An Arabidopsis Nucleoporin NUP85 modulates plant responses to ABA and salt stress

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Abstract

Several nucleoporins in the nuclear pore complex (NPC) have been reported to be involved in abiotic stress responses in plants. However, the molecular mechanism of how NPC regulates abiotic stress responses, especially the expression of stress responsive genes remains poorly understood. From a forward genetics screen using an abiotic stress-responsive luciferase reporter (RD29A-LUC) in the sickle-1 (sic-1) mutant background, we identified a suppressor caused by a mutation in NUCLEOPORIN 85 (NUP85), which exhibited reduced expression of RD29A-LUC in response to ABA and salt stress. Consistently, the ABA and salinity induced expression of several stress responsive genes such as RD29A, COR15A and COR47 was significantly compromised in nup85 mutants and other nucleoporin mutants such as nup160 and hos1. Subsequently, Immunoprecipitation and mass spectrometry analysis revealed that NUP85 is potentially associated with HOS1 and other nucleoporins within the nup107-160 complex, along with several mediator subunits. We further showed that there is a direct physical interaction between MED18 and NUP85. Similar to NUP85 mutations, MED18 mutation was also found to attenuate expression of stress responsive genes. Taken together, we not only revealed the involvement of NUP85 and other nucleoporins in regulating ABA and salt stress responses, but also uncovered a potential relation between NPC and mediator complex in modulating the gene expression in plants.

Author summary

Nuclear pore complex (NPC) mediates the traffic between nucleus and cytoplasm. This work identified NUCLEOPORIN 85 (NUP85) as an important factor for the expression of stress-responsive luciferase reporter gene RD29A-LUC in response to ABA and salt stress from a forward genetics screen. Mutation in NUP85 and other NPC components such as NUP160 and HOS1 resulted in decreased expression of several stress responsive genes
such as RD29A, COR15A and COR47. Proteomics data uncovered a list of putative NUP85 associated proteins. Furthermore, NUP85 was demonstrated to interact with MED18, a master transcriptional regulator, to control the expression of stress responsive genes. The study has added a new layer of knowledge about the diverse functions of NPC in abiotic stress responses.

Introduction

Nucleocytoplasmic transport plays vital roles in eukaryotic systems [1,2]. The exchange of macromolecules such as RNAs and proteins is predominantly regulated by highly conserved nuclear pore complexes (NPCs), which consist of multi-nucleopors (Nups) arranged in distinct sub-complexes [3,4]. Although the composition and functions of NPCs have been extensively characterized in yeast and vertebrate systems, our knowledge on the functions of plant NPCs remains poor.

Recent genetic screens have identified the involvement of several Nups from Arabidopsis and Lotus japonicus (lotus) in a variety of developmental processes, hormone signaling pathways and environment adaptation [3,5,6]. For instance, NUP160 (SUPPRESSOR OF AUXIN RESISTANCE1) and NUP96 (SUPPRESSOR OF AUXIN RESISTANCE3) have been indicated to play a role in auxin signaling as they were identified as suppressors of auxin-resistant 1 (axr1) mutants [7]. NUP160 was also shown to be involved in cold stress responses since the knockout of NUP160 impaired cold-induced expression the CBF3-LUC reporter gene and several endogenous genes; and thus resulted in hypersensitivity to chilling and freezing stresses [8]. Another important NPC component [9], HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1), which encodes a RING finger E3 ubiquitin ligase, is well-known as a negative regulator in the cold signaling [10]. Cold-responsive genes such as RESPONSIVE TO DESICCATION 29A (RD29A), COLD-REGULATED 47 (COR47), COLD-REGULATED 15A (COR15A) and KIN1 were reported to be induced to higher levels in hos1 mutants than in wild type plants [11,12]. HOS1 was further shown to attenuate cold signaling by ubiquitination and degradation of INDUCER OF CBF EXPRESSION 1 (ICE1), which encodes an important positive transcription factor critical for the cold-induction of C-REPEAT BINDING FACTORS (CBFs) [11–13]. Moreover, the mutation of NUP160, HOS1 and NUP96 caused early flowering phenotypes, indicating that NPCs are also involved in flowering time regulation [5]. Interestingly, only HOS1 was demonstrated to interact with specific chromatin such as the floral repressor FLOWERING LOCUS C (FLC) chromatin to regulate FLC transcription under low temperature [14–16].

Besides their involvement in hormonal and cold stress signaling and flowering time control, some Nups such as NUP160 and Seh1 are also important for disease resistance in Arabidopsis [17,18]. Similarly, mutation of either of NUP85, Seh1 and NUP133 led to defective responses to symbiotic microorganisms in Lotus japonicus [19,20]. Most recent genetic studies have revealed NUP85 (also termed as SBB1, suppressor of bak1 bkk1-1) as a suppressor for BRI1-ASSOCIATED KINASE 1 (BAK1) and BAK1-LIKE 1 (BBK1)-mediated cell death control [21]. These studies suggested that Nups also participate in plant defense and cell death control. Nevertheless, the biological functions of many Nups remain elusive in plants.

Recently, a proline-rich protein gene, SICKLE-1 (SIC-1), was isolated because sic-1 mutants exhibited enhanced expression of stress-inducible RD29A-LUC reporter in response to abiotic stresses such as cold and salt treatments [22]. To identify new regulatory components involved in the response to abiotic stresses, we performed a forward genetic screen after EMS
mutagenesis of the RD29Apro-LUC line in the sic-1 mutant background. NUP85 was identified as its mutation caused significantly reduced expression of RD29A-LUC in response to ABA and salt stress. Moreover, we discovered a list of putative NUP85 interacting proteins by affinity purification followed by mass spectrometry and further validated the interaction between NUP85 and MED18.

