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Distinct domains within the NITROGEN LIMITATION ADAPTATION protein mediate its subcellular localization and function in the nitrate-dependent phosphate homeostasis pathway

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Abstract

The NITROGEN LIMITATION ADAPTATION (NLA) protein is a RING-type E3 ubiquitin ligase that plays an essential role in the regulation of nitrogen and phosphate homeostasis. NLA is localized to two different subcellular sites, the plasma membrane and nucleus, and contains four distinct domains: i) a RING domain that mediates degradation of phosphate transporters at the plasma membrane; ii) an SPX domain that facilitates NLA's interaction with the phosphate transporters, and also exists in other proteins that regulate the nuclear transcription factors that control the phosphate starvation response pathway; iii) a linker domain that lies between the RING and SPX domains; and iv) a C-terminal domain, which, like the linker region, is of unknown function. Here we carried out a mutational analysis of NLA, which indicated that all the domains are not only essential for proper functioning of the protein, but also mediate its localization to the plasma membrane and/or nucleus, as well as to different subdomains within the nucleus. Overall, the results provide new insights to the distinct protein motifs within NLA and the role(s) that this protein serves at different subcellular sites with respect to the regulation of nitrogen-dependent phosphate homeostasis as well as other possible physiological functions.

Key words: Nitrate-dependent phosphate homeostasis, NITROGEN LIMITATION ADAPTATION, subcellular protein localization, RING domain, SPX domain.

Introduction

As a major component of most cellular macromolecules including proteins, nucleic acids and chlorophyll, nitrogen is essential for plant growth and development (Hawkesford et al. 2012). Consequently, plants subjected to low nitrogen conditions display numerous adaptive responses, including reduced photosynthetic rates and reduced biomass, early flowering, anthocyanin accumulation, and the remobilization of nitrogen from old to young tissues (Han et al. 2015; Nguyen et al. 2015; and references therein). In order to develop crop plants with improved nitrogen use efficiency, it is necessary to better understand the molecular mechanisms that allow plants to adapt to low nitrogen levels. While many studies have focused on the effects of short-term nitrogen removal or re-supply (Wang et al. 2004a; Scheible et al. 2004), other efforts have been aimed at understanding plant responses to long-term nitrogen stress, which is thought to reflect more accurately conditions in the field (Bi et al. 2007; Peng et al. 2007b; Kant et al. 2008; Tschoep et al. 2009; Sulprice et al. 2013; Allwood et al. 2015).

The *NITROGEN LIMITATION ADAPTATION (NLA)* gene of *Arabidopsis thaliana* was identified in a mutant screen performed under conditions of chronic limiting soil nitrogen for its role in nitrogen limitation adaptation (Peng et al. 2007a; 2007b; 2008). Notably, *nla* mutant plants grown under sufficient nitrogen conditions were indistinguishable from wild-type (WT) plants either by their phenotype or transcriptional profile. However, under limiting nitrogen conditions, the *nla* mutant plants did not show the typical low nitrogen responses, had less anthocyanin accumulation than WT and, perhaps most conspicuously, displayed yellowing of leaf tips and early necrotic senescence (Peng et al. 2007a; 2008).

To gain insight to the mechanisms underlying NLA functioning, Kant et al. (2011) used a second site-suppressor screen to identify mutants that restored the low-nitrogen adaptive response in the *Arabidopsis nla* mutant background. This screen revealed two suppressor

mutants harboring disruptions in the *PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (PHF1)* or *PHOSPHATE TRANSPORTER1.1 (PHT1.1)* gene. The *PHF1* gene encodes a membrane protein that facilitates the trafficking of PHT1.1 from the endoplasmic reticulum to the plasma membrane (Gonzalez et al. 2005), and PHT1.1 serves as a plasma membrane-localized transporter involved in the uptake of extracellular phosphate (Shin et al. 2004). These results suggested that *NLA* functioned, at least in part, by regulating phosphate homeostasis in plants. Indeed, in *nla* mutants, intracellular phosphate levels were nearly doubled those of WT, and cultivation of plants under low nitrogen conditions resulted in an increase in intracellular phosphate in WT plants that was increased further in *nla* mutant plants, resulting in phosphate toxicity and necrotic senescence (Kant et al. 2011). Hence, *NLA* acts as a major regulator of nitrate-dependent phosphate homeostasis.

The *NLA* protein is a member of the RING-HC-type E3 ubiquitin ligase family in *Arabidopsis* (Stone et al. 2005) and also contains a conserved SPX domain (named after the yeast *SYG1* and *Pho81* and human *XPR1* proteins), which is known to mediate protein-protein interactions, particularly in phosphate-dependent pathways (reviewed in Secco et al. 2012; Zhou et al. 2015; Qi et al. 2016). The SPX domain of *NLA* was shown to be sufficient for interaction with PHT1.1 and PHT1.4 at the plasma membrane (Lin et al. 2013), and *NLA* also recruited the ubiquitin-conjugase *PHO2* to promote their ubiquitin-mediated turnover (Park et al. 2014). Thus, *NLA* helps to suppress phosphate uptake by promoting the degradation of phosphate transporters. Furthermore, under conditions of low phosphate, the microRNAs *miR827* and *miR399* are induced and target the *NLA* and *PHO2* mRNA transcripts for degradation, resulting in decreased amounts of the corresponding proteins and subsequent increases in steady-state accumulation of phosphate transporters PHT1.1 and PHT1.4 (Bari et al. 2006; Hsieh et al. 2009; Kant et al. 2011; Lin et al. 2013). Some aspects of this phosphate starvation-signaling pathway

that regulate *NLA* appear to vary, however, between different plant species (Yue et al. 2017). Another recent study has shown that *NLA* also mediates the turnover of the plasma membrane-localized nitrate transporter *NRT1.7* (Liu et al. 2017), revealing a broader role for *NLA* in regulating both nitrogen and phosphate contents in plants.

There are also likely other functions of *NLA* beyond the regulation of plasma membrane transporters. For instance, initial subcellular localization studies of *Arabidopsis* *NLA* revealed that the protein was found at the plasma membrane as well as in the nucleus, nucleolus and nuclear speckles (Peng et al. 2007a); rice *NLA* was also shown to localize to the plasma membrane and subnuclear compartments (Lin et al. 2013). In addition, it was recently reported that *NLA* mediates susceptibility of plants to the cyst nematode *Heterodera schachtii* (Hewezi et al. 2016), and *NLA* has been shown to be required for the accumulation of salicylic acid and immune responses to *Pseudomonas syringae* (Yaeno and Iba 2008). These observations suggest there might be other, possibly conserved, roles for *NLA* in both the plasma membrane and nucleus. Additional evidence in support of this premise comes from a yeast 2-hybrid screen that identified ubiquitin conjugase protein 8 as an *NLA*-interacting protein, and bimolecular fluorescence complementation analysis revealed that these two proteins interacted specifically in nuclear speckles (Peng et al. 2007a). Previous studies have shown also that there is an extensive transcriptome response in *Arabidopsis* that is triggered by nitrogen limitation and regulated by *NLA* (Peng et al. 2007b), and given that other members of the plant SPX-domain-containing protein family are known to function, at least in part, in the nucleus by interacting (via their SPX domains) with transcription factors that control the phosphate starvation pathway (Wang et al. 2004b; Lv et al. 2014; Shi et al. 2014), *NLA* might serve an analogous role in the nucleus in terms of the regulation of nitrate-dependent phosphate homeostasis. While the latter possibility remains an open question, these observations, combined with the more established

role for NLA at the plasma membrane, suggests that NLA regulates different protein targets in different subcellular compartments.

