University of São Paulo
“Luiz de Queiroz” College of Agriculture

Characterization of NAC transcription factor family of *Tectona grandis* involved in
secondary cell wall deposition and stress response

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Dissertation presented to obtain the degree of Master in
Science. Area: Plant Physiology and Biochemistry

Piracicaba
2020

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Dedicated to my father Jorge Fernando Matias Cervantes, and my mother Maria Asunción Hurtado Vargas, who taught me to be a kind and moral person and to be strong beyond the difficulties.

I DEDICATED

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less"

Marie Curie

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RESUMO

Caracterização da família de fatores de transcrição NAC de *Tectona grandis* envolvidos na deposição de parede celular secundária e reposta à estresse

As proteínas NAC são uma das maiores famílias de fatores de transcrição específicos de plantas (TFs). Eles regulam diversos processos biológicos, incluindo a formação de parede celular secundária (SCW). A SCW é a principal componente da madeira e fonte de matéria-prima na indústria florestal. A espécie *Tectona grandis* (teca) é reconhecida por apresentar uma das madeiras mais valiosas do mundo devido às suas extraordinárias qualidades de cor, densidade e durabilidade. Estudos de genômica e transcriptômica de teca foram recentemente disponibilizados, essas informações permitiram identificar os fatores de transcrição NAC e analisá-los em relação a sua evolução e seu perfil de expressão durante a formação da madeira. Foram identificadas 130 proteínas NAC que são codificadas por 107 loci de genes. Essas proteínas foram classificadas em 23 clados da família NAC, juntamente com *Populus*, *Eucalyptus* e *Arabidopsis*. Os dados de expressão dos transcritos revelaram padrões de expressão temporal e espacial específicos para a maioria dos genes NAC de teca. O qRT-PCR indicou expressão de proteínas de Domínio Vascular NAC (VASCULAR NAC DOMAIN) envolvidas na formação de SCW em vasos do xilema de árvores jovens de 16 anos de idade. Ainda, foi caracterizado por expressão heteróloga o novo gene *TgNAC01* responsivo ao estresse salino. Foram geradas três linhas de tabaco transgênicas independentes que superexpressaram *TgNAC01*. A proteína *TgNAC01* foi demonstrada estar localizada no núcleo. A expressão relativa do gene *TgNAC01*, comprimento do caule e o teor de clorofila foram analisados nas plantas transgênicas com dois meses de crescimento em casa de vegetação. As plantas de tabaco que superexpressam *TgNAC01* mostraram maior crescimento, aumento na senescência foliar e maior tolerância ao estresse salino na concentração de 300 mM de NaCl em comparação com plantas controle (WT). O potencial hídrico, trocas gasosas e a fluorescência da clorofila foram utilizados para determinar a tolerância ao estresse salino. Neste trabalho de pesquisa, foi demonstrado que o fator de transcrição *TgNAC01* atua como um regulador positivo no desenvolvimento do xilema e como um ativador transcricional da regulação mediada pelo estresse salino e senescência foliar. Estes resultados abrem caminho para uma maior compreensão sobre os genes do fator de transcrição NAC na formação da madeira e na tolerância à estresse em teca, sendo potencialmente úteis para estudos futuros que visam melhorar a produtividade e a qualidade da madeira usando abordagens biotecnológicas.

Palavras-chave: Xilema secundário, Formação de madeira, Proteína NAC, Estresse salino

ABSTRACT

Characterization of NAC transcription factor family of *Tectona grandis* involved in secondary cell wall deposition and stress response

NAC proteins are one of the largest families of plant-specific transcription factors (TFs). They regulate diverse biological processes, including secondary cell wall formation (SCW). SCW is the main component of wood and source of raw material in forestry industry, *Tectona grandis* (teak) specie is recognized for presenting one of the most valuable woods in the world due to its extraordinary qualities of color, density and durability. Studies of teak genomics and transcriptomics were recently unveiled, producing information that allowed to identify teak NAC transcription factors and to analyse their evolution and expression profile during wood formation. It was identified 130 NAC proteins that were coded by 107 gene loci. These proteins were classified into 23 clades of the NAC family, together with *Populus*, *Eucalyptus*, and *Arabidopsis*. Data on transcription expression revealed specific temporal and spatial expression patterns for the majority of teak NAC genes. The qRT-PCR indicated expression of VASCULAR NAC DOMAIN proteins involved in SCW formation in xylem vessels of 16-year-old juvenile trees. In addition, it was characterized by heterologous expression, the novel salt stress-responsive *TgNAC01* gene. Three independent transgenic tobacco lines overexpressing TgNAC01 were generated. It was demonstrated that the TgNAC01 protein is localized only in the nucleus. Analyses of *TgNAC01* relative expression, stem length and chlorophyll content were evaluated in two months old transgenic plants growing in greenhouse. Tobacco plants overexpressing TgNAC01 showed higher stem length, an increase in leaf senescence, and tolerance to 300 mM of NaCl when compared to wild type (WT) plants. Water potential, gas exchange and chlorophyll fluorescence were used to determine the salt stress tolerance. In this research, it was demonstrated that TgNAC01 transcription factor acts as a positive regulator in xylem development and as a transcriptional activator mediated by salt stress and leaf senescence. These results open a way to further understanding of NAC transcription factor genes in *T. grandis* wood formation and stress tolerance, potentially being useful for future studies aiming to improve productivity and wood quality using biotechnological approaches.

Keywords: Secondary xylem; Wood formation; NAC protein; Salt stress

RESUMEN

Caracterización de la familia de factores de transcripción NAC de *Tectona grandis* envuelta en la deposición de la pared celular secundaria y respuesta a estrés

Las proteínas NAC son una de las mayores familias de factores de transcripción específicos de plantas (TF). Regulan varios procesos biológicos, incluida la formación de la pared celular secundaria (SWC). SCW es el componente principal de la madera y una fuente de materia prima en la industria forestal. La especie *Tectona grandis* (teca) es reconocida por tener una de las maderas más valiosas del mundo debido a sus extraordinarias cualidades de color, densidad y durabilidad. Recientemente se pusieron a disposición estudios sobre el genoma y el transcriptoma de teca, esas informaciones permitieron identificar los factores de transcripción NAC y analizarlos en relación a su evolución y su perfil de expresión durante la formación de la madera. Se han identificado 130 proteínas NAC que están codificadas por 107 loci de genes. Estas proteínas se clasificaron en 23 clados de la familia NAC, junto con *Populus*, *Eucalyptus* y *Arabidopsis*. Los datos de expresión de los transcritos revelaron padrones de expresión espacial y temporal específicos para la mayoría de los genes NAC de teca. La qRT-PCR indicó la expresión de proteínas de dominio vascular NAC (DOMINIO VASCULAR NAC) implicadas en la formación de SCW en vasos de xilema de árboles de 16 años. Además, el nuevo gen *TgNAC01* sensible al estrés salino se caracterizó por la expresión heteróloga. Se generaron tres líneas de tabaco transgénicas independientes que sobreexpresaron *TgNAC01*. Se ha demostrado que la proteína *TgNAC01* se encuentra en el núcleo. La expresión relativa del gen *TgNAC01*, la longitud del tallo y el contenido de clorofila se analizaron en plantas transgénicas con dos meses de crecimiento en un invernadero. Las plantas de tabaco que sobreexpresan *TgNAC01* mostraron un mayor crecimiento, una mayor senescencia de las hojas y una mayor tolerancia al estrés salino a una concentración de NaCl 300 mM en comparación con las plantas de control (WT). El potencial hídrico, el intercambio de gases y la fluorescencia de clorofila se usaron para determinar la tolerancia al estrés salino. En este trabajo de investigación, se demostró que el factor de transcripción *TgNAC01* actúa como un regulador positivo en el desarrollo del xilema y como un activador transcripcional de la regulación mediada por el estrés salino y la senescencia de las hojas. Estos resultados abren el camino a una mayor comprensión de los genes del factor de transcripción NAC en la formación de madera y en la tolerancia al estrés en la teca, siendo potencialmente útil para futuros estudios que apuntan a mejorar la productividad y la calidad de la madera utilizando enfoques biotecnológicos.

Palabras-clave: Xilema secundario, Formación de la madera, Proteína NAC, Estrés salino

1. INTRODUCTION

Teak (*Tectona grandis*) is a tropical tree from Southeast Asia belonging to the Lamiaceae family and Lamial order. The largest area of natural teak forests (46%) is located in India, but teak tree is also cultivated in other 36 countries for commercial purposes [1]. Teak is considered one of the most important tropical hardwood species due to its extreme durability, stability to adverse conditions, resistance to pests, chemicals and water, probably due to its high levels of quinones [2]. These characteristics make teak wood suitable for use in naval structures, floors, fine furniture and interior decorations. In Brazil, teak's plantation covers an extensive area (higher than 60 thousand ha) in the states of Mato Grosso, Roraima, Amazonas, Pará, Acre and Rondônia [3].

The understanding of the mechanisms involved in wood formation and the transcriptional network regulating this process is extremely important to improve teak productivity. The recently published teak genome collaborated to the characterization of transcription factors (TFs) from Terpene synthases (TPSs) protein family, involved in terpenoid biosynthesis in teak [4]. In addition, teak transcriptome has been elucidated in different tissues and conditions such as, vegetative development [5], lignified tissues formation [6], and in the adaptation to limited water supply [7].

Transcription factors (TFs) are proteins with DNA-binding domains that bind to specific sequences of DNA (promoter regions, enhancers of specific genes, domains of RNA polymerase II or other transcription factors) regulating the amount of messenger RNA (mRNA) produced by the gene. Specific amino acid side chains of DNA-binding domains in TFs can establish interactions to DNA target sequence through hydrogen bridges and Van der Waals interactions [8]. DNA curvatures are often required by TFs to bind target genes efficiently, therefore some transcription factors induce DNA bending [9], causing changes that facilitate other processes, such as DNA fusion or additional protein binding. TFs are subdivided into families according to their structure and by the form they interact with DNA[10].

NAC proteins are one of the largest families of TFs in plants, with 138 genes identified in *Arabidopsis thaliana*, 158 in *Oryza sativa* L. ssp. indica, 280 in *Nicotiana tabacum*, 269 *Glycine max*, 189 in *Zea mays*, 202 in *Eucalyptus grandis* and 289 in *Populus trichocarpa* [11]. The acronym NAC refers to the conserved domain present in *NO APICAL MERISTEM* (NAM) from *Petunia hybrida* E. Vilm., and from *ARABIDOPSIS TRANSCRIPTION FACTOR ACTIVATOR* (*ATAF1*, *ATAF2*) and *CUP-SHAPED COTYLEDON* (*CUC2*) from Arabidopsis [12]. The NAC domain has approximately 150-160 amino acids divided into five sub-domains, designated as domains A to E [13]. Their function is associated with nuclear localization, DNA binding and formation of homodimers or heterodimers with other proteins from the same family [14]. TFs from NAC family also have a highly divergent C-terminal region that function as a transcriptional regulatory region acting as an activator or repressor.

The NAC TFs undergo intensive post-translational regulation that includes ubiquitination, dimerization, phosphorylation or proteolysis, and microRNA regulation [15–17]. These regulatory steps enable these TFs to play multiple roles in most plant processes, including the formation of shoot apical meristem [18], flower development [19], cell division [20], leaf senescence [21], biotic and abiotic stress responses [22] and secondary cell wall formation [23].

Secondary cell wall (SCW) formation involves complex tissues and longitudinally and transversely arranged cell systems. The longitudinal system consists mainly of tracheids in gymnosperms, and vessels and fibers in angiosperms. The transversal system is composed of ray parenchyma cells. These systems derived from vascular cambium fusiform initials and ray initials, respectively [24]. Vessels and fiber form secondary xylem through developmental processes that includes cell division, cell expansion, secondary wall deposition, lignification and

programmed cell death [25]. These biological processes are controlled by a sophisticated network of transcription factors (TFs) that coordinately integrate developmental and environmental signals into SCW formation [26].

NAC and MYB transcriptional network regulating secondary cell wall formation have a hierarchical multitiered organization [27]. NAC TFs include the VNS proteins: SECONDARY WALL ASSOCIATED NAC DOMAIN1 (SND1/NST3), VASCULAR RELATED NAC DOMAIN (VND1-7), NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1), and SOMBRERO (SMB). The central element of the SCW network regulation is the VND7 protein. VND7 is the first-layer master switch regulating SCW formation [27], which includes both transcriptional up-regulation of enzymes involved in SCW biosynthesis, and the regulation of patterned SCW deposition [28]. VASCULAR NAC INTERACTING PROTEIN 2 (VNI2) was described as negative regulator of VND7 [29]. Following NAC TFs are the MYB SCW transcriptional regulation network. MYB46 and MYB83 were identified in Arabidopsis as second-layer master switch of SCW formation [30] (Figure 1).

Figure 1 Transcriptional network regulating secondary cell wall formation. A) Layer master switch NAC and MYB-based transcriptional regulation of secondary cell wall biosynthesis. B) Transcriptional regulatory network around VNS proteins, C) Transcriptional regulatory network around MYB proteins, involved in cellulose hemicellulose and lignin deposition, this model representation was based on Arabidopsis reports [27].

Some genes involved in development processes are also related to environmental adaptation in plants. Alterations in gene expression level or pattern may be by transcriptional changes and, may lead to stress tolerance depending of the tissue localization and the developmental stage of the plant [31,32]. NAC TFs play an important role in complex signaling networks during stress responses in plants. Phylogenetic analysis of NACs showed that most NAC TFs stress-sensitive appear to contain the NAC domain closely homologous [33,34]. On the other hand, stress-sensitive NACs exhibit greater diversity in expression patterns and participate in the regulation of a spectrum of responses to different abiotic stresses. NAC protein interact specifically with the consensus NAC recognition sequence (NACRS) of downstream genes promoter by binding to conserved NAC domain, or acting with others activators of downstream target genes in tissues where NAC proteins are present [35,36] (Figure 2).

Figure 2 Schematic representation of NAC Transcription factors regulation network during abiotic stress. Positive (arrows) and negative (bars) regulatory mechanisms are presented. Abbreviations: AMY, amylase; CBF, repeat link factor-C; COR, cold-responsive protein; DREB, dehydration-responsive binding protein; ERD, responsive early to dehydration; GST, glutathione S-transferase; LEA, abundant late embryogenesis; NACRS, NAC recognition sequence; OsPM1, *Oryza sativa* plasma membrane protein 1; RD, responsive to dehydration [16].

Salt stress produces two effects on plants: 1) osmotic stress, the consequence of high concentrations of solutes in the soil and low water potential in the root environment, 2) ion-specific stress, which results from the alteration of the k^+ / Na^+ ratio and the increase in Na^+ and Cl^- ion concentrations in the cell [37]. The activation of responses to osmotic stress has the same mechanisms of response to water stress. The regulatory mechanisms, ABA dependent and ABA- independent, are activated due to of difference in their NACRs promoter elements (Figure 1) [16]. Whole-NAC genome expression profiling studies in arabidopsis and rice have found NAC genes to be induced by salt stress. Complementary, studies done in arabidopsis showed that *ATAF1*-overexpression lines have increased sensibility to high salinity, and *ataf1-1* mutant increased sensitivity to ABA, compared to wild type plants [38]. In addition, in genetically modified rice with overexpression of the *OsNAC1* gene, there was a significant improvement in drought resistance and tolerance to high salinity levels in the vegetative stage. This transgenic line also showed high

sensitivity to abscisic acid, and slow loss of water due to increased closure of stomatal pores, in accordance with its high expression in the guard cells in rice [39].

Finally, NAC proteins are involved in the regulation of leaf senescence through changes in gene expression patterns, tissues structure and metabolism, including loss of photosynthetic activities and increased hydrolysis of proteins, membrane lipids, and nucleic acids [40]. These leaf senescence processes are accelerated by brassinosteroids, ethylene, jasmonic acid (JA), salicylic acid (SA), methyl jasmonate (MeJA), and abscisic acid (ABA), and delayed by gibberellic acid (GA), auxin and cytokinins [41]. ABA endogenous is responsible for up-regulation of genes associated with ABA-signaling. Moreover, treatment with exogenous ABA and salt stress induce expression of several senescence-associated genes (SAGs) known to accelerate leaf senescence [21,42,43], suggesting the connection of leaf senescence to ABA-signaling [44], and consequently, to NAC genes expression (Figure 1).

The purposes of this research were to unravel the NAC genes related to secondary cell wall formation and characterize the heterologous expression of the *TgNAC1* gene involved in the formation of secondary xylem, senescence and salt stress resilience in *Nicotiana tabacum*. For this study, a genome-wide identification and *in silico* expression analysis of teak NAC genes were done and are presented in chapter 1. In chapter 2, it is presented a functional analysis of the TF *TgNAC1* through the characterization of the transgenic line 35S:: *TgNAC1* in *N. tabacum* involved in salt stress tolerance by analysis of water potential, gas exchange and chlorophyll fluorescence.