Results

The nup85 mutation attenuates proRD29A-LUC reporter gene expression in response to ABA and salt stress

To identify new factors involved in abiotic stress responses, we performed a forward genetics screen using an EMS mutant population generated in the sic-1 mutant background with the stress-inducible proRD29A-LUC reporter gene. Putative mutants with altered luciferase (LUC) activities under abiotic stress treatments such as ABA treatment and salt stress were selected. One suppressor mutant was identified which exhibited reduced bioluminescence in response to ABA and salt stress compared to sic-1 plants (Fig 1A). As revealed in Fig 1A and 1B, the luminescence representing RD29A-LUC expression was highly induced in sic-1 by ABA and salt stress, whereas the luminescence was considerably weaker in the double mutant than sic-1 after either ABA or salt stress treatment. The luminescence intensities of the suppressor mutant were similar to those in the Col gl1 parental line harboring the proRD29A-LUC transgene (referred to as WT), which showed little LUC activity likely due to progressive transgene silencing [22,23]. After genetic mapping and whole genome re-sequencing, we discovered a mutation causing a premature stop codon in the 4th exon in NUP85 (AT4G32910), which encodes a nucleoporin protein (Fig 1C).

Gene expression data show that the LUC expression was highly induced in sic-1 by ABA, but this induction was impaired in the suppressor (i.e. sic-1 nup85) mutant (Fig 1D). The transcript level of endogenous RD29A was induced by ABA treatment in WT, sic-1 and the double mutant. However, the induction was significantly lower in the WT and sic-1 nup85 double mutant compared to that in sic-1 (Fig 1E). We also tested the expressions of other stress-responsive genes and the results showed that ABA-induced expression of COLD-REGULATED 15A (COR15A) and RD29B was lower in sic-1 nup85 double mutants than that in sic-1 mutant plants (S1 Fig).

To verify if the suppressor phenotype was caused by the NUP85 mutation, we cloned the genomic sequence of NUP85 with its native promoter and generated two independent complementation lines by transforming sic-1 nup85 double mutants. As shown in S2 Fig, the leaves of sic-1 nup85 double mutant were bigger than sic-1. The wild type NUP85 gene rescued the bigger leave size phenotype of the double mutant. We also examined the expression of RD29A-LUC reporter gene in the complementation lines after ABA and NaCl treatments (Fig 2). The diminished luminescence in sic-1 nup85 double mutant was rescued in the NUP85 complementation lines, which exhibited comparable LUC activity to sic-1. These genetic evidences demonstrated that the NUP85 mutation is responsible for the double mutant phenotypes.

The attenuated expression of stress responsive genes caused by NUP85 mutation

To investigate if NUP85 affects the expression of endogenous stress responsive genes in response to ABA and salt stress, we obtained two independent T-DNA insertion homozygous mutants, nup85-1 (SALK_133369) and nup85-2 (SALK_113274) (Fig 1C). PCR analysis confirmed that the two T-DNA insertion mutant lines are homozygous (S3A Fig). Gene expression data further showed that NUP85 expression was significantly lower in those two mutant lines.
compared to the Col-0 wild type (S3B Fig). We then treated the Col-0 wild type and two nup85 mutants with 50 μM ABA for 3h, and found that the expression of several stress responsive genes such as RD29A, COR15A and COR47 was significantly lower in the nup85 mutants than that in the wild type after ABA treatment (Fig 3A). In addition to ABA, we also investigated if high salinity induced expression of stress responsive genes was also altered by the NUP85
mutation. As shown in Fig 3B, salt stress induced expression of RD29A, COR15A and COR47 was evidently impaired in the two nup85 mutants after treated with 0.3M NaCl for 5h.

To better understand the genome-wide effects of NUP85, we performed RNA-sequencing experiments. Col-0 wild type and nup85-1 mutant seedlings were treated with mock or 50 μM ABA for 3 hours. Under mock treatment, there were 197 differentially expressed (DE) genes which showed more than 1.5-fold changes in nup85-1 mutant compared to Col-0 wild type plants (S1 Table). Gene ontology (GO) analysis revealed that the DE genes regulated by NUP85 under mock conditions were enriched in categories such as responses to stimulus, response to stress and response to hormone, indicating an important role of NUP85 in plant responses to environmental stresses (Fig 3C). Upon ABA treatment, there were totally 1389 ABA-responsive genes (759 ABA induced genes and 630 ABA repressed genes), whose expressions were significantly induced or repressed more than 4-fold changes by ABA treatments in Col-0 wild type (S2 Table), whereas there were 1357 ABA-responsive genes in nup85-1 mutants after ABA treatments with 982
Fig 3. The attenuated expression of stress responsive genes in nup85 mutants in response to ABA and salt stress. (A) The expression of stress responsive genes RD29A, COR15A and COR47 in Col-0 wild type and two alleles of nup85 mutants under mock and 50 μM ABA treatments for 3 h. The RNA was extracted from 7-day-old seedlings which were treated with mock or 50 μM ABA for 3 h. Data are mean values ±SD of three independent replicates. Asterisks indicate significant differences compared to WT Col under the same treatments. (B) The expression of RD29A, COR15A and COR47 in Col-0 wild type and two mutant alleles of nup85. The RNA was extracted from 7-day-old seedlings which were treated with mock or 0.3 M NaCl for 5 h. Data are mean values ±SD of three independent replicates. Significance between mean values were analyzed by student’s t test (* P < 0.05). Asterisks indicate significant differences compared to WT Col under the same treatments. (C) Gene ontology enrichment analysis of the differentially expressed (DE) genes regulated by NUP85 participates in abiotic stress responses.
overlapping genes in Col-0 wild type (S3 Table). Out of 1389 ABA responsive genes in Col-0, 178 ABA responsive genes including RD29B exhibited significantly altered expressions in nup85-1 mutants in comparison with Col-0 wild type, suggesting that about 13% of ABA responsive genes are regulated by NUP85 (S4 Table). GO analysis further revealed that those NUP85 regulated genes were also enriched at categories such as response to stimulus, response to chemical and response to abiotic stimulus (S4 Fig). It is possible that Nups may have redundant function in regulating ABA responsive genes since nup85 single mutants did not alter most ABA-responsive genes. The heat map generated with NUP85 regulated ABA-responsive genes in both Col-0 wild type and nup85-1 mutant showed that the induction of a group of ABA-responsive genes was partially suppressed by the NUP85 mutation (Fig 3D). In contrast, a small portion of DE genes were up-regulated in nup85-1 mutant compared to Col-0 wild type. These results indicate that NUP85 is involved in regulating gene expressions in response to ABA.