Given the complex subcellular targeting of NLA, the aim of the current study was to conduct a detailed mutational assessment of the distinct functional/structural domains in NLA to determine their role(s) in the protein's function and/or subcellular localization. Each of the mutant proteins was assessed to determine its subcellular localization, as well as its ability to functionally complement the low-nitrogen phenotype of *nla* mutant plants. Overall, our results indicate that all of the domains examined within the NLA sequence mediate, albeit to different extents, its localization to the plasma membrane and/or nucleus, and more specifically, to the nucleoplasm, nucleoli and nuclear speckles. Further, many of the mutant proteins lost the ability to functionally complement the *nla* mutation. Collectively, these studies will serve as a foundation for future efforts to characterize the molecular mechanisms by which NLA functions in different subcellular compartments to modulate nitrate-dependent phosphate regulation and other physiological processes in plants.

Materials and methods

Plant material, growth conditions and transformations

Arabidopsis (*Arabidopsis thaliana*) WT (Columbia-0) and *nla* mutant plants were grown with or without limiting nitrogen essentially as described in Peng et al. (2007a; 2008). Briefly, plants were cultivated in nutrient-free LB2 soil (Sun Gro Horticulture Canada Ltd.) and in controlled growth rooms at 16 h light/8 h dark, 22°C day/20°C night, light intensity 150 $\mu\text{E m}^{-2}\text{s}^{-1}$, and 75% relative humidity. Nutrient solution was applied once a week for four weeks (beginning on the day the seeds were sown) and contained 10 mM KH_2PO_4 , 2 mM MgSO_4 , 1 mM CaCl_2 , 0.1 mM Fe-EDTA, micronutrient solution (containing 50 μM H_3BO_3 , 12 μM MnSO_4 , 1 μM ZnCl_2 , 1 μM CuSO_4 , 0.2 μM Na_2MoO_4). The nutrient solution also contained nitrogen in the form of either 1 mM or 10 mM KNO_3 , which was previously shown for *Arabidopsis* to be a severe nitrogen-limiting condition or sufficient nitrogen content required for optimal plant growth and yield, respectively (Peng et al. 2007a; 2008).

nla mutant *Arabidopsis* plants were stably transformed via *Agrobacterium tumefaciens* (strain GV3101) with *NLA* constructs using the floral dip method (Clough and Bent 1998). See below for details on the construction of various *NLA* and *NLA* mutant-containing binary plasmids. Primary transformants were selected for the *bar* gene marker by spraying the T_1 seedlings with the herbicide Finale® (Bayer Crop Science). T_2 seeds were collected from eight to fifteen independent transgenic T_1 lines and were screened on media containing 10 mg L^{-1} glufosinate ammonium (Crescent Chemical) to identify potential homozygous lines. Both T_2 and homozygous T_3 lines were used for phenotypic testing in low nitrogen conditions (1 mM KNO_3). No obvious effects on plant growth and development were observed for any of the transgenic lines when grown in high nitrogen conditions (10 mM KNO_3). Three independent T_3 lines for each construct in the *nla* mutant background were confirmed by genotyping and reverse

transcription-polymerase chain reaction (RT-PCR) analysis and selected for further study; details on genotyping and RT-PCRs used to confirm the presence and expression of the *NLA* transgene, respectively, are available upon request.

Nicotiana bethamiana plants used for *A. tumefaciens*-mediated transient gene expression experiments were grown in regular, nutrient-rich soil (Sunshine Mix No. 4 [Sun Gro Horticulture Canada Ltd.]) at 28°C with a 16 h light/8 h dark cycle. Leaves of ~4-week-old plants were infiltrated with *A. tumefaciens* (strain GV3101) containing selected *NLA* constructs and then processed for microscopy (see below). Procedures for *A. tumefaciens* growth, transformation, and infiltrations have been described elsewhere (McCartney et al. 2005).

Plasmid Construction

Molecular biology reagents, unless otherwise indicated, were purchased from New England Biolabs and custom oligonucleotides were synthesized by Sigma-Aldrich. Total RNA was extracted from Arabidopsis plant tissues using TRIzol reagent according to the manufacturer's instructions (Invitrogen) and complementary DNA (cDNA) was synthesized from total RNA samples using a SuperScript IV First-Strand Synthesis System (ThermoFisher Scientific). All PCRs were carried out using TurboPfu polymerase (Stratagene). Sequence information for all primers used in gene cloning, genotyping, RT-PCRs, and plasmid construction is available upon request. All DNA constructs were verified using automated sequencing performed at the University of Guelph Genomics Facility.

Sequences encoding either full-length (WT) *NLA* (Arabidopsis Genome Initiative Accession No. At1g02860) or the *NLA*Δ236-281 mutant, which lacks the majority of the protein's RING domain sequence (i.e., residues 236-281) and was initially identified by Peng et al. (2007a) (and referred to therein as *NLA*^{RING}), were amplified via PCR from cDNA derived from WT or *nla*

leaf mRNA, respectively. PCR products were then subcloned sequentially into pDONR211 and the plant expression binary vector pEarleyGate100 (Earley et al. 2006) using Gateway technology (Invitrogen). The resulting plasmids, encoding non-tagged versions of NLA and NLA Δ 236-281 driven by the constitutive 35S cauliflower mosaic virus (CaMV) promoter, were used for stable transformation of Arabidopsis (see above).

Other binary plasmids encoding non-tagged NLA mutants used for stable transformation of Arabidopsis were constructed as follows. NLA^{NLS} and NLA^{C1C2} were generated using PCR-based site-directed mutagenesis with *NLA* cDNA as template and the appropriate oligonucleotide primers, followed by triple ligation of the two resulting PCR products as *AgeI-NotI* and *NotI-EcoRI* fragments into the *AgeI-EcoRI* sites of the binary vector pEGAD (Cutler et al. 2000). Thereafter, the NLA^{NLS} and NLA^{C1C2} sequences were PCR amplified and recombined into pDONR221 and subsequently into pEarleygate100. The NLA Δ 61-70, NLA Δ 135-142, NLA Δ 193-206 and NLA Δ 288-298 deletion mutations were constructed using a site-directed PCR-based mutagenesis procedure, whereby the *NLA* cDNA was amplified with the appropriate primers from pEGAD/*NLA* and introduced into pGEM-T Easy vector (Peng et al. 2007a). Thereafter, the modified *NLA* sequences were subsequently cloned into pDONR221 and pEarleygate100. Similarly, all other deletion *NLA* mutant constructs (i.e., NLA Δ 1-25, NLA Δ 161-190, NLA Δ 161-220, NLA Δ 204-220, NLA Δ 299-335) were cloned using Gateway technology, i.e., deletion mutants were generated via overlap PCR extension using *NLA* cDNA a template and the resulting PCR products were recombined into pDONR221 and then pEarleygate100.

