A novel family of plant nuclear envelope associated proteins.

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Statement of novelty

We describe a novel family of Nuclear Envelope Associated Proteins, NEAPs, which interact with each other, the SUN domain proteins and a putative transcription factor.

Abstract

This paper describes the characterisation of a new family of higher plant nuclear envelope associated proteins (NEAPs) that interact with proteins of the nuclear envelope. In the model plant *Arabidopsis thaliana*, the family consists of three genes expressed ubiquitously (*AtNEAP1-3*) and a pseudogene (*AtNEAP4*). NEAPs consist of extensive coiled-coil domains, followed by a nuclear localisation signal and a C-terminal predicted transmembrane domain. Domain deletion mutants confirm the presence of a functional nuclear localisation signal and transmembrane domain. AtNEAP proteins localise to the nuclear periphery as part of stable protein complexes, are able to form homo- and heteromers and interact with the SUN domain proteins AtSUN1 and AtSUN2, involved in the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex. An *A. thaliana* cDNA library screen identified a putative transcription factor called AtbZIP18 as a novel interactor of AtNEAP1, which suggest a connection between NEAP and chromatin. An *Atneap1 Atneap3* double knock out mutant showed reduced root growth and altered nuclear morphology and chromatin structure. Thus AtNEAPs are suggested as INM anchored coiled-coil proteins with roles in maintaining nuclear morphology and chromatin structure.

Key words:

Nuclear envelope, nucleoskeleton, chromatin, nuclear lamina, *Arabidopsis thaliana*, higher plant.

Introduction

The nuclear envelope (NE) in opisthokonts is closely associated with the proteins of the nuclear lamina and chromatin (Crisp et al., 2006; Evans et al., 2014). Proteins integral to the inner nuclear membrane (INM) link chromatin, the lamina and nuclear membranes. A key protein family involved in this process is the SUN domain protein family. SUN proteins interact with Klarsicht-Anc1-Syne1 Homology (KASH) domain proteins in the ONM, linking to the cytoskeleton, and to lamins in the nucleus, as part of the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex that spans the NE (Tzur et al., 2006). Interaction of the NE,

lamina and chromatin plays important roles in meiosis and mitosis, in chromatin positioning and silencing, in positioning nuclei and in signalling (Okada et al. 2005, Gonzalez-Suarez et al. 2009, Dechat et al. 2010, Smith et al., 2015). Previous studies have shown that SUN proteins are also present in plants and that they are also part of LINC complexes associating with plant-specific KASH proteins at the ONM and plant-specific nuclear filamentous proteins at the nuclear face of the NE (Moriguchi et al. 2005, Graumann et al. 2010, Murphy et al. 2010, Oda and Fukuda 2011, Graumann and Evans 2011, Graumann et al., 2014).

A nuclear lamina has been described underlying and closely associated with the INM in metazoan (Gruenbaum et al., 2005) and has been suggested to be present in plant nuclei, too (Fiserova et al., 2009). While the lamina of animal cells has been well characterised, that of plants is much less well described. The lamina of animal cells is comprised of lamins, type-5 intermediate filament proteins, and lamin associated proteins (reviewed by Wilson and Berk, 2010). Sequence homologues of mammalian lamins are not found in plants (Brandizzi et al., 2004; Meier, 2007; Graumann and Evans, 2010a, Koreny and Field 2016). However, there is a meshwork of proteins underlying and attached to the plant INM (Minguez and Moreno Diaz de la Espina, 1993; Masuda et al., 1997; Fiserova et al., 2009, Ciska and Moreno Díaz de la Espina, 2013; Sakamoto and Takagi, 2013). Proteins which may be components of this plant 'lamina' include the NMCPs (nuclear matrix constituent proteins) also known as LINC (little nuclei) and CRWN (crowded nuclei) in Arabidopsis (Masuda et al., 1993; Ciska et al., 2013; Ciska and Moreno Díaz de la Espina, 2013; Sakamoto and Takagi, 2013). They have multiple coiled-coil domains, form filamentous dimers and function in control of nuclear size, shape and heterochromatin organisation (Dittmer et al., 2007; van Zanten et al., 2011, 2012; Sakamoto and Takagi, 2013; Wang et al, 2013). Recently, interaction between AtCRWN1 and AtSUN1 and AtSUN2 has been suggested arguing in favour of an association between the plant lamina with the nuclear envelope (Graumann, 2014). AtKAKU4, a putative lamina component, has also been shown to be localised at the inner nuclear membrane and interacts with AtCRWN1 and AtCRWN4 (Goto et al., 2014). AtKAKU4 has been shown to affect nuclear shape and size.

In this study we describe members of a higher plant-specific family of nuclear-localised coiled-coil proteins that interact with SUN domain proteins at the nuclear periphery and suggest a role as putative bridges between NE and chromatin.

