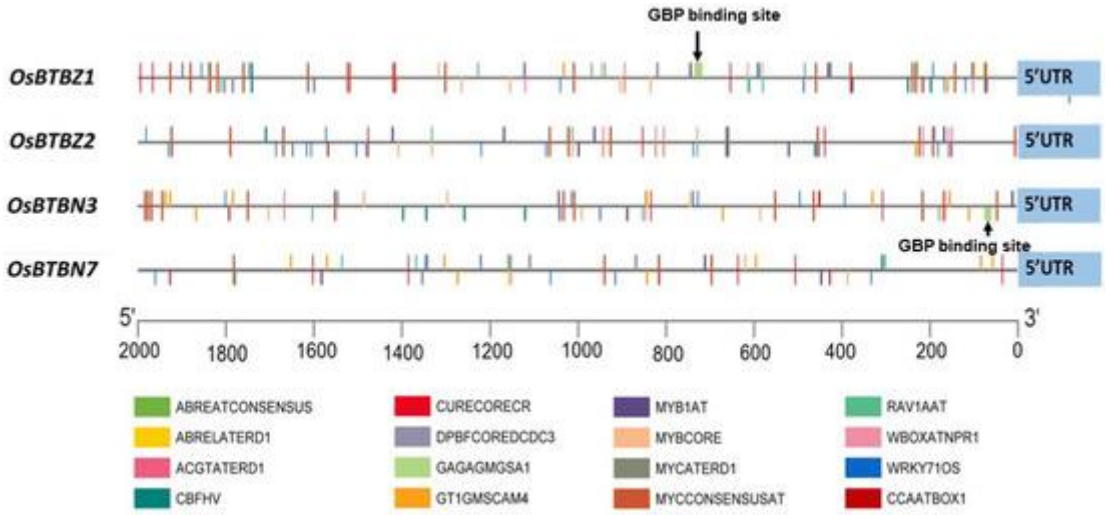


Figure 14. Prediction of the cis-elements related to stress in OsBTBZ1, OsBTBZ2, OsBTBN3, and the OsBTBN7 promoter

Table 7. The number of Stress-related cis-elements detected in *OsBTBZ1*, *OsBTBZ2*, *OsBTBN3*, *and OsBTBN7*.

Factor or Site Name	Signal Sequence	*OsBTBZ1*		*OsBTBZ2*		*OsBTBN3*		*OsBTBN7*		Functions
		Strand (+)	Strand (−)	Strand (+)	Strand (−)	Strand (+)	Strand (−)	Strand (+)	Strand (−)	
ABREATCONSENSUS	YACGTGGC	1	-	-	-	-	-	-	-	ABA-responsive element
ABRELATERD1	ACGTG	3	3	-	6	1	1	-	1	*ERD*-related gene (early responsive to dehydration)
ACGTATERD1	ACGT	5	5	8	8	4	4	1	1	Abiotic stresses (drought, salt); response to light
CBFHV	RYCGAC	1	3	2	2	-	4	-	-	Dehydration-responsive element (DRE) binding proteins (DREBs)
CCAATBOX1	CCAAT	-	2	-	1	1	1	-	2	Heat-shock element
CURECORECR	GTAC	5	5	3	3	3	3	5	5	Oxygen-responsive element
DPBFCOREDCDC3	ACACNNG	4	3	1	3	1	1	3	2	Dehydration and ABA response
GAGAGMGSA1	GAGAGAGAGAGAGAGAGA	2	-	-	-	-	1	-	-	GAGA binding protein (GBP) binding site
GT1GMSCAM4	GAAAAA	1	1	-	1	5	3	6	2	Plays a role in pathogen- and salt-induced SCaM-4 gene expression
MYB1AT	WAACCA	2	1	4	2	1	1	1	2	Element for the dehydration-responsive gene in *Arabidopsis*
MYBCORE	CNGTTR	5	6	2	4	3	3	1	1	Responsive to water stress, induced by dehydration stress
MYCATERD1	CATGTG	1	-	2	-	1	2	2	-	Necessary for *ERD1* expression, the binding site of the NAC protein
MYCCONSENSUSAT	CANNTG	16	16	10	10	12	12	4	4	Element for the dehydration-responsive gene in *Arabidopsis*
RAV1AAT	CAACA	5	4	2	1	-	3	6	1	Rosette leaves and the root-specific element, growth, and development, hormonal regulation brassinosteroid (BR); metabolism
WBOXATNPR1	TTGAC	2	2	1	3	1	1	2	1	Salicylic acid-induced WRKY DNA binding proteins
WRKY71OS	TGAC	8	6	3	11	8	2	3	4	Involved in gibberellic acid (GA), ABA-mediated pathways, and pathogen-related protein (PR)

3.3. OsBTBZ1 and OsBTBZ2 are in the same protein-protein interaction (PPI) network

The STRING database was used to investigate the protein-protein interaction (PPI) network of the four BTB proteins: OsBTBZ1, OsBTBZ2, OsBTBN3, and OsBTBN7 to select the best candidate OsBTB protein, which showed the highest number of connections with other proteins, for further characterization. OsBTBZ1 and OsBTBZ2 were connected, whereas OsBTBN3 and OsBTBN7 were dissociated. Furthermore, OsBTBZ1 and OsBTBZ2 were linked to other proteins, including CARM1, OS07T0626600-01, GCN5, and OS01T0884500-01. CARM1, OS07T0626600-01, and GCN5 are involved in chromatin remodeling. Modifying the chromatin architecture is necessary for the epigenetic control of gene expression, which does not involve alterations in DNA sequences, while increased chromatin compaction results in distinct higher-order structures (Kang et al., 2022). OS01T0884500-01 is a zP (CCCH-type) protein. Contrastingly, OsBTBZ1 is connected to OS03T0216600-01, a glucan 1,3-alpha-glucosidase, and is also connected with OsJ_06167, OsWRKY39, and OsJ_25984, a protein kinase (Szklarczyk et al., 2021), which mediates pathogen-associated molecular pattern (PAMP)-triggered responses (Figure 3.4).

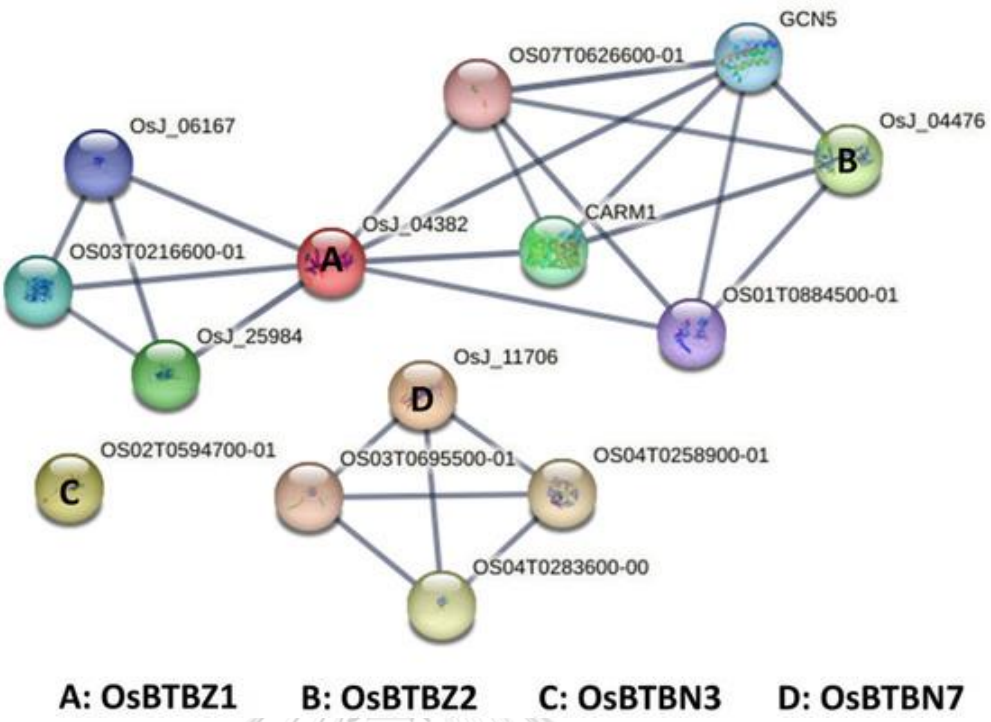


Figure 3.4 The protein-protein interaction (PPI) network assembly of LOC_Os01g66890 (A: OsBTBZ1), LOC_Os01g68020 (B: OsBTBZ2), LOC_Os02g38120 (C: OsBTBN3), and LOC_Os03g41350 (D: OsBTBN7) proteins, identified using STRING. The lines represent a high confidence PPI score of 0.7.

OsBTBN7 was associated with three uncharacterized proteins: OS03T0695500-01, OS04T0258900-01, and OS04T0283600-00, whereas OsBTBN3 was not associated with any other protein (Figure 3.4). Therefore, the functions of these two proteins were not investigated further. According to the PPI network prediction using STRING, OsBTBZ1 was predicted to interact with more proteins. Therefore, it was selected for further characterization.

3.4. *OsBTBZ1* is expressed in all plant tissues, especially in younger leaf sheaths

Because *OsBTBZ1* showed differential expression owing to salt stress at the seedling stage, 15-day-old seedlings were selected for monitoring the expression of this gene. High gene expression was detected in the young leaves and root tissues, and a relatively higher level of expression was detected in the young (first leaf) leaf sheath, compared to the young leaf blades.

However, the expression levels decreased in older leaves. In the oldest leaf of the 15-day-old seedlings, *OsBTBZ1* expression was lower in the leaf sheath than in the leaf blade. At the reproductive stage, *OsBTBZ1* was highly expressed in the leaf sheaths of flag leaves, as well as in the peduncles and spikelets (Figure 3.5).