N-terminal yellow fluorescent protein (YFP)-tagged versions of *NLA* constructs used in stable transformation of Arabidopsis and/or transient transformation of *N. benthamiana* leaves, including full-length and all mutant NLAs, were each generated by amplifying (via PCR and the appropriate primers) the corresponding *NLA* sequences from the pEGAD or pEarleyGate100

plasmids described above. Resulting PCR products were then introduced into the Gateway-compatible binary plasmid pEarlyGate104, which contains the 35S CaMV promoter, followed by the open reading frame (ORF) of the enhanced YFP (Clontech) and downstream recombination sites (Earley et al. 2006). Similarly, the C-terminal YFP-tagged version of full-length NLA (i.e., NLA-YFP) used in stable transformation of Arabidopsis was generated by amplifying (via PCR and the appropriate primers) the NLA ORF without its stop codon and then introducing the resulting PCR products into pEarlyGate101 (Earley et al. 2006). The pCAMBIA-based binary vector (<http://www.cambia.org/daisy/cambia/585.html>) containing the YFP ORF alone was provided by Dr. J. Mathur (University of Guelph).

Microscopy

Microscopic images of cells in leaves and roots from stably-transformed ~3-week-old Arabidopsis plants and in transiently-transformed leaves from ~4-week-old tobacco plants were acquired using a Leica DM RBE microscope equipped with a Leica 40X Plan Apochromat water-immersion objective, a Leica TCS SP2 spanning head, a Radium 405-nm laser, and the Leica LCS software package.

Processing of plant material for microscopy was performed as described elsewhere (Park et al. 2013; Cai et al. 2015) and DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride) staining of roots and leaves was carried out by immersing tissue sections in 1 μ M DAPI in water for 10-30 min at room temperature in the dark. YFP was excited with a 514-nm laser and the emission fluorescence signal was collected at 527-597 nm and DAPI was excited with a 405-nm laser and emission was collected at 410-550 nm. Images were acquired as individual single optical sections and single labeling experiments showed no detectable crossover at the settings used for data collection. All fluorescence images shown in individual figures are representative

of at least three separate experiments, including at least 50 independent (transient) transformations of tobacco leaf cells; and each cell was evaluated based on whether the fluorescence pattern attributable to the expressed (mutant) fusion protein was indicative of localization to the plasma membrane, nucleoplasm, nucleoli, and/or nuclear speckles. Figure compositions were generated using Adobe Photoshop CS (Adobe Systems).

Results

YFP-NLA is localized to both the nucleus and plasma membrane in stably-transformed Arabidopsis and transiently-transformed tobacco cells

Prior studies revealed that the function and localization of NLA was sensitive to the placement of fluorescent protein tags at either the N or C terminus. For instance, Arabidopsis NLA appended at its N terminus with the green fluorescent protein (i.e., GFP-NLA) localized to the plasma membrane, nucleoplasm, nucleoli, and nuclear speckles and functionally complemented the low nitrogen phenotype of *nla* mutant plants, while NLA-GFP, with the fluorescent protein tag at the C terminus, was unable to complement the phenotype and the fusion protein was undetectable (Lin et al. 2013). Consistent with these observations, we found that an N-terminal yellow-fluorescent-protein-tagged version of NLA (YFP-NLA), but not its C-terminal tagged counterpart, NLA-YFP, was able to functionally complement *nla* mutant plants with respect to adaptation to nitrogen limitation. That is, *nla* mutant plants stably-expressing YFP-NLA, like WT plants, showed the characteristic accumulation of anthocyanins and leaves remained turgid when grown under low nitrogen conditions (Fig. 1). The *nla* mutant plants stably-expressing NLA-YFP or YFP alone, on the other hand, showed the characteristic early senescence *nla* mutant phenotype (Peng et al. 2007a; 2008), which consists of yellowing of the tips of the leaves, wilting, and significantly less anthocyanin accumulation (Fig. 1). These data suggest that

YFP-NLA is functionally equivalent to NLA and, thus, suitable for investigating NLA localization and function. Indeed, similar also with previous observations for GFP-NLA (Lin et al. 2013), as well as more recently for YFP-NLA (Liu et al. 2017), we observed that YFP-NLA localized in leaf and root cells of stably-transformed *Arabidopsis nla* plants to both the plasma membrane and nucleus (Fig. 2A). This localization pattern was similar regardless of whether plants were grown in either low or high nitrogen conditions (data not shown). Furthermore, closer inspection of YFP-NLA within the nuclei of these cells revealed that the fusion protein, similar to previous reports for GFP-NLA (Lin et al. 2013), was distributed throughout the nucleoplasm and also enriched in nucleoli and nuclear speckles (Fig. 2B), both of which were usually distinct from the brightest DAPI-stained regions within the nucleus (Fig. 2B) that are considered to be chromocenters containing highly compacted pericentromeric heterochromatin (Fransz et al. 2002; Pontvianne et al. 2016). On the other hand, the nucleoli in these cells, which serve as sites of rRNA gene transcription, rRNA processing and ribosome biogenesis (reviewed in [Shaw and Brown 2012]), appear as a void in the DAPI staining (Fig. 2B) because of the lower concentrations of DNA in this region compared to the surrounding nucleoplasm (and chromocenters) (Pontvianne et al. 2016). Nuclear speckles, like nucleoli, are another type of nuclear body found in the interchromatin spaces and are generally considered to be storage and assembly sites where pre-mRNA splicing takes place in areas of active transcription (Spector and Lamond 2011; Reddy et al. 2012). Furthermore, like all subnuclear compartments, nucleoli and nuclear speckles are also considered to be complex and dynamic structures, whose size, number and shape, as well as protein constituents, vary from cell to cell and can change throughout plant growth and development, as well as in response to stress and changing nutritional levels (reviewed in Cheung and Reddy 2012; Guo and Fang 2014).

Given the localization of YFP-NLA to the plasma membrane and various sites within the nucleus, including the nucleoplasm, nucleoli and nuclear speckles (Fig. 2), we chose to further examine the subcellular distribution of YFP-NLA using the *Agrobacterium*-mediated tobacco leaf epidermal cell transient expression system (Sparkes et al. 2006; Zhou et al. 2006). This model plant cell system is considered to be especially useful for microscopic examination of protein localization because of the epidermal (pavement) cell's relatively large size, including its nucleus, and, in comparison to stable plant transformations, also provides a convenient means for examining a large number of different mutant versions of a protein(s) of interest.

As shown in the series of representative micrographs in Fig. 3A, transiently-expressed YFP-NLA in tobacco leaf epidermal cells was consistently localized to the plasma membrane and nucleus, including the nucleoplasm, nucleoli, and nuclear speckles. Indeed, based on examinations of ≥ 50 individual transformed cells from at least three separate infiltration experiments, YFP-NLA was localized at the plasma membrane and in the nucleoplasm in all (100%) cells examined and most cells also displayed localization of the fusion protein to nucleoli ($96 \pm 3\%$) and nuclear speckles ($95 \pm 5\%$) (refer to graph in Fig. 3A). Moreover, the YFP-NLA-containing nuclear speckles in tobacco epidermal cells, as in *Arabidopsis* cells (Fig. 2B), were mostly distinct from the DNA-rich, DAPI-stained regions of the nucleus (Fig. 3B). By contrast, there was no apparent localization of YFP alone to nuclear subdomains in transiently-transformed tobacco leaf cells; instead, YFP localized diffusely throughout the nucleoplasm and cytoplasm, including transvacuolar strands (Fig. 3C; compare with Fig. 3A and 3B), as expected. That is, since the molecular mass of YFP is less than the diffusion limit for the nuclear pore complex (i.e., 30-40 kDa) (Meier 2007), the protein can readily move into the nucleus, whereas, the nuclear localization of YFP-NLA, which has molecular mass ~ 60 kDa that well exceeds the

diffusion limit for the nucleus, implies that NLA relies on an active, signal-mediated process for nuclear import.