Methods

Seed stocks, plant growth and T-DNA mutants

All *A. thaliana* Transfer (T)-DNA insertion lines were ordered from the European *Arabidopsis* Stock Centre (Nottingham, UK) or ABRC, with the exception of the GABI-kat lines which were ordered from Bielefeld University (Germany). T-DNA lines were of the ecotype Col-0 and were established as homozygous lines. Seeds were germinated as described in Graumann et al., (2014) and grown in long day conditions (16 h light, 8 h dark at 18 ° C). Genotyping PCR was used for identification of homozygous T-DNA insertion lines and semi-quantitative RT-PCR to confirm absence of the corresponding mRNA (Table S1).

The SAIL_846_B07 homozygous line (*Atneap1*) was crossed with WiscDsLoxHS086_02C (*Atneap3*) and their *Atneap1 Atneap3* double heterozygous offspring were allowed to self-pollinate. Their seeds were collected and 24 seedlings were screened, several *Atneap1 Atneap3* homozygous mutant plants were identified and their progeny phenotyped (Table S1).

Membrane yeast two-hybrid system.

The Split-Ubiquitin based Membrane Yeast Two-Hybrid (MYTH) system (Snider et al., 2010a, b) was used essentially as described by Graumann et al., 2014 using the same bait and prey purchased from DUALSYSTEM Biotech (http://www.dualsystems.com). Prey constructs were cloned in the pPR3N (2μ, TRP1, AmpR) vector and bait constructs were cloned in the pBT3N (CEN, LEU2, KanR) vector. *AtNEAP* cDNA were fused to chimeric primers having 35 base pairs complementary to the linearized bait or prey plasmid on the 5′ ends, and 18 base pairs complementary to the N-terminus of *AtNEAP* cDNA on the 3′ end. *AtNEAP* cDNA were cloned in plasmid by 'gap-repair' homologous recombination in yeast (Oldenburg et al., 1997). After digestion by SfI1, prey or bait plasmids and cDNA were co-transformed into yeast in the 1:3 vector:insert ratio and successfully transformed clones were selected on test medium. Clones were then subjected to colony PCR, followed by extraction of the plasmid DNA and sequencing. *AtNEAP* containing bait vectors were verified for self-activation and only *AtNEAP1* and *AtNEAP2* baits that did not self-activate were used. Bait and prey vectors were allowed to co-transform in yeast. Presence of interaction was analysed by yeast growth on test medium (TM: YNB without Leu, Trp, Ade and His) at 30°C for more than 48 hours. The controls were grown on permissive medium (PM: YNB without Leu and Trp) in identical conditions as test medium. Clones were verified by colony PCR. The *A. thaliana* cDNA library containing 3.6 million fragments (DUALSYSTEM Biotech) cloned into the prey vector pDSL-Nx (2μ, TRP1, AmpR) was screened for novel interactors using the *AtNEAP1* bait. The library

consisted of cDNA from 6 day old etiolated seedlings as well as seedlings exposed to blue and far red light. A positive control prey included the yeast ER resident protein Ost1fused to the Nub portion of yeast ubiquitin in the pOst1–NubI (2μ, TRP1, AmpR) vector. Transformants from the screen were allowed to grow on highly restrictive medium (YNB without Leu, Trp, His, Ade) and as a backup on low stringency restrictive medium (YNB without Leu, Trp, His). Plasmid DNA was extracted from yeast colonies that grew on highly restrictive medium and sent for sequencing.

Phylogenetic reconstruction and evolution rate

AtNEAP coding sequences were used for phylogenic reconstruction and substitution rate calculation. Selected protein sequences were aligned with MUSCLE multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/muscle) and maximum likelihood analysis was performed with FastTree (http://www.microbesonline.org/fasttree) using default parameters. ω (the ratio of nonsynonymous/synonymous substitution rates) was determined using Codeml from the PaML package (Yang 2007).

RNA-Seq data mining

Already published RNA-Seq datasets from wild type Col-0 ecotype were used in order to monitor the expression of *AtNEAPs*. The Illumina RNA-Seq data are available at the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi) under accession numbers SRR1463325, SRR1463326 for epidermal cells from 10 day cotyledons, SRR1042766, SRR1042767 for primary roots from 7 day-old seedlings and SRR826283 from 10 day-old seedlings for guard cells. Reads from RNA-Seq libraries were mapped onto the candidate gene sequences allowing no mismatches using TOPHAt v 2.0.14 (Kim et al., 2013) using standard settings and maximum of multi hits set at 1, minimum intron length set at 15 bp, and maximum-intron length set as 6000 bp. Reads were summed up for each gene using HTseq-count with the overlap resolution mode set as intersection non-empty and with no strand-specific protocol (Anders et al., 2015). Transcription levels were normalised to *SAND* as for RT-qPCR and expressed in Reads per Kilobase of Exon Model (RPKM) per million mapped reads.

Domain prediction

Coiled coil domains were predicted using SMART COILS, PairCoil2 and Marcoil (Lupas et al, 1991; Dolerenzi and Speed, 2002; McDonnell et al., 2006, Letunic et al., 2012). NLSs were predicted using cNLS mapper and NLStradamus (Kosugi et al., 2009; Nguyen Ba at al., 2009).