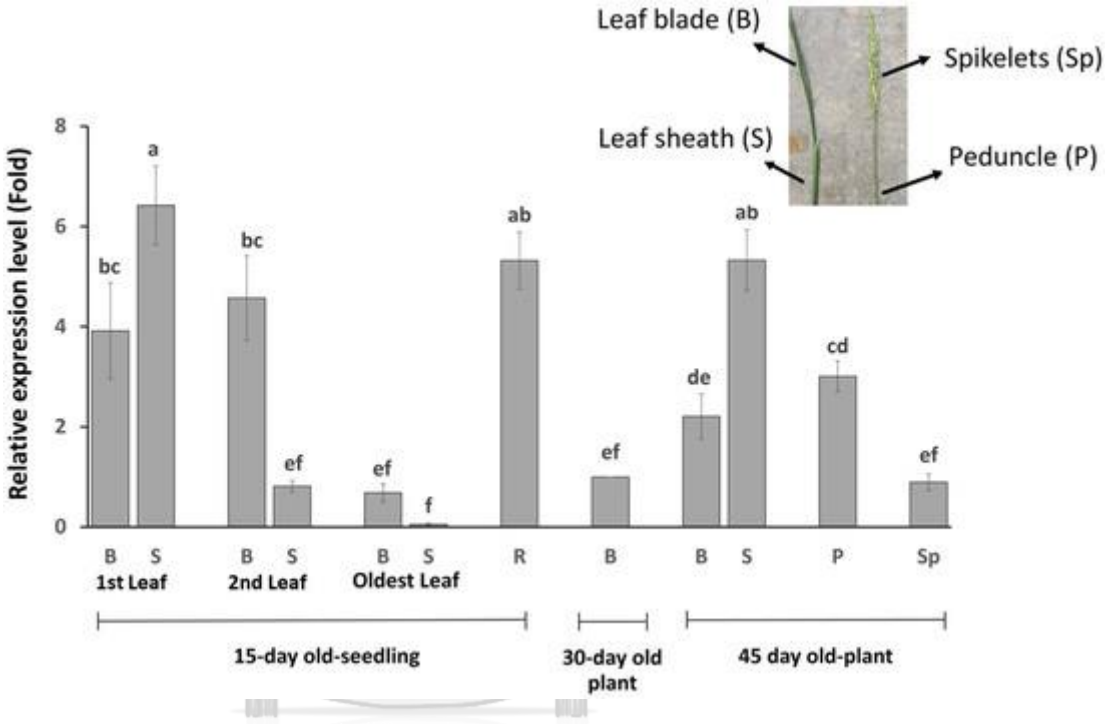


Figure 3.5. OsBTBZ1 gene expression in different tissues of "Nipponbare" rice. B = leaf blade; S = leaf sheath; P = peduncle; R = root; Sp = spikelet. The different lowercase letters above the bars mean the significant difference between means analyzed by Duncan's multiple range test at $p < 0.05$.

3.5. OsBTBZ1 is localized in the nucleus, suggesting the role of the transcription factor

To analyze the subcellular localization of the OsBTBZ1 protein, the CDS of *OsBTBZ1* was fused with *GFP* and transiently expressed in the epidermal cells of *Allium cepa*. *OsBTBZ1-GFP* expression resulted in fluorescence in the nucleus, whereas the GFP fluorescence of the control was observed outside the nuclear region (Figure 3.6). *IbBT4*, a *BTB* gene in *Ipomoea*, is also localized in the nucleus (Zhou et al., 2020a). A similar

result was reported by Weber and Hellmann (Weber & Hellmann, 2009), who examined the BPM1, BPM2, and BTB proteins in *Arabidopsis*. This is consistent with a transcription factor role for OsBTBZ1, which may regulate other genes that are responsible for salt tolerance.

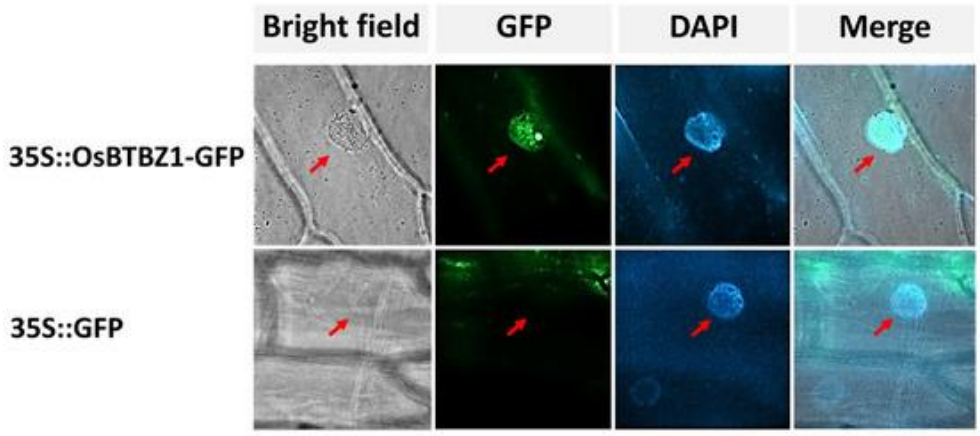


Figure 3.6 The subcellular localization of OsBTBZ1-GFP in onion (*Allium cepa*) epidermal cells. Red arrows point to the location of the nucleus.

3.6. Ectopic expression of OsBTBZ1 could revert the NaCl and ABA susceptibility of the *Atbt3 Arabidopsis* mutant at the germination stage

Chutimanukul et al. (2021) reported that the *Atbt3 Arabidopsis* mutant was more susceptible to salt stress. Therefore, to investigate the role of *OsBTBZ1* an ortholog to *AtBT3*, we fused it to the constitutive *CaMV35S* promoter and transformed it into the WT *Arabidopsis* and *Atbt3* mutant. We generated two lines (REV1 and REV2) in the *Atbt3* mutant background to test for complementation of salt sensitivity, along with two lines, OE1 and OE2, in the WT background to test for the effect of overexpression.

Germination tests were performed to investigate the effects of NaCl, ABA, and mannitol stress on *Arabidopsis* seed germination. The responses are shown in Figure 3.7 A–E. Under normal conditions, the seeds of all lines, comprising the WT, *Atbt3* mutant (*bt3*), REV1, REV2, OE1, and OE2, displayed no significant difference in germination rate (Figure 3.7 A,B).

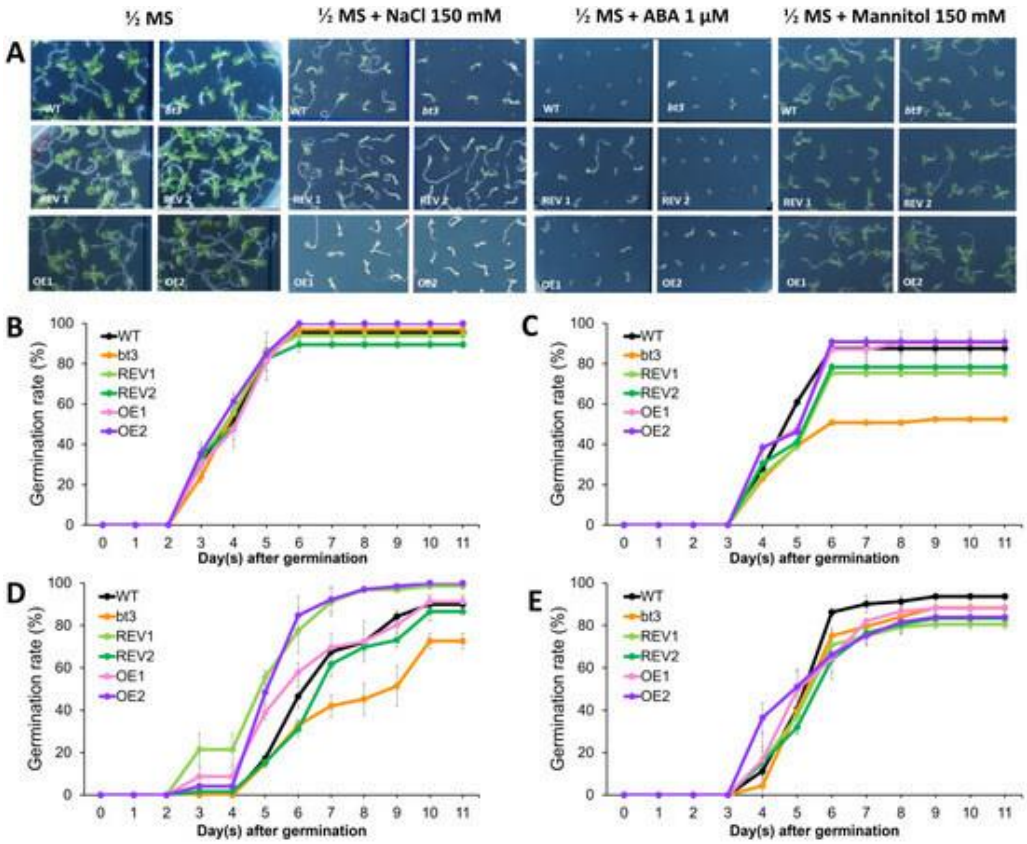


Figure 3.7. Germination tests of the wild-type (WT), mutant, and transgenic lines in several different media. (**A**) The lines of WT, bt3 mutant, REV1, REV2, OE1, and OE2 in ½ MS basal medium as a control, medium with NaCl 150 mM, ABA 1µM, and mannitol 150 mM (line mark = 1 cm). (**B**) Germination curve in the control medium (½ MS medium). (**C**) Germination curve in the ½ MS medium containing 150 mM NaCl. (**D**) Germination curve in the ½ MS medium containing 1 µM ABA. (**E**) Germination curve in ½ MS medium containing 150 mM mannitol.