The abiotic stress tolerance phenotypes of nup85 and other nucleoporin mutants

Since we have shown that NUP85 is important for the expression of several stress responsive genes in response to ABA and salt stress, we next examined if nup85 mutants may show altered stress phenotypes to ABA and salt stress. In plants, the NUP107–160 sub-complex consists of eight members, NUP160, NUP133, NUP107, NUP96, NUP85, NUP43, SEH1 and SEC13 [3,21,24]. Thus, we isolated homozygous mutant plants for the other seven nucleoporins and tested their stress phenotypes. As shown in S5 Fig, mutation of NUP43, NUP107, NUP133, NUP96, SEC13 and SEH1 did not cause obvious changes in their responses to ABA and salt stress when compared to Col-0 wild type control plants. The average root length of those mutants was similar to the Col-0 wild type control after being transferred to ABA and NaCl containing medium. Nevertheless, two alleles of nup85 mutants were slightly more sensitive to ABA and salt stress as their root length was shorter than the wild type control after being transferred to ABA and NaCl-containing medium (Fig 4A and 4B). In contrast, the root length of Col-0 wild type and nup85 mutants was comparable when they were transferred to control medium. In addition to the nup85 mutants, hos1 and nup160 mutants also showed increased sensitivity to ABA and salt stress as their root growth was significantly less compared to the Col-0 wild type after being transferred to the ABA and NaCl containing medium (Fig 4C and 4D). We also examined the responses of nup85-1 nup160 and nup85-1 hos1 double mutants to ABA and salt stress. As illustrated in Fig 5A, nup85-1, nup160, hos1 single mutants and those two double mutants all displayed hypersensitivity to ABA and NaCl because their root length was shorter than Col-0 wild type after being transferred to the indicated ABA and NaCl containing medium. The root length of the double mutants was not evidently shorter than the single mutant (Fig 5B), indicating a lack of additive phenotypes in the double mutant, and thus suggesting that the Nups may function in the same genetic pathway in regulation of the tested stress responses. These genetic results suggest that NUP85, NUP160 and HOS1 are involved in ABA and salt stress responses.

Expression of stress responsive genes is also attenuated in nup160 and hos1 mutants

To investigate if NUP160 and HOS1 affect the expression of stress responsive genes in response to ABA and salt stress, we treated Col-0 wild type, nup160 and hos1 mutants with mock,
50 μM ABA or 0.3 M NaCl. The results showed that ABA and salt stress induced expression of RD29A, COR15A and COR47 was expressively reduced in nup160 and hos1 mutants after ABA and salt treatment when compared to the Col-0 wild type (Fig 6A and 6B). Additionally, we also tested the expression of RD29B in the Col-0 wild type and those mutants. We found that the expression of RD29B was significantly inhibited in nup160, hos1 and nup85-1 mutants compared to Col-0 wild type (S6 Fig). These results suggest that HOS1 and NUP160 are also required for proper expression of stress responsive genes.

Identification of putative NUP85 interacting proteins

Recent proteomics studies have revealed more than 22 Nups in plants [24]. To better understand the biological functions of NUP85 and its associating proteins, we performed anti-MYC immunoprecipitation and subsequent mass spectrometry (IP-MS) in WT and Nup85pro: NUP85-MYC transgenic plants. From two independent IP-MS experiments, we totally
identified 55 putative NUP85 interacting proteins following two criteria to rule out the non-
specifically interacted proteins (1) proteins are present in the two independent replicates of
IP-MS data; (2) proteins that have unique peptides or significantly more peptides (at least

Fig 5. The ABA and salt hypersensitivity of nup85-1 nup160 and nup85-1 hos1 double mutants. (A) The root growth of Col-0 wild type, nup85-1, nup160, hos1 single mutants and nup85-1 nup160 as well as nup85-1 hos1 double mutants on control 1/2 MS and ABA or NaCl-containing MS medium. (B) Quantification of the primary root length of indicated genotypes at 7 days after transfer to control and ABA or NaCl-containing MS medium. Three-day-old seedlings from each genotype were transferred to the indicated media and primary root length was measured after 7 days. Values indicate means ± SD (n = 24). Asterisks indicate significant differences compared to WT Col under the same treatments. Significance between the mean values were analyzed with Student’s t test (* P < 0.05, ** P < 0.01).

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5-fold more) identified in NUP85 transgenic plants compared to WT samples. The full list of putative NUP85 interacting proteins are listed in S5 Table, NUP160, NUP133, NUP43, NUP96, NUP107 and Seh1 as well as Sec13 were present in the NUP85 immuno-complex with abundant unique peptides, confirming the conserved configuration of NUP107-160 sub-complex in plants. HOS1 was also present in the NUP85 immuno-complex with comparable abundance to other Nups in NUP107-160 complex. Besides Nups, some Transducin/WD40 repeat-like superfamily proteins such as Sec13A, which are known to be involved in mRNA and protein transport, were found in the NUP85 immuno-complex with comparable abundance to other Nups in NUP107-160 complex. Besides Nups, several mediator subunits including MED16, MED14 and MED18 were also precipitated by NUP85, suggesting that possible involvement of NUP85 and other Nups in transcriptional regulation. To validate some of the candidate interacting proteins, we cloned HOS1 and Sec13A, and tested their interactions with NUP85 in split-luciferase (LUC) complementation assays. The results indicated that NUP85 could directly interact with Sec13A but not HOS1 (S7 Fig).