Taken together, the localization of YFP-NLA to the plasma membrane and specific sites within the nucleus in both stably-transformed *Arabidopsis* and transiently-transformed tobacco cells (Figs. 2 and 3) not only reinforces the conclusions from previous studies that the NLA protein functions at different cellular sites in terms of its role(s) in plant adaptation to nitrogen and/or phosphate nutritional conditions (Peng et al. 2007a; Lin et al. 2013), it also confirms that tobacco leaf epidermal cells are a reliable model system for characterizing the subcellular localization of NLA.

Analysis of NLA protein domain mutants

To characterize the role of various domains within the NLA protein in determining subcellular localization, as well as their role(s) in the regulation of nitrogen homeostasis, we generated a number of site-specific and deletion mutants aimed at the four distinct functional/structural domains in the NLA protein, namely the SPX and RING domains, the intervening linker, and the C-terminal region. All of the NLA mutants examined are briefly summarized in Fig. 4, along with a diagrammatic representation of the NLA protein and its deduced amino acid sequence for reference purposes.

Among the NLA mutants constructed was NLA Δ 1-25. This mutant lacks the N-terminal 25 amino acid residues of the protein which also corresponds to the N-terminal portion of one of three 30-40 amino-acid-long subdomains (subdomains 1-3) found in the SPX domains of all SPX domain-containing proteins in plants (Secco et al. 2012), including NLA (residues 1-37, Fig. 4A and 4B, and Supplementary Fig. S1). Immediately downstream of the N-terminal 25 amino acid sequence, but still within in the conserved subdomain 1 region of the SPX domain in NLA, is

a putative bipartite-type nuclear localization signal (NLS) (residues 26-36; Fig. 4A and 4B). This sequence was the only predicted NLS identified by various *in silico* web-based programs (e.g., cNLS Mapper (Kosugi et al. 2009), Minimoto Miner (Balla et al. 2006) and LOCALIZER (Sperschneider et al. 2017), and is well conserved among all NLA proteins (Supplementary Fig. S1) and in some of the other SPX-domain-containing proteins in plants (Secco et al. 2012). Correspondingly, the NLA^{NLS} mutant was aimed at disrupting specifically the putative NLS, whereby all of the positively-charged residues within the candidate targeting signal sequence (underlined, -KKLKKILKRCR; refer also to Fig. 4B) were replaced with alanines within the context of the full-length NLA protein. Additional mutations to the SPX domain in NLA included NLA Δ 61-70 and NLA Δ 135-142, whereby conserved sequences within subdomains 2 and 3, respectively, of the SPX domain were deleted (Fig. 4B; refer also to Supplementary Fig. S1).

We also generated several deletion mutations within the so-called “linker region” of NLA (residues 155-230; Fig. 4A and 4B) that lies between the protein’s N-terminal SPX domain and downstream RING domain, including mutants that were devoid of either amino acid residues 161-190 (NLA Δ 161-190), 161-220 (NLA Δ 161-220), 204-220 (NLA Δ 204-220), or 193-206 (NLA Δ 193-206). Notably, two of these mutants, NLA Δ 161-220 and NLA Δ 193-206, contained a unique poly-proline sequence (residues 193-196; refer to Fig. 4B) that has the propensity to form a poly-proline type II helix, which in other proteins can mediate protein-protein or protein-nucleic acid interactions and play a major role in signal transduction and protein complex assembly (reviewed in Adzhubei et al. 2013), and was previously implicated as being important for NLA structure/function relationships (Peng et al. 2007a).

Two NLA mutants were also generated based on the RING domain, which lies in the C-terminal half of the protein (residues 231-280; refer to Fig. 4A and 4B) and mediates NLA’s ability to serve as a E3 ligase in the turnover of the phosphate transports PHT1.1 and PHT1.4 in

Arabidopsis (Lin et al. 2013; Park et al. 2014), as well as the recognition and ubiquitination of any other yet-to-be-identified target substrate proteins destined for turnover via the proteasomal pathway. These included NLA Δ 236-281, which lacks nearly the entire RING domain and corresponds to the same deletion mutant previously characterized by Peng et al (2007a), and NLA^{C1C2}, which consists of the two conserved cysteine residues within the functional site of the RING domain (i.e., residues 231 and 234; refer to Fig. 4B) being replaced with glycine residues in the context of full-length NLA. Consequently, this modified protein (NLA^{C1C2}), based on similar mutations in other well-characterized RING-domain proteins (Kraft et al. 2005; Stone et al. 2005; Carvalho et al. 2012), was expected to lack E3 ligase activity, ensuring that any potential NLA-interacting proteins were not modified and degraded by the proteasome.

Finally, two other mutations were aimed at regions within the C-terminal portion of the protein that extends beyond the RING domain (i.e., residues 281-335; Fig. 4A and 4B). Among these was NLA Δ 288-298, which includes amino acids corresponding to a leucine-rich (-LxxLL-) motif (Fig. 4B) that is conserved among other NLA homologs (Supplementary Fig. S1) and is known to mediate protein-protein interactions in other proteins associated with various aspects of transcriptional regulation (Billas and Moras 2013; Sparks et al. 2016; and references therein). NLA Δ 299-335 lacks the extreme C-terminal portion of the protein (i.e., residues 299-335 and immediately downstream of the LxxLL motif; Fig. 4B) and while this region does not contain any identifiable functional domain/motifs, it does possess an overall modest amount of sequence conservation among other NLA homologs (Supplementary Fig. S1), suggesting it is involved in NLA function. Indeed, as already mentioned, modifications to the C terminus, including addition of a fluorescent protein tag, e.g., NLA-YFP (Fig. 1) or NLA-GFP (Lin et al. 2013), abolishes NLA function, reinforcing the notion that this region is important for NLA activity.

As outlined in detail in the 'Materials and methods', sequences encoding each of the aforementioned mutant versions of NLA were subcloned into the appropriate plant expression binary vector and then stably introduced into the *nla* mutant background of Arabidopsis. At least three independent transgenic lines were then assessed in order to test for their ability to complement the early senescence phenotype in the *nla* mutant, using the same conditions for growth in limiting nitrogen as described above, i.e., low (1 mM KNO₃) versus high (10 mM KNO₃) nitrogen (refer to Fig. 1). In parallel, sequences encoding each NLA mutant were also subcloned into a binary vector appropriate for fluorescent fusion protein-based localization studies, whereby each mutant was transiently expressed as an N-terminal-appended YFP fusion protein in tobacco epidermal leaf cells and its subcellular localization assessed using confocal microscopy.