TM domains were predicted using ARAMEMNON and DAS (Cserzo et al., 1997; Schwacke et al., 2003).

Cloning and fluorescent protein fusions

The coding sequences of *AtNEAP1*, *AtNEAP2*, *AtNEAP3*, *AtbZIP18* and domain deletion mutants were amplified using the gene-specific primers listed in Table S2. Gateway *attB* flanking sequences were added to each of the constructs and gateway technology used for cloning in pDONR207 and afterwards into expression vectors pCAMBIA 1300, pK7CWG2 and pK7WGC2 as described by Graumann et al., 2014. The primers used to generate the domain deletions *AtNEAP3ΔCC1* (aa13-93 deleted), *AtNEAP3ΔCC2* (aa124-185 deleted), *AtNEAP3ΔNLS* (aa239-264 deleted) and *AtNEAP3ΔTM* (aa314-333 deleted) are listed in Table S2. Table S3 lists all expression vectors created in this study.

Transient expression and confocal microscopy.

Leaves of 5-6 week old *Nicotiana benthamiana* were infiltrated with agrobacteria carrying expression vectors for transient expression, as described by Sparkes et al (2006) and Graumann et al., (2014). All AtNEAP-FP fusions were infiltrated at an OD of 0.1, SUN-FP fusions were infiltrated at an OD of 0.03 together with p19 at an OD of 0.05. Tissue was imaged using a Zeiss (Welwyn Garden City, UK) LSM 510 META or an inverted LSM 510 confocal laser scanning microscope fitted with 40x, 63x and 100x oil immersion objectives.

Fluorescence recovery after photobleaching (FRAP) was used to investigate the mobility of AtNEAP 1-3 fluorescent protein fusions at the NE. FRAP was carried out as described by Graumann et al., (2007). Briefly, transiently expressing *N. benthamiana* lower epidermal leaf cells were treated with Latrunculin B to immobilise the nucleus and then imaged with the 514 nm laser to excite the YFP. Scanning transmission was kept low and bleaching performed at 100% transmission. The fluorescence was monitored in a constant sized region of interest pre and post bleach. The raw data was converted to percentage and mobile fractions and half times were calculated as described by Graumann et al. (2007 and 2010). Students t-test was used for statistical analysis; 30 nuclei per sample were photobleached.

***In planta* protein interaction studies**

Acceptor photobleaching fluorescence resonance energy transfer (apFRET) was used to detect *in planta* protein interactions. ApFRET was performed as described in Graumann et al. (2010) and Graumann (2014). Briefly, transiently expressing *N. benthamiana* leaf tissue was iamged as described in the previous section. YFP was excited with 514nm light and CFP with

458nm light. The YFP laser transmission was kept low during scanning to avoid photobleaching but was set at 100% during bleach. Five pre-bleach and five post-bleach scans were carried out in a constant sized ROI. Fluorescence intensity was monitored in the ROI and analysed using Microsoft Excel. For each sample, approximately 35 nuclei were used. Student's t-test was carried out for statistical analysis. FRET efficiency is given as percentage CFP fluorescence increase, expressed as mean ± standard error of mean (SEM) compared to a non-bleached control region;

Western blotting.

Total protein was extracted from infiltrated and non-infiltrated *N. benthamiana* leaves. Leaf material was immediately frozen in liquid nitrogen and ground with mortar and pestle. Ground material was collected in liquid nitrogen - cooled 15 ml tubes, to which 1 ml of protein extraction buffer (100 mM Tris, pH 6.8, 4.5 M urea, 1 M thiourea, 2% CHAPS, 0.5% Triton X-100, 10mM DTT, 1% Sigma protease inhibitor cocktail, benzonase, 0.52 µl ml^{-1} PMSF 5 µl) was added. Protein was precipitated using ice cold acetone, protein extract and trichloroacetic acid (8:1:1) and centrifuged for 15 min at 10,000 x g. The pellet was washed 2 x with ice cold acetone and dried before suspension in 100 µl of 1 x SDS buffer containing DTT and 8 M urea. The sample was separated on an 8% SDS-PAGE gel and transferred to Immobilon-P PVDF membrane (Millipore, Livingstone, UK) before blocking with 5% milk PBST and detection with Abcam (Cambridge UK) rabbit GFP antibody diluted 1;3000 in 5% milk PBST at 4^{o}C. Detection was with a goat anti-rabbit Cy5 conjugated antibody (Jackson Immunoresearch, Newmarket, UK) and imaged using a Bio-Rad ChemiDoc^{tm} imaging system.

Phenotype of AtNEAP1, AtNEAP3 and AtNEAP1/3 mutants.