The interaction between NUP85 and MED18

To investigate if there is an interaction between NUP85 and mediator subunits, we selected MED18, which has been reported to be involved in ABA signaling pathway [25,26]. As shown in Fig 7A, the co-expression of MED18-Cluc and NUP85-Nluc resulted in strong LUC activity

Fig 6. The impaired expression of stress responsive genes in nup160 and hos1 mutants in response to ABA and salt stress. (A)The expression of stress responsive genes RD29A, COR15A and COR47 in Col-0 wild type, nup160 and hos1 mutants under mock and ABA treatment. (B)The expression of RD29A, COR15A and COR47 in Col-0 wild type, nup160 and hos1 mutants under mock and salt treatments. RNA was extracted from 7-day-old seedlings which were treated with mock, 50 μM ABA or 0.3 M NaCl. Data are mean values ± SD of three independent replicates. Significance between mean values were analyzed by student’s t-test (* P<0.05, ** P<0.01). Asterisks indicate significant differences compared to WT Col under the same treatments.

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Fig 7. The interaction between NUP85 and MED18. (A) Split luciferase complementation assay in *Arabidopsis* protoplasts showing the interaction between NUP85 and MED18. Empty Cluc and PYL1-Cluc were used as negative controls. Approximately $1 \times 10^4$ protoplasts per sample were co-transformed with indicated plasmids. The split-LUC complementation assay was repeated three independent times with consistent results. (B) Co-immunoprecipitation assays in *Arabidopsis* protoplasts confirming the interaction between NUP85 and MED18. CDK8-HA was used as a control. (C) The relative gene expression of
in the split-LUC complementation assay. In contrast, the co-expression of NUP85-Nluc and empty Cluc or PYL1-Cluc did not cause any detectable LUC activities. Co-immunoprecipitation assay in protoplasts showed that MED18-HA was precipitated by NUP85-GFP (Fig 7B), further confirming the interaction between NUP85 and MED18. In addition, we also investigated the temporal and spatial expression patterns of \( \text{NUP85} \) and \( \text{MED18} \) in Arabidopsis electronic fluorescent pictograph (eFP) browser. As illustrated in S8 Fig, both \( \text{NUP85} \) and \( \text{MED18} \) are widely expressed in most of tissues such as seeds, leaves, and stems, especially in shoot apex and flowers with higher expression levels.

We next examined ABA and salt stress induced gene expression in Col-0 wild type and \( \text{med18} \) mutants (Fig 7C). Similar to \( \text{nup85} \) mutants, the ABA and salt stress induced expression of \( \text{RD29A}, \text{COR15A} \) and \( \text{COR47} \) was also inhibited in \( \text{med18} \) mutants relative to the wild type Col-0, signifying overlapping functions of \( \text{MED18} \) and \( \text{NUP85} \). To further explore the mechanism of how Nups regulate the expression of stress responsive genes, we examined \( \text{ABI5} \) expression in wild type Col-0, \( \text{nup85-1, nup160} \) and \( \text{hos1} \) as well as \( \text{med18} \) mutants under mock or ABA treatments. \( \text{ABI5} \) is not only one of the \( \text{MED18} \) targeted genes [25], but also a key regulator for the expression of stress responsive genes [27]. As illustrated in S9 Fig, \( \text{ABI5} \) expression was obviously inhibited in those \( \text{Nup} \) mutants and \( \text{med18} \) mutants after ABA treatments when compared to the wild type Col-0. Consistent with the interaction between \( \text{NUP85} \) and \( \text{MED18} \), \( \text{med18} \) mutants also displayed hypersensitivities to ABA and salt stress because their primary root length was shorter than Col-0 wild type after transferred to MS medium containing indicated concentrations of ABA and NaCl (Fig 8).

**Discussion**

Although core factors in abiotic stress signaling and responses have been identified with extensive genetic and biochemical studies in plants [28], abiotic stress responses remain intricate as many other factors yet to be discovered. In our present study, we have identified \( \text{NUP85} \) as a regulator of ABA and salt stress responsive genes. The mutation of \( \text{NUP85} \) impaired the ABA- and salt stress-induced expression of \( \text{RD29A} \) and several other stress-responsive genes, suggesting that \( \text{NUP85} \) may be a positive regulator for ABA and salt responses. Similarly, mutation of other Nups such as \( \text{HOS1} \) and \( \text{NUP160} \) also reduced the expression of stress responsive genes, thereby indicating that \( \text{NUP85} \) and other Nups components have overlapping functions in regulating the expression of stress responsive genes. Consistent with the altered expression of stress responsive genes, \( \text{nup85, nup160} \) and \( \text{hos1} \) mutants are hypersensitive to ABA and salt stress. Thus, our results indicate the involvement of Nups in ABA signaling and salt stress responses.

Currently, one of the best characterized NPC components is \( \text{HOS1} \), which has been shown to associate with the chromatin of \( \text{FLOWERING LOCUS C (FLC)} \) and the promoter region of \( \text{MIR168b} \), to regulate flowering time and miRNA biogenesis [15,23]. Nevertheless, how other Nups regulate gene expression remains largely unknown. Based on previous and our present studies, NPC components were suggested to play a role in gene expression regulation through various mechanisms. In fact, a number of previous studies have revealed that mutations in NPC components resulted in the accumulation of mRNAs in the nuclei [3,7,8,29,30], which could subsequently affect the expression of genes such as stress responsive genes in plants. Moreover, increasing evidence supported NPC as gene-gating organelles which could recruit
actively transcribed genes to them and regulate gene expression [31]. It is likely that transcription of group of stress responsive genes is dependent on certain NPC components in response to different environmental stresses. Our results showed that the expression of RD29A and several stress responsive genes was impaired at the transcriptional level in nup85, hos1 and nup160 mutants, indicating that these NPC components are required for high level transcription of the stress responsive genes in response to ABA and salt stress. Additionally, plant NPC was recently demonstrated to undergo conformational switch in response to pathogen infection, allowing significant activation of nucleocytoplasmic transport and multiple stress-related

Fig 8. The hypersensitivity of med18 in response to ABA and salt stress. (A) The root growth of Col-0 wild type and med18 mutants on control ½ MS and ABA or NaCl-containing MS medium at 7 days after transfer. (B) Quantification of the primary root length of Col-0 wild type and med18 mutants at 7 days after transfer to control and ABA or NaCl-containing MS medium. Three-day-old seedlings were transferred to the ½ MS or ABA/NaCl containing media and primary root length was measured after 7 days. Values indicate means ± SD (n = 20). Asterisks indicate significant differences compared to WT Col under the same treatments. Significance between the mean values were analyzed with Student’s t test (* P < 0.05).