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2. ANALYSIS OF NAC DOMAIN TRANSCRIPTION FACTOR GENES OF *TECTONA GRANDIS* INVOLVED IN SECONDARY CELL WALL DEPOSITION¹

Abstract

NAC proteins are one of the largest families of plant-specific transcription factors (TFs). They regulate diverse complex biological processes, including secondary xylem differentiation and wood formation. Recent genomic and transcriptomic studies of *Tectona grandis* (teak), one of the most valuable hardwood trees in the world, have allowed identification and analysis of developmental genes. In the present work, *T. grandis* NAC genes were identified and analyzed regarding to their evolution and expression profile during wood formation. We analyzed the recently published *T. grandis* genome, and identified 130 NAC proteins that are coded by 107 gene loci. These proteins were classified into 23 clades of the NAC family, together with *Populus*, *Eucalyptus*, and *Arabidopsis*. Data on transcript expression revealed specific temporal and spatial expression patterns for the majority of teak NAC genes. RT-PCR indicated expression of VND genes (*Tg11g04450-VND2* and *Tg15g08390-VND4*) related to secondary cell wall formation in xylem vessels of 16-year-old juvenile trees. Our findings open a way to further understanding of NAC transcription factor genes in *T. grandis* wood biosynthesis, while they are potentially useful for future studies aiming to improve biomass and wood quality using biotechnological approaches.

Keywords: tropical tree; wood formation; secondary growth

2.1. Introduction

NAC proteins are one of the largest families of plant-specific transcription factors (TFs), in which the DNA binding domain is highly conserved. The NAC designation is derived from the NO APICAL MERISTEM (NAM) gene from *Petunia hybrida* and from the *Arabidopsis thaliana* L. genes ATAF1–2 and CUP-SHAPED COTYLEDON (CUC2) [1]. Structures of NAC proteins are commonly divided into two regions, a conserved N-terminal DNA-binding domain and a variable C-terminal transcriptional regulatory region [2]. The N-terminal NAC domains comprise five (A–E) subdomains [2–4] located at the N-terminal. Usually, the subdomains contain 150–160 amino acid residues that are implicated in DNA binding, nuclear localization, and formation and localization of homodimers or heterodimers [2]. The C-terminal regions confer regulation of transcriptional activity [3,4]. NAC TFs have been analyzed at the genome-wide level in diverse species such as *Arabidopsis thaliana*, *Eucalyptus grandis*, *Populus trichocarpa* L. (black cottonwood), *Nicotiana tabacum* (tobacco), and *Glycine max* (soybean) [3,5–8].

Paleobotanical studies of tracheids resistant to degradation indicate that lignification and secondary cell wall (SCW) differentiation evolved in land plants in the early Devonian (about 415 million years ago) [9]. SCW biosynthesis requires coordinated expression of genes for cellulose, xylan, glucomannan, and lignin biosynthesis. A transcriptional network composed of NAC and MYB transcription factors regulates SCW biosynthetic pathways [10,11]. These closely related TFs function as master transcriptional switches that regulate downstream targets [12].

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Thus far, NAC TF genes were identified in all analyzed land plants, whereas eukaryotic algae species lack NAC TFs [13]. Comparative genomic and gene functional analyses have indicated that NAC TFs underwent an expansion in the lineage of vascular plants [13]. Also, tracheid evolution is associated with a burst of structural diversity [14], which is probably related to NAC genes controlling SCW biosynthesis in vascular plants, comprising seedless vascular plants, gymnosperms, and angiosperms [15]. Noticeably, plant species that are structurally less complex contain significantly fewer NAC genes, such as the model bryophyte *Physcomitrella patens* (32 NAC genes), and no NAC genes are found in Chlorophytes (*Ostreococcus lucimarinus*, *Ostreococcus tauri*, *Chlamydomonas reinhardtii*) [16]. Most likely, diversification of NAC genes began with the colonization of terrestrial environments by the ancestors of modern land plants [14]. NAC TFs have been associated with the regulation of a wide range of plant developmental processes acting as transcription activators or repressors. These plant developmental processes include ripening [17], cellular morphogenesis, signaling transduction [18], and establishment of the shoot apical meristem [4], floral organs [19], and lateral roots [20]. They are also involved in the signaling pathway induced during biotic and abiotic stresses [18,21,22], and in the regulation of leaf senescence [23]. As for secondary growth, NAC TFs have been described in the regulation of xylogenesis, fiber development, and secondary cell wall formation [24–32].

In *Arabidopsis*, NAC TFs represent the most upstream regulators in the transcriptional regulatory network that controls wood formation. Master regulators of this development are the VASCULAR NAC DOMAIN (VND1–VND7), NAC SECONDARY WALL THICKENINGS (NST1 and NST2), and SECONDARY WALL NAC DOMAIN TFs (SND1/NST3 and SND2) [10]. These NAC TFs regulate the expression of the MYB genes [11] during SCW formation [12]. Besides, the PROTEIN SOMBRERO (SMB) TF and its two close homologs, BEARSKIN1 (BRN1) and BRN2, are related to the VND/NST TFs in this SCW regulatory network [33]. In this same *Arabidopsis* regulatory network, the VND-INTERACTING 2 (VNI2) TF is described to negatively regulate VND7 [34]. *Tectona grandis* L.f. (teak), a worldwide attractive tropical tree with valuable wood of high economic importance in industrial forestry, is also a plant species of interest to better understand the SCW biosynthesis and wood formation. Teak's secondary xylem biosynthesis is a complex molecular process that involves several genes and environmental factors. Recent genomic and transcriptomic analyses of *T. grandis* have allowed to improve the understanding of the xylogenesis role in biomass increase and wood quality [35–38], even though *T. grandis* NAC transcription factors have not yet been identified. The identification and characterization of teak NAC TFs are fundamental to elucidate their regulation role in vascular tissue differentiation and wood formation, as well as future biotechnological studies aiming to improve biomass and wood quality.

Here, we report a genome-wide identification, phylogenetic profile, gene structure, chromosome localization, phylogenetic relationship, and expression profiles of *T. grandis* NAC genes related to wood formation. In addition, tissue-specific expression patterns of 13 teak NAC TF genes homologous to *Arabidopsis* in their response to wood formation were investigated by quantitative real-time RT-PCR (RT-qPCR). The data represent a step forward to the understanding of plant NAC transcription factors and the regulation of secondary cell wall biosynthesis and wood formation in *T. grandis*.

2.2. Materials and Methods

2.2.1. Database Search and Phylogenetic Profiles

The *Tectona grandis* NAC genes were compared to the model species *Eucalyptus grandis*, *Populus trichocarpa*, and *Arabidopsis thaliana* in which NAC genes were already identified and analyzed [3,5,6]. Deduced proteomes of *Eucalyptus grandis* [39] and *Populus trichocarpa* [40] were obtained from Phytozome v12, and The Arabidopsis Portal [41] was used for *A. thaliana* proteins. As for *T. grandis*, the proteins were accessed in the Dryad database [38]. We considered that any protein carrying the NAM domain was a member of the NAC family of TFs. The NAM domain was searched and identified using the profile Hidden Markov Model for this domain from PFAM (Accession Number PF02365). This profile was downloaded from Pfam Database (<http://pfam.sanger.ac.uk/>) and a hmm-search was performed with the HMMer software v3.2.1 'hmmsearch'. Hit scores higher than 21.2 (gathering cutoff from PFAM specific for the NAM model) were considered true positives and selected for further analyses. A diagram of the phylogenetic relationship among teak and 28 species was constructed with the tree generator PhyloT (<http://phylot.biobyte.de/index.html>) based on the NCBI taxonomy IDs. The APGIII classification system was used to manually indicate the phylogenetic orders of the flowering plants species in the tree [42]. As for *Clorophyta*, *Bryophyta*, and *Licophyta*, the classification was done according to Novikiv and Barabas-Krasni [43].

2.2.2. Phylogenetic Analysis of NAC Transcriptional Factors

Non-redundant peptide sequences of NAC domain proteins (Pfam ID: NAM; PF02365) from *Tectona grandis* (116), *Populus trichocarpa* (196), *Arabidopsis thaliana* (153), and *Eucalyptus grandis* (190), previously identified (section 2.1), were aligned using MAFFT v7.407 in the auto mode [44]. Poorly aligning regions from the multiple sequence alignment were removed using TrimAl v1.4, in the “automated1” mode [45]. Phylogenetic inference under the Maximum Likelihood approach was carried out with IQ-Tree v1.6.9 [46], with the options “m MFP -st AA -seed 12345 -lmap 65400 -alrt 1000 -bb 1000”. The best evolutionary model for phylogenetic inference was also estimated within IQ-Tree with the option -m MFP (Model Finder Plus), by computing the log-likelihoods for many different evolutionary models against an initial parsimony tree and choosing the model that minimizes the Bayesian information criterion (BIC) [47]. Branch support was assessed with the Shimodaira–Hasegawa (SH)-like approximate likelihood ratio test [46] and ultrafast bootstrap [48], both with 1000 replicates. The multi-species phylogenetic tree of NAC TFs was reconciled with the species tree, using Notung 2.9 in order to infer the most likely root and the history of gene duplications in the NAC gene family [49]. Briefly, the species tree–gene tree reconciliation process tries to account for the differences between the species tree and the gene tree, with the gene-level processes of gene duplication and gene losses.

2.2.3. Chromosomal Location

The teak NAC genes were mapped on chromosomes in accordance with the whole genome of this species (<https://datadryad.org/stash/dataset/doi:10.5061/dryad.77b2422>) [38]. Of the 18 teak chromosomes, 17 near-complete pseudomolecules were generated with one chromosome present as two chromosome arm scaffolds [38]. Chromosomal locations of the identified teak NAC genes were extracted from the general feature format (GFF) file provided with the genome sequence and visualized in the Integrative Genomics Viewer (IGV v2.7.0) [50]. Note that for the purpose of the visualization of NAC genes on teak chromosomes, the pseudomolecules 18 and 19 were joined into a single chromosome.

2.2.4. Gene Structure and Conserved Motifs

Exon–intron structures of *T. grandis* NAC genes were analyzed and illustrated with the Gene Structure Display Server (GSDS) (<http://gsds.gao-lab.org/index.php>) by comparison of coding sequence (CDS) regions with genomic DNA sequences of this species [38]. The software MEME v5.0.5 (MEME—<http://meme-suite.org/index.html>) was employed for the detection of conserved motifs with the following parameters: Distribution of motif occurrences, zero or one per sequence; minimum width, 6, maximum width, 50; maximum number of motifs, 10; and optimum motif width, ≥ 6 and ≤ 116 [51]. The subcellular localization of the identified NAC proteins was predicted by using an online analysis tool from Molecular Bioinformatics Center v2.5 (<http://cello.life.nctu.edu.tw/>) and BUSCA (<http://busca.biocomp.unibo.it>)

2.2.5. In Silico Gene Expression Profiling

The RAW RNASeq data from *T. grandis* were accessed in the Sequence Read Archive (SRA-NCBI) under the accession number SRP059970. These data were previously generated by our group [36] and comprise various plant tissues in three plant developmental stages. The teak RAW RNASeq data were processed with BBDuk2 [52] in order to remove low-quality regions from the reads, remainders of adapter sequences, and ribosomal RNA. Salmon v1.0.0 [53] was employed to estimate expression values, as transcripts per million (TPM), using as reference the predicted cDNAs from the *T. grandis* genome [38]. Expression values were imported into R [54] with the tximport package [55] and summarized to gene level. Gene expression values for each gene were transformed using Z-scores, i.e., the expression value of a gene in a given condition was subtracted from the average gene expression and expressed as standard deviation of the mean, and then visualized as heatmaps using the pheatmap package.

2.2.6. Expression Analysis of Marker Genes for Secondary Cell Wall Formation by RT-qPCR

Expression patterns of genes involved in wood formation were analyzed in samples of teak stem collected from 4-year-old plants grown in the greenhouse, and sapwood samples collected from 16- and 64-year-old trees grown in a field in Piracicaba, São Paulo State, Brazil (Latitude: 22°42'23"S, Longitude: 47°37'7"W, 650 m above sea level). This is the same population of trees used for the previously reported transcriptional profile mentioned in Section 2.5 [36]. In this expression analysis, new tissue samples were collected with a Pressler borer at DBH, 4 years apart of the first sampling [36]. Frozen tissue samples (0.6 g) were ground to a fine powder in liquid nitrogen using a sterilized mortar and pestle. Total RNA was extracted following the Trizol (Invitrogen, city, country) protocol. RNA was quantified in a NanoDrop 2000 spectrophotometer (Thermo Scientific, city, country) and RNA integrity was examined by gel electrophoresis. Total RNA was treated with DNase I (Promega) and then used for cDNA synthesis using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, city, country), according to the

manufacturer's instructions. Quantitative RT-PCR (qPCR) reactions were conducted in 12.5 μ L total volume using a Platinum Sybr Green Supermix (Invitrogen), and ran in an ABI 7500 qPCR thermocycler (Applied Biosystems, city, country). Expression data were normalized using the $2^{-\Delta C_t}$ method. The constitutive *Elongation Factor-1 alpha* (EF-1 *alpha*) housekeeping gene was used as internal control [56]. We used three biological repetitions and two technical repetitions. The statistical analysis was performed using Prism v8 software (GraphPad, city, country). Normality of data was confirmed by the Shapiro–Wilk test, which indicated that the data follow normal distribution. Statistical significance was determined by one-way ANOVA, with Tukey post-hoc analysis ($p > 0.1$). The analyzed genes are key regulators of SCW formation in Arabidopsis (Additional file 1).

2.3. Results and Discussion

2.3.1. Identification and Evolution Analyses of NAC Family Transcription Factors

The analysis of teak NAC TF encoding genes identified 130 NAC proteins that are coded by 107 loci. We analyzed, with CD-HIT, the 130 teak NAC proteins and identified 14 that were 100% identical to another protein in the original set. Thus, we generated a set of non-redundant proteins (at 100% identity), which has 116 proteins, and used that set for phylogenetic analysis. This procedure was also carried out for each species in the phylogenetic analyses. Each gene was annotated as TgNAC0XX, where Tg refers to the initials of *Tectona grandis* and XX refers to the position of the gene in the chromosomes. Detailed information of teak's NAC family genes, including gene locus, given code, accession numbers, and similarities to their Arabidopsis orthologues, is listed in Additional file 2.

NAC TF genes have been identified in all analyzed land plants, but so far, they have not been found in eukaryotic algae species [14] (Additional file 7). The NAC multigene family is highly variable among plant species. Presumably, NAC TFs first appeared in bryophytes such as *Physcomitrella patens* and, most likely, they were responsible for the expansion of the NAC genes named VNS (VND-NST/SND-SMB-related proteins) in land plants [13,57]. *Populus* (Rosidae) contains large numbers of NAC genes in a relatively small genome size compared to *Eucalyptus grandis* and *Glycine max*, both containing large numbers of NAC genes in larger genome sizes. Expansion of NAC genes in various plant species is probably the result of multiple events of gene duplication. In the *Populus* lineage, for example, whole genome duplication and multiple segmental and tandem duplication events may have contributed to the expansion of the NAC family [40]. As for *T. grandis*, the small number of duplication events could be one of the reasons for the relatively small number of identified NAC genes (107). As depicted in Additional file 8, only 36 gene duplication events were inferred for *T. grandis*, whereas they were 77 in Arabidopsis, 114 in Eucalyptus, and 98 in *Populus*. It is also curious that only 31 NAC genes were found in the coniferous *Pinus taeda*, a species with a large genome, possibly due to the high content of pseudogenes in conifer genomes [58]. Besides, only two genes of the VASCULAR NAC DOMAIN (VND) family were identified in *Pinus taeda*. This finding could be interpreted as evidence of the importance of co-option and expansion of the VND gene family during the evolution of angiosperms [59].

2.3.2. Phylogenetic Analysis of the NAC Gene Family

Evolutionary relationships were examined among non-redundant NAC protein sequences of *T. grandis* (116), *Populus trichocarpa* (196), *Eucalyptus grandis* (190), and *Arabidopsis thaliana* (153). A rooted tree was constructed and

maximum-likelihood algorithm separated the NAC family proteins into 23 distinct clades (NAC-a–NAC-w), as represented in Figure 3. *T. grandis* NAC proteins were identified in 14 of the 23 clades (NAC-h–NAC-s, NAC-u, and NAC-w).

Figure 3 Maximum-likelihood phylogenetic tree rooted of NAC domain proteins of *T. grandis*, *Populus*, *Arabidopsis*, and *Eucalyptus*. The NAC family proteins were clustered in 23 distinct clades (NAC-a–NAC-w). Members of *T. grandis* (116), *Populus* (196), *Arabidopsis* (153), and *Eucalyptus* (190) NAC protein family were denoted with green, brown, black, and blue letters, respectively. Bootstrap values are described in the branch points.

Clade NAC-s clustered the largest group of conserved genes that consisted of *T. grandis* (16 genes), *Eucalyptus* (19 genes), *Populus* (34 genes), and *Arabidopsis* (34 genes) sequences (Figure 3). Clades NAC-d, -e, and -f comprised exclusively *Populus* NAC genes and that is consistent with the evidence of major gene duplication events in this species [40]. In general, NAC members were interspersed among the majority of the clades, which indicates expansion of NAC genes previously to the evolutionary divergence of *T. grandis*, *Populus*, *Arabidopsis*, and *Eucalyptus*.

