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Arabidopsis NITROGEN LIMITATION ADAPTATION regulates ORE1 homeostasis during senescence induced by nitrogen deficiency

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Nitrogen is an important macronutrient in plants and its deficiency induces rapid leaf senescence. Two genes, ORE1 and NITROGEN LIMITATION ADAPTATION (NLA), have been implicated in regulating the senescence process but their relationship is unclear[1,2]. Here, we show that nla and pho2 (also known as ubc24) plants develop rapid leaf senescence under nitrogen-starvation condition, whereas ore1 and nla/ore1 and pho2 (ubc24)/ore1 plants stay green. These results suggest that ORE1 acts downstream of NLA and PHO2 (UBC24). NLA interacts with ORE1 in the nucleus and regulates its stability through polyubiquitination using PHO2 (UBC24) as the E2 conjugase. Our findings identified ORE1 as a downstream target of NLA/PHO2 (UBC24) and showed that post-translational regulation of ORE1 levels determines leaf senescence during nitrogen deficiency.

Leaf senescence is a controlled developmental process involving modulation and reprogramming of gene expression[3–5]. During this process, chlorophylls and major chloroplast proteins are degraded with accompanying decline in photosynthetic capacity. In addition to being age-dependent leaf senescence is also affected by interactions between the growth stage of plants and biotic/abiotic factors[3,6–9].

Nitrogen is one of the most important macronutrients in plants and its starvation induces leaf senescence[9–11]. The NAC protein ORE1 is a key transcription factor regulating age-dependent leaf senescence in *Arabidopsis*. ORE1 overexpression in transgenic plants induces early senescence, whereas *ore1* shows delayed senescence compared to wild type (WT)[12]. Furthermore, *miR164* regulates age-induced leaf senescence by targeting *ORE1* messenger RNA cleavage and *ORE1* transcript levels are elevated or reduced in *mir164abc* triple mutant or miR164b overexpression lines, respectively[2,8]. However, the role of ORE1 in leaf senescence induced by abiotic stresses, especially nitrogen deficiency, has not yet been characterized. One gene that plays a key role in early senescence under nitrogen deficiency is *NLA*[13]. NLA is an E3 ubiquitin ligase using PHO2 (also known as UBC24) as its E2 conjugase[14] and *nla* plants display rapid senescence when starved of nitrogen[1,13,15]. The relationship between ORE1 and NLA was hitherto unknown. Here, we show that ORE1 associates with NLA in the nucleus and is targeted by NLA/PHO2 (UBC24) for regulated proteolysis. ORE1 levels determine the progression of leaf senescence under nitrogen starvation.

Leaf senescence is induced by nitrogen deficiency[9]. To investigate the regulatory functions of *NLA*, *PHO2* (*UBC24*) and *ORE1* in leaf senescence, we examined the phenotype of three *Arabidopsis* null mutants, *nla*, *pho2* (*ubc24*) and *ore1*, as well as NLA and ORE1 overexpression plants under nitrogen deficiency. Delayed senescence phenotype was observed in *ore1* and NLA overexpression plants compared to WT. By contrast, leaves of *nla*, *pho2* (*ubc24*) and ORE1 overexpression plants turned yellow, showing leaf growth inhibition owing to nitrogen starvation (Fig. 1a).

To determine the epistatic relationship between *ORE1* and *NLA/PHO2 (UBC24)* we generated plants of different genotypes. Double mutants of *nla*/*ore1* and *pho2* (*ubc24*)/*ore1* displayed similar delayed senescence phenotype as the single *ore1* mutant indicating that *ORE1* acts downstream of *NLA*/*PHO2* (*UBC24*). Consistent with this finding, NLA overexpression in *ore1* background had little impact on leaf senescence, whereas ORE1 overexpression in *nla* mutant background increased the severity of senescence (Fig. 1a). By genotyping, we confirmed that *nla*/*ore1* and *pho2* (*ubc24*)/*ore1* double mutants are indeed functional knockout lines of both genes (Supplementary Fig. 6). Supplementary Table 2 lists the primer set used for genotyping.