The SPX domain is required for the proper functioning of NLA as well as its localization to and within the nucleus

As shown in Fig. 5A, deletion of the first 25 amino acids of the SPX domain in Arabidopsis NLA (NLA Δ 1-25) or mutation of the putative bipartite NLS (NLA^{NLS}) both disrupted the ability of the protein to functionally complement the *nla* mutation, resulting in wilting and yellowing of leaves similar to that observed in the negative control transgenic plants described in Fig. 1. Inspection of subcellular localizations revealed that YFP-NLA Δ 1-25 still localized efficiently to the plasma membrane in almost all tobacco leaf epidermal cells examined, similar to full-length YFP-NLA (Fig. 3), but in only approximately half (49 $\pm$ 14%) of the cells did YFP-NLA Δ 1-25 localize to the nucleoplasm, with even less in nucleoli and none in nuclear speckles (Fig 5B). Mutation of the putative NLS in NLA (i.e., NLA^{NLS}), on the other hand, did not appear to influence localization to the nucleoplasm or to the plasma membrane (Fig. 5C), but did disrupt targeting to nuclear

speckles and nucleoli, albeit in a manner opposite to that observed for YFP-NLA Δ 1-25. That is, YFP-NLA^{NLS} was not enriched in the nucleoli in any of the cells examined and in only a relatively few of the cells (25 $\pm$ 13%) did the mutant fusion protein localize to nuclear speckles (Fig. 5C). Taken together, these data indicate that the N-terminal portion of the SPX domain is necessary for NLA localization to subnuclear compartments, but is not required for targeting to the plasma membrane.

As shown also in Fig. 5A, the two other SPX domain mutants of Arabidopsis NLA examined, NLA Δ 61-70 and NLA Δ 135-142, did not complement the *nla* mutant phenotype in Arabidopsis either. These mutants harbor deletions of sequences that are relatively well conserved in the SPX subdomains 2 and 3, respectively, of all SPX domain-containing proteins, including plant NLAs (Secco et al. 2012; refer also Supplementary Fig. S1). Both of the corresponding YFP-NLA mutant proteins localized to the plasma membrane and nucleoplasm in a manner similar to full-length YFP-NLA (Fig. 5D and 5E), but YFP-NLA Δ 61-70 showed reduced targeting to nucleoli (Fig. 5D), while YFP-NLA Δ 135-142 showed reduced targeting to both nuclear speckles and nucleoli (Fig. 5E). Furthermore, at least some of the YFP-NLA Δ 61-70 and YFP-NLA Δ 135-142-containing nuclear speckles, unlike those observed in cells expressing full-length YFP-NLA (Fig. 3B), also partially colocalized with the DAPI-stained regions of the nucleus (Fig. 5D and 5E), implying that these sequences in the NLA SPX domain are involved in mediating the protein's proper subnuclear localization to nuclear speckles that are normally distinct from heterochromatic chromocenters.

Collectively, the results presented in Fig. 5 indicate that the N-terminal region of NLA contains targeting information that is required for its proper localization to subnuclear compartments, and in some cases, to the nucleoplasm. Further, none of the mutant proteins

were able to functionally complement the low-nitrogen phenotype of *nla* mutant plants, despite their continued ability to target to the plasma membrane.

The linker region in NLA is important for NLA function and localization to the nucleolus

As shown in Fig. 6A, the linker region that lies between the SPX and RING domains in the NLA protein is important for its function, since three of the mutants with deletions within this region, namely NLA Δ 161-190, NLA Δ 161-220 and NLA Δ 204-220, were all unable to complement the low-nitrogen phenotype of *nla* mutant plants. By contrast, NLA Δ 193-206, which includes deletion of the poly-proline sequence within the linker region (refer to Fig. 4B), was able to complement the *nla* mutant phenotype, as evidenced by the lack of any pronounced yellowing at the tips of the leaves of NLA Δ 193-206-transformed *nla* mutant plant grown in low nitrogen conditions (Fig. 6A; compare with images of the YFP-NLA-transformed *nla* mutant plants shown in Fig. 1).

While all four mutations within the linker region of NLA did not affect localization of the mutant proteins to the plasma membrane and nucleoplasm (Fig. 6B-E), each of the mutants displayed a significant reduction in their localization to the nucleolus, albeit relatively less so ($46\pm 18\%$) for YFP-NLA Δ 193-206 (Fig. 6B-6E). Furthermore, while all four mutant fusion proteins also localized to nuclear speckles in all or most of the cells examined, the sizes of the nuclear speckles in YFP-NLA Δ 193-206-transformed cells were often conspicuously smaller (Fig. 6E). These latter observations suggest that the ability of NLA to localize to nuclear speckles relies at least in part on residues 193-206, and given that this region contains a poly-proline sequence that in other proteins is known to mediate protein complex assembly (Adzhubei et al. 2013), it may be that this region of NLA facilitates protein self-assembly and/or interactions with other proteins in nuclear speckles.

We also observed that for at least three of the NLA linker region mutants, namely YFP-NLA Δ 161-190, YFP-NLA Δ 161-220 and YFP-NLA Δ 204-220, the nuclear speckles, similar to those in YFP-NLA Δ 61-70- and YFP-NLA Δ 135-142-transformed cells (Fig. 5D and 5E), often colocalized with DAPI-stained chromocenters (Fig. 6B-6D). While it is not clear if the localization of these three NLA linker region mutants to heterochromatin-containing regions of the nucleus is relevant to NLA function or is perhaps an indirect consequence of their respective mutations, their similarities in terms of their inability to complement the *nla* mutant phenotype (Fig. 6A) and mislocalization in tobacco leaf cells (Fig. 6B-6D), suggests that the linker region of NLA is important for overall conformation of the protein as opposed to the involvement of specific motifs and/or residues. Moreover, the observation that the NLA Δ 193-206 does not disrupt NLA function (Fig. 6A) implies that the poly-proline sequence deleted in this mutant is not essential for the protein's proper functioning during low-nitrogen conditions.

The RING domain is essential for NLA function and is required for its proper localization within the nucleus

The *nla* mutation previously identified by Peng et al. (2007a) consists of a deletion of almost the entire the RING domain in NLA (i.e., residues 236-281; refer to Fig. 4B) and, hence, was referred to in this study as NLA Δ 236-281. As shown in Fig. 7A, NLA Δ 236-281, as well as the mutant NLA^{C1C2}, wherein the first two (of four) conserved catalytic cysteine residues of the RING domain motif were replaced with glycine residues (Fig. 4B), were both unable to complement the *nla* mutant phenotype, reinforcing the notion that a functional RING domain is essential for NLA to act as an E3 ubiquitin ligase. This conclusion is also consistent with the observation that another RING domain mutant of NLA, NLA^{C279A}, whereby the third conserved cysteine residue at position

279 in NLA RING domain motif was replaced with an alanine, resulted in decreased degradation of the phosphate transporter PHT1.1 (Lin et al. 2013).