Expression of *OsBTBZ1* could enhance the germination rate of *bt3* mutant lines. When 150 mM NaCl was applied to 0.5× MS medium, the WT germination percentage was reduced to 88%, whereas the *bt3* mutant line showed only 54% germination after 6 d of germination. The ectopic expression of *OsBTBZ1* in the *bt3* mutants reversed this effect. Both REV1 and REV2 had germination rates of 70% after 9 d of germination. Moreover, the OE1 and OE2 lines, which expressed *OsBTBZ1* in the WT, had a germination rate similar to that of the WT (Figure 3.7 A,C).

The application of ABA to the medium delayed seed germination in all lines. On 0.5× MS medium supplemented with ABA, no germination occurred

in the WT, *bt3* mutant, and REV2 after 4 d of germination (Figure 3.7 A,D). Later, after 5 d of incubation, the germination rate of all lines increased and reached a maximum after 10 d. A 100% germination rate was detected in REV1 and OE2, while WT showed an 86% germination rate. The lowest germination rate under ABA was found in the *bt3* mutant (73%). The ectopic expression of *OsBTBZ1* could revert ABA susceptibility in the *bt3* mutant line by increasing the germination rate to over 80% in both REV1 and REV2. In the WT background, *OsBTBZ1* expression enhanced germination, and, after 5 d of germination, approximately 40% germination was detected in both OE1 and OE2, whereas WT showed a germination rate of <20%. Although WT and OE1 showed similar levels of germination (73%) after 10 d of germination, OE2 showed 100% germination (Figure 3.7 A,D).

To discern the precise function of the *OsBTBZ1* gene, it is essential to evaluate its role in germination under mannitol-induced conditions, which will help in determining whether it is solely responsive to salt or has a broader function within the osmotic regulation mechanisms. Therefore, 150 mM mannitol was added to the MS medium as a germination test. A slight reduction in the WT seed germination percentage was detected. The mutation of *AtBt3* increased the sensitivity to mannitol stress, as shown by the significantly lower germination percentage of the *Atbt3* mutant. However, the ectopic expression of *OsBTBZ1* did not reverse the inhibition of mannitol-induced germination (Figure 3.7 A, B, E).

Based on these results, we can conclude that *OsBTBZ1* can reverse the *Atbt3* mutation under salt and ABA stress, but not under drought stress that is induced by mannitol.

3.7. *OsBTBZ1* enhanced the salt and ABA tolerance in transgenic *Arabidopsis*

To investigate whether *OsBTBZ1* enhanced the stress tolerance of plants, the fresh weight, root length, and photosynthetic pigment content under stress conditions were measured. At the beginning of the experiment (0 d), seedlings of the WT, *bt3* mutant, REV1, REV2, OE1, and OE2 lines showed

similar fresh weights (Supplementary Figure S1). After 6 d of the experiment, the *bt3* mutant had a significantly lower fresh weight than the WT. Ectopic expression could reverse the phenotype of the *bt3* mutant to a fresh weight similar to that of the WT under normal conditions, as shown by the phenotypes of REV1 and REV2 (Figure 3.8A,B). Moreover, the root lengths of all the lines were similar under normal conditions (Figure 3.8A,C).

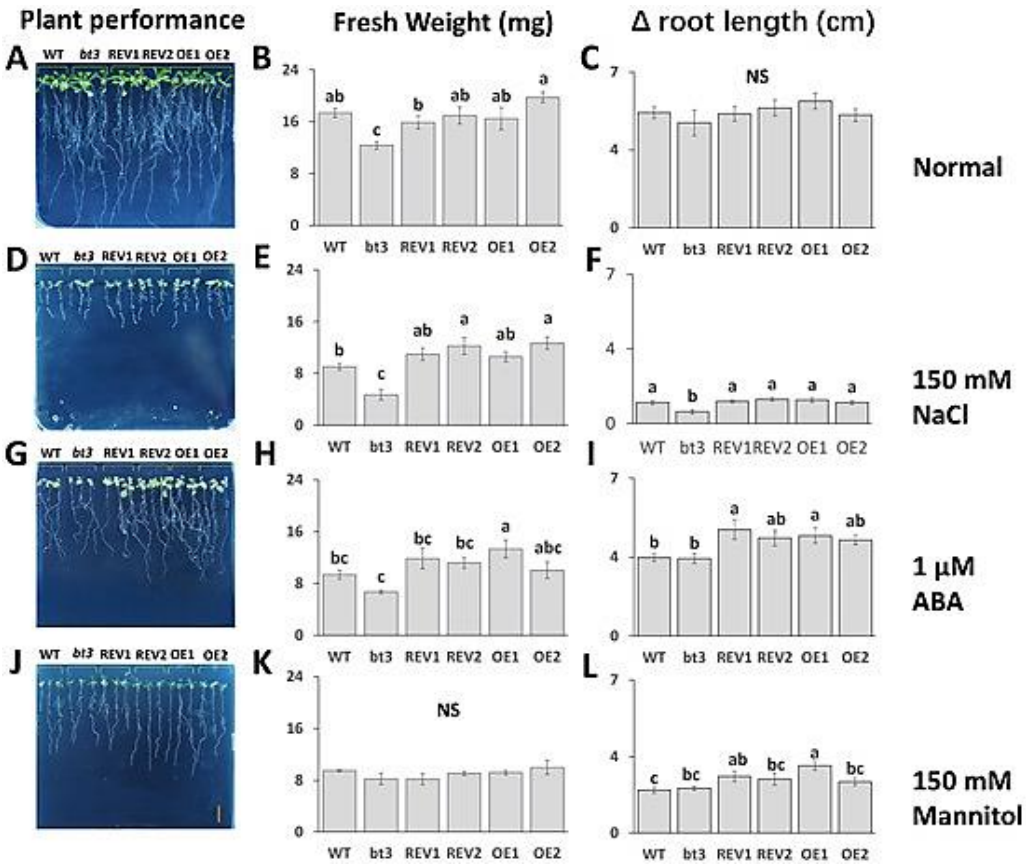


Figure 3.8. Growth (**A**, **D**, **G**, **J**) WT, *bt3* mutant, and transgenic lines of 7-day-old Arabidopsis seedlings in various conditions: normal (control), supplemented with 150 mM NaCl, 1 µM ABA, or 150 mM mannitol on day 6 of stress exposure. (**B**, **E**, **H**, **K**): fresh weight of plants; (**C**, **F**, **I**, **L**): Δ root length of plants. The different letters above the bars represent the significant difference in means at $p < 0.05$ and NS represents no significant difference.

Salt stress and ABA treatments reduced the fresh weight and root length of all lines (Figure 3.8D–I). The fresh weight of WT was reduced by 50%, whereas that of the *Atbt3* mutant decreased by more than 60%. This indicated that the *Atbt3* mutant was more susceptible to salt stress. The revertant lines, REV1 and REV2, could reverse NaCl susceptibility by showing a significantly higher fresh weight than the *Atbt3* mutant after 6 d of the NaCl treatment. OE1 and OE2 also had higher fresh weights than WT (Figure 3.8E). A similar response could also be detected via the root-length response under salt stress (Figure 3.8F). The effect of 1 µM of ABA on *Atbt3* growth was not as strong as that of the NaCl treatment. Approximately 50% of the fresh weight was reduced in the ABA-treated *Atbt3* mutant after 6 d, whereas a similar reduction in the root length of the WT and mutant was detected. The ectopic expression of *OsBTBZ1* in both the WT and mutant backgrounds reversed these effects (Figure 3.8G–I). Mannitol reduced the fresh weight and root length of all lines; however, no significant difference in fresh weight was detected (Figure 3.9J–L). Conversely, the ectopic expression of *OsBTBZ1* enhanced the root length (Figure 3.8L).

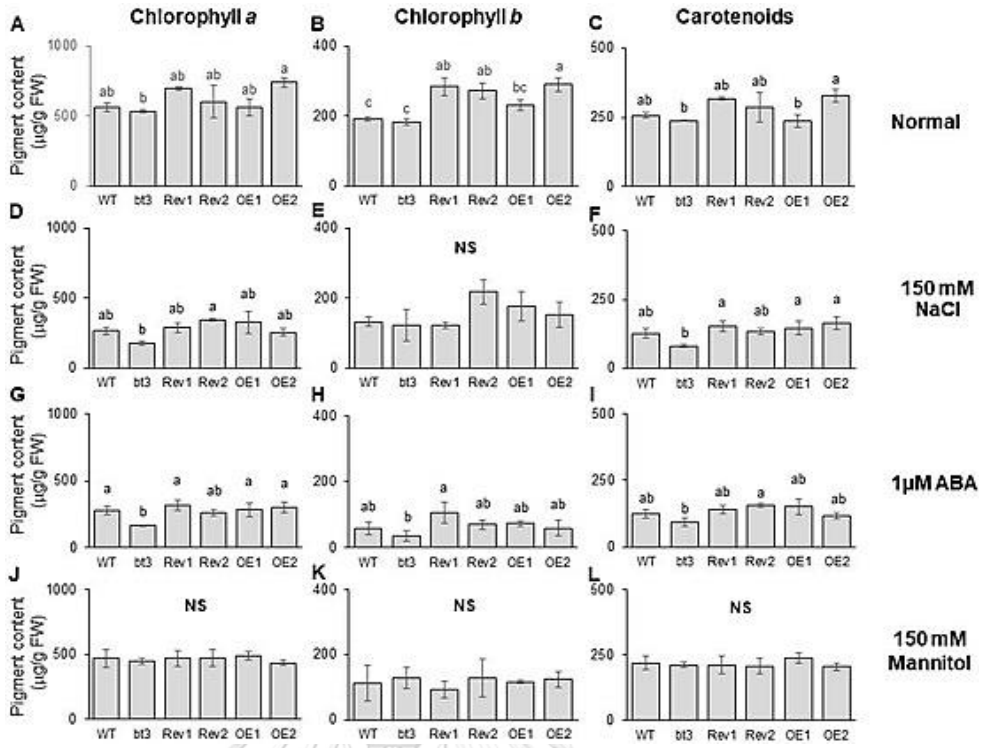


Figure 3.9. Contents of pigments, namely, Chl a, (**A**, **D**, **G**, **J**), Chl b, (**B**, **E**, **H**, **K**), and carotenoids (**C**, **F**, **I**, **L**) of 13-day-old Arabidopsis seedlings treated with 150 mM NaCl, 1 μM ABA, or 150 mM mannitol for 6 d. The data were collected from the WT and *bt3* complemented lines with the OsBTBZ1 gene in the *bt3* mutant background (REV1 and REV2) and the *OsBTBZ1* ectopic expression line with a WT background (OE1 and OE2). The different letters above the bars represent the significant difference in means at $p < 0.05$ and NS represents no significant difference.