Noticeably, NAC genes with the same functions tended to fall into a same clade, as previously reported [60]. For instance, clade NAC-p grouped SCW biosynthesis genes involved in xylem vessels biosynthesis (*VND4* (*Tg15g08390*, *Tg16g07170*), *VND1* (*Tg09g04510*, *TgUn296g00020*), *VND7* (*Tg03g10560*, *Tg03g10970*, *Tg18g00740*)) and

in biosynthesis of fibers (*NSTI* (*Tg05g19210*, *Tg05g19290*, *Tg11g07410*) and *SMB* (*Tg02g10510*)). As for VNI genes, both VNI1 (*Tg09g02550*) and VNI2 (*Tg10g07670*, *Tg11g14670*, *Tg16g09090*, *Tg15g04300*, *Tg16g09080*) genes were located in clade NAC-n, with the exception of *Tg12g02970* (VNI2), that was placed in clade NAC-w. The SND2 genes (*Tg02g15550*, *Tg17g02630*) were clustered in clade NAC-q (Figure 3).

2.3.3. Gene Structure and Conserved Motifs of Teak NAC Genes

Variations in gene and protein structure are the basis of evolution in multigene families [61]. A structural analysis of teak NAC genes and proteins was performed in order to gain information on structural diversity among them. In the phylogenetic tree, the analyzed proteins were clustered according to the presence of conserved motifs, as shown in Figure 4A. The color of protein names refers to the clades in Figure 3. Exon–intron structure of TgNAC coding sequences was also analyzed individually (Figure 4B). NAC members of a same clade shared similar gene length and exon–intron structure.

In total, 10 divergent motifs were localized and named as motifs 1–10 (Figure 4C, Additional file 3). The motifs 1–3 and 5–7 were recognized as NAC subdomains in *Eucalyptus*, as described previously [5,62]. Although motifs 4 and 8 have already been described in *Eucalyptus* [5] and *Populus* [6], respectively, they were not recognized as NAC. The motifs 9 and 10 were not described until this study. As expected, most phylogeny-based groups displayed common motifs with the same alignment and position. Thus, NAC proteins with similar gene structures and motifs tended to cluster in the same clade. Clade NAC-q contains the proteins VNS, VNDs (VND1, VND4, and VND7), NST1, and SMB that have very conserved structures (Figure 4A,C). The gene SMB is among the groups of NAC genes present in early land plants, such as the bryophyte *Physcomitrella patens* [63]. Proteins that were clustered in the clades NAC-o, NAC-s, and NAC-q also shared high similarity in their structures, as shown in Figure 4C. The shortest teak TgNAC gene (*TgUn720g00010*) is 544 bp long, and the two longest genes (*Tg12g03010* and *Tg09g02550*) are 6 kb in size. Most *T. grandis* genes contained introns in their sequences, except for *Tg10g05820*, *Tg11g02730*, *Tg12g02970*, and *TgUn296g00020* (Figure 4B). The subcellular localization of the identified NAC proteins was predicted by using the online analysis tools Molecular Bioinformatics Center v2.5 (<http://cello.life.nctu.edu.tw/>) and BUSCA (<http://busca.biocomp.unibo.it>). *T. grandis* NAC proteins were located in the nucleus, cytoplasm, chloroplast, plasma membrane, endomembrane system, and mitochondria. Such subcellular localization is similar to those of various plant species, such as *Zea mays* and *Cucumis sativus*, in which NAC TFs are also located in diverse cell compartments (Additional file 4) [64,65].

Figure 4 Phylogenetic relationship, gene structure, and motif composition of *T. grandis* NAC genes. (A) Maximum-likelihood phylogenetic tree rooted of 116 teak NAC proteins was constructed using Q-Tree v1.6.9 and ultrafast bootstrap, with 1000 replicates. (B) Exon-intron structure represented in kilobases (Kb) scale of 107 TgNAC genes. (C) Schematic representation of the conserved motifs in the NAC proteins from teak. Green boxes and black lines represent exons and introns, respectively.

2.3.4. Chromosomal Location

Of the 18 teak chromosomes, 17 were generated as near-complete pseudomolecules, and one of them as two chromosome arm scaffolds [38]. Integrative Genomics Viewer (IGV v2.7.0) was used for assembling the 18 chromosomes (Chr) and to localize the 107 NAC genes, as represented in Figure 5. Three of the 107 genes (TgUn272g00030, TgUn296g00020, TgUn720g00010), identified by using the published *T. grandis* genome [38], were located in scaffolds that have not been placed within a chromosome; therefore, they were represented in a box at the bottom right-hand corner of Figure 5. The largest number of TgNAC genes was located on Chr 11 and consisted of 11 TgNAC genes. Other TgNAC genes were distributed among 16 of the remaining chromosomes, as follows: 9 on Chr2, 8 on Chr3, 7 on chromosomes 1, 6, 12, and 15, and in lower numbers among the other teak chromosomes. No

NAC gene was identified on Chr4. Likewise, unequal distributions of NAC TF genes across chromosomes and highest gene density in distal regions have been reported in barley and wheat [66,67].

Figure 5 Location and distribution of 107 TgNAC genes on 18 *T. grandis* chromosomes. The box at the bottom right-hand side represents three TgNAC genes that were not placed within any of the chromosomes and were present in orphan scaffolds. TgNAC genes were mapped according to their genomic position in the pseudomolecules (Additional file5). The scale on the left-hand side is represented by megabit (Mb).

Many of the teak NAC genes were located adjacent to each other (less than 10,000 bp apart), as, for instance, Tg01g08840 and Tg01g08850 on Chr1, Tg03g17880 and Tg03g17890 on Chr3, Tg06g07740 and Tg06g07750 on Chr6, Tg07g01830 and Tg07g01840 on Chr7, Tg12g02960 and Tg12g02970 on Chr12, and Tg16g09080 and Tg16g09090 on Chr16 (Figure 5, Additional file 5). Most likely, these closely adjacent genes could have resulted from duplication events. In wheat, single loci are found more frequently in proximal regions of the chromosomes, while gene duplications often occur in the distal regions [67].

2.3.5. In Silico Gene Expression Profiles of Teak NAC Genes

Expression profiles of 107 TgNAC genes were assessed in order to gain insights into their transcript accumulations. Transcript abundance analyses were carried out for diverse tissues of teak, including primary (leaves, flowers, roots, seedling) and secondary (branch and stem secondary xylem of 12- and 60-year-old plants) tissues [36]. Of the 107 analyzed TgNAC genes, 94 were expressed in the tissues and conditions represented in Figure 6. The heatmap based on hierarchical clustering of expression levels revealed 10 putative NAC groups (A–J).

Figure 6 Hierarchical clustering of expression profiles of TgNAC genes in diverse *T. grandis* tissues. The 94 genes were clustered into 10 groups (A–J). Plant tissues (branch of 12- and 60-year-old trees; stem of 12- and 60-year-old trees, flowers, leaves, roots, and seedlings) were clustered according to the type tissue. The color scale on the left-hand side represents transcript per million (TPM). Gene expression values for each gene were transformed using Z-scores. Higher expression levels are represented in red, and lower expression levels are denoted in blue (Additional file 6). The TgNAC TF genes marked in red were selected for expression analysis.

Transcript abundance of the teak NAC genes in group A (*Tg09g02930-XND1*, *Tg09g02550-VNI1*, and *Tg15g04300-VNI2*) was higher in secondary tissues. These TgNAC genes are homologous to Arabidopsis NAC genes involved in the SCW biosynthesis (Figure 6). In group F, TgNAC genes were preferentially transcribed in branches of 12-year-old trees. Only the genes *Tg01g12810-ANAC075* and *Tg02g05550-SND2* were homologous to the Arabidopsis NAC genes recognized to be involved in secondary tissue formation. As for group G, there are 13 NAC genes highly expressed in secondary tissues, from which only two (*Tg0519210-NST1* and *Tg02g15550-SND2*) are homologous to Arabidopsis genes involved in SCW formation. Most genes in the remaining groups were preferentially expressed in primary tissues (Figure 6). These data reveal several *T. grandis* NAC genes that seem to be involved in secondary growth and that will be better characterized and understood in further studies.

In Figure 6, the NAC genes marked in red were selected for qRT-PCR expression analysis due to their homology to Arabidopsis NAC genes involved in SCW formation. They are (*VND1-Tg09g04510*; *VND2-Tg11g04450*; *VND4-Tg15g08390*; *VND4-Tg15g11670*; *VND4-Tg16g07170*; *VND7-Tg03g10970*; *NST1-Tg05g19210*; *VNI2-Tg15g04300*; *XND1-Tg08g13700*; *SND2-Tg02g15550*; *ANAC075-Tg01g12810*; *ANAC33-Tg02g10510*; and *ANAC70-Tg03g13770*). Several of these genes showed high transcript abundance in teak primary tissues (roots, leaves, and flowers), suggesting function divergence from the split between both lineages.

2.3.6. Expression Analysis of Marker Genes for Secondary Cell Wall Formation at Different Tree Ages

The expression pattern of teak NAC genes that are homologous to Arabidopsis genes involved in SCW biosynthesis was analyzed to verify their functionality during teak wood formation in tissues of trees of different ages. Tissue samples were analyzed by quantitative real-time RT-PCR (RT-qPCR) in order to verify the expression of 13 selected TgNAC TF genes in stem from 4-year-old teak plants and in sapwood of 16- and 64-year-old teak trees (Figure 6). These 13 genes were selected based on their regulatory activity in the SCW formation pathway and programmed cell death, both important events during wood formation (Figure 7) [68].

Figure 7 Expression levels of selected TgNAC genes using RT-qPCR. The relative expression of 13 selected NAC genes of *T. grandis* was normalized to the reference gene TgEF-1 α in stem of 4-year-old trees and sapwood of 16- and 64-year-old trees. Bars represent standard errors (SEs) of three biological replicates and two technical replicates. The p-values are shown, and different letters represent statistically different means. Relative expression of the genes: (a) VND1–Tg09g04510, (b) VND2–Tg11g04450, (c) VND4–Tg15g08390, (d) VND4–Tg15g11670, (e) VND4–Tg16g07170, (f) VND7–Tg03g10970, (g) NST1–Tg05g19210, (h) VNI2–Tg15g04300, (i) XND1–Tg08g13700, (j) SND2–Tg02g15550, (k) ANAC075–Tg01g12810, (l) ANAC33–Tg02g10510, (m) ANAC70–Tg03g13770.

The process of wood formation comprises a series of consecutive events, starting with cell division in the vascular cambium and then followed by cell expansion and secondary cell wall deposition. The wood formation process ends in programmed cell death and heartwood formation [68]. Several transcription factors regulate the entire wood formation process, mainly the ones of the NAC and MYB families [10]. Among the NAC transcription factors regulating SCW deposition, VASCULAR-RELATED NAC-DOMAIN1-7 (VND1–VND7 proteins) are the main regulators of xylem vessel cell differentiation (Figure 7) [69]. In the present work, we analyzed the expression of four

In plant cells, the majority of NAC TFs are transcriptional activators, although transcriptional repressors are also present in the NAC gene family [74]. VND-INTERACTING2 (VNI2) is described in Arabidopsis as a transcriptional repressor that regulates differentiation of xylem cells by interacting with VND proteins and possibly with other NAC domain proteins [34]. We analyzed the expression of the *T. grandis* *Tg15g04300* gene, which is homologous to the *VNI2* in Arabidopsis (Figure 7h). There were no significant differences in the *Tg15g04300*

expression among the teak trees of different ages. The VNI2 transcription factor represses the expression of genes regulated by VND7, which is a master regulator of xylem vessel differentiation [34]. In the analysis, also the expression of VND7 homologous (*Tg03g10970*) was not altered during teak developmental stages, which indicates that perhaps VNI2 does not act as a repressor, or the repression may be occurring in all the teak stages analyzed.

Another repressor of secondary cell wall deposition is the XYLEM NAC DOMAIN1 (XND1) transcription factor (Figure 7). This gene is highly expressed in xylem and inhibits secondary cell wall deposition and autolysis in xylem vessels [75]. XND1 negatively regulates expression of genes involved in both programmed cell death and lignocellulose synthesis during secondary cell wall formation [29,76]. Interestingly, the expression of the teak repressor *Tg08g13700—XND1* (Figure 7i) was low in 4- and 16-year-old teak plants, but it was significantly higher in 64-year-old trees. These results corroborate the hypothesis that the secondary cell wall deposition is lower in older trees than in faster-growing younger trees. Apparently, the XND1 molecular mechanism of action is not related to VND genes [77]. The NAC protein named SECONDARY WALL-ASSOCIATED NAC DOMAIN2 (SND2) is involved in the regulation of cellulose and hemicellulose biosynthesis (Figure 8). SND2 seems to occupy a subordinate place in the central layer of the transcriptional network of secondary cell wall formation [78]. There was no significant difference among the teak plants analyzed for the expression of the *Tg02g15550-SND2* gene (Figure 7j) among the teak plants analyzed, which could be acting in a secondary role in the secondary cell wall formation [78].

Another NAC transcription factor, the ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 75 (ANAC075) is also involved in secondary cell wall biosynthesis and it is an upstream regulator of VND7 [75]. ANAC075 overexpression induced ectopic secondary cell wall formation in Arabidopsis tissues through increasing expression of VND7 [75,79]. Similarly, in *Populus*, higher expression of PNAC127 (homologous of ANAC075) was observed in differentiating xylem [6]. No difference in the expression of the teak *ANAC075* homolog *Tg01g12810* (Figure 7k) was observed in our analysis. Probably, the other NAC transcription factors have a stronger influence than *Tg01g12810* during the SCW formation in the *T. grandis* tissues analyzed.

We also analyzed TFs ANAC033 and ANAC070 that are known to regulate programmed cell death, the last step of secondary growth. The highest expression of the *ANAC070* teak homologue (*Tg03g13770*) was observed in 16- and 64-year-old trees (Figure 7m). This result indicates that this gene might have a key function in programmed cell death of *T. grandis* secondary tissues. While, the evaluation of *ANAC033* (*Tg02g10510*) homologue expression, showed no statistically significant difference between the three ages analyzed (Figure 7l). Apparently, these two Arabidopsis homologous proteins have a more prominent role in regulating lateral root cap formation than in regulation of secondary cell wall deposition [80].

2.4. Conclusions

Here we presented a genome-wide analysis of *T. grandis* NAC domain genes with identification and characterization of NAC transcriptional factors homologous to Arabidopsis and that are related to SCW formation. Analysis of teak NAC TFs encoding genes identified 130 NAC proteins, from which 116 represented non-redundant proteins coded by 107 loci. These identified 116 *T. grandis* NAC proteins were phylogenetically clustered into 23 distinct clades in evolutionary relationships with Arabidopsis, *Populus*, and *Eucalyptus* trees. Many of the TgNAC genes were located adjacent to each other, indicating that they might have resulted from duplication events. Most genes in groups A and F were preferentially expressed in secondary tissues, where they presented higher transcript abundance. As for the remaining groups, most NAC genes were highly expressed in flowers, roots, leaves, and seedlings. Considering the

regulation function of the NAC genes in SCW biosynthesis, 13 NAC genes were analyzed for their expression in various tissues of three developmental stages of teak trees. Data from RT-qPCR revealed highest expression of VND genes *VND2-Tg11g04450* and *VND4-Tg15g08390* in tissues of 16-year-old trees. VND TFs are related to xylem vessels formation, indicating that these two VND genes (*Tg11g04450* and *Tg15g08390*) possibly participate in the regulation of *T. grandis* SCW deposition. In addition, the NAC homologous repressor XND1 (*Tg08g13700*) presented highest expression in 64-year-old tissues and gave support to the hypothesis of lower secondary cell wall deposition in older trees. Data reported in the present work contribute to the understanding of structure and functionality of NAC transcription factor genes in *T. grandis* and they are potentially useful for future studies aiming to improve biomass and wood quality using biotechnological approaches.

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Additional files

Additional file 1. Primers used for gene expression analysis by RT-qPCR.

Gene	Reference Number	Primers
<i>ANAC075</i> ^a	Tg01g12810	FW - 5' TCCACGTCCCAAGAACCATG 3' RV - 5' GCAGCCCATGATGACTTCCT 3'
<i>ANAC33</i> ^b	Tg02g10510	FW - 5' ACGGGCAGAAGACTGATTTGG 3' RV - 5' TGGAAAGGTACAACGGGCTC 3'
<i>SND2</i> ^c	Tg02g15550	FW - 5' CATCTCCGCTGATCCCGAAT 3' RV - 5' ATTCCTCGTCGCTGCTAACC 3'
<i>VND7</i> ^d	Tg03g10970	FW - 5' CCCACAATGATGACTTAGATGCTC 3' RV - 5' CGTTCCAACCTTGGAGAAGGA 3'
<i>ANAC70</i> ^e	Tg03g13770	FW - 5' TGAGAGTGCAAGTCCAGCAG 3' RV - 5' ACAAGGCGATCCCATTTCGTT 3'
<i>NST1</i> ^f	Tg05g19210	FW - 5' CCACAGGGACACGCACTAAT 3' RV - 5' GGGGCACGACCTTTGTAGAA 3'
<i>XND1</i> ^g	Tg08g13700	FW - 5' GCCATCCAGATGTCATCCCA 3' RV - 5' TCGACCCCTATAGTCTGCCA 3'
<i>VND1</i> ^h	Tg09g04510	FW - 5' ATCCCACAACCTGGTGTGCA 3' RV - 5' CCTTTTCGTTGGTGGCATGG 3'
<i>VND2</i> ⁱ	Tg11g04450	FW - 5' CGTTCAATCTATGATGCCACTGG 3' RV - 5' AAGTGTACCTGTTCTCCTCCCC 3'
<i>VNI2</i> ^j	Tg15g04300	FW - 5' CCCCTTGCCAGCTTCCATAA 3' RV - 5' TGTTGCCTTCCAGTAGCCAG 3'
<i>VND4</i> ^k	Tg15g11670	FW - 5' TTCTTCCACAATTCGACGG 3' RV - 5' GCCAAACAGTCCTCTTCCAT 3'
<i>VND4</i> ^l	Tg15g08390	FW - 5' GCAATTTGCAGTCATCAGGA 3' RV - 5' GGGATGCCACAAACTTGTCT 3'
<i>VND4</i> ^m	Tg16g07170	FW - 5' CGAGCCCCAAATGGACAGAA 3' RV - 5' TCCTTGTGGTTGCCATCCTC 3'

^aARABIDOPSIS NAC-DOMAIN CONTAINING PROTEIN 75, ^bARABIDOPSIS NAC-DOMAIN CONTAINING PROTEIN 33, ^cSECUNDARY WALL-ASSOCIATED NAC DOMAIN 2, ^dVASCULAR-RELATED NAC-DOMAIN 7, ^eARABIDOPSIS NAC-DOMAIN CONTAINING PROTEIN 70, ^fNAC SECUNDARY WALL THICKENING PROMOTING FACTOR, ^gXYLEM NAC DOMAIN 1, ^hVASCULAR-RELATED NAC-DOMAIN 1, ⁱVASCULAR-RELATED NAC-DOMAIN 2, ^jVDN-INTERACTING 2, ^kVASCULAR-RELATED NAC-DOMAIN 4, ^lVASCULAR-RELATED NAC-DOMAIN 4, ^mVASCULAR-RELATED NAC-DOMAIN 4.