T-DNA lines were obtained for *AtNEAP1* (SAIL_846_B07), NASC number CS837770) and *AtNEAP3* (WiscDsLoxHs086_02C). For general observation of phenotype, seeds were germinated and grown in 16 hours light at 21^{0}C and 8 hours dark at 18^{0}C. 10-12 days old seedlings were transplanted on Levington F2S compost mixed with perlite in 5 x 5 cm pots. Wild type and mutant plants were grown simultaneously in controlled conditions and germination efficiency, plant vigour and fertility were carefully observed. For root growth analysis, seedlings were grown on half-strength MS agar on vertical plates and scanned on days 3, 7, 10 and 14 after germination. Nuclear morphology and chromatin organisation was determined with NucleusJ as described by Poulet et al., (2015).

Results

NEAPs in the plant kingdom

Four members of a family of proteins, designated AtNEAP for Arabidopsis thaliana Nuclear Envelope Associated Protein (At3g05830, AtNEAP1; At5g26770, AtNEAP2; At1g09470, AtNEAP3 and At1g09483, AtNEAP4) were initially identified in a bioinformatics screen searching for the presence of coiled-coil domains, a nuclear localisation signal and a C-terminal hydrophobic domain (based in a previous description of AtNEAP1 Lu, 2011; Figure S1). Among this small protein family, AtNEAP1 had been annotated as a nuclear intermediate filament (IF) like protein in the UniProtKB/TrEMBL database by Colter and Sanders (1996).

The NEAPs identified in *A. thaliana* are members of the gene family HOM03D003386 (PLAZA 3.0, http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_dicots/) with 54 members for which no function is assigned. Additional analysis of the gene family reveals representatives in the magnoliophyta and gymnosperms (Figure S2). The cladogram is organised with AtNEAP1, 2, 3 and 4 forming adjacent sub branches clustering with other crucifer homologues (*Brassica rapa*, *Capsella rubella*, *Arabidopsis lyrata* and *Thellungiella parvula*); monocot NEAPs group together in three sub-branches. The gymnosperm *Picea abies* has two representatives while the basal angiosperm *Amborella trichopoda* has a single representative (AtR_00045G00720).

Three members of the family, AtNEAP1-3 display similar size (349, 335 and 336 amino acids respectively) while AtNEAP4 is smaller (112 amino acids; Fig. 1). AtNEAP4 shares highest sequence homology with the C-terminus of AtNEAP3 and may be a truncated gene duplication of the common ancestor of *AtNEAP3* and *4*. This hypothesis is strongly supported by the fact that AtNEAP4 is associated with the AtNEAP3 sub branch in the phylogenetic data (Figure S2). Analysis of the expression levels (Figure S3) and evolution rates of the AtNEAPs using the PAML software (Yang et al., 2007) suggest that AtNEAP4 is a pseudogene as analysis of the *AtNEAP* orthologous show an increase of the accumulation of non-synonymous mutations in *AtNEAP4* (Figure S3). Therefore, this study was focused on AtNEAP1, AtNEAP2 and AtNEAP3. AtNEAPs 1-3 show a characteristic domain structure (Fig. 1A, Figure S1), with a variable, long N-terminal domain containing two or three coiled-coils (aa54-184 and aa221-266 AtNEAP1; aa54-185 and aa220-298 AtNEAP2; aa13-93, aa124-185 and aa220-306 AtNEAP3) predicted using SMART, COILS, PairCoil2 and Marcoil (Lupas et al., 1991; Delorenzi and Speed, 2002; McDonnell et al., 2006; Letunic et al., 2012) and a conserved C-terminus comprising an NLS, and hydrophobic domain close to the C-terminus (Fig. 1A). The bipartite NLS predicted by cNLS mapper and NLStradamus (Kosugi et al., 2009, Nguyen Ba

et al., 2009) is located at the N-terminus of the coiled-coil domain nearest to the C-terminus (KTK-X9-RR and KTK-X16-KKK; aa239-264 AtNEAP1, aa238-263 AtNEAP2 and aa239-264 AtNEAP3; Fig. 1A and Figure S1). The C-terminus of AtNEAPs 1-3 ends in a characteristic motif ending in the hydrophobic domain (aa324-345 AtNEAP1; aa311-331 AtNEAP2; aa314-333 AtNEAP3) followed by SxR where x is K (AtNEAP1, AtNEAP2) or R (AtNEAP3). In monocots this is typically xKR, where x is either A or T. The TM domains of AtNEAP1-3 show a high level of sequence homology (Figure S1).

Western blot analysis of YFP-AtNEAP1, YFP-AtNEAP2 and YFP-AtNEAP3 proteins expressed transiently in *N. benthamiana,* indicates that the relative molecular mass of YFP-AtNEAP1 and YFP-AtNEAP2 were approximately 60kDa, while YFP-AtNEAP3 was larger at 65kDa, giving AtNEAP1 and AtNEAP2 a relative molecular mass of 34kDa and AtNEAP3 of 38kDa, smaller than their predicted masses of 41, 38 and 39 kDa (Fig. 1B). Extraction of AtNEAPs required the presence of a high concentration of urea and of detergent (CHAPS and Triton x-100) indicating the NEAPs are highly insoluble and may explain the aberrant molecular mass obtained.