We also compared the photosynthetic pigment levels. On the first day of the experiment, all lines had similar levels (Supplementary Figure S2). Under normal conditions, after 6 d, *OsBTBZ1* ectopic expression significantly enhanced the Chl *b* content in the *Atbt3* mutant, showing a tendency to enhance the Chl *a* and carotenoid contents in the WT (Figure 3.9A–C).

Salt stress (150 mM NaCl) reduced the Chl *a*, Chl *b*, and carotenoid contents in all lines. Strong reductions in the Chl *a* and carotenoid contents were detected in the *Atbt3* mutant. However, these effects were reversed by *OsBTBZ1* expression, as shown by the REV1 and REV2 pigment contents. The ectopic expression of *OsBTBZ1* in the WT did not show significant levels

of Chl *a*, Chl *b*, and carotenoid contents when compared to the pigment content of the WT (Figure 3.9D–F).

ABA significantly decreased the Chl *a*, Chl *b*, and carotenoid contents in all lines (Figure 3.9G–I), with a much stronger effect on the Chl *b* content (Figure 3.9B, E, F). The *Atbt3* mutant was more susceptible to ABA treatment in terms of the photosynthetic pigment content. An approximately 60–75% reduction in Chl *a* and Chl *b* content was detected in the *Atbt3* mutant, respectively, after ABA treatment, whereas in the WT, an approximately 40–60% reduction in Chl *a* and Chl *b* contents was found (Figure 3.9G,H), along with a reduction in the carotenoid content (Figure 3.9I). The ectopic expression of *OsBTBZ1* reversed these effects.

Furthermore, treatment with 150 mM of mannitol caused a similar reduction in the photosynthetic pigment content in all lines (Figure 3.9J–L). Based on the earlier investigation into growth and photosynthetic pigment contents, the expression of *OsBTBZ1* in the WT and *Atbt3* mutant background could confer abiotic stress tolerance under salt and ABA stress and showed fewer effects under drought stress when treated with 150 mM of mannitol.

4. Discussion

The BTB domain genes are part of a large gene family that has various roles in plant responses to abiotic stresses, ubiquitination, and development. In the present study, *OsBTBZ1*, *OsBTBZ2*, *OsBTBN3*, and *OsBTBN7* were induced by salt stress at various stages, suggesting roles in the salt stress response in both the seedling and booting stages. The *BTB* genes in other species have also been reported to be involved in salt stress. For example, the *A. thaliana* stress-induced BTB protein 1 (AtSIBP1) is a positive regulator of salinity responses in *Arabidopsis* (Wan et al., 2019). CaBPM4 (*Capsicum annuum* BTB-POZ and MATH domain protein) from pepper is upregulated during salt-stress exposure (He et al., 2019). The SlBTB18 in tomatoes contains the TAZ domain and its expression increases dramatically under cold, salt, and oxidative stress (Li et al., 2018). The *AtBt3* gene, which was in the same cluster as *OsBTBZ1*, has been reported to play a crucial role in gametophyte development in *Arabidopsis* (Robert et al., 2009). Chutimanukul et al. (2021) demonstrated

that the *Atbt3* mutant lines were more susceptible to salt stress than the WT. Moreover, over two dozen different protein domains were associated with the BTB, five of which (MATH, Kelch, NPH3, ion transport, and the zF domains) were much more frequent than the others(Perez-Torrado et al., 2006). Furthermore, the combination of the BTB domain with the TAZ domain is only observed in plants (Gingerich et al., 2007).

The other motif was NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3), which was present in OsBTBN3 and OsBTBN7. The BTB–NPH3 proteins, also called NPH3/RPT2-like (NRLs) proteins, are plant-specific BTB/POZ proteins18. NRLs contain an N-terminal BTB domain and a C-terminal NON-PHOTOTROPHIC HYPOCOTYL 3 (NPH3) domain; some members contain an additional C-terminal coiled-coil domain (Ban & Estelle, 2021). NPH3, a BTB NPH3 family member in *Arabidopsis*, functions as a CRL3 substrate adaptor and regulates the ubiquitylation of phototropin1 (phot1) in response to different blue-light intensities. The dephosphorylation of NPH3 when stimulated by blue light may also be crucial for phot1-dependent phototropism (Roberts et al., 2011). Proteins containing NPH3 are abundantly localized in the plasma membrane and interact with phototropins and blue light receptor kinases (Inada et al., 2004).

Evidence from a putative cis-element analysis and PPI showed that *OsBTBZ1* and *OsBTBZ2* are related to the stress response. CARM1, GCN5, and OS07T0626600-01 are involved in post-translational modifications such as phosphorylation, glycosylation, acetylation, succinylation, carbonylation, S-nitrosylation, and Tyr-nitration, which can alter the epigenetic status of plants (Hashiguchi & Komatsu, 2016). CARM1 histone-arginine methyltransferase, methylates (mono- and asymmetric dimethylation), and the guanidino nitrogens of arginyl residues in several proteins are involved in DNA packaging, transcription regulation, and mRNA stability. They can also be recruited to the promoters upon gene activation to methylate histone H3 and activate transcription via chromatin remodeling. Protein arginine methyltransferases (PRMTs), which are equivalent to CARM1 in mammals, were reported to be related to salt tolerance

in *Arabidopsis*. Prmt5 and prmt4a; 4b *Arabidopsis* mutants display an alteration in salt-stress tolerance (Hernando et al., 2015). Both OsBTBZ1 and OsBTBZ2 were predicted to directly interact with protein arginine methyltransferases, suggesting that both OsBTBZs are involved in salt-stress tolerance. The histone acetyltransferase GCN5 functions in acetylation of histone H3, which provides a specific tag for epigenetic transcriptional activation. GCN5 operates in concert with certain DNA-binding transcriptional activators that act via the formation of large multiprotein complexes to modify chromatin. Zheng et al. (2019) reported that GCN5 plays an important role in cell wall integrity and salt tolerance in *Arabidopsis*. The wheat *TaGCN5* gene can complement the *Atgcn5* mutation, leading to the restoration of the salt-tolerant phenotype in the mutant line. This information suggested that OsBTBZ1 and OsBTBZ2 could be involved in chromatin remodeling and epigenetic regulation in the salt-tolerant phenotype in rice. It was also reported that the histone deacetylase, HDA710, regulated salt tolerance in rice via ABA signaling (Ullah et al., 2020). This is consistent with our results in this research that the complementation of *OsBTBZ1* in the *Atbt3* mutant background could restore the susceptible phenotypes of the *Atbt3* mutant under ABA treatment (Figure 3.7, Figure 3.8 and Figure 3.9). OS07T0626600-01 is a putative MYST-like histone acetyltransferase 1 (histone acetyltransferase), which may be involved in transcriptional activation. The involvement of regulation via acetylation and chromatin remodeling is consistent with an earlier report, which stated that the acetylation levels of histone H3 at K9 in maize increase during salt stress (Li et al., 2014). OS01T0884500-01 is another protein in the PPI of OsBTBZ1 that belongs to a group of protein-like zF (CCCH-type). In general, zinc fingers are of the C2H2-type or CCCC-type, being grouped by the configuration of cysteine and histidine. The less prevalent CCCH zF proteins are crucial for controlling plant stress responses (Han et al., 2021).

OsBTBZ1 is also associated with OS03T0216600-01, a defense response protein elicited by PAMPs. This suggests an interaction between the responses to biotic and abiotic stresses. OsJ_06167, a putative WRKY

transcription factor (TF), is one of the TFs that are involved in many biotic and abiotic stress regulations. Epigenetic, retrograde, and proteasome-mediated regulations enable WRKYs to attain dynamic cellular homeostatic reprogramming (Phukan et al., 2016). Li et al. (2015) reported that the overexpression of *SpWRKY1* in tobacco resulted in enhanced salt and drought stress tolerance by reducing lipid peroxidation, enhancing antioxidant enzyme activity, and maintaining photosynthesis. The promoter of *OsBTBZ1* also had a motif that is associated with the WRKY protein. WRKY71OS and WBOXATNPR1 are involved in various stress responses and are mediated by gibberellic acid, ABA, and salicylic acid (Trivedi et al., 2013). In rice, WRKY13 binds to multiple cis-elements to regulate abiotic and biotic stress (Xiao et al., 2013). OsJ_25984 belongs to the protein kinase superfamily. Kinases are necessary for signal transduction in many aspects of cellular regulation and metabolism. The regulation of plant growth and development and the plant's responses to stress conditions involve protein kinases such as mitogen-activated protein kinase cascades, receptor-like kinases, sucrose nonfermenting1-related protein kinases, and calcium-dependent protein kinases (Chen et al., 2021).