Additional file 2. Teak NAC gene family.

Gene locus	Gene Code	Arabidopsis locus description	Arabidopsis ortholog locus	Score	E-value
Tg01g00150	TGNAC001	ANAC071	AT4G17980.1	288	2E-32
Tg01g01920	TGNAC002	ANAC071	AT4G17980.1	301	2E-34
Tg01g08840	TGNAC003	ANAC087	AT5G18270.1	702	7E-91
Tg01g08850	TGNAC004	ANAC087	AT5G18270.1	704	1E-91
Tg01g11480	TGNAC005	ANA072, RD26	AT4G27410.2	460	6E-55
Tg01g12810.t1	TGNAC006	ANAC075	AT4G29230.1	1205	1E-164
Tg01g12810.t3	TGNAC007	ANAC075	AT4G29230.1	1181	1E-160
Tg01g17760	TGNAC008	ANAC090	AT5G22380.1	606	3E-80
Tg02g02730	TGNAC009	ANAC087	AT5G18270.1	746	4E-98
Tg02g05550	TGNAC010	ANAC075	AT4G29230.1	1309	1E-179
Tg02g09900	TGNAC011	ANAC042	AT2G43000.1	686	7E-91
Tg02g09660	TGNAC012	ANAC021	AT1G56010.2	762	1E-101
Tg02g10410	TGNAC013	ANAC020	AT1G54330.1	750	1E-100
Tg02g10510	TGNAC014	ANAC033	AT1G79580.1	706	2E-92
Tg02g14690	TGNAC015	ANAC025	AT1G61110.1	729	8E-97
Tg02g15550	TGNAC016	ANAC073, SND2	AT4G28500.1	890	1E-121
Tg02g15570	TGNAC017	ANAC029, NAC-like, activated by AP3/PI	AT1G69490.1	309	2E-36
Tg03g08530	TGNAC018	ANAC002, ATAF1	AT1G01720.1	933	1E-128
Tg03g10560	TGNAC019	ANAC030, VND7	AT1G71930.1	726	4E-96
Tg03g10970	TGNAC020	ANAC030, VND7	AT1G71930.1	726	4E-96
Tg03g13770	TGNAC021	ANAC070	AT4G10350.1	807	1E-107
Tg03g18100	TGNAC022	ANAC020	AT1G54330.1	199	6E-20
Tg03g17880	TGNAC023	ANAC101, VND6	AT5G62380.1	207	9E-20
Tg03g17890	TGNAC024	ANAC032	AT1G77450.1	161	2E-14
Tg03g18120	TGNAC025	ANAC102	AT5G63790.1	199	7E-19
Tg05g04990	TGNAC026	ANAC038	AT2G24430.2	772	1E-103
Tg05g10730	TGNAC027	ANAC074	AT4G28530.1	685	6E-90
Tg05g12200	TGNAC028	ANAC035	AT2G02450.2	855	1E-113
Tg05g19210	TGNAC029	ANAC043, NSTI	AT2G46770.1	896	1E-120
Tg05g19290	TGNAC030	ANAC043, NSTI	AT2G46770.1	889	1E-119
Tg06g07710	TGNAC031	ANAC002, ATAF1	AT1G01720.1	432	1E-50
Tg06g07670	TGNAC032	ANAC072, RD26	AT4G27410.2	432	1E-52
Tg06g07740	TGNAC033	ANAC072	AT4G27410.2	228	4E-23
Tg06g07750	TGNAC034	ANAC019	AT1G52890.1	216	9E-22
Tg06g11580	TGNAC035	ANAC035	AT2G02450.2	849	1E-112
Tg06g16210	TGNAC036	ANAC041	AT2G33480.1	407	7E-50
Tg06g17630	TGNAC037	ANAC014	AT5G24590.2	699	1E-86
Tg07g01830	TGNAC038	ANAC052	AT3G10490.2	742	1E-95

Tg07g01840	TGNAC039	ANAC053	AT3G10500.1	913	1E-117
Tg07g09430	TGNAC040	ANAC002, ATAF1	AT1G01720.1	691	2E-92
Tg07g12160	TGNAC041	ANAC028	AT1G65910.1	1187	1E-157
Tg08g06610.t1	TGNAC042	ANAC017	AT1G34190.1	1059	1E-139
Tg08g06610.t2	TGNAC043	ANAC017	AT1G34190.1	332.8	5.6E-107
Tg08g06620	TGNAC044	ANAC017	AT1G34190.1	1077	1E-142
Tg08g07120	TGNAC045	ANAC003	AT1G76420.1	768	1E-101
Tg08g13700	TGNAC046	ANAC104, XND1	AT5G64530.1	518	1E-68
Tg08g17690	TGNAC047	ANAC036	AT2G17040.1	680	5E-91
Tg09g04510	TGNAC048	ANAC037, VND1	AT2G18060.1	1072	1E-147
Tg09g02550.t1	TGNAC049	ANAC082, VNI1	AT5G09330.3	692	1E-85
Tg09g02550.t2	TGNAC050	ANAC082, VNI1	AT5G09330.3	692	4E-87
Tg09g02550.t6	TGNAC051	ANAC082, VNI1	AT5G09330.3	693	5E-87
Tg09g02930	TGNAC052	ANAC104, XND1	AT5G64530.1	426	2E-54
Tg09g03360	TGNAC053	ANAC002, ATAF1	AT1G01720.1	908	1E-124
Tg09g15630	TGNAC054	ANAC009	AT1G26870.1	785	1E-102
Tg09g15920	TGNAC055	ANAC100	AT5G61430.1	879	1E-118
Tg10g01380	TGNAC056	ANAC057	AT3G17730.1	847	1E-117
Tg10g05660	TGNAC057	ANAC100	AT5G61430.1	930	1E-126
Tg10g05820	TGNAC058	ANAC060	AT3G44290.1	162	7E-14
Tg10g06080	TGNAC059	ANAC032	AT1G77450.1	127	2E-09
Tg10g07670	TGNAC060	ANAC083, VNI2	AT5G13180.1	456	1E-57
Tg11g07410	TGNAC061	ANAC043 NSTI	AT2G46770.1	808	1E-107
Tg11g02730	TGNAC062	ANAC033	AT1G79580.1	98	0,000007
Tg11g02740	TGNAC063	ANAC052	AT3G10490.2	86	0,00001
Tg11g03710	TGNAC064	ANAC100	AT5G61430.1	939	1E-128
Tg11g04450	TGNAC065	ANAC076, VND2	AT4G36160.1	166	1E-14
Tg11g05530	TGNAC066	ANAC058	AT3G18400.1	775	1E-103
Tg11g07580	TGNAC067	NAC TRANSCRIPTION FACTOR-LIKE 9, NTL9	AT4G35580.2	652	9E-85
Tg11g10750	TGNAC068	ANAC008	AT1G25580.1	1237	1E-170
Tg11g12110	TGNAC069	ANAC009	AT1G26870.1	805	1E-105
Tg11g11960	TGNAC070	ANAC029, NAC-like, activated by AP3/PI	AT1G69490.1	789	1E-107
Tg11g14670	TGNAC071	ANAC083, VNI2	AT5G13180.1	593	3E-78
Tg12g02960	TGNAC072	ANAC083, VNI2	AT5G13180.1	169	4E-15
Tg12g02970	TGNAC073	ANAC083, VNI2	AT3G10480.2	184	2E-16
Tg12g03010	TGNAC074	ANAC050	AT3G10480.2	747	5E-96
Tg12g03030	TGNAC075	ANAC053	AT3G10500.1	1027	1E-135
Tg12g08930.t1	TGNAC076	ANAC104, XND1	AT5G64530.1	486	9E-64
Tg12g08930.t3	TGNAC077	ANAC104, XND1	AT5G64530.1	319	6E-39
Tg12g10590	TGNAC078	ANAC054/CUC1	AT3G15170.1	106	0,00007
Tg12g17130.t1	TGNAC079	ANAC036	AT2G17040.1	773	1E-104

Tg12g17130.t2	TGNAC080	ANAC036	AT2G17040.1	582	9E-76
Tg13g00820.t1	TGNAC081	ANAC042	AT2G43000.1	649	1E-85
Tg13g00820.t2	TGNAC082	ANAC042	AT2G43000.1	433	3E-54
Tg13g06720	TGNAC083	ANAC098, CUC2	AT5G53950.1	808	1E-107
Tg13g07150.t1	TGNAC084	ANAC072, RD26	AT4G27410.2	959	1E-131
Tg13g07150.t2	TGNAC085	ANAC072, RD26	AT4G27410.2	909	1E-123
Tg13g09540	TGNAC086	ANAC090	AT5G22380.1	568	2E-74
Tg13g09550	TGNAC087	ANAC090	AT5G22380.1	576	1E-75
Tg13g12990	TGNAC088	ANAC081	AT5G08790.1	346	2E-41
Tg14g02070	TGNAC089	ANAC040	AT2G27300.1	595	6E-75
Tg14g02200	TGNAC090	ANAC090	AT5G22380.1	647	3E-87
Tg14g03970	TGNAC091	ANAC056	AT3G15510.1	324	7E-36
Tg14g11050.t1	TGNAC092	ANAC074	AT4G28530.1	711	2E-94
Tg14g11050.t2	TGNAC093	ANAC074	AT4G28530.1	714	2E-94
Tg15g01040	TGNAC094	ANAC008	AT1G25580.1	719	1E-91
Tg15g02070	TGNAC095	ANAC0100	AT5G61430.1	878	1E-118
Tg15g04300	TGNAC096	ANAC083, VNI2	AT5G13180.1	744	1E-101
Tg15g08390	TGNAC097	ANAC007, VND4	AT1G12260.1	211	5E-21
Tg15g10730	TGNAC098	ANAC009	AT1G26870.1	759	1E-98
Tg15g11030	TGNAC099	ANAC029, NAC-like, activated by AP3/PI	AT1G69490.1	830	1E-113
Tg15g11670	TGNAC100	ANAC007, VND4	AT1G12260.1	979	1E-133
Tg16g02600	TGNAC101	ANAC039	AT2G24430.2	640	3E-83
Tg16g07170	TGNAC102	ANAC007, VND4	AT1G12260.1	979	1E-133
Tg16g09080	TGNAC103	ANAC083, VNI2	AT5G13180.1	422	1E-52
Tg16g09090	TGNAC104	ANAC083, VNI2	AT5G13180.1	447	2E-56
Tg16g13420	TGNAC105	ANAC057	AT3G17730.1	589	6E-78
Tg17g02630	TGNAC106	ANAC073, SND2	AT4G28500.1	871	1E-118
Tg17g08390	TGNAC107	ANAC072, RD26	AT4G27410.2	899	1E-122
Tg17g09060	TGNAC108	ANAC098, CUC2	AT5G53950.1	697	4E-90
Tg17g08380	TGNAC109	ANAC056	AT3G15510.1	891	1E-120
Tg18g00740	TGNAC110	ANAC030, VND7	AT1G71930.1	780	1E-104
Tg18g04440	TGNAC111	ANAC041	AT2G33480.1	149	6E-12
Tg18g09420	TGNAC112	ANAC032	AT1G77450.1	503	1E-58
Tg18g11300.t1	TGNAC113	ANAC090	AT5G22380.1	573	3E-75
Tg18g11300.	TGNAC114	ANAC090	AT5G22380.1	425	1E-53
TgUn272g00030	TGNAC115	ANAC002, ATAF1	AT1G01720.1	939	1E-129
TgUn296g00020	TGNAC116	ANAC037, VND1	AT2G18060.1	253	3E-28
TgUn720g00010	TGNAC117	ANAC104, XND1	AT5G64530.1	366	2E-46

Additional file 3. Teak NAC protein motifs. Logos of 10 motifs identified in TgNAC proteins using MEME, the E-value, motif consensus, and name annotation based previously published work (* *Eucalyptus* [5] motif consensu and ** *Populus* [6] motif consensu) are presented.

Motif	E – value	Motif consensu	Name anotation
<p>Motif 1</p>	1.90e-136	motif3*	NAC subdomain C
		motif4*	NAC subdomain D
<p>Motif 2</p>	3.93e-146	motif1*	NAC subdomain A
<p>Motif 3</p>	2.21e-173	motif6*	NAC subdomain D
<p>Motif 4</p>	1.01e-281	Motif9*	–
Motif 5	5.16e-281	motif2*	NAC subdomain B

Additional file 4. Cellular localization of *Tectona grandis* NAC proteins.

Protein ID	Localization BUSCA	Localization CELLO
Tg01g00150	nucleus	nucleus
Tg01g01920	nucleus	nucleus
Tg01g08840	nucleus	nucleus
Tg01g08850	nucleus	nucleus
Tg01g11480	nucleus	nucleus
Tg01g12810	nucleus	nucleus
Tg01g17760	nucleus	nucleus
Tg02g02730	nucleus	nucleus
Tg02g05550	nucleus	nucleus
Tg02g09660	nucleus	nucleus
Tg02g09900	nucleus	nucleus
Tg02g10410	nucleus	nucleus
Tg02g10510	nucleus	cytoplasm
Tg02g14690	nucleus	nucleus
Tg02g15550	nucleus	nucleus
Tg02g15570	chloroplast	nucleus
Tg03g08530	nucleus	nucleus
Tg03g10560	nucleus	nucleus
Tg03g10970	nucleus	nucleus
Tg03g13770	nucleus	nucleus
Tg03g17880	nucleus	cytoplasm
Tg03g17890	nucleus	plasma membrane
Tg03g18100	chloroplast	chloroplast
Tg03g18120	nucleus	cytoplasm
Tg05g04990	nucleus	nucleus
Tg05g10730	nucleus	nucleus
Tg05g12200	nucleus	nucleus
Tg05g19210	nucleus	nucleus
Tg05g19290	nucleus	nucleus
Tg06g07670	nucleus	nucleus
Tg06g07710	nucleus	nucleus
Tg06g07740	nucleus	nucleus
Tg06g07750	nucleus	nucleus
Tg06g11580	nucleus	nucleus
Tg06g16210	nucleus	nucleus
Tg06g17630	endomembrane system	nucleus
Tg07g01830	nucleus	nucleus
Tg07g01840	endomembrane system	nucleus
Tg07g09430	nucleus	nucleus
Tg07g12160	endomembrane system	nucleus
Tg08g06610	endomembrane system	nucleus

Tg08g06620	endomembrane system	nucleus
Tg08g07120	nucleus	nucleus
Tg08g13700	nucleus	nucleus
Tg08g17690	nucleus	nucleus
Tg09g02550	nucleus	nucleus
Tg09g02930	nucleus	cytoplasm
Tg09g03360	nucleus	nucleus
Tg09g04510	nucleus	nucleus
Tg09g15630	nucleus	nucleus
Tg09g15920	nucleus	nucleus
Tg10g01380	nucleus	nucleus
Tg10g05660	nucleus	nucleus
Tg10g05820	nucleus	nucleus
Tg10g06080	nucleus	extracellular space
Tg10g07670	nucleus	nucleus
Tg11g02730	nucleus	cytoplasm
Tg11g02740	extracellular space	cytoplasm
Tg11g03710	nucleus	cytoplasm
Tg11g04450	nucleus	cytoplasm
Tg11g05530	nucleus	nucleus
Tg11g07410	nucleus	nucleus
Tg11g07580	nucleus	cytoplasm
Tg11g10750	nucleus	nucleus
Tg11g11960	Nucleus	nucleus
Tg11g12110	nucleus	nucleus
Tg11g14670	nucleus	nucleus
Tg12g02960	nucleus	nucleus
Tg12g02970	nucleus	nucleus
Tg12g03010	nucleus	nucleus
Tg12g03030	endomembrane system	nucleus
Tg12g08930	nucleus	nucleus
Tg12g10590	nucleus	nucleus
Tg12g17130	nucleus	cytoplasm
Tg13g00820	nucleus	nucleus
Tg13g06720	nucleus	nucleus
Tg13g07150	nucleus	nucleus
Tg13g09540	Nucleus	nucleus
Tg13g09550	Nucleus	nucleus
Tg13g12990	Nucleus	nucleus
Tg14g02070	plasma membrane	nucleus
Tg14g02200	Nucleus	cytoplasm
Tg14g03970	Nucleus	nucleus
Tg14g11050	Nucleus	cytoplasm
Tg15g01040	nucleus	nucleus

Tg15g02070	nucleus	nucleus
Tg15g04300	nucleus	nucleus
Tg15g08390	nucleus	nucleus
Tg15g10730	nucleus	nucleus
Tg15g11030	nucleus	nucleus
Tg15g11670	nucleus	nucleus
Tg16g02600	nucleus	nucleus
Tg16g07170	nucleus	nucleus
Tg16g09080	nucleus	nucleus
Tg16g09090	nucleus	nucleus
Tg16g13420	nucleus	nucleus
Tg17g02630	nucleus	nucleus
Tg17g08380	nucleus	nucleus
Tg17g08390	nucleus	nucleus
Tg17g09060	nucleus	plasma membrane
Tg18g00740	nucleus	nucleus
Tg18g04440	nucleus	nucleus
Tg18g09420	extracellular space	nucleus
Tg18g11300	nucleus	nucleus
TgUn296g00020	chloroplast	cytoplasm
TgUn272g00030	nucleus	nucleus
TgUn720g00010	nucleus	nucleus

Additional file 5. Genomic location of NACs in the genome of *Tectona grandis*.