In terms of subcellular localization, the NLA Δ 236-281 and NLA^{C1C2} mutations appeared to have distinct effects on localization of the corresponding mutant YFP-fusion proteins within the nucleus of transiently-transformed tobacco leaf cells. YFP-NLA Δ 236-281 localized to the plasma membrane, nucleoplasm and the nucleolus (Fig. 7B) in a similar manner as YFP-NLA, but was absent in nuclear speckles in almost all cells examined. By contrast, YFP-NLA^{C1C2} was excluded from the nucleolus in most cells, but accumulated in nuclear speckles that partially colocalized with DAPI-stained heterochromatin (Fig. 7C), indicating that the RING domain of NLA is required for its proper subnuclear localization and perhaps its function therein.

The C terminus of NLA is required for localization to the plasma membrane

As shown in Fig. 8A, deletion of residues 288-298 from NLA (NLA Δ 288-298), which contains a leucine-rich (-LxxLL-) motif known to mediate protein-protein interactions in other proteins (Plevin et al. 2005), had no apparent effect on the ability of NLA to complement the *nla* mutant phenotype, indicating that this region is not required for the function of NLA under low nitrogen conditions. Furthermore, subcellular localization studies revealed reduced targeting to the nuclear speckles and a further reduction to the nucleolus, while targeting to the plasma membrane and nucleoplasm was preserved (Fig. 8B). This subcellular distribution pattern was also similar to that of YFP-NLA Δ 193-206, which is the only other mutant protein that retained the ability to functionally complement the *nla* low-nitrogen phenotype (Fig. 6E). Deletion of the C-terminal sequence further downstream of the leucine-rich motif (NLA Δ 299-335) abolished the ability of NLA to functionally complement the *nla* mutant (Fig. 8A), and also reduced targeting to both the plasma membrane and nuclear subcompartments (Fig. 8C).

Discussion

The availability of nitrogen and phosphate for proper plant growth and development is among the main environmental factors impacting crop productivity world-wide and research to date aimed at addressing this issue has revealed that plants have evolved a complex array of physiological and metabolic strategies in order to cope when either or both of these macronutrients are limiting (Vidal et al. 2008, 2010; Gruber et al. 2013; Baligar and Fageria 2015; Han et al. 2015; Beatty et al. 2016). Underlying these adaptations are a series of cellular signaling pathways that mediate the interplay between nitrogen and phosphate homeostasis and rely on NLA as central regulator (Peng et al. 2008; Kant et al. 2011; Lin et al. 2013).

NLA functions in several different ways to maintain phosphate and nitrogen homeostasis, including at both the post-transcriptional and post-translation levels and via at least two key functional domains within the protein (see Lin et al. 2013; Park et al. 2014; Yue et al. 2017 and references therein). In phosphate-replete conditions, NLA promotes the ubiquitin-dependent degradation of the PHT1.1 and PHT1.4 phosphate transporters at the plasma membrane via its N-terminal SPX domain, which mediates the interaction with the transporters, and its C-terminal RING domain, which possess ubiquitin E3 ligase activity (Lin et al. 2013; Park et al. 2014). Likewise, NLA mediates the interaction and subsequent turnover of the nitrate transporter NRT1.7 at the plasma membrane in nitrogen-sufficient conditions (Liu et al. 2017). When nitrogen or phosphate is limited, NLA is post-transcriptionally repressed by microRNAs, alleviating the negative regulation of transporter abundance at the plasma membrane (Lin et al. 2013; Park et al. 2014; Nguyen et al. 2015). NLA is also localized to the nucleus (Peng et al. 2007; Lin et al. 2013) and although its function there remains an open question, the fact that several other members of SPX-domain protein family in plants known to be involved in phosphate homeostasis and signalling are also localized to the nucleus (Zhou et al. 2015), suggests that NLA

has protein targets in this subcellular compartment. Indeed, in a recent study on the role of NLA in the plant defense response to nematode infection, NLA was shown to interact with a number of potential protein substrates involved in a wide range of biological processes and molecular functions, including pathogenesis-related proteins, MAP kinases, and transcription factors (Hewezi et al. 2016).

Here, we extended the understanding of NLA structure/function relationships by employing a mutational analysis aimed at identifying the regions of the Arabidopsis NLA protein required to complement the *nla* mutant phenotype in plants grown under limiting nitrogen conditions and those involved in mediating its localization to different subcellular compartments, including the plasma membrane and specific regions within the nucleus. While the SPX domain was shown to be sufficient for targeting to both the plasma membrane and nucleus (Lin et al. 2013) and for mediating NLA's interaction with phosphate and nitrate transport proteins at the plasma membrane (Lin et al. 2013; Liu et al. 2017), NLA appears to possess additional targeting information involved in its localization to the plasma membrane other than the SPX domain. For instance, we demonstrated that for all but one of the NLA mutants examined (i.e., YFP-NLA Δ 299-335; Fig. 8), the localization to the plasma membrane was preserved, indicating that while the SPX and RING domains in NLA mediate interaction and ubiquitination, respectively, of the phosphate and nitrate transporters at the plasma membrane, the C-terminal portion of the protein plays a distinct role in this aspect of NLA subcellular trafficking. In line with this conclusion and the results obtained for YFP-NLA Δ 299-335, the appendage of GFP to the C-terminal end of full-length NLA disrupted its localization to the plasma membrane (Peng et al. 2007a), presumably by sterically blocking the protein's C-terminal targeting information and/or altering the overall conformation of the protein. Furthermore, we showed that the C terminus of NLA was important also for its function since

NLA-YFP was unable to complement the *nla* mutant phenotype in Arabidopsis (Fig. 1). It will now be interesting to determine the nature of the C-terminal-dependent targeting of NLA, and whether it contains sequence-specific information required for targeting to the plasma membrane, or alternatively, if it plays primarily a role in maintaining the overall structure of the protein, thereby allowing the SPX domain to facilitate plasma membrane localization through its interaction with the transporter proteins.

While the results of this study also indicate that the NLA protein, within the nucleus, preferentially localizes to the nucleoplasm, nucleolus and nuclear speckles, the targeting information responsible for this nuclear and subnuclear localization appears to be complex. For instance, NLA appears to contain other nuclear targeting signals separate from the putative bipartite NLS identified within the SPX domain (residues 26-36; Fig. 4), since modification of this region (i.e., NLA^{NLS}) abolished subnuclear targeting, including to both nucleoli and nuclear speckles, but still allowed for targeting to the nucleoplasm (Fig. 5). On the other hand, deletion of the N-terminal 25 amino acids in NLA (i.e., YFP-NLA Δ 1-25) disrupted targeting to the nucleoplasm, at least in approximately half of the cells examined (Fig. 5), suggesting that this region contains targeting information necessary for the proper localization to the nucleus. While the precise nature of the potential nuclear targeting signal in the N terminus of NLA and/or elsewhere within the protein is unknown, there is a general appreciation that there are many NLSs that do not match the canonical NLSs (Kosugi et al. 2009), and it is also not uncommon for proteins to rely on multiple, sometimes redundant targeting signals to mediate their nuclear localization (Hunter et al. 2014; Fang et al. 2017). Further, the sequences responsible for subnuclear localization, in general, are poorly defined (Emmott and Hiscox 2009; Spector and Lamond 2011), and still remain to be identified in NLA, if they even exist. Another possibility is that the localization of NLA to the nucleus and/or subnuclear compartments, such as the

nucleolus or nuclear speckles, is not mediated exclusively by a sequence-specific, *cis*-acting targeting motif(s), but by either direct or indirect protein-protein interactions with other proteins that do contain an NLS or specific targeting motifs for the nucleolus/nuclear speckles. This type of targeting mechanism is commonly referred to as “piggy-back” targeting (reviewed in Wagstaff and Jans, 2009) and relies on not only specific targeting motifs, but the protein’s ability to interact and assemble with other proteins, and perhaps do so, as further discussed below, in a dynamic manner that varies depending on the different states or needs of the cell. Regardless of the mechanism by which NLA localizes to and within the nucleus, the data presented in this study suggest that this process is important for its ability to function in low nitrogen adaptation. Alternatively, the possibility also exists that localization to subnuclear compartments is not required for NLA function in low nitrogen conditions, but rather those NLA mutants that targeted to the plasma membrane (e.g., YFP-NLA Δ 1-25 and YFP-NLA^{NLS}; Fig. 5) are properly localized, but not functional at this location due to the mutations in their N termini.