The putative cis-element analysis also showed interesting results, summarizing the function of *OsBTBZ1*. The ABA-responsive element (ABRE) is the most conserved cis-element in plants (Nakashima & Yamaguchi-Shinozaki, 2013; Yamaguchi-Shinozaki & Shinozaki, 2005). The ABRE cis-elements control the transcriptional regulation of several genes in response to cytosolic Ca^{2+} (Kaplan et al., 2006). CAMTA12 enhances drought tolerance in soybean and *Arabidopsis* by binding to the ABRE cis-elements (Noman et al., 2019). Many TFs regulate both abiotic and biotic stress, such as cold, drought, heat, and salinity, by binding to different cis-elements (Cheng et al., 2013). The OsBTBZ1 promoter contains putative MYB-binding sites that are attached to the MYB transcription factor. MYB cis-elements, including MYBCORE, and MYB1AT, were detected in the promoter regions of *OsBTBZ1*, *OsBTBZ2*, *OsBTBN3*, and *OsBTBN7*; however, the number of elements varied. They play essential roles in the regulation of many genes

related to biotic and abiotic stresses (Ma & Constabel, 2019). ABRE and MYC play important roles in the ABA-induced activation of biotic and abiotic genes (Li et al., 2020). MYC also responds to drought stress (Lekklar et al., 2019). For example, AtMYC2 and AtMYB2 specifically interact with MYB recognition sites to regulate the ABA genes related to biosynthesis and signaling (Abe et al., 2003). In another study, a chromatin immunoprecipitation assay and effector-reporter coexpression assays of *Nicotiana tabacum* confirmed the relationship of *MYB* and *WRKY* cis-elements with the promoters of peroxidase, superoxide dismutase, and phenylalanine ammonia-lyase to regulate abiotic stress (Guo et al., 2018). Additionally, MYB- and WRKY-related cis-elements were found to regulate the transcription of auxin-regulated genes (Berendzen et al., 2012). Similarly, the dehydration-responsive element (*DRE*) is also an essential cis-element that regulates the drought response and other abiotic stresses in plants. DREB-responsive genes are regulated by the *DREB* cis-elements to mediate stress responses in plants (Baral, 2019; Jangale et al., 2019; Lata & Prasad, 2011). In banana plants, DREBs mitigate heat and drought stress (Baral, 2019), whereas in soybean plants, the cis-elements of *DREBs* are important for proline accumulation to mediate the plant's response to salt stress (Nguyen et al., 2019).

OsBTBZ1 was selected to confirm its role in salt tolerance by generating the revertant lines REV1 and REV2 in the *Atbt3 Arabidopsis* mutant. Upon adding 150 mM of mannitol, the expression of the *OsBTBZ1* gene could increase the tolerance of the transgenic lines to salt stress more than the mutant line, but not to drought stress (Figure 3.7, Figure 3.8 and Figure 3.9). This suggested that the function of *OsBTBZ1* was more specific to the salt stress response than to osmotic stress. Phenotypic complementation of the mutant line by the expression of *OsBTBZ1* was also observed under the ABA treatment, suggesting that the mechanism via the ABA-dependent pathway is involved in *OsBTBZ1* functioning, which is consistent with the model proposed by Chutimanukul et al. (2021)

5. Conclusions

Our study indicates a clear role for *OsBTBZ1* in salt tolerance in *Arabidopsis*. A role in salt tolerance in rice is consistent with the higher expression of this gene in the salt-tolerant line, CSSL16, compared to the original genetic background, KDML105 rice (Chutimanukul et al., 2021). It is also consistent with the location of this gene on the salt tolerance QTL on chromosome 1 (Chutimanukul, 2018b; Chutimanukul et al., 2021). Therefore, this study can support the use of this gene and QTL for the improvement of salt tolerance in rice.

6. Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms241914483/s1

CHAPTER IV
CONCLUSION

The transcriptomic approach in rice using two rice lines with close genetic relationships, but different salt tolerance ability, gives the new insight in identifying the salt-tolerant genes. The Integration of Gene Co-expression Network (GCN), Clustering coefficient (CC), and Weighted Gene Co-expression Network (WGCN) analyses in conjunction with Single Nucleotide Polymorphism (SNP) information facilitates the precise delineation of nine genes implicated in salt tolerance in rice. The nine genes from three methods are *LOC_Os01g61010 (OsNodulin), LOC_Os01g64870, LOC_Os01g66890* (*OsBTBZ1*), *LOC_Os01g67370, LOC_Os01g72210* (*OsERD*), *LOC_Os01g71190 (OsPSB28), LOC_Os01g73110, LOC_Os04g03050* (*OsSub34*), and *LOC_Os06g46799* (*OsPeroxidase*). Furthermore, *LOC_Os01g66890* (*OsBTBZ1*) and *LOC_Os01g72210* (*OsERD*).

Validation by employing qRT-PCR was conducted to confirm the expression level of nine candidates genes. Notably, during early stress responses, significant upregulation of *LOC_Os01g64870*, *OsBTBZ1*, *LOC_01g67370*, *OsPeroxidase*, *OsERD*, *LOC_01g73110*, and *OsSub34* in CSSL16 compared to KDML105 was observed. These genes exhibited more than 15-fold induction in the early response. OsPSB28 showed fluctuating expression in the early response. In the late response, *Nodulin*, *LOC_Os01g64870*, *OsBTBZ1*, *LOC_Os0167370*, and *PSB28* significantly increased in CSSL16 but decreased in KDML105 after 3 days of salt stress. After 6 days, *ERD*, *LOC_Os01g73110*, and *peroxidase* maintained elevated expression in both lines, while *OsSub34* decreased more in CSSL16 than in KDML105. These results suggest the potential involvement of the examined genes in rice salt tolerance.

OsBTBZ1 was selected to confirm its role in salt tolerance by generating the revertant lines (REV1 and REV2) in the *Atbt3 Arabidopsis* mutant and over expressed line (OE1 and OE2) in the wildtype lines. Under salt stress (150 mM NaCl), Chl *a*, Chl *b*, and carotenoid contents

decreased in all lines. The *Atbt3* mutant showed significant reductions, particularly in Chl *a* and carotenoid contents, which were counteracted by *OsBTBZ1* expression. ABA treatment markedly reduced Chl *a*, Chl *b*, and carotenoid contents across all lines, with a more significant effect on Chl *b* content. The *Atbt3* mutant exhibited higher susceptibility to ABA treatment, with a 60–75% reduction in Chl *a* and Chl *b* content. *OsBTBZ1* expression reversed these effects. Furthermore, mannitol treatment at 150 mM induced a similar reduction in weight, root length, and photosynthetic pigment content across all lines. Moreover, *OsBTBZ1* expression in the WT and *Atbt3* mutant backgrounds conferred abiotic stress tolerance under salt and ABA stress, with fewer effects observed under drought stress induced by 150 mM mannitol.

The phenotyping result indicates that the role of *OsBTBZ1* is more targeted towards responding to salt stress rather than osmotic stress. The observed phenotypic restoration of the mutant line through the introduction of *OsBTBZ1* expression also occurred under ABA treatment, indicating the involvement of the ABA-dependent pathway in *OsBTBZ1* function.

APPENDIX

Amount of differentially expressed genes

Table 8 The number of differentially expressed genes in leaves at seedling stage, second leaf, and flag leaf at booting stage under salt stress condition

Stage of leaf	Number of gene		Total
	Up-regulated gene	Down-regulated gene	
Seedling leaf	238	273	511
Second leaf	240	280	520
Flag leaf	271	313	584

Table 9 Modified standard evaluation score (SES) of visual salt injury at seedling stage (Gregorio et al., 1997).

Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips or few leaves whitish and rolled	Tolerant
5	Growth severely retarded; most leaves rolled; only a few are elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dry; some plants dying	Susceptible
9	Almost all plants dead or dying	Highly susceptible

Reagent and medium

1. Composition of a half strength of Murashige and Skoog

MS powder	2.215 g

The weighted composition placed in glass beaker and added with 900 mL of distilled water. The adjustment of the pH was conducted to obtain 5.8 by the usage of 1 N NaOH. After pH value adjusted, distilled water was added until 1000 mL. The homogenized mixture subsequently autoclaved for 25 min at 120°C then stored at room temperature.

Note : For selection of transgenic line, an antibiotic was added.
Hygromycin (Final concentration 25 µg/mL)

2. Composition of Luria Bertani (LB) broth

Tryptone	1.0 g
Yeast extract	0.5 g
NaCl	1.0 g

The weighted composition placed in glass beaker and added with 80 mL of distilled water. The adjustment of the pH was conducted to obtain 7.0 by the usage of 1 N NaOH. After pH value adjusted, distilled water was added until 100 mL. The homogenized mixture subsequently autoclaved for 25 min at 120°C then stored at room temperature.

Note : for cloning purpose, the antibiotic was added before used

A. Ampicillin (final concentration 50 µg/mL) for pFLC1_*OsBTBZ1* cloning
B. Kanamycin (final concentration 50 µg/mL) for pENTR_*OsBTBZ1* cloning
C. Spectinomycin (final concentration 50 µg/mL) for pGWB511_*OsBTBZ1*, pGWB511_*OsBTBZ1*, pGWB511_*OsBTBZ1_GFP* cloning

3. Composition of Luria Bertani (LB) solid

Tryptone	1.0 g
Yeast extract	0.5 g
NaCl	1.0 g
Agar	1.5 g

The weighted composition placed in glass beaker and added with 80 mL of distilled water. The adjustment of the pH was conducted to obtain 7.0 by the usage of 1 N NaOH. The agar was added and distilled water was added to obtain 100 mL. The homogenized mixture subsequently autoclaved for 25 min at 120°C then stored at room temperature.