Figure 1a shows that plants of various genotypes produced different numbers of leaves owing to developmental differences on nitrogen-deficient medium. We compared chlorophyll content of leaves 1–3, leaves 4–6 and leaves 7–10 of various plants (Fig. 1b–d). Changes in chlorophyll content of leaves 1–3 and leaves 4–6 were very similar. Overexpression of ORE1 resulted in a lower chlorophyll content, whereas *ore1* produced more chlorophyll compared with WT. The opposite results were obtained with *nla* and NLA overexpression. Similarly, *pho2* (*ubc24*) also displayed lower chlorophyll content compared to WT. Seedlings of ORE1 overexpression/*nla* and ORE1 overexpression/*pho2 (ubc24)* were similar to those of ORE1 overexpression, *nla* and *pho2* (*ubc24*). Analysis of *nla*/*ore1* and *pho2 (ubc24)*/*ore1* showed that ORE1 is epistatic. Consistent with this observation, overexpression of NLA in *ore1* background had little effect on chlorophyll content and double-overexpression plants (NLA overexpression/ORE1 overexpression) had similar chlorophyll content as WT. Similar results were obtained for leaves 7–10 except that differences in chlorophyll content were not as striking. In addition, plants of *nla*, *pho2 (ubc24)* and ORE1 overexpression had similar chlorophyll content as WT. This is probably due to the mobilization of resources from older leaves to support growth of the younger leaves.

We monitored expression levels of senescence-related genes, *RNS3*, *SINA1*, *BFN1*, *SAG29* and *VNI2*, which are direct ORE1 targets under nitrogen-sufficient or -deficient conditions (Fig. 2).

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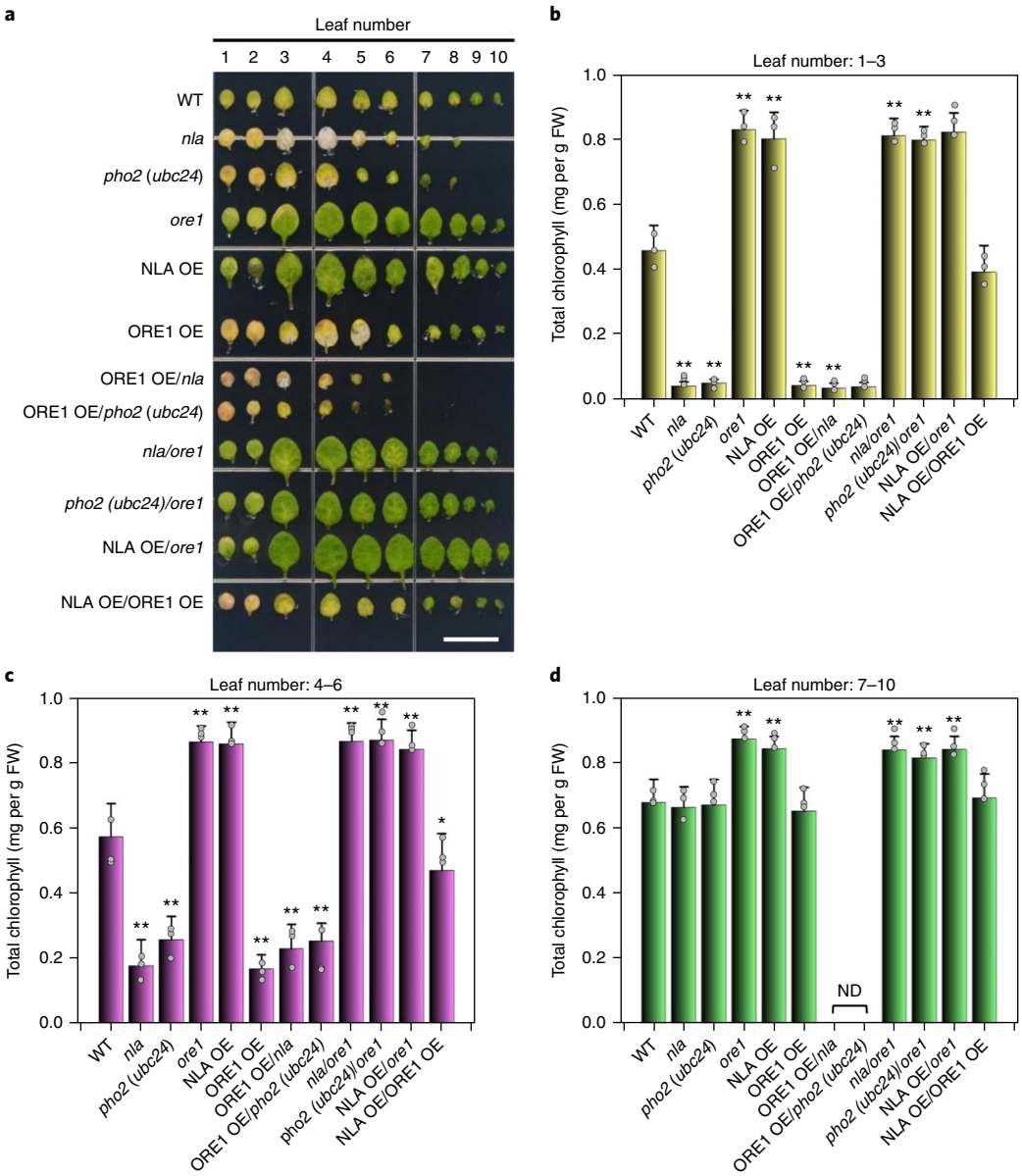