Gene_ID	Pseudomolecule	Gene Start (pb)	Gene End (pb)
Tg01g00150	01	100651	102109
Tg01g01920	01	1272763	1274227
Tg01g08840	01	6650521	6652802
Tg01g08850	01	6655440	6657402
Tg01g11480	01	8960430	8962139
Tg01g12810	01	10560086	10565923
Tg01g17760	01	17473607	17475180
Tg02g02730	02	2368292	2370614
Tg02g05550	02	5861368	5866533
Tg02g09660	02	11444805	11448869
Tg02g09900	02	11642247	11644252
Tg02g10410	02	12125444	12127643
Tg02g10510	02	12212042	12216373
Tg02g14690	02	15067485	15068720
Tg02g15550	02	15691858	15695487
Tg02g15570	02	15707390	15708003
Tg03g08530	03	10338456	10340163
Tg03g10560	03	11813720	11815838
Tg03g10970	03	12085352	12087581
Tg03g13770	03	13974114	13977147
Tg03g17880	03	17073920	17075388
Tg03g17890	03	17083750	17089834
Tg03g18100	03	17311080	17312660
Tg03g18120	03	17324513	17325847
Tg05g04990	05	3563051	3567504
Tg05g10730	05	8509349	8512085
Tg05g12200	05	11165686	11168157
Tg05g19210	05	17430830	17432430
Tg05g19290	05	17510868	17512817
Tg06g07670	06	5426077	5427523
Tg06g07710	06	5461617	5463672
Tg06g07740	06	5481308	5482406
Tg06g07750	06	5484991	5486584
Tg06g11580	06	9451036	9453886
Tg06g16210	06	15389293	15391097
Tg06g17630	06	16308386	16311887
Tg07g01830	07	1417889	1422065
Tg07g01840	07	1426175	1430978
Tg07g09430	07	9986626	9988534
Tg07g12160	07	13653345	13657814
Tg08g06610	08	4671777	4675732

Tg08g06620	08	4676644	4679788
Tg08g07120	08	5045264	5048014
Tg08g13700	08	13606310	13607704
Tg08g17690	08	16640344	16641591
Tg09g02550	09	2108466	2115424
Tg09g02930	09	2532679	2534247
Tg09g03360	09	3008524	3010544
Tg09g04510	09	4929000	4931759
Tg09g15630	09	15581846	15583632
Tg09g15920	09	15875987	15877920
Tg10g01380	10	825541	828407
Tg10g05660	10	4133932	4135739
Tg10g05820	10	4296708	4297518
Tg10g06080	10	4539325	4547787
Tg10g07670	10	6369428	6370530
Tg11g02730	11	2364113	2364779
Tg11g02740	11	2369832	2370644
Tg11g03710	11	3287731	3289306
Tg11g04450	11	4346122	4347447
Tg11g05530	11	6258532	6261052
Tg11g07410	11	9693204	9695369
Tg11g07580	11	9919320	9922886
Tg11g10750	11	13053622	13058541
Tg11g11960	11	13937913	13939282
Tg11g12110	11	14064504	14066034
Tg11g14670	11	15902286	15903495
Tg12g02960	12	2124414	2125307
Tg12g02970	12	2127026	2127923
Tg12g03010	12	2159430	2165896
Tg12g03030	12	2169965	2174339
Tg12g08930	12	9005829	9007473
Tg12g10590	12	10658980	10659737
Tg12g17130	12	15364039	15365527
Tg13g00820	13	506214	507523
Tg13g06720	13	6415767	6418101
Tg13g07150	13	7167495	7169625
Tg13g09540	13	10758767	10760459
Tg13g09550	13	10784952	10787577
Tg13g12990	13	13515197	13517742
Tg14g02070	14	1278566	1284409
Tg14g02200	14	1361124	1362421
Tg14g03970	14	2885920	2887672
Tg14g11050	14	10646580	10650310
Tg15g01040	15	920180	923969

Tg15g02070	15	2810419	2812071
Tg15g04300	15	6384214	6385961
Tg15g08390	15	9676357	9680089
Tg15g10730	15	11502116	11503715
Tg15g11030	15	11702280	11703828
Tg15g11670	15	12098199	12100357
Tg16g02600	16	1973134	1976925
Tg16g07170	16	6673219	6677803
Tg16g09080	16	10385023	10386605
Tg16g09090	16	10395308	10397373
Tg16g13420	16	14015643	14018968
Tg17g02630	17	2124067	2126017
Tg17g08380	17	8935438	8937265
Tg17g08390	17	8955890	8957571
Tg17g09060	17	9535173	9536792
Tg18g00740	18	434746	436426
Tg18g04440	18	3341645	3345250
Tg18g09420	18	6931431	6933095
Tg18g11300	18	8596625	8598152
TgUn296g00020	Scaffold_Un296	36115	36486
TgUn272g00030	Scaffold_Un272	23446	25033
TgUn720g00010	Scaffold_Un720	19039	19583

Additional file 6. Transcript abundances of *Tectona grandis*

Gene_ID	Seedling s ^a	Roots b	Leaves ^c	Flower s ^d	Stem 12yr.1 ^e	Stem 12yr.2 ^f	Stem 60yr.1 g	Stem 60yr.2 h	Branc h 12yr.1 ⁱ	Branc h 12yr.2 ^j	Branc h 60yr.1 k	Branch 60yr.2 ^l
Tg09g02930	1.14	14.62	3.00	2.10	2.88	3.24	26.13	8.98	26.30	40.57	56.25	60.54
Tg09g02550	15.68	37.60	50.74	44.27	39.15	30.50	44.52	62.54	96.97	48.77	73.94	63.96
Tg15g04300	27.19	89.45	21.17	74.12	115.99	107.31	133.21	195.30	273.85	165.66	171.05	162.73
Tg16g02600	0.30	1.28	0.19	1.05	1.02	3.26	1.30	1.39	0.65	3.18	1.32	0.12
Tg06g07670	0.00	0.00	0.00	0.16	0.00	0.44	0.00	0.00	0.00	0.00	0.00	0.00
Tg06g11580	5.54	5.59	13.40	2.02	16.98	11.45	11.15	11.72	5.17	6.42	9.33	10.22
Tg14g02070	6.91	6.08	17.59	12.89	16.65	17.35	19.33	26.57	13.52	13.53	29.66	23.38
Tg13g00820	4.45	3.00	0.84	0.72	1.74	1.98	0.67	0.00	2.82	0.85	1.85	2.14
Tg13g09550	0.52	0.51	0.00	0.20	0.37	0.30	0.00	0.00	0.39	0.00	0.00	0.21
Tg02g14690	28.51	4.63	27.27	18.34	0.00	0.21	0.76	1.87	16.73	0.97	1.58	2.13
Tg01g17760	1.71	0.48	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg01g08840	0.59	0.22	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg02g15570	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg15g10730	0.19	1.78	0.60	0.14	0.88	0.18	0.20	1.10	1.62	0.19	0.99	0.69
Tg03g10970	1.00	3.73	3.28	0.66	0.00	0.00	0.00	0.00	0.51	0.00	0.00	0.00
Tg03g13770	0.35	2.96	1.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg12g17130	1.18	21.98	4.66	1.61	1.39	1.58	0.54	0.20	2.71	0.34	1.61	0.73
Tg05g04990	1.34	19.87	0.23	4.50	0.00	0.38	0.00	0.00	0.00	0.00	0.00	0.09
Tg08g13700	1.91	38.65	0.16	4.20	0.00	0.00	0.00	0.00	0.81	1.58	0.16	0.00
Tg11g02730	0.00	1.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg11g14670	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg01g08850	0.62	2.18	0.80	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.00
Tg09g15630	2.41	6.26	0.09	0.22	0.00	0.00	0.00	0.00	0.54	0.00	0.00	0.00
Tg02g10510	0.84	3.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg11g12110	0.17	0.76	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg11g05530	0.30	4.63	0.00	2.27	0.18	0.00	0.00	0.00	0.19	0.00	0.00	0.00
Tg11g04450	0.47	2.27	0.00	0.66	0.00	0.00	0.00	0.00	1.40	0.00	0.00	0.00
Tg02g02730	12.30	66.67	26.91	39.17	0.32	0.38	0.29	0.32	33.50	2.32	1.96	4.53
Tg14g11050	0.53	2.50	0.46	0.71	1.62	1.98	0.82	1.34	14.71	15.34	4.46	5.10
TgUn272g00030	0.76	0.54	0.00	0.00	0.25	0.21	1.13	0.00	2.13	1.85	0.00	0.13
Tg11g07580	9.67	18.14	15.66	23.50	14.74	20.90	29.76	14.71	87.53	19.41	27.98	27.64
Tg01g12810	0.64	2.50	4.57	2.88	1.01	0.20	1.77	2.48	23.14	2.52	4.37	6.31
Tg02g05550	0.31	1.82	1.98	2.97	0.20	0.08	1.86	0.60	9.05	0.79	1.43	2.46
Tg05g10730	5.88	3.58	0.59	3.67	0.95	1.70	1.76	0.24	13.45	2.91	0.25	0.28
Tg11g11960	53.41	57.75	10.13	21.68	0.88	0.00	1.20	2.84	296.03	3.42	4.49	5.63
Tg02g09660	26.62	23.09	4.39	53.19	0.37	0.18	0.00	0.00	169.37	0.39	2.22	3.04
Tg02g09900	5.91	12.81	3.11	1.00	0.35	0.99	0.65	0.00	21.72	0.15	0.57	0.75
Tg15g11030	8.85	112.13	2.99	6.82	16.74	23.20	27.62	7.59	248.02	56.38	11.24	7.56
Tg18g09420	8.12	20.44	36.26	28.97	7.26	5.31	9.81	12.54	30.17	10.72	14.60	13.61
Tg12g03030	34.60	66.63	70.97	67.51	20.75	20.03	25.90	22.35	67.57	20.67	25.46	27.54

Tg10g05660	10.62	48.13	22.42	39.82	0.15	0.48	0.84	0.30	87.41	0.39	0.67	0.66
Tg18g04440	13.25	45.66	27.07	24.54	2.14	1.25	4.39	6.94	52.08	6.22	4.80	6.72
Tg08g06620	9.27	15.04	12.16	14.47	5.90	6.76	9.33	11.38	20.15	13.32	9.65	10.84
Tg12g03010	47.12	60.89	61.95	55.07	25.81	21.35	34.92	48.40	108.10	62.95	36.70	31.55
Tg07g01830	5.55	10.16	3.77	10.84	4.79	3.61	6.04	7.19	8.18	10.39	5.54	5.56
Tg05g19210	0.00	0.20	0.00	0.60	0.00	0.00	0.00	0.00	0.45	0.00	0.00	0.00
Tg07g01840	12.28	45.52	17.02	51.50	6.43	5.72	6.95	7.36	52.87	4.95	9.01	9.70
Tg10g01380	0.87	0.99	1.09	2.89	0.00	0.00	0.00	0.00	1.57	1.25	0.35	0.38
Tg17g02630	1.18	1.52	2.35	17.84	0.18	0.00	0.67	0.18	5.20	0.47	0.10	0.45
Tg02g15550	0.82	5.06	3.10	29.42	0.45	0.24	2.62	5.16	13.37	4.10	5.50	6.73
Tg17g08380	1.47	2.98	0.77	73.93	0.91	0.87	2.88	2.01	42.10	1.10	2.41	2.11
Tg06g07710	0.00	0.00	0.15	0.09	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg05g12200	1.85	0.49	9.90	0.41	1.15	0.00	0.00	0.49	0.34	0.00	0.00	0.00
Tg18g11300	8.38	5.31	49.29	6.23	0.00	0.00	0.00	0.00	3.05	0.00	0.00	0.14
Tg13g09540	0.10	0.41	2.74	0.63	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg06g07740	0.00	0.16	0.62	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg09g03360	20.17	155.52	640.42	14.49	3.65	1.83	1.25	1.91	13.88	3.74	3.52	3.10
Tg14g02200	0.90	31.92	114.66	0.45	0.00	0.21	0.00	0.00	1.11	0.00	0.14	0.16
Tg06g17630	15.48	57.89	329.98	38.15	19.40	15.43	20.15	20.18	52.79	26.55	27.95	27.50
Tg08g17690	3.68	10.42	63.94	6.65	0.00	0.54	0.21	0.00	4.57	0.00	0.12	0.41
Tg07g09430	38.58	208.89	1.150.97	18.87	6.21	7.76	3.38	3.34	52.09	11.76	6.21	7.45
Tg08g06610	27.56	63.79	108.05	20.41	11.40	8.33	20.05	17.99	42.23	12.76	18.79	18.31
Tg09g04510	1.24	2.04	3.40	1.59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11
Tg03g08530	138.50	155.50	444.30	127.98	10.76	9.71	12.97	15.67	80.78	23.05	14.06	20.71
Tg14g03980	0.00	0.00	4.79	4.80	1.81	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg06g16210	9.88	13.62	31.56	14.82	0.43	0.17	0.79	1.30	22.27	2.03	11.19	15.95
Tg07g12160	0.72	0.43	1.62	0.50	0.00	0.00	0.00	0.00	1.56	0.12	0.67	0.42
Tg16g09090	11.40	2.79	26.90	6.19	0.00	0.00	0.00	0.00	26.80	0.43	3.75	4.25
Tg17g08390	42.81	44.32	266.29	72.71	1.72	2.10	3.88	5.35	148.93	11.15	15.37	19.90
Tg13g07150	46.39	13.61	310.16	36.67	21.62	18.72	25.92	40.52	187.71	52.85	53.62	57.24
Tg09g15920	35.20	16.60	18.58	71.84	10.58	10.36	20.71	44.33	32.06	56.84	54.08	62.53
Tg16g09080	1.85	2.67	2.24	3.42	0.00	0.00	0.00	0.00	1.61	0.68	1.39	5.07
Tg16g07170	1.40	2.56	3.33	3.26	0.00	0.00	0.00	0.00	0.20	0.16	0.00	0.47
Tg10g07670	1.18	1.94	1.51	2.97	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg15g08390	1.27	1.48	1.82	2.79	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08
Tg18g00740	0.40	1.28	0.75	2.09	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00
Tg11g03710	1.81	4.75	1.25	7.62	0.00	0.00	0.00	0.18	2.10	0.00	0.10	0.23
Tg03g10560	0.11	0.47	0.00	0.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg11g10750	15.08	29.03	10.07	28.36	3.53	2.23	5.07	7.62	5.67	3.95	4.14	5.87
Tg08g07120	0.08	0.00	0.21	1.73	0.42	0.00	0.39	0.00	0.00	0.00	0.11	0.00
TgUn720g00010	0.00	0.00	0.00	14.01	2.03	0.00	1.59	0.00	0.00	0.00	0.00	0.00
Tg17g09060	0.18	0.48	0.11	4.81	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.29
Tg15g11670	0.00	0.00	0.00	0.71	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg12g02970	0.00	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Tg01g11480	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
g10g06080	0.00	0.00	0.00	1.37	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg12g08930	0.33	3.83	0.80	45.32	1.61	1.15	0.38	0.41	0.43	0.35	0.92	0.76
Tg11g07410	0.71	2.30	0.32	41.64	0.00	0.14	0.00	0.00	1.25	0.00	0.26	0.70
Tg13g06720	0.85	2.86	0.29	14.12	0.00	0.00	0.68	0.48	0.26	0.00	0.00	0.00
Tg15g02070	1.15	4.34	1.11	16.57	0.87	1.53	0.80	0.69	2.19	2.87	1.07	1.32
Tg05g19290	0.00	0.75	0.00	2.49	0.00	0.14	0.00	0.00	0.19	0.00	0.00	0.00
Tg15g01040	0.41	0.09	0.17	0.54	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.42
Tg02g10410	1.24	0.23	0.11	1.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tg16g13420	0.13	0.00	0.00	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Sequence Read Archive (SRA) codigs of RNAseq samples used; ^aseedlings_SRR2080097; ^broots_SRR2080096; ^cleaves_SRR2080095; ^dflowers_SRR2080094; ^estem12yr1_SRR2080131; ^fstem12yr2_SRR2080132; ^gstem60yr1_SRR2080137; ^hstem60yr2_SRR2080138; ⁱbranch12yr1_SRR2080148; ^jbranch12yr2_SRR2080149; ^kbranch60yr1_SRR2080150; ^lbranch60yr2_SRR2080151.