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signaling pathways [32]. The study raised the possibility that NPC components may facilitate the transcription of stress responsive genes in response to various environmental stresses by controlling the transport of critical signaling components and transcriptional regulators between the nuclei and cytoplasm.

In addition to these evidence, proteomics data from our studies further revealed several putative NUP85 interactors, which not only broadened our understanding of NPC functions in plants, but also indicated some potential mechanisms of how NPC components regulate the gene expression. It is worth noting that all the proteins identified from our proteomics data are putative NUP85 interacting proteins until they are validated by further experiments. Our IP-MS results suggested that NUP85 forms a complex with HOS1 and seven other nucleoporins located in the NUP107-160 sub-complex in plants, thus confirming the conserved configuration of NUP107-160 sub-complex in eukaryotic cells. Additionally, some Transducin/WD40-repeat proteins were co-purified with NUP85 including Sec13A, which are involved in assembling NPC domains, signal transduction and mRNA and protein transport [24]. Another interesting discovery from our proteomics data was that several mediator subunits were also present in the NUP85 immuno-complex, providing an alternative way for NUP85 and other Nups to regulate transcription. Recent studies in yeast cells showed that nuclear pore-associated TREX-2 complex directly interacts with mediators to regulate gene expression through the RNA Pol II transcription machinery [33]. However, whether NPC could regulate transcription through the mediator complex remains unknown in plants. Our data suggested the possibility of NUP85 and other NPC components in accessing the core transcriptional machinery through modulating the mediator complex and RNA Pol II association. Among the mediator subunits co-purified by NUP85, MED18 has been known to be involved in ABA signaling, flowering control and plant defense [25,34]. The ABA and salt stress induced expression of several stress responsive genes is significantly attenuated in med18 mutants, which was similarly observed in nup85 mutants. Importantly, we further demonstrated that NUP85 and MED18 are both important for the expression of ABI5, which could activate the expression of downstream stress responsive genes [35]. In summary, our study illustrates that NUP85 and some Nups such as NUP160 and HOS1 regulate stress-responsive gene expression through cooperating with mediator complex. Thus, our findings provide new insights into the role of Nups in ABA signaling and salt responses.

Materials and methods

Plant materials, growth conditions and stress phenotypic analysis

To screen for new regulatory components involved in abiotic stresses, we first performed EMS mutagenesis on sic–1 mutants expressing RD29Apro-LUC as described in [23]. The mutants with significant reduced luminescence in response to 100 μM ABA or 200 mM NaCl were selected. The wild type (WT) for LUC assays refers to Col-0 ecotype with the gl–1 mutation harboring the proRD29A-LUC transgene. For abiotic stress phenotypic analysis and gene expression, WT refers to Col-0. The T-DNA insertion mutants nup85-1 (SALK_133369C), nup85-2 (SALK_113274C), nup160 (SALK_126801C), hos1 (SALK_069312C), nup43 (SALK_095344C), nup107 (SALK_057072), nup96 (SALK_135920C), nup133 (SALK_092608C), sec13 (SALK_045825C), seh1 (SALK_022717C) and med18 (SALK_027178C) were in Col-0 background and were obtained from the Arabidopsis Information Resource Center (ABRC). nup85-1 nup160 double mutant was described in [36]. nup85-1 hos1 double mutant was generated by crossing the nup85-1 and hos1 single mutant. The seeds were surface sterilized and sown on half Murashige and Skoog (MS) medium containing 1% sucrose and 0.8% agar. After 2 days in 4°C, the plates were moved to growth chamber under photoperiod of 16 h light/8 h dark.
Root growth inhibition assays were performed as described previously [37]. For post-germination root growth assays, 3 or 4-days-old seedlings were first germinated on vertical half MS medium and then were transferred to ABA (20 μM) or NaCl (100 mM) containing medium and the primary root growth was documented and quantitatively measured at 7 days after transfer.

Generation of transgenic plants
To generate NUP85 complementation lines, the genomic sequence of NUP85 with about 2 kb upstream sequence before its start codon was amplified by high-fidelity DNA polymerase (PrimeSTAR HS DNA Polymerase, Clontech). The PCR product was first inserted into pENTR vector (Invitrogen), and then was transferred to destination vector pGWB-16 (MYC tag) via LR reaction. After verified by sequencing, the construct was introduced into Agrobacterium GV3101 for plant transformation. All indicated constructs were transformed by floral dip method [38]. The homozygous NUP85 complementation lines were used for affinity purification.

 Luciferase imaging
Twelve-day-old seedlings of wild-type, sic–1 and sic–1 nup85 were sprayed with distilled water or indicated concentrations of ABA and NaCl solutions for 3h and 5h, respectively. The LUC images were captured using a low-light video imaging system with WinView software.

Map-based cloning and whole genome sequencing
Map-based cloning was performed as described [23]. The F2 mutants with suppressed LUC phenotype were selected by genotyping for sic-1 mutant. The results indicated that the mutation was located in chromosome 4 between 12,980 Kb and 16,500 Kb. Whole genome sequencing was performed, and a mutation was found in At4g32910 locus.

Quantitative real-time PCR
RNA was extracted from 7-day-old seedlings by RNase plant mini kit (QIAGEN) followed by DNase (Turbo) digestion. For testing gene expressions, 1 μg total RNA was used for reverse transcription using M-MLV reverse transcriptase (Promega) according to the manufacturer’s instructions. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on a CFX96 real-time PCR detection system (Bio-Rad). Actin 2 (ACT2) was used as the internal reference for all reactions. The relative gene expression value was calculated as described previously [39]. All primers were listed in S6 Table.