Note : for cloning purpose, the antibiotic was added before plating

A. Ampicillin (final concentration 50 µg/mL) for pFLC1_*OsBTBZ1* cloning
B. Kanamycin (final concentration 50 µg/mL) for pENTR_*OsBTBZ1* cloning
C. Spectinomycin (final concentration 50 µg/mL) for pGWB511_*OsBTBZ1*, pGWB511_*OsBTBZ1*, pGWB511_*OsBTBZ1_GFP* cloning

4. Composition of TBE buffer

Tris base	54 g
Boric acid	27.5 g
Ethylenediaminetetraacetic acid (EDTA) (pH 8.0) 0.5 M	20.0 ml

The mixture were then added with distilled water until 1 L

5. Composition of Diethylpyrocarbonate (DEPC) 0.1% treated water

DEPC	1mL
ddH_2O	~ 1000 mL

6. Composition of WP solution (Vajrabhaya and Vajrabhaya, 1991)

Macro elements:

KNO_3	580 mg
$CaSO_4$	500 mg
$MgS0_4.7H_2O$	450 mg
Triple super phosphate	250 mg
$(NH_4)_2SO_4$	100 mg

Microelements:

Na_2EDTA[a]	160 mg
$FeSO_4.7H_2O$[a]	120 mg
$MnSO4.H_2O$	15 mg
H_3BO_3	5 mg
$ZnSO_4.7H_2O$	1.5 mg
KI	1.00 mg
$Na_2MoO_4.2H_2O$	0.10 mg
$CuSO_4.5H_2O$	0.05 mg
$CoCl_2.6H_2O$	0.05 mg
H_2O	800 mg

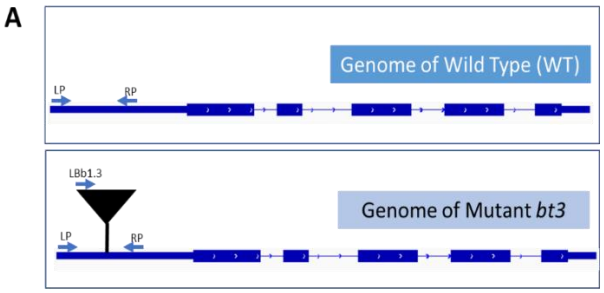
Stir with a magnetic stirrer, add 2 mL of $FeSO_4.7H_2O$ and adjust the volume to 1 L with water.

[a]Preparation of 30 g/L $FeSO_4$ stock

Na_2EDTA	40 g
$FeSO_4.7H_2O$	30 g

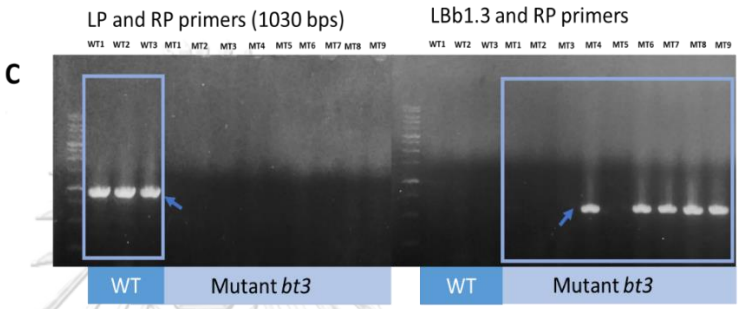
Stir each chemical solution with a magnetic stirrer and adjust the volume to I000 mL with water.

Homozygosity Test



B

Primer	Sequence	Tm(oC)
LB1.3-TDNA	ATTTTGCCGATTTCGGAAC	53
LP-ATBT3	GATATAACCCCGCGGTATACC	57
RP-ATBT3	TCGAACAGTGACACGAACAAG	57

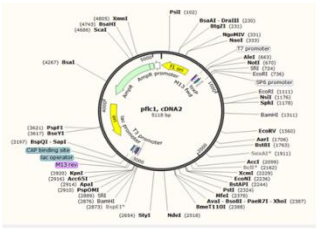


Insertion chr1 1707059 BP+RP_product size = 482-782

Figure 21. Homozigocity test. (A) position of TDNA insertion; (B) Primer for homozigocity test; (C) Electrophoregram of PCR product.

Vector construction using Gateway system

1. Validation of obtained cDNA in the original construct



https://www.dna.affrc.go.jp/distribution/distdna.html

2 ul cloning in *E.coli, plasmid extraction*

Digestion with RE

Restriction Enzyme Double Digestion

Steps

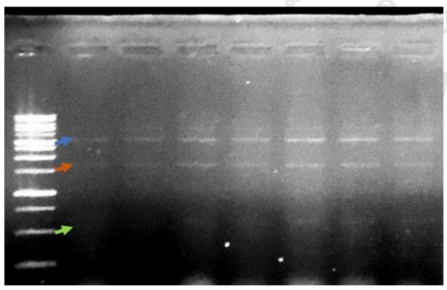
1. Set up reaction as follows:

COMPONENT	50 µl REACTION
DNA	1 µg
10X NEBuffer 2.1	5 µl (1X)
PstI	1.0 µl (or 10 units)
Nuclease-free Water	to 50 µl

2. Incubate at 37°C for 5-15 minutes as PstI is Time-Saver qualified.
3. Add 1.0 µl (or 10 units) of SfiI
4. Incubate at 50°C for 5-15 minutes as SfiI is Time-Saver qualified.

http://nc2.neb.com/NEBcutter2/cutshow.php?name=2d27cf6b-

http://nebcloner.neb.com/?fbclid=IwAR21pnkeWzaEHflbu3-fz8vJ9uwwvHr-YitFX2i70swu2FDLrn26t8eEBq4#!/protocol/re/double-temp/SfiI,PstI



SfiI (724) – PstI (2324) =1600 bps
PstI (2324) – SfiI (2889) = 565 bps
SfiI (2889) - SfiI (724) = 2829 + 724 = 3553 bps

Sequencing 1 : The colony that has those three fragments

Fw M13 : GTAAAACGACGGCCAG
F primer : ACAATCGCAGATTCTTTGGAGC
G primer : CGTGTTTTGACGGAAGATGGC
H primer : AAAGAGCAGTCAGGCCGTTT