Expression data gained from Genevestigator (Toufighi et al., 2005) and from RNAseq data mining reveals that *AtNEAP1* and *AtNEAP2* are expressed at medium levels in most tissues including primary root, leaf epidermis and guard cells. *AtNEAP3* is expressed at low levels in the leaf epidermis and guard cells but at higher levels in the primary root (Fig. S3).

NEAPs localise to the nuclear periphery

Localisation of the NEAP family to the nucleoplasm or inner nuclear envelope was suggested by the presence of a bipartite NLS (Fig. 1) and confirmed using fluorescent protein fusions in transient expression in *N. benthamiana* leaves. YFP-NEAPs 1-3 localise to the nuclear periphery, surrounding chromatin labelled with histone H2B-CFP (Fig. 1C). Transiently expressed YFP-AtNEAP1-3 were also used to study the mobility of the proteins at the NE by FRAP in *N. benthamiana* leaves (Fig. 2). YFP-AtNEAP1 and YFP-AtNEAP2 have significantly lower ($p<0.001$) mobile fractions (20.6±1.8% and 17.7±1.5%, respectively) compared to YFP-AtNEAP3 (46.9±5.3%; Fig. 2). Similarly, the half time is significantly higher ($p<0.05$) for YFP-NEAP3 (9.5±3.5 sec) then YFP-AtNEAP1 and YFP-AtNEAP2 (3.6±0.17 sec and 2.3±2.4 sec, respectively). The significant differences in mobility of AtNEAP3 suggest that binding interactions differ between AtNEAP homologues, with AtNEAP1 and AtNEAP2 being most tightly bound. The mobile fractions of the AtNEAP1 and AtNEAP2 are comparable to other NE

proteins such as the AtSUNs and NE-associated proteins such as AtCRWN1 (Graumann et al., 2014; Graumann 2014).

Domain function was studied using deletion and truncation mutants of AtNEAP3 (Fig. 3). Deletion of the first coiled-coil domain (aa13-93; YFP-AtNEAP3ΔCC1) resulted in nucleoplasmic fluorescence while deletion of the second coiled coil domain (aa124-185; YFP-AtNEAP3ΔCC2) had no effect on localisation (Fig. 3A-B). Deletion of the NLS (aa239-264; YFP-AtNEAP3ΔNLS) resulted in cytoplasmic fluorescence (Fig. 3A-B). Finally, deletion of the predicted TM domain (aa314-333; YFP-AtNEAP3ΔTM) resulted in nucleoplasmic fluorescence (Fig. 3A-B). The presence of CC1 and the TM domain are therefore important in localising the protein from nucleoplasm to nuclear periphery, while the presence of the NLS is required to target the protein to the nucleus from the cytoplasm. Interestingly, co-expression of the domain deletions with full length CFP-AtNEAP3 resulted in co-localisation at the NE (Fig. 3C). This suggests that NE-localised CFP-AtNEAP3 can interact with all four domain deletion mutants and "rescue" them to the NE.

AtNEAP proteins interact to form homomers and heteromers

The effect on localisation of the AtNEAP3 domain deletion mutants upon co-expression with full length AtNEAP3 suggests the possibility that AtNEAP3 is able to interact with itself. To test interactions between the AtNEAPs, apFRET and MYTH were used. Firstly, YFP- and CFP fusions of the NEAPs were co-expressed transiently to show that all AtNEAPs co-localised at the nuclear periphery (Fig. 4A).

The co-localisation of the AtNEAPs was used to measure apFRET efficiency (E_F; Fig. 4B). There was no significant increase ($p>0.1$) in AtNEAP1-CFP fluorescence post YFP-AtNEAP1 bleach, indicating that AtNEAP1 does not interact with itself in this system (Fig. 4B). However, both AtNEAP2 and AtNEAP3 showed a significant ($p<0.001$) interaction with themselves with AtNEAP2-CFP (E_F 21.3±1.7%) and AtNEAP3-CFP (E_F 18.4±1.9%), respectively (Fig. 4B). Furthermore, bleaching YFP-AtNEAP3 also led to a significant ($p<0.001$) increase in fluorescence of co-expressed AtNEAP1-CFP and AtNEAP2-CFP with calculated E_F of 16.6±1.5% and 18.6±1.4% respectively (Fig. 4B). Bleaching YFP-AtNEAP1 also led to a significant ($p<0.001$) increase in co-expressed fluorescence of AtNEAP2-CFP (E_F 10.2±1.1%; Fig. 4B). Thus AtNEAP1, AtNEAP2 and AtNEAP3 interact with each other *in planta* although AtNEAP1 does not strongly self-interact.