Fig. 1 | Leaf senescence phenotype in various genotypes under nitrogen-deficient condition. a, Two-week seedlings (24 per genotype) were transferred onto nitrate-deficient medium and leaf senescence phenotype was recorded after 2 weeks for WT, *nla*, *pho2* (*ubc24*), *ore1*, *NLA* overexpression, *ORE1* overexpression, *nla/ore1*, *ORE1* overexpression/*nla*, *NLA* overexpression/*ore1*, *NLA* overexpression/*ORE1* overexpression, *pho2* (*ubc24*)/*ore1* and *ORE1* overexpression/*pho2* (*ubc24*). Experiments were repeated three times with similar results. Scale bar, 1 cm. OE, overexpression. **b**,**c**,**d**, Total chlorophyll content was analysed in leaf numbers 1–3, 4–6 and 7–10 from 48 plants per indicated genotype. Data are means ± s.d. $n = 3$ (biologically independent experiments) and individual data points as overlays. Supplementary Table 3 shows *P* values. Asterisks indicate statistically significant difference compared with WT. $^{*}P < 0.05$, $^{**}P < 0.01$; two-tailed *t*-test; ND, no statistical difference. FW, fresh weight.

Expression of these five target genes was induced by nitrogen deficiency in WT[5]. In *nla* and *pho2* (*ubc24*) the induction level was 2–4-fold higher than in WT, but gene induction in *ore1* and NLA overexpression plants was reduced by 2–10-fold compared to WT. These marker gene results are consistent with the senescence phenotype seen. By contrast, in ORE1 overexpression plants, expression of the five target genes was already induced under nitrogen-sufficient condition and a further twofold induction was observed on nitrogen starvation. These results show that ORE1 and NLA are positive and negative regulators, respectively, of leaf senescence induced by nitrogen starvation (Fig. 2a,b). In ORE1 overexpression plants in *nla* or *pho2* (*ubc24*) background, expression of these genes was highly induced under both nitrogen-sufficient and -deficient conditions. By contrast, there were no obvious changes in the expression levels of these five marker genes in *nla*/*ore1*, *pho2* (*ubc24*)/*ore1*, NLA overexpression/*ore1* and NLA overexpression/ORE1 overexpression plants. On the other hand, under nitrogen-deficient condition, marker gene expression in ORE1 overexpression/*nla* and ORE1 overexpression/*pho2* (*ubc24*) was threefold higher than that under sufficient condition. These results support the epistatic relationship between ORE1 and NLA and further demonstrate that NLA/PHO2 (UBC24) negatively regulates ORE1 upon nitrogen starvation (Fig. 2c,d).

The genetic relationship between ORE1 and NLA raises the question of their biochemical relationship. Yeast two-hybrid assays show ORE1–NLA interaction in yeast cells (Supplementary Fig. 1).