Figure 22 The validation flowchart of original vector with *OsBTBZ1* cDNA

2. The insertion in donor vector

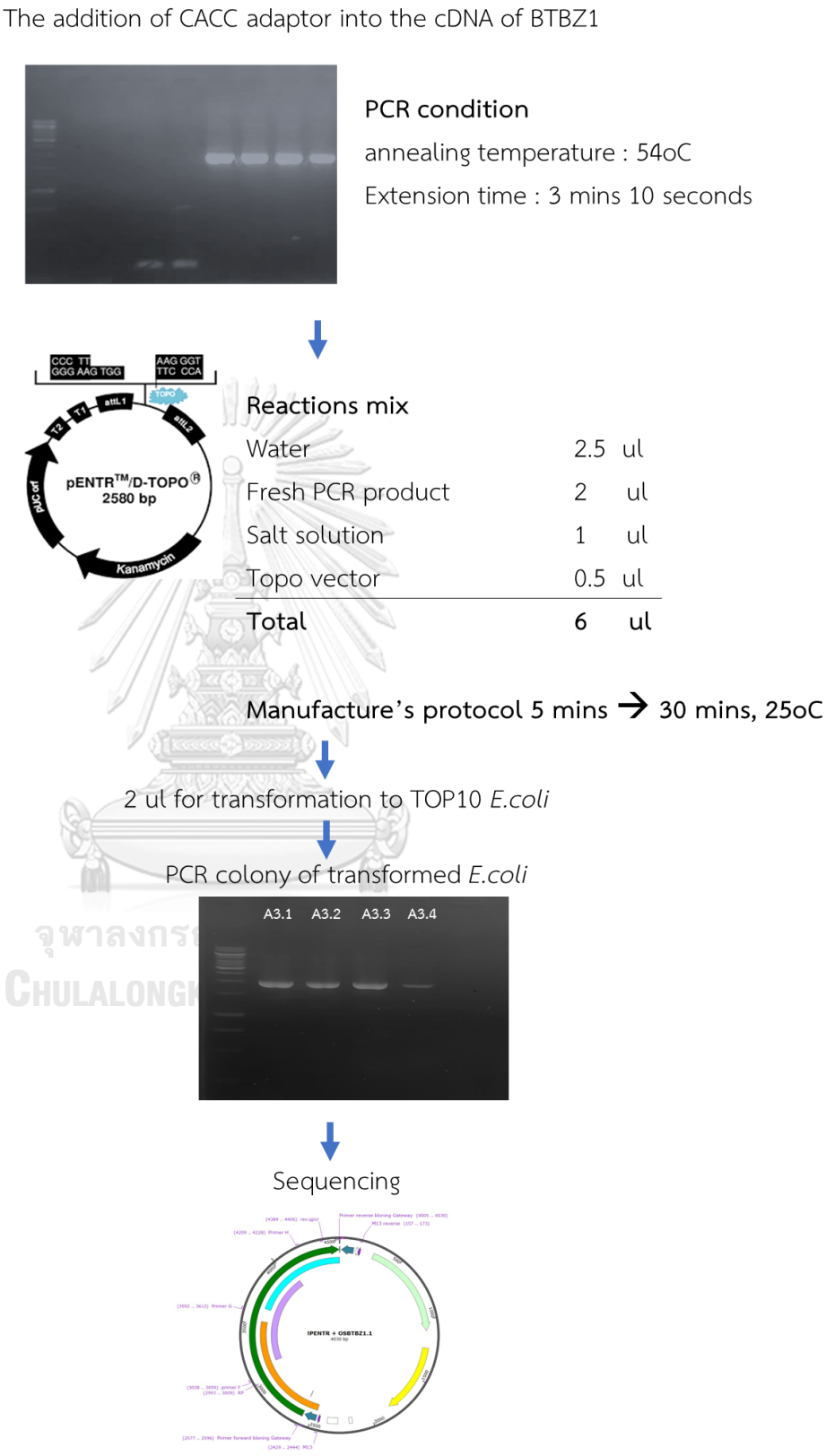


Figure 23 The insertion of *OsBTBZ1* cDNA to donor vector

3. LR clonase reaction from donor vector to destination vector

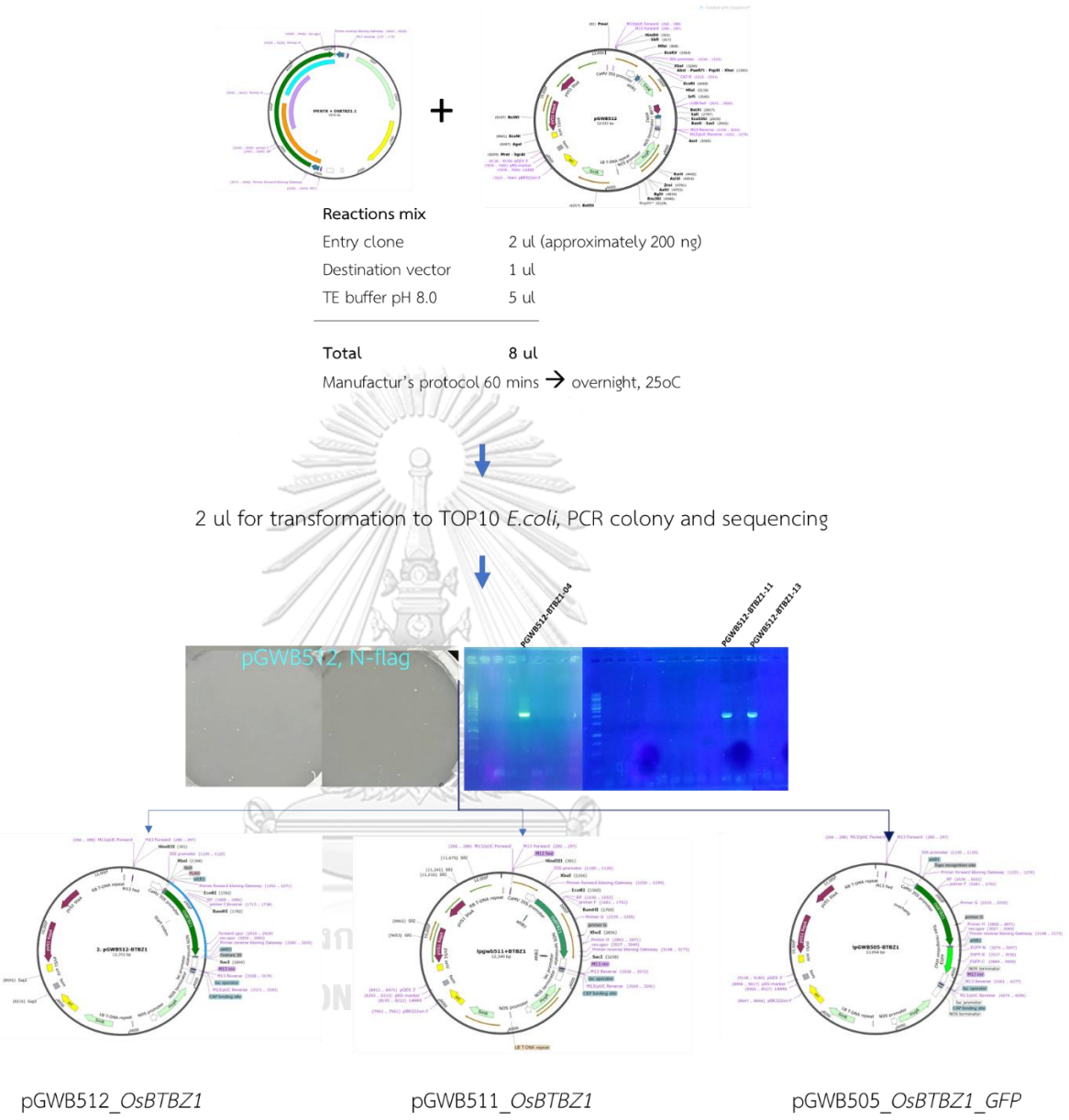


Figure 24 The flowchart of LR clonase reaction

Sequencing result of pGWB512_OsBTBZ1

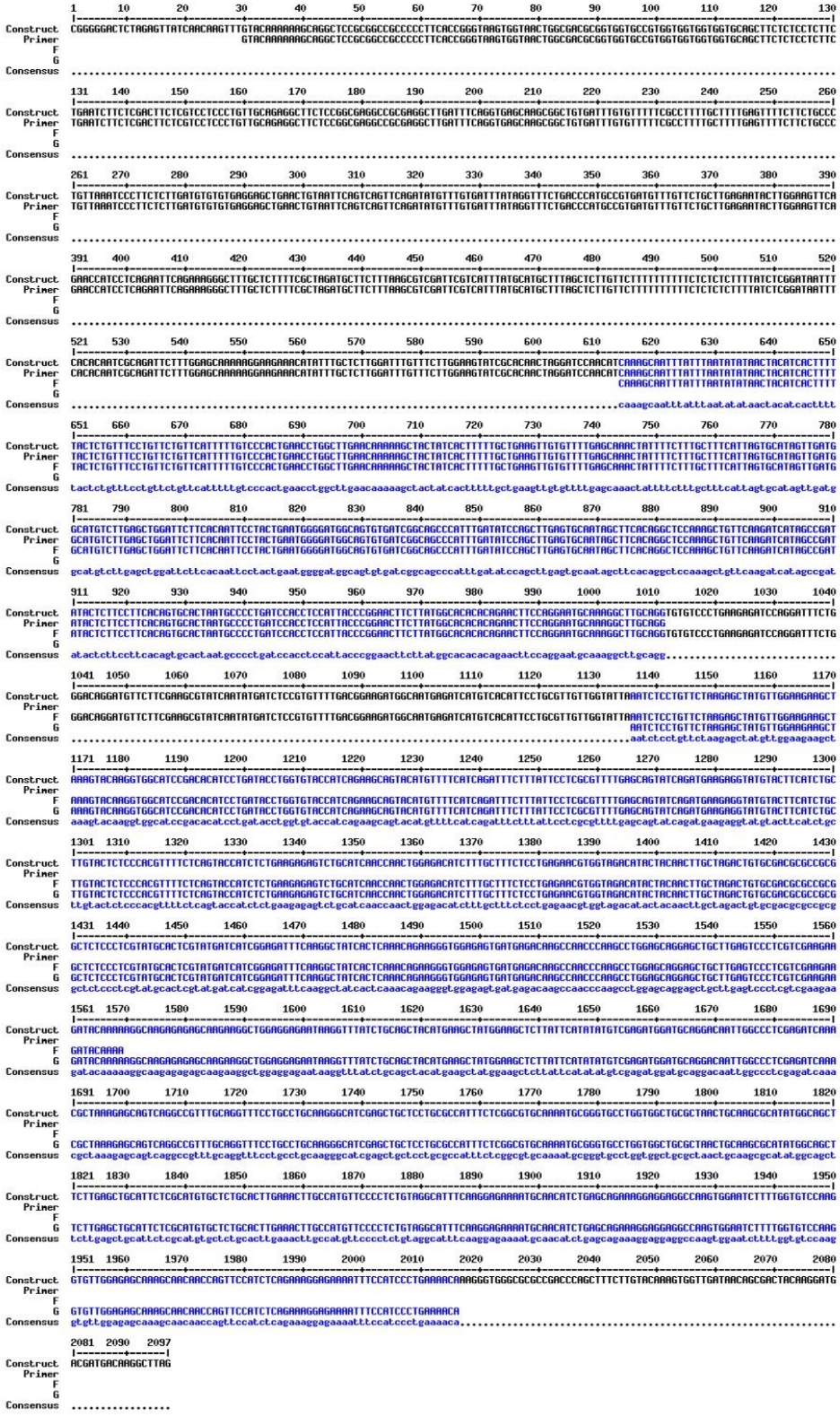


Figure 25 The sequencing result of pGWB512_OsBTBT1

Sequencing result of pGWB511_OsBTBZ1

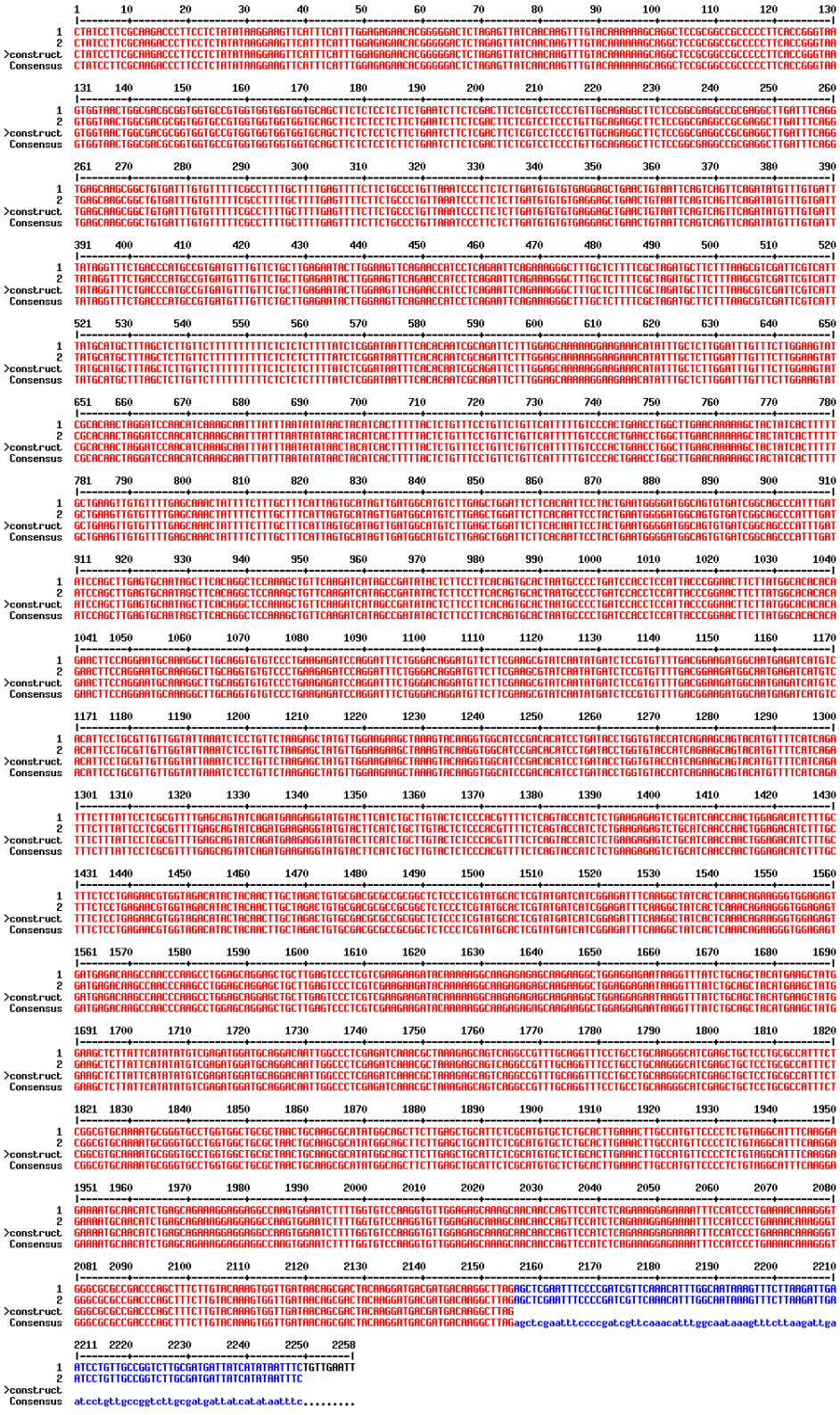


Figure 26 The sequencing result of pGWB511_OsBTBT1

Sequencing result of pGWB505_OsBTBZ1_GFP

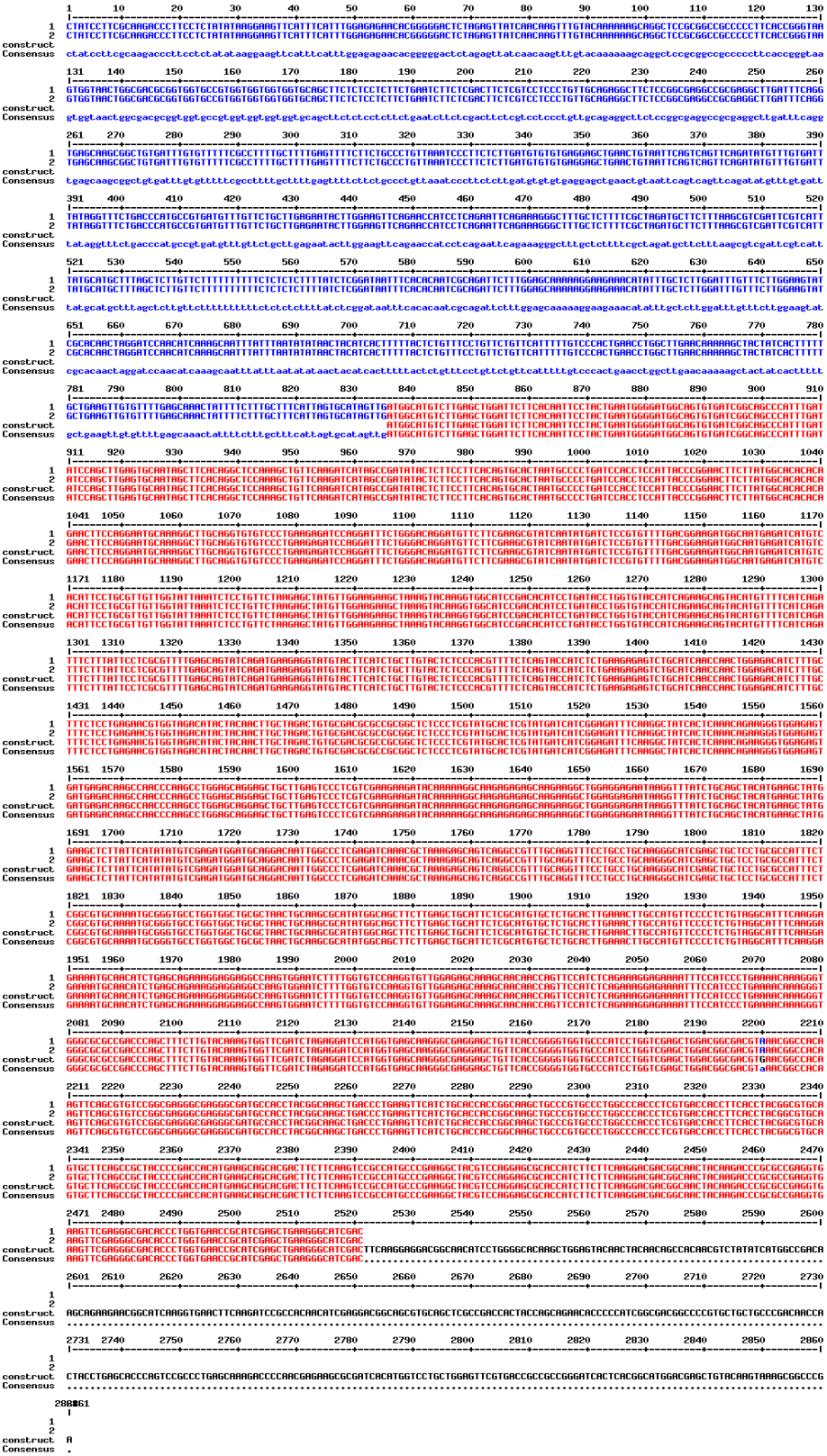


Figure 27 The sequencing result of pGWB505_OsBTBT1_GFP

Phenotyping of transgenic lines in 0-day

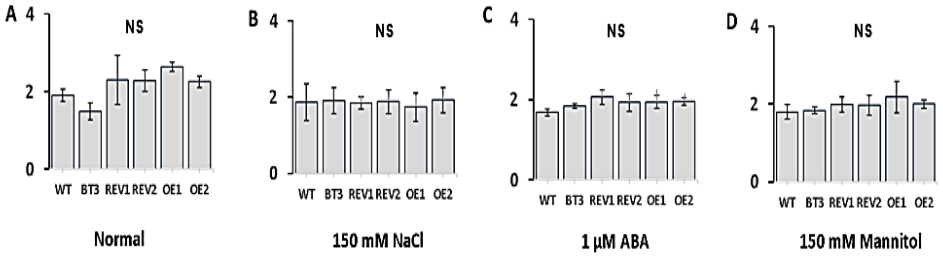