As apFRET relies on transient expression of proteins, we also performed a Membrane Yeast Two Hybrid (MYTH) system to confirm NEAP-NEAP interactions identified by apFRET. Two bait vectors containing *AtNEAP1* and *AtNEAP2* were used. *AtNEAP3* was discarded as it activates detection in the absence of prey. When yeast containing the AtNEAP1 bait were transformed with AtNEAP1, AtNEAP2 and AtNEAP3 prey vectors, all transformations successfully yielded colonies on restrictive medium (Figure S5A), confirming the AtNEAP1-AtNEAP2 and AtNEAP1-AtNEAP3 interaction identified *in vivo*.

AtNEAP proteins interact with other nuclear envelope proteins

The mobility studies indicated that YFP-AtNEAP1 and YFP-AtNEAP2 have similar mobile rates as the SUN domain proteins. As the SUN proteins are a well characterised group of NE proteins and part of nucleo-cytoskeletal bridging complexes, we wanted to explore the possibility whether the AtNEAPs can associate with AtSUNs. For this, combinations of N-terminal YFP fusions of AtSUN1 and AtSUN2 were co-expressed with N- terminal CFP fusions of AtNEAP1, AtNEAP2 and AtNEAP3 in *N. benthamiana* leaves, which revealed that AtNEAPs co-localise with AtSUN1 and AtSUN2 at the NE (Fig. 5A).

In planta interactions between co-localised CFP-NEAPs and YFP-SUNs were tested using apFRET. Bleaching YFP-AtSUN1 led to a significant ($p<0.005$) increase in fluorescence of co-expressed CFP-AtNEAP1, CFP-AtNEAP2 and CFP-AtNEAP3 with average E_F of 6.9±0.7%, 7.8±0.7% and 3.9±0.4%, respectively (Fig. 5A). Similarly, bleaching YFP-AtSUN2 led to a significant ($p<0.0001$) increase in fluorescence of co-expressed CFP-AtNEAP1, CFP-AtNEAP2 and CFP-AtNEAP3 with average E_F of 18.4±1.4%, 14.4±0.9%, and 26.9±1.9%, respectively. This shows that all three NEAPs can interact with AtSUN1 and AtSUN2 in planta.

Interactions between SUNs and NEAPs were also confirmed using MYTH. Yeast containing AtNEAP1 and AtNEAP2 bait were transformed with AtSUN1 and AtSUN2. The growth of colonies on restrictive medium confirmed the interactions of AtNEAP2 with AtSUN1 and AtSUN2 but was not detected with AtNEAP1 (Figure S5B). The ability of NEAPs and SUNs to interact with each other, indicates that AtNEAPs may also be associated with nucleo-cytoskeletal bridging complexes in plants.

AtNEAP1 interacts with a transcription factor

The MYTH assay was also employed to screen the *A. thaliana* cDNA library for novel AtNEAP1 interaction partners. Briefly, 3.6 million cDNA fragments were screened for

interactors of AtNEAP1 bait, 25 colonies were selected and sent for sequencing. Nine of the 25 colonies sequenced returned a single gene, At2g40620, a basic-leucine zipper (*AtbZIP28*) transcription factor (Jakoby et al. 2002). In order to confirm its nuclear localisation, fluorescent protein fusion of the coding sequence of *AtbZIP18* under the CaMV 35S promoter was expressed transiently in *N. benthamiana*. YFP-AtbZIP18 was localised to the nucleoplasm and cytoplasm (Fig. 6A). When co-expressed with YFP-AtbZIP18, CFP-AtNEAP1 failed to accumulate at the nuclear periphery and was found to co-localise with the YFP-bZIP18 in the nucleoplasm (Fig. 6B). The nucleoplasmic co-localisation with YFP-bZIP18 was also seen with AtNEAP2 and AtNEAP3 (data not shown). AtbZIP18 is therefore a potential *in vivo* interaction partner for the AtNEAPs.

Functional analysis of NEAPs

To investigate putative functions of the AtNEAPs, we used T-DNA knock out lines and focused on tissues AtNEAP1-3 appeared expressed at higher levels – primary root, leaf epidermis and guard cells (Figure S4). All single and the double *neap* mutant lines germinated normally (95-100% germination). No significant difference was observed in root growth in single insertion lines, but in contrast, the *atneap1 atneap3* double knock out showed significantly reduced primary root growth from day 2 to day 8 post-germination (Fig. 7A-C). Nuclear morphology and chromatin organisation of pavement cells (PC) and guard (GC) cells for the cotyledon epidermis were examined for *atneap1*, *atneap3* and *atneap1 atneap3* mutants. Nuclear volume appeared increased in pavement cells of all three mutants (Fig. 7D). Chromocentre volume was decrease in all mutants in both pavement and guard cells (Fig. 7E). In addition, the *atneap3* single mutant also had reduced relative heterochromatin fraction (RHF; Tessadori et al 2007) in both cell types (Fig. 7F) while the number of chromocentres appeared increased in pavement cells (Fig. 7G). The latter indicates that chromocentre organisation is disrupted in the *atneap3* single mutant with smaller but more numerous chromocentres. indicating some impact on nuclear organisation. RT-PCR showed that both single mutants were complete knock out mutants (Fig. 7H).