Additional file 7. Phylogenetic profile of 28 plant species and numbers of identified NAC proteins. Ploidy level, the plant genome size (Gb), the number of chromosomes and total number of proteins identified in each genome. The genomes were annotated and assembled in Phytozome12 (<https://phytozome.jgi.doe.gov>). Subscripts on NAC proteins number refers to the Genome Reference of each species. The references are presented below.

Additional file 8. Phylogenetic and duplication events in Arabidopsis, *Eucalyptus*, *Populus*, and *T. grandis*.

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3. OVEREXPRESSION OF THE *TECTONA GRANDIS* *TGNAC01* REGULATES GROWTH, LEAF SENESCENCE AND CONFERS SALT STRESS TOLERANCE IN TRANSGENIC TOBACCO PLANTS.

ABSTRACT

NAC transcription factors play critical roles in xylem secondary development and in regulation of stress response in plants. Recently, *Tectona grandis* (teak) NAC proteins related to secondary cell wall development were characterized. However, any teak transcription factors have been studied by heterologous expression so far. We characterized the novel stress-responsive *TgNAC01* gene. The *TgNAC01* protein was shown to be localized in the nucleus by stable expression in *Nicotiana tabacum*. Three independent transgenic tobacco lines overexpressing of *TgNAC01* were generated (35S:*TgNAC01*-3, 35S:*TgNAC01*-4, 35S:*TgNAC01*-6). After two months of growth in the greenhouse, *TgNAC01* relative expression, stem length and chlorophyll content were evaluated in the transgenic lines. Tobacco plants overexpressing *TgNAC01* showed an increase in leaf senescence, growth and salt tolerance compared to wild type (WT) plants. The stress tolerance among the tobacco lines analyzed was proportional to the rate of *TgNAC01* expression. Water potential, gas exchange and chlorophyll fluorescence were used to determine the salt stress tolerance. The lines 35S:*TgNAC01*-3 and 35S:*TgNAC01*-6, under salt stress (300 mM NaCl), showed higher photosynthesis rate, stomatal conductance, transpiration, carboxylation efficiency, instantaneous water use efficiency and decrease in water potential, compared to WT plants. Our data demonstrate that *TgNAC01* transcription factor acts as a transcriptional activator of the stress-mediated regulation of leaf senescence.

Keywords: Secondary cell wall; Wood formation; Plant stress, Transcription factor

3.1. INTRODUCTION

The plant vascular system plays an important role in the adaptive mechanisms of land plants, providing mechanical support and transport of water, nutrients, hormones and other signaling molecules. These adaptive processes involve organization of tissues and cells led by signal molecules, such as auxins [1], cytokinins [2], brassinosteroids [3]. Also, transcription regulatory networks are essential for the organization of vascular tissues, as the auxin-*TARGET OF MONOPTEROS 5* (*TMO5*), *LONESOME HIGHWAY* (*LHW*), cytokinin-*TARGET LONELY GUY 4* (*LOG4*) [4], MYB and NAC genes [5]. The NAC gene family, has the name derived from the *NO APICAL MERISTEM* (*NAM*) gene from *Petunia hybrida* and from the *Arabidopsis thaliana* L. *ATAF1-2* and *CUP-SHAPED COTYLEDON 2* (*CUC2*) genes [6]. This protein family has important roles in senescence regulation [7], cell division and wood formation [8–10], and biotic and abiotic stress responses [11–14].

The differentiation of xylem cells in the vascular system (i.e. wood formation) of *Arabidopsis* involves drastic cytological changes, which has been well reported previously [10]. Vessels and fibers form the secondary xylem through developmental processes that includes cell division, cell expansion, secondary wall deposition, lignification and programmed cell death [15]. These biological processes are controlled by a sophisticated network of transcription factors (TFs) that coordinately integrate developmental and environmental signals into secondary cell wall (SCW) formation [16]. Wood formation has been found to be led by a particular group of NAC transcription factors, denominated VNS proteins, i.e. *VASCULAR RELATED NAC-DOMAIN 1-7* (*VND1-7*), *NAC SECONDARY*

WALL THICKENING PROMOTING FACTOR 1 AND 2 (*NST1/3*), *SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 1-2* (*SND1-2*) and *SMB* (*Sombrero*) [17–19]. In the same regulation network, *ANAC075* is a putative regulator of *VND7* [20] and *VASCULAR NAC INTERACTING 2* (*VNI2*) is a negative regulator of *VND7* in Arabidopsis [21].

Furthermore, modifications at the *NAC* gene expression level result in transcriptional changes leading to stress tolerance [22,23]. *VNI2* gene has been related to ABA-mediated response under salt stress in leaves by gene regulation of *COLD-REGULATED* (*COR*) and *RESPONSIVE TO DEHYDRATION* (*RD*) in Arabidopsis [24]. Other *NAC* proteins related to stress response have been reported such as *ANAC032*, an abscisic-acid-responsive *NAC* that showed higher expression under several stress conditions when compared to controls, as oxidative, light, osmotic and salinity stress [25]. Also, overexpression of *ANAC019*, *ANAC055* and *ANAC072* significantly enhanced drought tolerance in Arabidopsis [26,27]. The overexpression of *NAC57* from *Populus trichocarpa* improved salt tolerance in transgenic Arabidopsis [28]. Also, *PeNAC036* from *Populus euphratica* increased tolerance to salt and drought in Arabidopsis [29].

Also, *NAC* family has been reported as one of the largest among transcription factors, with a significant regulation on leaf senescence [30,31]. Half of the known Arabidopsis *NAC* members (65 of 113 genes) showed expression changes during leaf aging [32]. Several studies characterized *ANAC016* [31], *ANAC029/AtNAP* [33], *ANAC059/ORS1* [34] and *ANAC092/ORE1* [35] as positive *NAC* regulators, and *ANAC042/JUB1* [36] and *ANAC083/VNI2* [24] as negative regulators of leaf senescence. This biological process is accompanied by transcriptional reprogramming of a large number of genes, including *Senescence-Associated Genes* (*SAGs*) [36]. Moreover, physiological responses during leaf senescence such as chlorophyll (Chl) degradation and ABA-signaling have been previously reported [30,37].

The molecular processes involved in teak's secondary xylem biosynthesis have been analyzed in recent years through transcriptome and genome studies. The teak chromosomal genome was assembled, allowing the characterization of the Terpene Synthase protein family, responsible to defend woody tissues from pests and pathogens [38]. In addition, teak transcriptome was assembled and studied using different tissues and conditions [39]. This study used lignified tissues, which allowed the characterization of several *MYB* transcription factors and heat-shock proteins. These publications based on *T. grandis* genetics have allowed the understanding of how certain genes regulate xylogenesis. Recently, six putative orthologous of *TgNAC01* and one orthologous of *ANAC083* (*VNI2*) from Arabidopsis were identified in teak, they are all related to secondary growth [40]. In order to broaden the understanding of the *NAC* transcription factors, transgenic tobacco lines were generated to study the role of *TgNAC01* in leaf senescence and salt stress.

3.2. MATERIALS AND METHODS

3.2.1. Phylogenetic analysis and evolutionary profile of *TgNAC01* gene

The *TgNAC01* protein sequence was obtained from the teak transcriptome (NCBI accession number MH003855.1) [39], and aligned to teak *NAC* proteins previously identified [40] by MAFFT v7.407 (<https://mafft.cbrc.jp/alignment/software/>) using the auto mode. A phylogenetic tree with 116 teak *NAC* genes and *TgNAC01* was made using MEGA 7.0 (<https://www.megasoftware.net/>). In addition, an unrooted phylogenetic tree was prepared with 25 homologous proteins from 22 species, including Chlorophyta, Bryophytas, Lycophytas,

Gymnosperms and Angiosperms [41] selected by using BLASTp in the Plant Transcription Factor DataBase v.5.0 (<http://planttfdb.cbi.pku.edu.cn/>) with higher score and lower E-value per species. Also including the Tg15g04300.t1 member of the NAC-N group previously selected in the first phylogenetic tree based on teak NAC genes [40]. The initial tree was obtained as a matrix of pairwise distances using Neighbor-Joining (NJ), BioNJ algorithms, the JTT model and selected the topology with superior log likelihood value. It was used the maximum-likelihood method and poisson correction model with 1000 replicates to represent the evolutionary history of the taxa. It was employed the software MEME v5.0.5 (MEME-<http://meme-suite.org/index.html>) for the detection of conserved motifs using the following parameters: distribution of motif occurrences, zero or one per sequence, 20 maximum number of motifs, and a range of ≥ 6 and ≤ 25 for optimum motif width. It was used the motif database FIMO v. 5.1.1. (<http://meme-suite.org/tools/fimo>) in auto mode parameters for motif search.

3.2.2. Construction of the *TgNAC01* overexpression vector and Plant Transformation

The full-length coding sequence (CDS) of *TgNAC01* was amplified using cDNA from teak secondary xylem and primers containing the “CACC” sequence in the N-terminal region (Additional file 1). In one construct the stop codon from the amplicon was excluded to allow the expression of the reporter gene (EGFP) already in the destination vector pK7FWG2 to observe the subcellular localization of the gene. Another construct was prepared with the stop codon, cloned into pENTR™/D-TOPO® cloning vector following plasmid recombination via LR clonase in the pK7FWG2 destination vector in frame and downstream from CaMV35S promoter using the gateway system, following the manufacturer's instructions (Thermo Fisher, USA). The vectors were confirmed the position and integrity of the sequence by restriction enzymes digestion on DNA electrophoresis, PCR and DNA sequencing. The two vectors prepared containing the p35S:*TgNAC01* were used to transform *Agrobacterium tumefaciens* strain EAH105. Seedlings of *Nicotiana tabacum* cv. Petit Havana SR1 were grown in glass jars with half concentration of MS salts, under 16/8 h light/dark photoperiod at 25°C. Leaves discs from thirty-days-old tobacco plants were used as explant for the plant transformation process via *A. tumefaciens* [42].

3.2.3. Subcellular localization analysis

The *TgNAC01* gene without the stop codon was cloned into the pK7FWG2 vector in frame with the Green Fluorescent Protein (EGFP) at the C-terminal portion using the same procedure described for vector construction. The pK7FGW2 empty vector was used as control. Thirty-days-old tobacco leaves were transformed with the empty and *TgNAC01*-EGFP vectors via *Agrobacterium tumefaciens* [42]. The fluorescence visualization was checked in root differentiation zone on 7-days-old transgenic tobacco line (T1-first generation) using a FV1000 Olympus FluoView (Olympus, Tokyo, Japan) with an argon laser. Excitation wavelength was calibrated between 488 and 509 nm. For the subcellular localization prediction, it was used CELLO v.2.5 (<http://cello.life.nctu.edu.tw/>) and WoLF PSORT (<https://wolfsort.hgc.jp/>), and for transmembranal prediction it was used HMMTOP (<http://www.enzim.hu/hmmtop/index.php>).

3.2.4. Characterization of TgNAC01 Tobacco Transgenic Lines

The second generation (T₂) of the tobacco TgNAC01 lines were screened from planting seeds on half strength MS medium supplemented with 100 g L⁻¹ Kanamycin (Kan). Green Lines which segregated in a 3:1 (Kan-resistant:Kan-susceptible) rate were selected as transgenic lines. Seeds from T₂ generation on selective medium 100% resistance to kanamycin were selected as homozygous transgenic lines. DNA from those selected lines was isolated using the CTAB method [43]. Transgenic lines were confirmed by PCR with specific primers (Additional file 1) of the *TgNAC01* gene and the *neomycin phosphotransferase II (nptII)* gene to confirm the insertion of the target gene and the kanamycin resistance, respectively.

3.2.5. Salt stress treatment in the transgenic tobacco plants

Transgenic tobacco lines were grown in 5L pots containing soil composed mainly of substrate, under maximum luminosity incidence of 200 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ (at noon), maximum temperature for exhaust fans activation of 27°C, and 60% of controlled humidity by an automatic water curtain system in greenhouse. It was used the relative water content (RWC) in the pots estimated by the gravimetric method calculated as the difference between the soil weight after drainage and soil weight after drying. The irrigation volume was calculated from the relative water content and evapotranspiration data following the Penman-Monteith equation [44]. Tobacco crop coefficient (K_c) = 0.8 was calculated following previous studies [45]. Different concentrations of NaCl (0, 150 and 300 mM) were applied along with the quantity of water for seven days. We set up three biological replicates for each treatment. The second fully expanded leaf from the apical meristem was collected from each plant for further gene expression analysis.

3.2.6. Leaf water potential and gas exchange measurements in the transgenic tobacco plants

The average water potential was measured at 4:00 a.m. (predawn) and 1:00 p.m. (midday) using a Pressure chamber (PMS Instrument Company, Model 1000, Albany SE, USA) on the 4th fully expanded leaves. The RWC was calculated as $(\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight}) \times 100$ [46]. Physiological parameters were measured at day 0 and after 7 days of stress. It was performed analyzes of net CO₂ assimilation (photosynthesis) rate (A), stomatal conductance to water vapor (gs), transpiration (E), and carboxylation efficiency (A/Ci). All measures were done in the second pair of fully expanded leaves below the apex and using three replicates. Instantaneous water use efficiency (WUE_{ins}, mmol mol^{-1}) was defined as the ratio of A/E, and intrinsic water use efficiency (WUE_{int}, $\mu\text{mol mol}^{-1}$) as the ratio of A/gs [47]. All the leaf gas exchange measurements were performed between 08:00 a.m. and 12:00 p.m., at 25°C, with a portable infrared CO₂/H₂O gas analyzer (IRGA) (LCpro-32 070, ADC Bioscientific Ltd., Great Amwell, U.K.) equipped with a broad leaf chamber. The Photosynthetic Response to Light Intensity (P.R.L.I) was measured on leaves exposed to a CO₂ concentration of 400 ppm. We used a Photosynthetically Active Radiation (P.A.R.) of 1,200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 25°C, as previously calculated [48].

3.2.7. Chlorophyll and fluorescence measurements

Chlorophyll fluorescence measurements were obtained from 8:30 a.m. to 12:00 p.m. with a portable JUNIOR-PAM Chlorophyll fluorometer (Walz, Effeltrich, Germany). The quantum efficiency of PSII (Φ_{PSII}) was measured with a Saturation Pulse ($10,000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, 800 ms) applied after 3 min of illumination at $1,050 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ of actinic light (AL) in dark-adapted leaves. The Φ_{PSII} was determined using the equation $\Phi_{PSII} = (F'_m - F_s) / F'_m$, where F'_m is the maximum fluorescence obtained with a light-saturating pulse and F_s is the steady-state fluorescence in the light [49]. The Electron Transport Rate (ETR) was calculated with the equation $\text{ETR} = \Phi_{PSII} * \text{PPFD} * \alpha * \beta$, where PPFD is the photosynthetic photon flux density, α is the leaf absorptance, β is the distribution of absorbed energy between the two photosystems (assumed to be 0.5) and Φ_{PSII} is the quantum efficiency of the photosystem II [50]. The non-photochemical quenching was calculated with the equation $\text{NPQ} = (F_m - F'_m) / F'_m$ [51,52]. Chlorophyll index was obtained using a chlorophyll-meter (atLEAF, Wilmington, USA). We measured all the chlorophyll traits using the second fully expanded leaf from the apical meristem.

3.2.8. Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted from leaves of tobacco transgenic lines using the TRIzol reagent (ThermoFisher, USA) to examine the *TgNAC01* gene expression. Total RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and its integrity was examined by electrophoresis. The RNA was treated with DNase I (Promega, USA) and cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System for RT-qPCR (Invitrogen, USA), according to the manufacturer's instructions. Quantitative real-time PCR (RT-qPCR) reactions were conducted in 12,5 μL of total volume using a Platinum Sybr Green Supermix (Invitrogen, USA), and run in an ABI 7500 qPCR thermocycler (Applied Biosystems, USA). Expression data was normalized using the $2^{-\Delta\text{Ct}}$ method, the constitutive *Elongation Factor-1 alpha* (*EF-1 alpha*) housekeeping gene was used as internal control [53]. The primers were designed using the primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>) (Additional file 1). We used three biological replicates and two technical repetitions for each transgenic line.