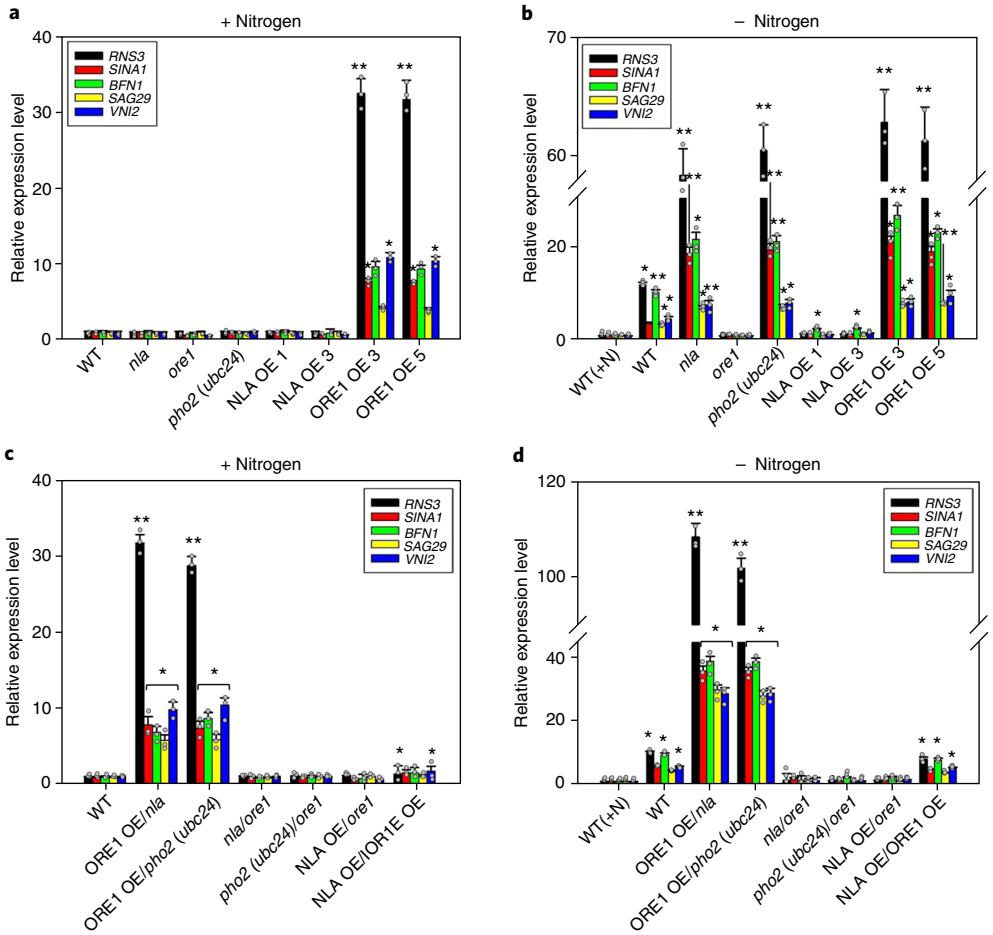

Fig. 2 | ORE1 target gene expression in various genotypes under nitrogen-sufficient or -deficient condition. a,**b**, Expression levels of *RNS3, SINA1, BFN1, SAG29* and *VIN2* in two-week seedlings of WT, *nla*, *pho2* (*ubc24*), *ore1*, NLA overexpression (OE) and ORE1 overexpression transferred and grown under nitrogen-sufficient (**a**) or -deficient (**b**) conditions for one week. N, nitrogen. **c**,**d**, Expression levels of *RNS3, SINA1, BFN1, SAG29* and *VIN2* in two-week seedlings of WT, ORE1 overexpression/*nla*, ORE1 overexpression/*pho2* (*ubc24*), *nla/ore1*, *pho2* (*ubc24*)/*ore1*, NLA overexpression/*ore1* and NLA overexpression/ORE1 overexpression transferred and grown under nitrogen-sufficient (**c**) or -deficient (**d**) conditions for one week. All expression data were relative to *ACT2* transcripts. Twenty seedlings were used per genotype. The expression level of WT (+ nitrogen) was set at 1. Data are means ± s.d. $n = 3$ (biologically independent experiments) and individual data points as overlays. Supplementary Table 4 shows *P* values. Asterisks indicate statistically significant difference compared with WT (+ nitrogen). $^{*}P < 0.05$, $^{**}P < 0.01$; two-tailed *t*-test.

This interaction was confirmed by in vitro pull-down assays (Supplementary Fig. 2a), which further demonstrated that ORE1 was bound to the NLA N-terminal fragment (amino acids 1–167) containing the SPX domain (Supplementary Fig. 2c). This binding is specific as no association was detected between ORE1 and SINAT5, which is a RING E3 ligase for NAC1[16] (Supplementary Fig. 2b). To confirm the specificity of the NLA target, we investigated using in vitro pull-down and ubiquitination assays between NLA and NAC1. We found that although NLA interacted with NAC1 (Supplementary Fig. 5a), the E3 ligase was unable to polyubiquitinate the latter (Supplementary Fig. 5b).