RNA-sequencing and data analysis
Wild type and nup85 mutant seeds were germinated on 1/2 MS medium in growth chamber at 24°C for 7 days and were treated with mock or 3 hr of 50 μM ABA at room temperature. The total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer’s instruction and RNA-sequencing were carried out by the Core Facility of Genomics in Shanghai Plant Stress Biology Center, China. Quality control was checked using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc). RNA-Seq reads were trimmed using the fastx_trimmer command FASTX-Toolkit (hannonlab.cshl.edu/fastx_toolkit/index.html) with parameter “--1 11 -l 80” before alignment. Trimmed reads were mapped to Arabidopsis reference genome (TAIR10) using TopHat2 using ”—b2-very-sensitive” option [40]. Read count for each gene were obtained using featureCounts [41]. Differentially expressed (DE) genes were identified using DESeq2 [42]. NUP85-regulated genes were defined under the criteria: 1)
genes were differentially expressed after ABA in Col-0 with 4-fold change; 2) gene expressions in *nup85* mutants were at least 1.5-fold higher or lower than those in wild type after ABA treatments. GO enrichment analysis were performed at http://geneontology.org/.

**Affinity purification and mass spectrometry**

For affinity purification of NUP85 and its associated proteins, about two grams of 12-day-old *NUP85pro: NUP85-MYC* transgenic seedlings were collected and same amount of WT seedlings was used as a negative control. Total protein extraction and affinity purification were performed as described previously [39]. The protein supernatants were incubated with 30 μL of anti-MYC agarose beads (Abcam), which had been pre-equilibrated with lysis buffer. After incubation at 4°C with rotation for 4 hours, the agarose beads were washed four times with lysis buffer followed by one time wash with 1 mL of PBS buffer. The agarose beads were finally resuspended in 100 μL of PBS buffer. After trypsin digestion, the mass spectrometer was operated in the data-dependent mode in which a full MS scan (from m/z 350–1500 with the resolution of 30,000 at m/z 400), followed by the 10 most intense ions being subjected to collision-induced dissociation (CID) fragmentation. CID fragmentation was performed and acquired in the linear ion trap (normalized collision energy (NCE) 30%, AGC 3e4, max injection time 100 ms, isolation window 3 m/z, and dynamic exclusion 60 s) according to [43]. The raw files were searched directly against the *Arabidopsis thaliana* database (TAIR10) with no redundant entries using Proteome Discover 2.1. Precursor mass tolerance was set at 10 ppm, and the MS/MS tolerance was set at 0.6 Da. The searches were performed with trypsin digestion and allowed a maximum of two missed cleavages on the peptides analyzed from the sequence database. The false discovery rates for proteins and peptides were set at 0.01, and the minimum peptide length was six amino acids. To identify significantly changed proteins from IP-MS results, we performed two biological replicates. The putative interacting proteins of NUP85 were selected based on two criteria: (1) proteins were identified in both replicates of IP-MS data or (2) proteins that have unique peptides or significantly more peptides in NUP85 transgenic plants than in control samples.

**Split Luciferase (LUC) complementation**

The full-length coding sequences of *NUP85, HOS1, Sec13A* and *MED18* were amplified by PCR using primers listed in S2 Table. The PCR products were first cloned into pENTER vector (Invitrogen) and then transferred to nLUC/cLUC vectors via LR reactions. Split-LUC complementation assay was performed in *Arabidopsis* protoplasts per [39]. The reconstitute LUC activity was detected in the dark by cooling camera. The image and quantification of LUC activities were analyzed with Winview software.

**Co-Immunoprecipitation assay**

The NUP85-GFP and MED18-HA or CDK8-HA were transiently co-expressed in *Arabidopsis* protoplasts per [39]. After transformation, the protoplasts were collected and suspended in 1 mL lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM DTT, 0.1 (v/v) Triton X-100 and Protease Inhibitor Cocktail (Sigma-Aldrich) in ice for 20 min. The lysis was then centrifuged at 13000 rpm for 15 min at 4°C and was incubated with pre-equilibrant GFP-Trap beads (Chromo Tek) for at least 4 hours with continuous rotation. The beads were washed at least four times with lysis buffer at 4°C and boiled in 4× SDS loading buffer for 10 min. Protein samples were separated by SDS-PAGE and were further detected with polyclonal anti-HA (Abcam) and anti-GFP antibody (Roche).
Supporting information

S1 Fig. The expressions of COR15A and RD29B in WT, sic-1 and sic-1 nup85 double mutants under mock and ABA treatment. RT-qPCR analysis showed that the expressions of ABA responsive genes COR15A and RD29B were significantly lower in sic-1 nup85 double mutants when compared to sic-1. Values represent means ± SD (n = 3).

S2 Fig. The leaf size and morphology of WT, sic-1, sic-1 nup85 double mutants and NUP85 complementation lines. (A) The typical leaves detached from indicated 5-week-old plants grown in soil. (B) The morphology of indicated genotypes grown in soil under normal growth conditions.

S3 Fig. Verification of nup85 T-DNA insertion mutants. (A) genomic DNA PCR showing the homozygous of nup85 mutants. (B) The relative gene expression levels of NUP85 in Col-0 wild type and two lines of nup85 mutants. The RNA was extracted from leaves of 4-week-old plants in soil. Data represent means value ± SD (n = 3). Significance between mean values were analyzed by student’s t test (* P < 0.05). Asterisks indicate significant differences compared to WT Col under the same treatments.

S4 Fig. Gene ontology enrichment analysis of NUP85 regulated ABA responsive genes (p value < 0.05).

S5 Fig. The root length of Col-0 wild type and nucleoporin mutants under control, ABA and salt stress. The root length was documented at 7 days after seedlings transfer to ½ MS plates, 20 μM ABA or 100 mM NaCl MS plates At least twelve 3-day-old seedlings from each genotype were transferred and root length was measured after 7 days. The experiments were repeated two independent times. Values indicate means ± SD (n = 24).

S6 Fig. The expression of RD29B in Col-0 wild type, hos1, nup160 and nup85-1 mutants under mock and ABA treatments. Values represent means ± SD (n = 3). Asterisks indicate significant differences compared to WT Col under the same treatments. Significance between mean values were analyzed by student’s t test (* P < 0.05).