While it is also not clear what function(s) NLA may serve within the nucleolus, if at all, this subnuclear localization is in line with other previous findings (Kant et al. 2011; Lin et al. 2013; Park et al. 2014; Nguyen et al. 2015) and suggests a role for NLA and microRNA-mediated post-transcriptional regulation as a mechanism for modulating phosphate uptake under low nitrate conditions. Additional studies are also needed to address the potential functional significance of NLA’s localization to nuclear speckles. There are several types of nuclear speckles that vary depending on their protein constituents (Reddy et al. 2012), but all of which are generally similar in terms of their number, size and distribution with the NLA-labeled speckles observed in this study, as well as the inherent variability that can exist in these structures from cell to cell. Resolving the identity of the NLA-containing nuclear speckles via, for instance, colocalization studies with various marker proteins known to localize to different types of

nuclear speckles (Lorkovic et al. 2008), may provide some further indication as to what role(s) NLA may be serving in these areas of the nucleus.

Another intriguing possibility is that the regulatory mechanisms underlying NLA localization to nuclear speckles and perhaps other subnuclear compartments may be differentially regulated depending on the tissue/cell types, nitrogen or phosphate levels, and/or light/dark conditions. For instance, other studies have shown that proteins that localize to nuclear speckles, including the splicing regulator proteins in plants, sometimes do not or only partial colocalize to the same nuclear areas depending on the cell type and physiological conditions, presumably because these proteins can shuttle between and associate with nuclear speckles and/or different areas within the nucleus based on their ability to self-assemble and/or interact with other proteins (Tillemans et al. 2005; Reddy et al. 2012). Interestingly, we observed that some NLA mutants (e.g., YFP-NLA Δ 61-70, YFP-NLA Δ 135-142, NLA Δ 161-190, NLA Δ 161-220 and NLA Δ 204-220; Fig. 5 and 6) localized to nuclear speckles that colocalized with DAPI-stained heterochromatic chromocenters, whereas other NLA mutants localized to nuclear speckles that were distinct from heterochromatic chromocenters. Furthermore, all of the former NLA mutants were also unable to complement the *nla* mutant phenotype, suggesting that the alterations in the nuclear speckles of mutant proteins might be a primary reason for the observed loss-of-function. However, as already mentioned, we cannot rule out the possibility of loss of function of these mutant proteins at the plasma membrane. Regardless, understanding how NLA localization within the nucleus and to the plasma membrane, and perhaps any dynamic interplay that takes place between these two subcellular destinations, reflects the role of NLA in mediating the interactions between nitrate levels and phosphate homeostasis will be an important next step.

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Figure captions

Fig. 1. Complementation of the low-nitrogen-induced early senescence phenotype by YFP-NLA. WT (i.e., non-transformed) plants or *nla* mutant plants stably transformed either with YFP-NLA, NLA-YFP, or YFP alone were grown in either high (10 mM KNO₃) or low (1 mM KNO₃) nitrogen conditions. NLA-YFP- and YFP alone-transformed *nla* mutant plants grown in low nitrogen displayed characteristic early senescence symptoms, i.e., leaf tip yellowing and necrosis and leaf wilting, similar to the *nla* mutant phenotype, whereas YFP-NLA-transformed *nla* mutant plants, similar to WT, display a hyper-accumulation of anthocyanin in low nitrogen conditions indicative of functional complementation. No obvious differences were observed among plant lines grown in high nitrogen conditions (10 mM KNO₃).

Fig. 2. Subcellular localization of YFP-NLA in stably transformed *Arabidopsis nla* mutant plants. Representative confocal microscopy images of YFP-NLA in **(A)** leaf epidermal (pavement) or mesophyll cells or root tip cells and in **(B)**, shown at higher magnification, nuclei in the same cell types of *Arabidopsis nla* mutant plants grown in low (1 mM KNO₃) nitrogen conditions. Similar subcellular localization patterns were observed for YFP-NLA in plants grown in high (10 mM KNO₃) nitrogen conditions (data not shown). Shown also in (A) and (B) are the corresponding brightfield (BF) images and in (B) the DAPI and merged images. Solid and open arrowheads in (B) indicate examples of YFP-NLA localization to nuclear speckles and nucleoli, respectively. Note also in (B) the general lack of co-localization between YFP-NLA-containing nuclear speckles and nucleoli and DAPI-stained regions of the nucleus corresponding to putative heterochromatic chromocenters. Bars = 20 µm in (A) and 5 µm in (B).

Fig. 3. Subcellular localization of YFP-NLA to the plasma membrane and nucleoplasm, nucleolus, and nuclear speckles in tobacco leaf cells. Representative confocal microscopy images of transiently-expressed YFP-NLA **(A)** or YFP alone **(C)** in tobacco leaf epidermal (pavement) cells and in **(B)** and **(D)**, shown at higher magnification, YFP-NLA or YFP alone in the nucleus of a transformed cell. Shown also in (A) is a graph summarizing the localization results for YFP-NLA as the percentage of epidermal cells wherein YFP-NLA localized to the plasma membrane (PM), nucleoplasm (Nu), nuclear speckles (Ns), and/or nucleoli (No). Averages and standard deviations were calculated from ≥ 50 YFP-NLA-transformed cells examined in at least three infiltration experiments. Shown also in (A), (B) and (D) are the corresponding DAPI and brightfield (BF) images, and in (B) the merge of the YFP-NLA and DAPI images. Solid and open arrowheads in (A) and (B) indicate examples of YFP-NLA localization to nuclear speckles and nucleoli, respectively. Bars = 20 μm in (A) and (C), 2 μm in (B) and (D).

Fig. 4. Arabidopsis NLA and summary of NLA mutants examined in this study. **(A)** Schematic diagram illustrating the domain organization of Arabidopsis NLA. Numbers denote the specific amino acids derived from full-length NLA (1-335 residues) that delineate the protein's SPX and RING domains, which are colored light red and blue, respectively. Also indicated are the protein's intervening linker region and C-terminal domain (C-term). **(B)** Deduced amino acid sequence of Arabidopsis NLA. The polypeptide sequence was obtained from the Arabidopsis Genome Initiative (At1g02860) and numbers at the far left and right of each row represent specific amino acid residues in full-length NLA. As in (A), the SPX and RING domains are highlighted light red and blue, respectively. Also indicated are sequences corresponding to the SPX subdomains 1-3 in NLA (residues 1-36, 58-84, and 118-145, respectively) and conserved in all SPX-domain-containing proteins (based on Secco et al. 2012; refer also to Supplementary Fig.