An NLA-GFP fusion protein was previously shown to display nuclear localization in onion epidermal cells[15] and an ORE1-GFP fusion protein also accumulated in nuclei of *Arabidopsis*[2]. However, other investigations found that NLA is also localized to plasma membranes where it controls the degradation of plasma membrane-localized phosphate transporters in *Arabidopsis*[14,17].

We used a bimolecular fluorescence complementation (BiFC) system[18,19] to determine the subcellular site of interaction between NLA and ORE1 in vivo. Figure 3a shows that coexpression of *35S:NLA-cYFP* and *35S:ORE1-nYFP* reconstituted a clear yellow fluorescent protein (YFP) signal in nuclei of *Nicotiana benthamiana* leaves. No signal was detected with *35S:NLA-cYFP/35S:nYFP* and *35S:cYFP/35S:ORE1-nYFP*. This NLA–ORE1 interaction in vivo was further verified by pull-down assays using extracts prepared from WT, *nla* and ORE1 overexpression plants (Fig. 3b).

An NLA–UBC8 interaction was previously observed in yeast as well as in plant cells[15]. However, a different report showed that the E3 ubiquitin ligase activity of NLA specifically requires PHO2 (UBC24)[14] and no activity was detected with UBC8. The in vitro and in vivo association of ORE1 and NLA led us to hypothesize that NLA regulates leaf senescence through polyubiquitinating ORE1 and mediating its degradation. Therefore, we performed in vitro ubiquitination assays using NLA (E3), PHO2 (UBC24) (E2) and ORE1 (substrate). Figure 3c shows that, indeed, ORE1 was polyubiquitinated by NLA in a reaction dependent on E1, E2 and E3 enzymes.

MicroRNAs (miRNAs) play important roles in regulating nutrient metabolism in plants[20–23]. In particular, *miR164* has been shown to cleave *ORE1* mRNA in age-dependent senescence, but how this miRNA is regulated under nitrogen-starvation condition is unclear.

A previous report suggested that leaf senescence regulated by *miR164* was specific to carbon starvation and *miR164* expression