3.2.9. Statistical analyses

The experiment was established as a completely randomized design with three replicates. Differences between means for the physiological parameters were assessed through an ANOVA, using an univariate general linear model and the standard error of the difference between means (SED) for the comparisons. The gene expression (RT-qPCR) data was subjected to statistical analysis using the Student's t test. The salt stress assay was analyzed with the model $y = \mu + \text{Pb} + \text{Qc} + \text{Sd} + \epsilon$, where y is the vector of each physiological trait, μ is the constant of the general mean for each trait, b is the vector for the transgenic lines and WT effect, c is the vector of NaCl concentration effect, d is the vector of interaction between genotypes (transgenic lines and WT) and salt treatment, and ϵ is the vector of error effect with $\epsilon \sim N(0, \sigma)$. The capital letters represent the incidence matrices for each of the effects. Differences

between means were assessed through an ANOVA, using an univariate general linear model, and the Tukey test with a 99% of significance level for the mean comparisons

3.3. RESULTS

3.3.1. Phylogeny and evolution of TgNAC01

The TgNAC01 gene has 492 nucleotides as coding sequence, and encodes a protein with 164 amino acids, and a typical NAC domain divided into four sub-domains (A, B, C and D) [54] (Figure 9A). Teak NAC phylogenetic tree showed that TgNAC01 is located in the NAC-N group of teak NAC protein family [40] sharing the same taxa with Tg15g04300.t1 (Additional file 2). Multiple sequence (Clustal) analysis of TgNAC01 protein showed 63% of similarity with the Tg15g04300.t1 teak protein. However, after comparing the TgNAC01 protein with the same amino acids from the N-Terminal portion of the Tg15g04300.t1 protein, both presented 96% of similarity (158 of 164 amino acids) (Additional file 3). The constructed unrated phylogenetic tree allowed grouping TgNAC01 with Potri.0001G061200.2 of poplar and Eucgr.H03387.1.p of Eucalyptus (tree species). It has VNI2 Arabidopsis protein as most recent common ancestor into eudicots monophyletic group and the kfl003330190 protein of charophyte green algae *klebsormidium flaccidum* as possible group paraphyletic of TgNAC01 protein (Figure 9B). The TgNAC01 homologous protein motifs showed five sub domains located in N-terminal region, which is conserved in all species (Figure 9C), being able to vary in C-terminal region protein. Therefore, it was observed that Tg15g04300.t1 is the most recent common ancestor of TgNAC01 in teak and VNI2 protein presents regions rich in proline (P), glutamate (E) or aspartic acid, serine (S), and threonine (T) defined as PEST motifs (Figure 9D). However, TgNAC01 protein has not presented this motif suggesting that TgNAC01 protein possibility has different biological role compared to VNI2 protein. The [MEKVSFVKNGV] motif located in N-terminal region is found only in eudicots and *Amborella trichopoda* suggesting that VNI2 homologous protein have appeared around the divergence of amborellales (Figure 9E).

Figure 9 Evolutionary relationship and protein multiple alignment of VNI2 proteins from different species. A) NAC sub domains of TgNAC01 protein, B) Molecular evolution of TgNAC01 (VNI2 homologous) compared with 22 species (25 proteins). The phylogenetic tree was constructed using the Likelihood method, person correction and 1,000 bootstraps using Mega 7.0. C) Structure motifs of VNI2 proteins. C) Logo PEST motif D) Logo corresponding to the most conserved motifs of the VNI2 homologous protein in eudicots.

3.3.2. Subcellular localization of TgNAC01

Two tobacco transgenic lines of 35S:TgNAC01-EGFP were obtained by Agrobacterium transformation (supplementary data). One line was used to analyse the subcellular localization of TgNAC01 protein (Figure 10A). GFP fluorescence of the 35S:TgNAC01-EGFP protein was observed only in the nucleus, which was also confirmed by Differential Interference Contrast (DIC) (Figure 10B). This result reinforces the computational prediction, which indicates that TgNAC01 is localized in the nucleus (Additional file 4 and 5) and TgNAC01 protein has no transmembrane motifs (Additional file 6). The absence of NAC WITH TRANSMEMBRANE MOTIF1 (NTM1) reinforces the result that TgNAC01 protein is only located in the nucleus. Moreover, the 35S:EGFP fluorescence was

observed in both nucleus and cytoplasm (Figure 10C). As expected, the WT tobacco plants showed no fluorescence (Figure 10A).

Figure 10 Subcellular localization of TgNAC01:GFP in the roots of transgenic tobacco seedlings. Root elongation zone cells of 7-day-old seedlings of a tobacco line expressing 35S:TgNAC01-EGFP, WT and 35S:EGFP were observed in DIC contrasting and fluorescence excitation. (A) WT roots with no fluorescence. (B) The TgNAC01-13 transgenic line showing GFP fluorescence in the nucleus. (C) 35S:EGFP showing GFP fluorescence in nucleus and cytoplasm.

3.3.3. Characterization of TgNAC01 transgenic tobacco plants

Among the eight independent 35S:TgNAC01 transgenic lines generated, three of them 35S:TgNAC01-3, 35S:TgNAC01-4, and 35S:TgNAC01-6 were homozygous in T2 generation under kanamycin selection (50 mg L^{-1}). The insert was confirmed on those three lines by PCR (Additional file 7). The three transgenic lines showed significant shorter root length compared to WT plants (Figure 11A). Note that the 35S:TgNAC01-1 presented longer root length when compared to the other two transgenic lines. Previous studies have found that VNI2 (TgNAC01 homologous) acts as a negative regulator in the development of the protoxylem possible due to the PEST motif, which is involved

in the post translational control of VND7 activity [21]. Surprisingly, TgNAC01 did not show the PEST motif. This could probably mean that the PEST motif does not have a significant role for the root development and elongation related to TgNAC01 protein. The 35S:TgNAC01-3 line showed significant higher relative expression when compared to other two transgenic lines (Figure 11B).

Figure 11 Characterization of TgNAC01 transgenic lines of tobacco. A) Root length of 35S:TgNAC01 transgenic lines and WT plants seven days after germination. B) Relative expression of *TgNAC01* of leaves from transgenic lines. Means correspond to three measurements. Standard error of the mean (SEM) is shown as bars. Letters explain differences between means for root length that 99% of confidence. Asterisks explain differences between means for relative expression at 99% of confidence.

3.3.4. Role of TgNAC01 in growth and leaf senescence

Difference in stem length was observed after one month under optimal conditions in the greenhouse. The 35S:TgNAC01-3 line showed 30% longer stem length compared to WT, while 35S:TgNAC01-4 and 35S:TgNAC01-6 lines did not present significant statistical differences (Figure 12A-B). The difference in length could be explained with a previous study describing higher *TgNAC01* gene expression in secondary xylem of stem (50-fold) and branch (20-fold) when compared to leaves and roots in teak [55]. Senescence of basal leaves was observed after one month of growth in the 35S:TgNAC01-3 transgenic line. Also, chlorophyll content was significantly lower after two months of growth in the 35S:TgNAC01-3 transgenic line when compared to the other lines and control plants, showing leaf senescence for this line (Figure 12C-D). These results suggest that *TgNAC01* gene is related to plant senescence. Following the hypothesis that *TgNAC01* overexpression in tobacco plants accelerates the process of senescence and may affect other physiological responses, the transgenic lines were submitted to different salt stress treatments.

Figure 12 Phenotype, growth and leaf senescence of 35S:TgNAC01-1, 35S:TgNAC01-4, 35S:TgNAC01-6 tobacco transgenic lines and WT sixty-days-after germination A) Phenotype of 35S:TgNAC01 lines and WT at day 60 after germination, B) Growth of 35S:TgNAC01 lines and WT measured at day 30 and 60 after germination, C) TgNAC01 transgenic lines of tobacco and WT sixty-days-after germination, D) Chlorophyll index measured at day 30 and 60 after germination. Values represent the means $\pm$ SD calculated using three replicates. Differences between means were considered to be significant at $P < 0.01$ by Tukey multiple comparison tests, calculated for one and two months separately. Lowercase letters represent the differences among transgenic lines and WT plants.

3.3.5. Role of TgNAC01 under NaCl stress

alt treatments of 150 and 300 mM affected growth of WT and transgenic lines in the seventh day of stress induction, except for plants of line 35S:TgNAC01-3 (Figure 13A). The transgenic line 35S:TgNAC01-3 plants showed higher growth even after 7 days under 300 mM of salt stress compared to WT and the other transgenic lines (Figure 13B). Leaf water potential (ψ_w) was measured after 7 days under salt stress during predawn and midday. Basal potential (predawn) showed a decrease (more negative values) in all transgenic lines when compared to control plants under salt stress conditions. The minimum values for water potential in predawn occurred in WT plants under 300 mM of NaCl salt stress. However, significant differences in water potential parameters were not observed during the stress period between WT plants and transgenic lines under salt stress measured onmidday.

Figure 13 Phenotype, leaf water potential and chlorophyll index of 35S:TgNAC01-1, 35S:TgNAC01-4, 35S:TgNAC01-6 tobacco transgenic lines and WT under salt stress. (A) Phenotype of 35S:TgNAC01 lines and WT after 7 days under three NaCl concentrations. (B) Stem length (cm) measured at day 0 and after 7 days on salt stress, leaf water potential (MPa) measured after 7 days of salt stress and Chlorophyll index measured at day 0 and after 7 days on salt stress. Values represent the means $\pm$ SD calculated using three replicates. Differences between means were considered to be significant at $P < 0.01$ by Tukey multiple comparison tests, calculated for day 0 and 7 separately. Capital letters represent the difference due to the effect of the treatment (salt concentration) and lowercase letters represent the differences among transgenic lines and WT plants.

3.3.5.1. Gas exchange parameters of tobacco transgenic lines overexpressing TgNAC01 under salt stress

Alterations in photosynthetic parameters under abiotic stress are indicators of plant physiological state and how is the response to adverse factors. Indeed, gas exchange parameters in transgenic lines were measured to find out if the photosynthetic activity decreases due to both leaf senescence and *TgNAC01* overexpression. The results showed that photosynthesis (A), stomatal conductance (gs), transpiration rate (E) and carboxylation efficiency (A/Ci) decreased proportionally to the increase of NaCl concentration in all plants (Figure 14A-D). The 35S:TgNAC01-3 transgenic line showed the lowest values of gas exchange when no salt stress was applied (Figure 14A-D). In general, transgenic lines showed a decrease of photosynthesis, stomatal conductance, transpiration and carboxylation efficiency rate after 7 days of salt stress compared to WT plants (Figure 14). It is noticed that, the 35S:TgNAC01-4 transgenic line presented the most significant decrease of A, gs, E and A/Ci after 7 days of 300 mM of salt stress compared to the other transgenic lines and WT plants (Figure 14A-D). While the analyses of instantaneous WUE increased in all conditions (with and without salt stress) in all transgenic lines and WT plants (Figure 14E), intrinsic WUE increased in WT and transgenic lines only under intrinsic WUE increased in all transgenic lines and WT 150 and 300 mM of salt stress (Figure 14F).

Figure 14 Gas exchange parameters under salt stress in tobacco transgenic lines and WT plants. A) Photosynthetic rate (A). B) Stomatal conductance (gs). C) Transpiration rate (E). D) Carboxylation efficient (A/ intercellular CO₂ concentration). E) Instantaneous water use efficiency (WUEins). F) Intrinsic water use efficiency (WUEint). Values represent the means $\pm$ SD calculated using three replicates. Differences between means were considered to be significant at $P < 0.01$ using the Tukey multiple comparison tests. Capital letters represent the difference due to the effect of the treatments (salt concentration) and lowercase letters represent the difference among transgenic lines and WT plants.

3.3.5.2. Chlorophyll fluorescence of tobacco transgenic lines overexpressing TgNAC01 under salt stress

No statistical significant differences in chlorophyll fluorescence parameters were observed during the stress period between WT plants and transgenic lines under salt stress (Figure 15). Although the electron transport rate (ETR) did not show significant differences between control plants and transgenic lines associated with salt concentrations (Figure 15A), all plants showed a slightly and general reduction in the ETR after 7 days under salt stress, reaching the lowest values at 300 mM NaCl especially on WT plant (Figure 15A). In accordance with the ETR results, the non-photochemical quenching (NPQ) values did not show significant differences between treatments at the end of the stress period, but WT plants exposed to 300 mM NaCl showed an increase of NPQ (Figure 15B). The maximum quantum efficiency of PSII photochemistry (F_v/F_m) and the PSII operating efficiency [$Y(II)$] increased in all condition and genotypes (Figure 15C-D). The response of the WT plants under saline stress was lower compared to the

transgenic lines for both ETR and Y (II) (Figure 15A-D). The increase of NPQ in high salt stress condition could have a role in preventing photo-damage in transgenic lines and WT plants.

Figure 15 Chlorophyll fluorescence parameters under salt stress in tobacco transgenic lines and WT plants. (A) Electron transport rate (ETR). (B) Non-photochemical quenching (NPQ). (C) Maximum quantum yield of PSII photochemistry. (D) Effective photochemical quantum yield of PSII (YII). Values are the means $\pm$ SD calculated using three replicates. Differences between means were considered to be significant at $P < 0.01$ using Tukey multiple comparison tests. Capital letters represent the difference due to the effect of the treatment (salt concentration) and lowercase letters represent the difference among transgenic lines and WT plants.

3.4. DISCUSSION

3.4.1. TgNAC01 protein evolution and localization

NAC protein family has been exclusively described in land plants, since they have not been identified neither in single red alga (*Cyanodioschyzon merolae*), glaucophytes (*Cyanophora paradoxa* and *Glaucocystis nostochinearum*) nor chlorophytes (*Ostreococcus tauri*, *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, and *Volvox carteri*), it may suggest that NAC family may have appeared during the Klebsormidiales divergence [56]. Consistently, we found that the closest ancestors to TgNAC01 would be kfl003330190 of *Klebsormidium flaccidum* (filamentous green algae) and VNI homologous proteins from the Amborellales plant order (Figure 9B). TgNAC01 protein belongs to the NAC-N group related to the NAC protein family, as previously described [40], and it is likely to have been originated from gene duplication events, sharing a near evolutionary origin with the Arabidopsis *AT2G33480.1* (ANAC83-VNI2) gene. In general, the NAC family is conserved among different plant species in its N-terminal portion, and shares motifs with the eudicots VNI homologous proteins [25]. The evolution of proteins has been traditionally related to improved or diversified

molecular functions, and the protein function is directly related to protein structure [57]. We found a conserved region between TgNAC01 protein and VNI2 homologous, particularly at the first five motifs of the protein alignment (Figure 9C-E). Nevertheless, TgNAC01 protein does not present the PEST proteolysis target motif [TITDLNLLPSSPSSD] (Figure 9C-D), which is present in the C-terminal region of the Arabidopsis VNI2 protein. This motif is known to promote rapid protein degradation [58] and it is possible that it may be responsible for the degradation of the VND7 negative regulator [21]. The lack of the PEST motif in TgNAC01 could mean that this protein might not be a negative regulator in the xylem differentiation. Furthermore, computational predictions and the absence of NTM1 indicated that TgNAC01 protein might be located in the nucleus (Additional file 4 and 5). In Arabidopsis and rice, a total of 13 and 6 NAC proteins, respectively, were reported to have the NTM1 region [59]. Location of TgNAC01 in the nucleus was further confirmed by its heterologous expression in tobacco (Figure 10b). Previous studies in stem-differentiating xylem of *P. trichocarpa* detected six VNDs being exclusively overexpressed in the nucleus [60]. Also, Arabidopsis protoplasts showed overexpression of the *VNI2* gene in the nucleus [24].

3.4.2. *TgNAC01* has a role in growth and regulation of senescence

Xylem cell differentiation consists in four biological processes: secondary cell wall (SCW) deposition, programmed cell death, autolysis and lignification [15]. The *TgNAC01* overexpression could affect the SCW deposition since this transcription factor has been demonstrated to be a master switch that presents higher expression in teak secondary xylem when compared to other tissues [55]. Consequently, it may affect the radial stem expansion through periclinal division, resulting in a greater stem length [61]. Knowing that *TgNAC01* expression was higher in lignified tissues when compared to leaves [55], it is presumed that overexpression of this gene increases lignification and plant height. Overexpression of *SND2* in Arabidopsis led to an increase in xylem fiber wall thickening and consequently in height [62]. The *NAC15* gene was identified as regulator of cell wall thickening during fiber development in *Populus* [10,63]. NAC15 protein also plays a significant role in wood formation when it was overexpressed in transgenic tobacco plants [64]. In agreement with our results, *Populus NAC13* overexpression in transgenic tobacco plants showed a positive regulation under salt stress leading to a higher plant growth compared to control plants [65]. The significant role of NAC transcription factors in regulating leaf senescence has been reported previously [66,67]. NAC proteins are involved in the regulation of leaf senescence through changes in gene expression patterns, structure and metabolism, including loss of photosynthetic activities and increases hydrolysis of proteins, membrane lipids, and nucleic acids [68]. Previous studies in Arabidopsis showed that overexpression of *AtNAP/ANAC029* and *ORE1/ANAC092* NAC-related transcription factors promoted leaf senescence [33]. *VNI2*, a NAC-related gene, presented higher expression in senescent leaves compared to healthy ones [24]. It is possible that *TgNAC01* overexpression in transgenic tobacco plants accelerated leaf senescence (Figure 12) through ABA-signaling as a saline stress response. The strong relationship between ABA-signaling and leaf senescence has been well described previously [30,69,70].