Figure A.8 Fresh weight of 7-day old Arabidopsis seedling of WT, bt3 mutant, and transgenic lines (REV1, REV2, OE1, OE2) grown in various conditions; normal (control) (A), Supplemented with 150 mM NaCl (B), 1 µM ABA (C) or 150 mM mannitol (D) on day 0 of stress exposure. NS = no significant difference among means of the treatments.

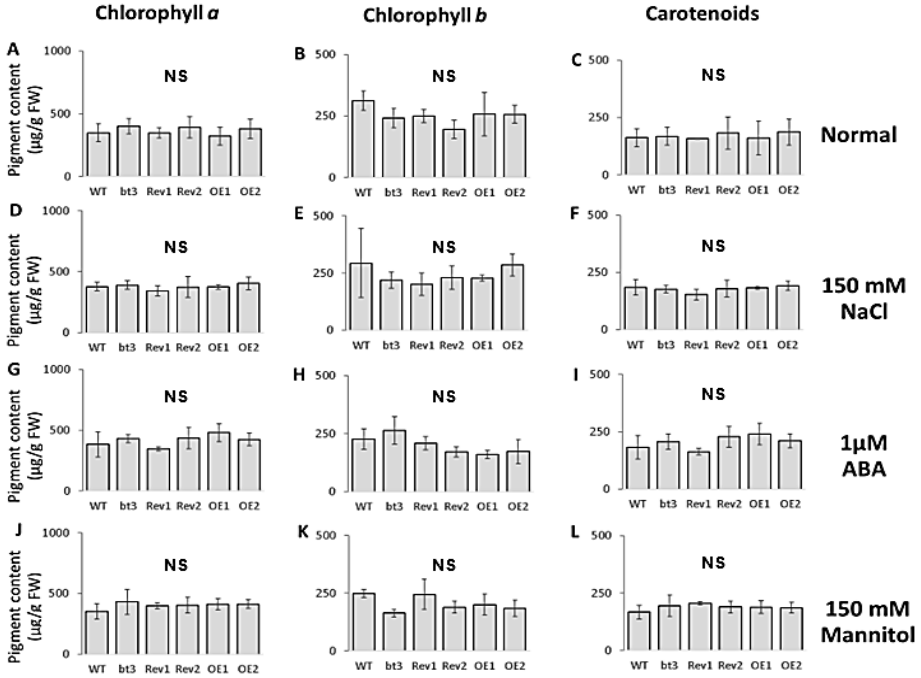


Figure A.9 Contents of pigments, namely, Chl a, (**A**, **D**, **G**, **J**), Chl b, (**B**, **E**, **H**, **K**), and carotenoids (**C**, **F**, **I**, **L**) of 7-day-old Arabidopsis seedlings treated with 150 mM NaCl, 1 µM ABA, or 150 mM mannitol for 6 d. The data were collected from the WT and bt3 complemented lines with the OsBTBZ1 gene in the bt3 mutant background (REV1 and REV2) and the OsBTBZ1 ectopic expression line with a WT background (OE1 and OE2). The different letters above the bars represent the significant difference in means at $p < 0.05$ and NS represents no significant difference.

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