Discussion

The members of the family designated AtNEAP1-4 and characterised in this paper are plant-specific proteins associated with the inner nuclear envelope. Structurally, they are

predominantly coiled-coil proteins, with an active NLS and a predicted C-terminal transmembrane domain; together, these localise the proteins at the INM, predicted to be orientated with the coiled-coil domains in the nucleoplasm. As a full proteome of the plant INM is yet to be identified, the AtNEAPs are part of a small group of characterised plant INM proteins. Another well characterised group of INM proteins are the SUN domain proteins, which are part of nucleo-cytoskeletal bridging complexes. The ability of AtNEAPs to interact with AtSUN1 and AtSUN2 strongly indicates that AtNEAPs are also part of these LINC complexes and may be involved in some LINC functions. Interestingly, plant LINC complex components such as AtSUNs and AtCRWN have been shown to regulate nuclear morphology (Dittmer et al., 2007; Graumann et al., 2014; Poulet et al., 2015). The nuclear morphology changes observed in the AtNEAP1 and AtNEAP3 knock out mutants support the hypothesis that the AtNEAPs may also be involved in this process. Similarly, a reduction in primary root length, as observed here for the AtNEAP1-AtNEAP3 double knock out, have previously been reported for the plant KASH protein AtTIK, also an interactor of AtSUNs (Graumann et al., 2014). This raises the question whether AtTIK, AtNEAP1 and AtNEAP3 may be part of LINC complexes involved in cellular events that effect root growth. Certainly, *AtNEAP1* and *AtNEAP3* are expressed in this tissue and *AtNEAP3* at higher levels then in other tissues. Interestingly, AtNEAP3 in particular, appears to have a function in chromatin organisation judging by the mutant's effects on chromocentre organisation and relative hetrochromatic fraction. While it remains unclear, how AtNEAP3 is linked to chromatin, a more direct association with chromatin has been identified for AtNEAP1. The interaction of AtNEAP1 with chromatin is suggested by the identification of a DNA binding leucine zipper transcription factor, AtbZIP18, as an interaction partner by MYTH and by altered localisation of CFP-AtNEAP1 resulting from co-expression with YFP-AtbZIP18. This is first evidence, that in plants LINC complexes are also associated with chromatin. The functional significance of the AtNEAP1-AtbZIP18 interaction will be explored in future studies.

Our *in vivo* and *in planta* interaction data shows that all three AtNEAPs are able to homomerise and heteromerise. All NEAPs have extensive coiled coils and it could be hypothesised that they play a role in mediating NEAP-NEAP interactions. Though, at least for AtNEAP3 the first coiled coil domain is not required as the YFP-AtNEAP3ΔCC1 mutant relocalises together with full length CFP-AtNEAP3 at the NE. Interestingly, AtNEAP1 and AtNEAP2 appear to be more tightly anchored at the INM then AtNEAP3 indicating that they might be involved in different binding or protein complexes. This is also supported by the different expression patterns of AtNEAP1-3, where AtNEAP1 and 2 appear more highly expressed then AtNEAP3. The

observation that all three AtNEAPs have reduced mobility at the NE, comparable to other NE and NE-associated proteins, indicates that they are functional components of the NE.

Taken together, the AtNEAPs are a novel family of nuclear envelope proteins and our identification and initial characterisation of the NEAP family adds one more component to the rapidly developing story of the plant nuclear envelope, nucleoskeleton and chromatin interactome and will provide a basis for further understanding the way in which the plant nucleus is structured and functions.

Supplementary Data

Supplementary figures (Figures S1-S6) include phylogenetic data, expression data, MYTH interactions, AtCRWN co-expression and supplementary tables (Table S1-S3) list primers and fluorescent protein fusions generated in this study.

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Figure legends

Fig. 1. AtNEAP structure and expression. A) Schematic representation of AtNEAP1, 2, 3 and 4 protein organisation. Coiled-coil domains (orange rectangles), NLS (grey boxes) and transmembrane domain (green ovals). The sequence and position of the bipartite NLS and conserved C-terminal motif are indicated. **B)** Western blot of protein extracts from *N. benthamiana* leaves transiently expressing YFP-AtNEAP1-3 in the presence of p19, resolved on an 8% SDS-PAGE gel and detected with an anti-YFP antibody. Red arrows highlight the NEAP bands. YFP-AtNEAP1 and YFP-AtNEAP2 have a relative molecular mass of approximately 60kDa while YFP-AtNEAP3 is approximately 65 kDa. The net relative molecular mass of AtNEAP1, 2 and 3 was approximately 34, 33 and 38kDa respectively. **C)** Confocal micrographs showing N-terminal YFP fusions of AtNEAP proteins (green) and histone H2B-CFP (magenta) transiently expressed in *N. benthamiana* leaf epidermal cells in the presence of p19. All three NEAPs localize at the nuclear periphery surrounding chromatin labelled by histone H2B-CFP. Scale bar = 10 µm.