3.4.3. *TgNAC01* confers salt stress tolerance

The three transgenic lines studied presented increase in leaf water potential in predawn when compared to WT plants (Figure 3C), which suggest that these plants under salt stress could counteract the excessive loss of water [71,72]. It has been well reported that salt accumulation in the root zone may cause a decrease in osmotic potential,

and consequently, a decrease in the leaf water potential [73]. Likewise, midday leaf water potential increased due to stomatal opening and transpiration ratio (Figure 13B). The 35S:TgNAC01-3 transgenic line showed the least negative value compared to 35S:TgNAC01-4 and 35S:TgNAC01-6 lines, and WT plants. Consecutively, *TgNAC01* overexpression increased water loss tolerance in transgenic tobacco plants (Figure 14C-F). Similar results regarding the reduction of water loss were observed when *TbNAC7* gene from *Tamarix hispida* was overexpressed in Arabidopsis [72]. *VNI2* overexpression under high salinity positively regulated *COR/RD* genes mediated by a transcriptional activation with a subsequent tolerance to saline stress [24]. Overexpression of *CarNAC3* and *CarNAC6* transcription factors from *Cicer arietinum* in poplar transgenic lines conferred tolerance to high salt stress, while WT plants showed withered leaves and stopped their growth [74]. Transgenic lines showed less decrease of A, gs, E and A/Ci after 7 days of salt stress compared to WT plants (Figure 14). Consequently, a decrease in carboxylation efficiency rate indicates that the salt stress condition affects photosynthesis due to a metabolic limitation associated with a decrease in the rubisco carboxylase activity [75]. Previous studies have shown that several NAC transcription factors regulate stomatal movements via ABA signaling, mediating the PEG-induced drought response [76]. The observation in this study helps to infer that overexpression of *TgNAC01* (Figure 13C) could improve water use efficiency. In general, 35S:TgNAC01-3 and 35S:TgNAC01-6 transgenic lines showed a better response under salt stress in terms of the photosynthetic rate (A), stomatal conductance (gs), transpiration ϵ and carboxylation efficiency (A/Ci) in spite of presenting a higher leaf senescence compared to the WT plants and the 35S:TgNAC01-4 transgenic line (Figure 14). However, despite the fact that 35S:TgNAC01-3 line started with a lower gas exchange response ($5.41 \mu\text{mol}(\text{CO}_2)\text{m}^{-2}\text{s}^{-1}$ of A, $0.06 \text{ mmolm}^{-2}\text{s}^{-1}$ of gs and $4.34 \mu\text{mol}(\text{H}_2\text{O})\text{m}^{-2}\text{s}^{-1}$ of E) when compared to the other transgenic plants and control plants under 300 mM of NaCl. The 35S:TgNAC01-3 line gas exchange values was higher ($4.24 \mu\text{mol}(\text{CO}_2)\text{m}^{-2}\text{s}^{-1}$ of A, $0.06 \text{ mmolm}^{-2}\text{s}^{-1}$ of gs, $2 \mu\text{mol}(\text{H}_2\text{O})\text{m}^{-2}\text{s}^{-1}$ of E) than control plants ($1.75 \mu\text{mol}(\text{CO}_2)\text{m}^{-2}\text{s}^{-1}$ of A, $0.03 \text{ mmolm}^{-2}\text{s}^{-1}$ of gs, $1.42 \mu\text{mol}(\text{H}_2\text{O})\text{m}^{-2}\text{s}^{-1}$ of E) on the seventh day of 300 mM NaCl concentration. This result may suggest that the more *TgNAC01* expression and leaf senescence increases, the more salt stress tolerance occurs. A similar result was described when the NAC-related *AtJUB1* gene was overexpressed in tomato plants under high salinity conditions, and transgenic plants were able to maintain biomass and physiological parameters better than the WT plants [77]. Transgenic tobacco lines overexpressing *TsNAC1* from *Thebellungiella 71alophila* showed slower growth and better survival after exposure to drought stress, high-salt stress when compared to control plants [78]. The opposite result was observed when *PeNAC045* overexpression in *Populus* showed lower A, gs and E compared to WT plants, with a further reduction in drought and salt tolerance [29]. In previous studies, it has been reported that the electron transport rate (ETR) proportionally increases with carbon fixation, so stress conditions can decrease both parameters drastically [79]. This result supports the low photosynthesis values observed in WT plants (Figure 14A). In accordance with the ETR results, the non-photochemical quenching (NPQ) showed higher values of WT plants exposed to 300 mM NaCl (Figure 15). The NPQ represents heat dissipation and plant use it as a mechanism of photo-protection, state transition and photo-inhibition [80–82]. It has been described that NPQ increases under stress conditions such as high light intensity or photoinhibition, salinity, heavy metal toxicity, drought or chilling [83].

3.4.4. Proposed model for the molecular process related to senescence and abiotic stress

The results obtained in this research showed that WT tobacco plants were the most affected under salinity stress, while the transgenic lines present a better tolerance under salt stress conditions (Figure 12). Based on previous studies [24,55] and the results presented here, we are proposing a model representing the role of *TgNAC01* in teak (Figure 16). *TgNAC01* appears as a bifunctional transcription factor, which acts as a positive regulator in the secondary xylem development and as a transcriptional activator in the salt stress-mediated regulation of leaf senescence. Under salt stress, *TgNAC01* confers tolerance through an ABA-mediated response (Figure 16, green squares). There by, teak plantlets experience different physiological responses, as a positive response mediated by *TgNAC01*, such as an increase in photosynthesis rate, leaf water potential, stomatal conductance, carboxylation efficiency, and a decrease in photoinhibition and water loss (Figure 16, green squares). Also, *TgNAC01* has shown a role in leaf senescence, and it is expected that is an ABA-mediated process, activated by an age-dependent senescence signal, as found in previous studies [24] (Figure 16, red squares). Finally, *TgNAC01* has been related to have a role in the deposition and differentiation of the secondary cell wall [55].

Figure 16 Proposed model for the *TgNAC01* roles in teak. Green squares show the salt stress tolerance promoted by *TgNAC01*, and the physiological response in teak. Red squares explain the leaf senescence led by *TgNAC01*. Purple square explains the role of *TgNAC01* in secondary cell wall deposition, previously reported [55]. Continuous squares show processes that have been studied in teak and in research. Dotted squares showed functions previously reported in other species, but not in teak yet [24].

3.5. CONCLUSIONS

The amino acid sequence of *TgNAC01* protein is conserved among species in its N-terminal portion, specifically in the region that characterizes the NAC family name. *TgNAC01* protein and VNI2 from *Arabidopsis* are

homologous NAC proteins although TgNAC01 protein does not present the PEST motif, which is present in VNI2, possible motif responsible for conferring negative regulation of xylem development consequently TgNAC01 may have an opposition function. *TgNAC01* gene is expressed only in the nucleus, and does not present transmembrane motifs, which is in accordance with its role as transcription factor. Transgenic tobacco plants showed increased senescence in basal leaves compared to WT plants, and this result was more evident in the chlorophyll content of sixty-days-old plants, suggesting that *TgNAC01* gene is related to senescence. The 35S:TgNAC01-3 and 35S:TgNAC01-6 transgenic lines had a better response under salt stress, showing higher photosynthetic rate, stomatal conductance, transpiration and carboxylation efficiency in spite of presenting increase in leaf senescence when compared to WT plants and 35S:TgNAC01-4 line. Finally, this study presents *TgNAC01* as a bifunctional transcription factor, which acts as a positive regulator of the secondary xylem development and as a transcriptional activator in stress-mediated regulation of leaf senescence.

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Additional files

Additional file 1. PRIMERS RT-PCR

Primers	Primer sequences (5' – 3')
<i>nptII</i>	F: GAGGCTATTCGGCTATGACTG
	R: TCGACAAGACCGGCTTCCATC
<i>TgNAC01</i> Overexpression	F: CACCATGGAGAAAGTTAGCCTTCTGAAGAAT
	R: AGCCACCAATTCAAATCTCACTTGTGCT
<i>TgNAC01</i> Fusion-EGFP	F: CACCATGGAGAAAGTTAGCCTTCTGAAGAAT
	R: TACAGCCACCAATTCAAATCTCACTTGTGCT
<i>TgNAC01</i> RT PCR	F: GGTCAAACAGGGCTACAGTTTC
	R: ACCAATTCAAATCTCACTTGTGC
35SProm	F: CGCACAATCCCACTATCCTTC
35STerm	R: TGCCTGCAGGTCACCTGGATTTTGGTTT TAGGAA
EF-1A RT PCR	F: TGAGATGCACCACGAAGCTC
	R: CCAACATTGTCACCAGGAAGTG
VirG	F: CTGGCGGCAAAGTCTGAT
	R: TGTCGTAAACCTCCTCGT
EGFP	F: AGCTGACCCTGAAGTTCATCTG
	R: GGTGTTCTGCTGGTAGTGGTC

Additional file 2. Phylogenetic tree of teak NAC proteins and TgNAC01. The phylogenetic tree was constructed using the Likelihood method, person correction and 1,000 bootstraps using Mega 7.0.

Additional file 3. CLUSTAL alignment of TgNAC01 and Tg15g04300 teak proteins

CLUSTAL format alignment by MAFFT (v7.452)

```

TgNAC01      MEKVSLLKNGVLRPPGFRFHPTDEELVVQYLKRKVLSCPLPASIPEVDVCKSDPWDLP
Tg15g04300   MEKVSLLKNGVLRPPGFRFHPTDEELVVQYLKRKVLSCPLPASIPEVDVCKSDPWDLP
*****

TgNAC01      GDSEQERYFFSTREIKYPNGNRSNRATVSGYWKATGLDKQIVSTRSHQIVGMKKTLVFYR
Tg15g04300   GDSEQERYFFSTREIKYPNGNRSNRATVSGYWKATGLDKQIVSTRSHQIVGMKKTLVFYR
*****

TgNAC01      GKPPKGCRTDWIMHEYRLITAQNSAITSPQAKNLAQVRFELVA-----
Tg15g04300   GKPPKGCRTDWIMHEYRLITAQNSAITSPQAKNLAQENWVLCRIFLKRRNSKTDDEAAAM
*****

TgNAC01      -----
Tg15g04300   QNSGRATGAVPVFYDFMAKERADLNLLPANSSSGSSGITQISSRNESDDHEESSCNSFP

TgNAC01      -----
Tg15g04300   TSFRRNLCS

```

Additional file 4. Prediction of the subcellular localization of TgNAC01 by Cello

SeqID: TgNAC01 protein [*Tectona grandis*]

Analysis Report:

SVM	LOCALIZATION	RELIABILITY
Amino Acid Comp.	Mitochondrial	0.527
N-peptide Comp.	Mitochondrial	0.525
Partitioned seq. Comp.	Extracellular	0.460
Physico-chemical Comp.	Mitochondrial	0.272
Neighboring seq. Comp.	Nuclear	0.411

CELLO Prediction:

Localization	Value
Mitochondrial	1.664 *
Nuclear	1.057 *
Extracellular	0.891
Cytoplasmic	0.768
Chloroplast	0.411
Peroxisomal	0.072
Lysosomal	0.038
PlasmaMembrane	0.037
Cytoskeletal	0.021
ER	0.016
Vacuole	0.013
Golgi	0.012

Additional file 5. Prediction of the subcellular localization of TgNAC01 by WOLF

Id	site	length
TgNAC01	nucl	163
At2g39470.1	chlo	238
At5g25390.1	nucl	186
BCA6_ARATH	cyto	356
RP3B_ARATH	nucl	319
THS1_ARAHY	cyto	389
At3g17590.1	nucl	240
THS3_ARAHY	cyto	389
At3g60830.1	nucl	363
PODK_MESCR	chlo	949
K125_TOBAC	cysk	1006
At1g77090.1	chlo	260
FERB_ALOMA	chlo	98
AXR1_ARATH	nucl	540

Additional file 6. Transmembrane prediction of TgNAC01 protein by HMMTOP

Protein: TgNAC01
Length: 163
N-terminus: OUT
Number of transmembrane helices: 0
Transmembrane helices: 0

Total entropy of the model: 17.0131
Entropy of the best path: 17.0131

The best path:

seq	MEKVSLLKNG	VLRLPPGFRF	HPTDEELVVQ	YLKRKVLSCP	LPASIIPEVD	50
pred	0000000000	0000000000	0000000000	0000000000	0000000000	
seq	VCKSDPWDLP	GDSEQERYFF	STREIKYPNG	NRSNRATVSG	YWKATGLDKQ	100
pred	0000000000	0000000000	0000000000	0000000000	0000000000	
seq	IVSTRSHQIV	GMKKTLVFYR	GKPPKGCRTD	WIMHEYRLIT	AQNSAITSPQ	150
pred	0000000000	0000000000	0000000000	0000000000	0000000000	
seq	AKNLAQVRFE	LVA	163			
pred	0000000000	000				

Additional file 7. A) Agarose gel electrophoresis (1%) of PCR product of 35S:TgNAC01 transgenic lines. Ladder: 1kb Plus DNA Ladder (Invitrogen). 0.9 µg/lane, 0.9% agarose stained with ethidium bromide; Lane 1 – 7: tobacco transgenic lines; Lane 9: WT plant; Lane 10: E.coli DNA plasmidial with the 35S:TgNAC01-EGFP construction. Lane 11. H2O miliQ. B) Nine-days-old 35S:TgNAC01-3, 35S:TgNAC01-4, 35S:TgNAC01-6, 35S:TgNAC01-9 transgenic lines and WT tobacco seedlings, in a medium selection with 50 mg L⁻¹ kanamycin

Additional file 8. Agarose gel electrophoresis (1%) of PCR product of 35S:TgNAC01 transgenic lines. Ladder: 1kb Plus DNA Ladder (Invitrogen). 0.9 µg/lane, 0.9% agarose stained with ethidium bromide; Lane 1 – 8: tobacco transgenic lines; Lane 9 and 10: WT plants; Lane 10: E.coli DNA plasmidial with the 35S:TgNAC01 construction. Lane 11. H2O miliQ.

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4. GENERAL CONCLUSIONS

The present work is the second study of molecular characterization of complete teak gene families, since Terpeno Synthase genes were characterized in the teak genome report. In this research, using bioinformatics tools with molecular biology approaches such as cloning, gene expression in addition to physiological parameters, a genome-wide analysis of teak NAC domain protein with identification of NAC transcriptional factors homologous to Arabidopsis and the NAC protein related to SCW formation were characterized. In total, it was identified 130 NAC proteins, from which 116 represented non-redundant proteins coded by 107 loci. These identified 116 *T. grandis* NAC proteins were phylogenetically clustered into 23 distinct clades in evolutionary relationships with Arabidopsis, *Populus*, and *Eucalyptus*. In accordance to other studies, NAC protein family showed to be highly conserved among land plant species and the NAC protein diversification processes occurred from seed plants. Many of the teak NAC genes were located adjacent to each other, indicating that they might have resulted from duplication events. Most genes in groups A and F were preferentially expressed in branch and stem of 16 and 64 years old tree, where they presented higher relative expression.

Based on the regulation function of the NAC protein in SCW biosynthesis, 13 NAC genes were selected for genetic expression in various tissues of three developmental stages of teak trees. Data from RT-qPCR revealed highest expression of VND proteins *VND2-Tg11g04450* and *VND4-Tg15g08390* in tissues of 16-year-old trees. VND TFs are related to xylem vessels formation, indicating that these two VND genes (*Tg11g04450* and *Tg15g08390*) possibly participate in the regulation of *T. grandis* SCW deposition. In addition, the NAC homologous repressor XND1 (*Tg08g13700*) presented highest expression in 64-year-old tissues and gave support to the hypothesis of lower secondary cell wall deposition in older trees.

The amino acid sequence of TgNAC01 protein is conserved among species in its N-terminal portion, specifically in the region that characterizes the first five functional motifs. Tg NAC01 is agrupaed in teak NAC-N clade together to VNI2 arabidopsis protein. Comparative analysis of TgNAC01 homologous proteins showed a funtional motif that is shared only in eudicots and *Amborella trichopoda* suggesting that VNI2 homologous protein have appeared around the divergence of amborellales. TgNAC01 protein does not present the PEST motif, which is present in VNI2 arabidopsis protein, which is a negative regulator of xylem development. Although TgNAC1 is homologus to VNI2 they do not have the same function in teak.

TgNAC01 protein does not present transmembrane motifs and in silico prediction reveled that TgNA01 protein is located in the nucleus, this prediction is confimated by stable and constitutive expression in tobacco, indicating that *TgNAC01* gene is expressed only in the nucleus. Transgenic tobacco plants showed increased senescence in basal leaves compared to WT plants, and this result was more evident in the chlorophyll content of sixty-days-old plants, suggesting that *TgNAC01* gene is related to senescence. The 35S:TgNAC01-3 and 35S:TgNAC01-6 transgenic lines had a significant tolerance response under salt stress, showing higher photosynthetic rate, stomatal conductance, transpiration and carboxylation efficiency in spite of presenting greater leaf senescence compared to WT plants and 35S:TgNAC01-4 line. This study presents *TgNAC01* as a bifunctional transcription factor, which acts as a positive regulator of the secondary xylem development and as a transcriptional activator in stress-mediated regulation of leaf senescence.

The present teak NAC protein characterization joins the only three species of trees reported (*Populus*, *Pinus* and *Eucalyptus*). In addition, the TgNAC01 characterization is the first study by expression heterologous in teak, being considerably relevant. The data obtained can be used in applied and basic science along to biotechnological approaches. Since, it is known that some NAC proteins are the first layer master switch in transcriptional network

regulating wood formation, and are responsible for transcriptional control during abiotic stress. Contributing to understanding of the molecular mechanism in woody plants.