S7 Fig. NUP85 interacts with Sec13A but not HOS1. Split-LUC complementation assays showing the interactions between NUP85 and Sec13A or HOS1 in Arabidopsis protoplasts. Approximately 1×10⁴ protoplasts per sample were co-transformed with indicated plasmids. The split-LUC complementation assay was repeated three independent times with similar results.

S8 Fig. The expression patterns of NUP85 and MED18 in Arabidopsis eFP Browser. The snapshot of tissue specific expression patterns of MED18 and NUP85 from Arabidopsis eFP Browser.

S9 Fig. The relative expression of ABI5 in wild type, nup85-1, nup160 and hos1 under mock or ABA treatments. Values indicate means ± SD (n = 3). Asterisks indicate significant
differences compared to WT Col under the same treatments. Significance between the mean values were analyzed with Student’s t test ( * P < 0.05).

(TIF)

S1 Table. The list of differentially expressed genes regulated by NUP85 under mock conditions.
(XLSX)

S2 Table. The list of ABA responsive genes identified in Col-0 wild type.
(XLSX)

S3 Table. The list of ABA responsive genes identified in nup85-1 mutants.
(XLSX)

S4 Table. The list of ABA responsive genes regulated by NUP85 in response to ABA.
(XLSX)

S5 Table. The list of putative NUP85 interacting proteins identified from IP-MS experiments.
(XLSX)

S6 Table. The list of primers used in the study.
(XLSX)

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References
1. Gorlich D, Kutay U (1999) Transport between the cell nucleus and the cytoplasm. Annu Rev Cell Dev Biol 15: 607–680. https://doi.org/10.1146/annurev.cellbio.15.1.607 PMID: 10611974
2. Weis K (2003) Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. Cell 112: 441–451. PMID: 12600309

3. Parry G (2015) The plant nuclear envelope and regulation of gene expression. J Exp Bot 66: 1673–1685. https://doi.org/10.1093/jxb/erv023 PMID: 25680795

4. Alber F, Dokudovskaya S, Veenhoff LM, Zhang W, Kipper J, et al. (2007) The molecular architecture of the nuclear pore complex. Nature 450: 695–701. https://doi.org/10.1038/nature06405 PMID: 18046406

5. Xu XM, Meier I (2008) The nuclear pore comes to the fore. Trends Plant Sci 13: 20–27. https://doi.org/10.1016/j.tplants.2007.12.001 PMID: 18046406

6. Meier I, Brkljacic J (2009) The nuclear pore and plant development. Curr Opin Plant Biol 12: 87–95. https://doi.org/10.1016/j.pbi.2008.09.001 PMID: 18938103

7. Parry G, Ward S, Cernac A, Dharmasiri S, Estelle M (2006) The Arabidopsis SUPPRESSOR OF AUXIN RESISTANCE proteins are nucleoporins with an important role in hormone signaling and development. Plant Cell 18: 1590–1603. https://doi.org/10.1105/tpc.106.041566 PMID: 16751346

8. Dong CH, Hu X, Tang W, Zheng X, Kim YS, et al. (2006) A putative Arabidopsis nucleopore, AtNUP160, is critical for RNA export and required for plant tolerance to cold stress. Mol Cell Biol 26: 9533–9543. https://doi.org/10.1128/MCB.01063-06 PMID: 17030626

9. MacGregor DR, Penfield S (2015) Exploring the pleiotropy of hos1. J Exp Bot 66: 1661–1671. https://doi.org/10.1093/jxb/erv022 PMID: 25697795

10. Ishitani M, Xiong L, Lee H, Stevenson B, Zhu JK (1998) HOS1, a genetic locus involved in cold-responsive gene expression in arabidopsis. Plant Cell 10: 1151–1161. PMID: 9668134

11. Lee H, Xiong L, Gong Z, Ishitani M, Stevenson B, et al. (2001) The Arabidopsis HOS1 gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo—cytoplasmic partitioning. Genes Dev 15: 912–924. https://doi.org/10.1101/gad.866801 PMID: 11297514

12. Dong CH, Agarwal M, Zhang Y, Xie Q, Zhu JK (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. Proc Natl Acad Sci U S A 103: 8281–8286. https://doi.org/10.1073/pnas.0602874103 PMID: 16702557

13. Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, et al. (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. Genes Dev 17: 1043–1054. https://doi.org/10.1101/gad.1077503 PMID: 12672693

14. Jung JH, Park JH, Lee S, To TK, Kim JM, et al. (2013) The cold signaling attenuator HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1 activates FLOWERING LOCUS C transcription via chromatin remodeling under short-term cold stress in Arabidopsis. Plant Cell 25: 4378–4390. https://doi.org/10.1105/tpc.113.118364 PMID: 24220632

15. Jung JH, Seo PJ, Park CM (2012) The E3 ubiquitin ligase HOS1 regulates Arabidopsis flowering by mediating CONSTANS degradation under cold stress. J Biol Chem 287: 43277–43287. https://doi.org/10.1074/jbc.M112.394338 PMID: 23135282

16. Lazaro A, Mouriz A, Pineiro M, Jarillo JA (2015) Red Light-Mediated Degradation of CONSTANS by the E3 Ubiquitin Ligase HOS1 Regulates Photoperiodic Flowering in Arabidopsis. Plant Cell 27: 2437–2454. https://doi.org/10.1105/tpc.15.00529 PMID: 26373454

17. Roth C, Wiemer M (2012) Nucleoporins Nup160 and Seh1 are required for disease resistance in Arabidopsis. Plant Signal Behav 7: 1212–1214. https://doi.org/10.4161/psb.21426 PMID: 22902705

18. Wiemer M, Cheng YT, Imkamp J, Li M, Wang D, et al. (2012) Putative members of the Arabidopsis Nup107-160 nuclear pore sub-complex contribute to pathogen defense. Plant J 70: 796–808. https://doi.org/10.1111/j.1365-313X.2012.04928.x PMID: 22288649