Fig. 2. Fluorescence recovery curves of YFP-AtNEAP1 (red), YFP-AtNEAP2 (blue) and YFP-AtNEAP3 (green) obtained after photo bleaching *in planta*. Time zero denotes time of bleach. Result of an unpaired t-test showed that the maximum fluorescence recovery of YFP-AtNEAP3 was significantly ($p<0.0001$) higher than YFP-AtNEAP1 and YFP-AtNEAP2.

Fig. 3. Domain deletion mutants of AtNEAP3. **A)** Schematic presentation of domain deletion constructs AtNEAP3ΔCC1, AtNEAP3ΔCC2, AtNEAP3ΔNLS and AtNEAP3ΔTM highlighting which amino acids are not present in the respective constructs. **B)** and **C)** Domain deletion constructs were fused to YFP at the N-terminus and transiently expressed in *N. benthamiana* leaf epidermal cells in the presence of p19. **B)** Confocal micrographs of single expression showing nuclear localisation of YFP-AtNEAP3ΔCC1 and YFP-AtNEAP3ΔTM, cytoplasmic localisation of YFP-AtNEAP3ΔNLS and nuclear rim localisation of YFP-AtNEAP3ΔCC2. **C)** Confocal micrographs of full length CFP-AtNEAP3 co-expressed with the domain deletion mutants show that mutant localisation is rescued to the NE. Scale bar = 10µm.

Fig. 4. Interactions between AtNEAPs as measured by apFRET. **A)** Confocal micrographs of transiently co-expressed YFP- and CFP-AtNEAPs demonstrating co-localisation at the nuclear periphery. Scale bars = 10µm. **B)** apFRET of c-localised AtNEAPs; changes in CFP fluorescence in a bleached (red) vs non-bleached (pink) region of YFP fluorescence. A significant increase in CFP fluorescence indicates interaction *in planta*. In each case the upper partner is YFP-NEAP while the lower partner is CFP-NEAP. Values are percentage mean ±

standard error of the mean and compared to unbleached control region (n=30). Paired t-test was performed between bleached and non-bleached populations ($p<0.001$)

Fig. 5. *In planta* interactions between AtSUNs and AtNEAPs A) Confocal micrographs showing the co-localisation of N-terminal YFP (green) fusions of AtSUN1 and AtSUN2 with N-terminal CFP (magenta) fusions of AtNEAP1, AtNEAP2 and AtNEAP3, expressed transiently in the presence of p19. Scale bar = 10 µm. AtSUNs and AtNEAPs co-localised at the NE. **B)** apFRET of co-localised AtSUNs and AtNEAPs; changes in CFP fluorescent in a region of bleached (red) versus a control non bleached (pink) region of YFP fluorescence. A significant increase in CFP fluorescence indicates interaction *in planta*. In each case, the upper partner is a N-terminal YFP AtSUN construct, while the lower partner is a N-terminal CFP NEAP construct. Values are expressed as percentage mean ± SEM (n=30). Paired t-test was performed between the bleached and non-bleached populations (*$p<0.005$). **D)**.

Fig. 6. Subcellular localisation of AtbZIP18. Confocal micrographs showing YFP-AtbZIP18 expressed transiently in *N. benthamiana* leaves in the presence of p19; **A)** during single expression, the protein is localised in the nucleoplasm and cytoplasm; **B)** co-expression with CFP-AtNEAP1 shows the two proteins co-localised in the nucleoplasm. Scale bar = 10µm.

Fig. 7. Analysis of AtNEAP1 and AtNEAP3 T-DNA knock out mutants. **A-C)** Root growth assays comparing primary root length of (**A**) *neap1,* (**B**) *neap3 and* (**C**) *neap1/3* double mutant lines versus WT Col-0 in 1-8 day old seedlings. Values are shown with mean ± standard error of the mean and an unpaired t-test was performed where *$p<0.05$ was statistically significant (n = 30). **D-G)** Box plots show the nuclear volume (**D**), chromocentre (CC) volume (**E**), Relative Heterochromatic Fraction (RHF; **F**) and number of chromocentres (**G**) for mutant and wild type nuclei of guard cells (GC); pavement cells (PC) of 10 d cotyledons of wild type (Col 0) and mutant (*atneap1, atneap3* and *atneap1 atneap3).* **H)** Scheme of *AtNEAP1* and *AtNEAP3* genes with insertion sites and locations of the primers used for RT-PCR. **I)** RT-PCR experiment performed on Col 0 and *atneap1 atneap3.* Negative controls (RT-) are presented where no MMLV-Reverse Transcriptase was added. An actin gene was used as positive control.

Fig. 8. Schematic representation of the NEAPs localised in the Inner Nuclear Membrane (INM) and interacting with AtSUN, a component of the LINC complex and AtbZIP18 a putative transcription factor linked to chromatin.