S1). Lines and labels above specific sequences represent those that were either deleted or altered (i.e., specific amino acid substitutions) in the various NLA mutant constructs; refer to (C) for additional details. Sequences corresponding to the putative bipartite NLS, conserved sequences in the SPX domain, the poly-proline sequence in the linker region, the two conserved cysteine residues (C1C2) in the RING domain, and the leucine-rich motif in the C-terminal domain are also in bold. **(C)** List of names and the descriptions of the various NLA mutant constructs examined in this study. The numbers in the name of each mutant construct denote the specific amino acid residues that were deleted from both non-tagged and YFP-tagged NLA, and the resulting mutant (fusion) proteins were then assessed for their ability to functionally complement the Arabidopsis *nla* mutant in stably transformed *nla* mutant plants grown under low nitrogen conditions, and their subcellular localization (via confocal microscopy) in tobacco epidermal leaf cells determined using transiently transformed YFP-tagged proteins, respectively.

Fig. 5. Functional complementation and subcellular localization studies using Arabidopsis NLA proteins harbouring mutations in the SPX domain. **(A)** Arabidopsis *nla* mutant plants were stably transformed either with NLA Δ 1-25, NLA^{NLS}, NLA Δ 61-70, or NLA Δ 135-142 and grown in either high (10 mM KNO₃) or low (1 mM KNO₃) nitrogen conditions. Refer to Fig. 4 for additional information on each mutant. **(B-E)** Representative confocal images showing the localization of the YFP-NLA mutant proteins in transiently-transformed tobacco leaf epidermal (pavement) cells, including, at higher magnification in the three panels on the right, their localization within the nucleus of a transformed cell. Shown also for the higher magnified images in (B-E) are the corresponding DAPI and brightfield (BF) images. Solid and open arrowheads indicate examples of localization of the YFP-NLA mutant proteins to nuclear speckles and/or nucleoli, respectively. Also indicated with solid arrowheads are examples of DAPI-stained structures that colocalize

with YFP-NLA-containing nuclear speckles. Note that, other than in (E), low and high-magnified images are of different transformed cells and sometimes vary in terms of the subcellular localization of the mutant protein (refer to [B]). Bars in (B-E) = 20 μm (left panel) and 2 μm (second panel from left). Also shown at the far right in (B-E) is a graph summarizing the localization results for each YFP-NLA mutant as the percentage of cells wherein the mutant protein localized to the plasma membrane (PM), nucleoplasm (Nu), nuclear speckles (Ns), and/or nucleoli (No). Averages and standard deviations were calculated from ≥ 50 transformed epidermal cells examined in at least three infiltration experiments.

Fig. 6. Functional complementation and subcellular localization studies using Arabidopsis NLA proteins harbouring mutations in the linker domain. **(A)** Arabidopsis *nla* mutant plants were stably transformed either with NLA Δ 161-190, NLA Δ 161-220, NLA Δ 204-220 or NLA Δ 193-206 and grown in either high (10 mM KNO₃) or low (1 mM KNO₃) nitrogen conditions. Refer to Fig. 4 for additional information on each mutant. **(B-E)** Representative confocal images and summary of localization results of the corresponding YFP-NLA mutant proteins (as indicated by panel labels) in transiently-transformed tobacco leaf epidermal cells, as described in the legend for Fig. 5. Bars in (B-E) = 20 μm (left panel) and 2 μm (second panel from left).

Fig. 7. Functional complementation and subcellular localization studies using Arabidopsis NLA proteins harbouring mutations in the RING domain. **(A)** Arabidopsis *nla* mutant plants were stably transformed either with NLA Δ 236-281 or NLA^{C1C2} and grown in either high (10 mM KNO₃) or low (1 mM KNO₃) nitrogen conditions. Refer to Fig. 4 for additional information on each mutant. **(B)** and **(C)** Representative confocal images and summary of localization results of the corresponding YFP-NLA mutant proteins (as indicated by panel labels) in transiently-transformed

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Fig. 8. Functional complementation and subcellular localization studies using Arabidopsis NLA proteins harbouring mutations in the C-terminal domain. **(A)** Arabidopsis *nla* mutant plants were stably transformed either with NLA Δ 288-298 or NLA Δ 298-335 and grown in either high (10 mM KNO₃) or low (1 mM KNO₃) nitrogen conditions. Refer to Fig. 4 for additional information on each mutant. **(B)** and **(C)** Representative confocal images and summary of localization results of the corresponding YFP-NLA mutant proteins (as indicated by panel labels) in transiently-transformed tobacco leaf epidermal cells, as described in the legend for Fig. 5. Bars in (B) and (C) = 20 μm (left panel) and 2 μm (second panel from left).

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Fig. 1. Hannam et al.

Fig. 1. Complementation of the low-nitrogen-induced early senescence phenotype by YFP-NLA. WT (i.e., non-transformed) plants or *nla* mutant plants stably transformed either with YFP-NLA, NLA-YFP, or YFP alone were grown in either high (10 mM KNO₃) or low (1 mM KNO₃) nitrogen conditions. NLA-YFP- and YFP alone-transformed *nla* mutant plants grown in low nitrogen displayed characteristic early senescence symptoms, i.e., leaf tip yellowing and necrosis and leaf wilting, similar to the *nla* mutant phenotype, whereas YFP-NLA-transformed *nla* mutant plants, similar to WT, display a hyper-accumulation of anthocyanin in low nitrogen conditions indicative of functional complementation. No obvious differences were observed among plant lines grown in high nitrogen conditions (10 mM KNO₃).

85x152mm (300 x 300 DPI)

Fig 2. Hannam et al.

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150x101mm (300 x 300 DPI)

Fig 3. Hannam et al.

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119x122mm (300 x 300 DPI)

Fig 4. Hannam et al.

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80x149mm (300 x 300 DPI)

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Fig 5. Hannam et al.

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Representative confocal images showing the localization of the YFP-NLA mutant proteins in transiently-transformed tobacco leaf epidermal (pavement) cells, including, at higher magnification in the three panels on the right, their localization within the nucleus of a transformed cell. Shown also for the higher magnified images in (B-E) are the corresponding DAPI and brightfield (BF) images. Solid and open arrowheads indicate examples of localization of the YFP-NLA mutant proteins to nuclear speckles and/or nucleoli, respectively. Also indicated with solid arrowheads are examples of DAPI-stained structures that colocalize with YFP-NLA-containing nuclear speckles. Note that, other than in (E), low and high-magnified images are of different transformed cells and sometimes vary in terms of the subcellular localization of the mutant protein (refer to [B]). Bars in (B-E) = 20 μm (left panel) and 2 μm (second panel from left). Also shown at the far right in (B-

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147x211mm (300 x 300 DPI)

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Fig 6. Hannam et al.

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150x200mm (300 x 300 DPI)

Fig 7. Hannam et al.

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147x116mm (300 x 300 DPI)

Fig 8. Hannam et al.

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147x113mm (300 x 300 DPI)