19. Saito K, Yoshikawa M, Yano K, Miwa H, Uchida H, et al. (2007) The Arabidopsis nucleoprotein NUP85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in Lotus japonicus. Plant Cell 19: 610–624. https://doi.org/10.1105/tpc.106.046938 PMID: 17307929

20. Kanamori N, Madsen LH, Radutoiu S, Frantescu M, Quistgaard EM, et al. (2006) A nucleopore is required for induction of Ca2+ spiking in legume nodule development and essential for rhizobial and fungal symbiosis. Proc Natl Acad Sci U S A 103: 359–364. https://doi.org/10.1073/pnas.050883103 PMID: 16407163

21. Du J, Gao Y, Zhan Y, Zhang S, Wu Y, et al. (2016) Nucleocytoplasmic trafficking is essential for BAK1- and BKK1-mediated cell-death control. Plant J 85: 520–531. https://doi.org/10.1111/tpj.13125 PMID: 26775605

22. Zhan X, Wang B, Li H, Liu R, Kalia RK, et al. (2012) Arabidopsis proline-rich protein important for development and abiotic stress tolerance is involved in microRNA biogenesis. Proc Natl Acad Sci U S A 109: 18198–18203. https://doi.org/10.1073/pnas.1216199109 PMID: 23071326
23. Wang B, Duan CG, Wang X, Hou YJ, Yan J, et al. (2015) HOS1 regulates Argonaute1 by promoting transcription of the microRNA gene MIR168b in Arabidopsis. Plant J 81: 861–870. https://doi.org/10.1111/tpj.12772 PMID: 25619693

24. Tamura K, Fukao Y, Iwamoto M, Haraguchi T, Hara-Nishimura I (2010) Identification and characterization of nuclear pore complex components in Arabidopsis thaliana. Plant Cell 22: 4084–4097. https://doi.org/10.1105/tpc.110.079497 PMID: 21189294

25. Lai Z, Schluttenhofer CM, Bhide K, Shreve J, Thimmmapuram J, et al. (2014) MED18 interaction with distinct transcription factors regulates multiple plant functions. Nat Commun 5: 3064. https://doi.org/10.1038/ncomms4064 PMID: 24451981

26. Liao CJ, Lai Z, Lee S, Yun DJ, Mengiste T (2016) Arabidopsis HOOKLESS1 Regulates Responses to Pathogens and Abscisic Acid through Interaction with MED18 and Acetylation of WRKY33 and ABI5 Chromatin. Plant Cell 28: 1662–1681. https://doi.org/10.1105/tpc.16.00105 PMID: 27317674

27. Finkelstein RR, Lynch TJ (2000) The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. Plant Cell 12: 599–609. PMID: 10670247

28. Zhu JK (2016) Abiotic Stress Signaling and Responses in Plants. Cell 167: 313–324. https://doi.org/10.1016/j.cell.2016.08.029 PMID: 27716505

29. Germain H, Qu N, Cheng YT, Lee E, Huang Y, et al. (2010) MOS11: a new component in the mRNA export pathway. PLoS Genet 6: e1001250. https://doi.org/10.1371/journal.pgen.1001250 PMID: 21203492

30. Chinnusamy V, Gong Z, Zhu JK (2008) Nuclear RNA export and its importance in abiotic stress responses of plants. Curr Top Microbiol Immunol 326: 235–255. PMID: 18630756

31. Blobel G (1985) Gene gating: a hypothesis. Proc Natl Acad Sci U S A 82: 8527–8529. PMID: 3866238

32. Gu Y, Zebell SG, Liang Z, Wang S, Kang BH, et al. (2016) Nuclear Pore Permeabilization Is a Convergent Signaling Event in Effector-Triggered Immunity. Cell 166: 1526–1538 e1511. https://doi.org/10.1016/j.cell.2016.07.042 PMID: 27569911

33. Schneider M, Hellerschmied D, Schubert T, Amlacher S, Vinayachandran V, et al. (2015) The Nuclear Pore-Associated TREX-2 Complex Employs Mediator to Regulate Gene Expression. Cell 162: 1016–1028. https://doi.org/10.1016/j.cell.2015.07.059 PMID: 26317468

34. Zheng Z, Guan H, Leal F, Grey PH, Oppenheimer DG (2013) Mediator subunit18 controls flowering time and floral organ identity in Arabidopsis. PLoS One 8: e53924. https://doi.org/10.1371/journal.pone.0053924 PMID: 23326539

35. Brocard IM, Lynch TJ, Finkelstein RR (2002) Regulation and role of the Arabidopsis abscisic acid-insensitive 5 gene in abscisic acid, sugar, and stress response. Plant Physiol 129: 1533–1543. https://doi.org/10.1104/pp.005793 PMID: 12177466

36. Parry G (2014) Components of the Arabidopsis nuclear pore complex play multiple diverse roles in control of plant growth. J Exp Bot 65: 6057–6067. https://doi.org/10.1093/jxb/erus346 PMID: 25165147

37. Fuji H, Zhu JK (2009) Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. Proc Natl Acad Sci U S A 106: 8380–8385. https://doi.org/10.1073/pnas.0903144106 PMID: 19420218

38. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743. PMID: 10069079

39. Zhu Y, Schluttenhofer CM, Wang P, Fu F, Thimmmapuram J, et al. (2014) CYCLIN-DEPENDENT KINASE8 differentially regulates plant immunity to fungal pathogens through kinase-dependent and -independent functions in Arabidopsis. Plant Cell 26: 4149–4170. https://doi.org/10.1105/tpc.114.128611 PMID: 25281690

40. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, et al. (2013) TopHat2: accurate alignment of transcripts in the presence of insertions, deletions and gene fusions. Genome Biol 14: R36. https://doi.org/10.1186/gb-2013-14-4-r36 PMID: 23618408

41. Liao Y, Smyth GK, Shi W (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30: 923–930. https://doi.org/10.1093/bioinformatics/btt656 PMID: 24227677

42. Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15: 550. https://doi.org/10.1186/s13059-014-0550-0 PMID: 25516281