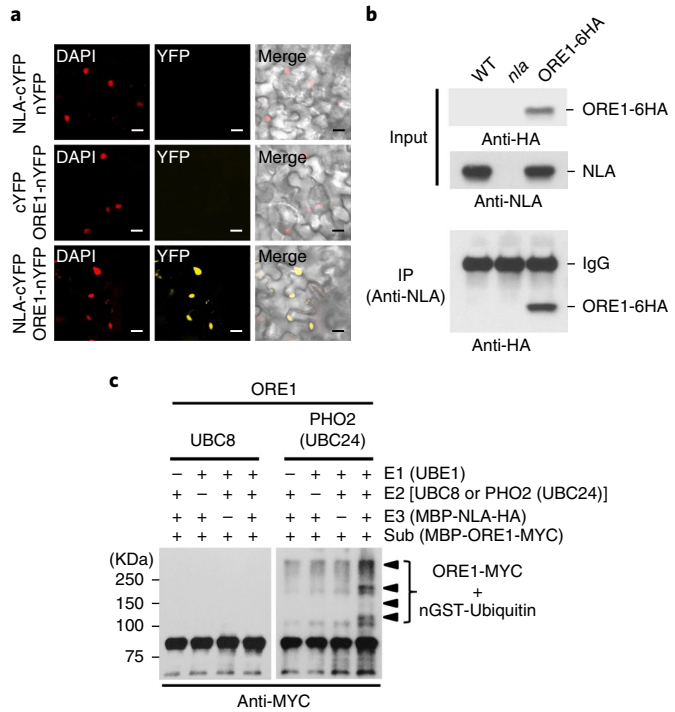

Fig. 3 | ORE1 interacts with NLA in vivo and its polyubiquitination is controlled by NLA and PHO2 (UBC24). a, BiFC analysis of interaction between ORE1 and NLA in tobacco. Reconstituted YFP signals were detected in nuclei of leaf cells when ORE1-nYFP was transiently coexpressed with NLA-cYFP in *N. benthamiana* leaves infiltrated with *Agrobacterium* suspensions. Localization is shown by merging DAPI, YFP and DIC images (Merge). Confocal images were taken two days after infiltration. Nuclei were stained by DAPI. DAPI, 4,6-diamidino-2-phenylindole; DIC, differential interference contrast. Scale bars, 10 μm. **b**, NLA interaction with ORE1 in vivo was detected by coimmunoprecipitation with full-length proteins using anti-NLA antibody in two-week-old WT, *nla* and ORE1 overexpression (35S:ORE1-HA) plants under nitrogen-sufficient condition. Pulled-down proteins were analysed by western blots using anti-HA antibody. IP, immunoprecipitation. **c**, PHO2 (UBC24), but not UBC8, supports ORE1 polyubiquitination. For in vitro ubiquitination assay, the reaction mix contained 25 ng UBE1 (rabbit E1), 100 ng UBC8 or PHO2 (UBC24) (E2), 500 ng MBP-NLA-HA (E3), 500 ng MBP-ORE1-MYC (substrate) and 8 μg GST-Ubiquitin. Polyubiquitinated ORE1 was analysed by western blot using anti-MYC antibody. $n = 3$ (biologically independent experiments).

was not responsive to nitrogen[24]. We examined transcript levels of *miR164* and *ORE1* using quantitative PCR with reverse transcription (RT-qPCR) and ORE1 protein levels by western blot in plants of various genotypes under nitrogen-starvation condition. One day of nitrogen starvation triggered a striking decrease of *miR164* levels with little increase in *ORE1* mRNA level in WT, *nla* and *pho2* (*ubc24*) plants (Supplementary Fig. 3). Little change in *miR164a/b/c* levels was seen upon further growth on nitrogen-deficient medium for six additional days. However, during this period there was a 15–20-fold increase in *ORE1* mRNA levels in all 3 genotypes probably due to transcriptional activation. These results suggest that *miR164* is not a regulator of *ORE1* mRNA levels under nitrogen-starvation condition. Supplementary Fig. 3d shows a 2.5-fold increase of ORE1 protein in WT at 6 days on nitrogen-deficient medium; by contrast, ORE1 protein levels increased by about 5-fold in the 2 mutants. These results suggest that given comparable *ORE1* transcript levels ORE1 protein level is determined by NLA (E3) and PHO2 (UBC24) (E2).

To investigate the requirement of NLA (E3) and PHO2 (UBC24) (E2) for ORE1 degradation in vivo under full nutrient or nitrogen-deficient conditions, we transformed WT, *nla* and *pho2* (*ubc24*) mutant plants with *35S:ORE1–6MYC* and investigated ORE1 protein stability using cycloheximide (CHX) to block new protein synthesis. Figure 4a shows that in WT plants without MG132 ORE1 protein levels decreased in a time-dependent manner, under nitrogen-deficient condition but not under nitrogen-sufficient condition. Moreover, the decrease in ORE1 protein levels induced by nitrogen deficiency was blocked in *nla* and *pho2* (*ubc24*) background (Fig. 4b,c). Taken together with the in vitro polyubiquitination data, our results show that NLA and PHO2 (UBC24) mediate ORE1 degradation in vivo under nitrogen deficiency.

Previous work showed that NLA is constitutively active and degrades phosphate transporter in the presence of high phosphate[14]. When phosphate becomes limiting, *NLA* mRNA is cleaved by the induced *miR827* and this relieves the post-translational repression. Here, we propose that NLA functions upstream of ORE1 and provide biochemical and molecular evidence that under nitrogen-deficient condition NLA, together with PHO2 (UBC24), mediates ORE1 polyubiquitination and destruction and maintains it at a low level to delay rapid leaf senescence.

NLA mRNA and *PHO2 (UBC24)* mRNA are targeted by *miR827* and *miR399*, respectively, for post-transcriptional cleavage. Under phosphate-deficient conditions, levels of these two miRNAs are elevated along with the concomitant decease of mRNA levels of *NLA* and *PHO2 (UBC24)*[25–29]. By contrast, under nitrogen-starvation condition, expression levels of these two miRNAs decreased by 3–6-fold[1,22,24], allowing the expressed NLA and PHO2 (UBC24) proteins to destabilize ORE1.

Supplementary Fig. 4 shows that, under nitrogen-deficient condition, *ORE1* mRNA levels were elevated 15–20-fold as a result of transcriptional activation, but there was also a 2-fold increase in *NLA* and *PHO2* (*UBC24*) mRNA levels. Therefore, the twofold increase of ORE1 protein after six days on nitrogen-deficient medium is probably due to the increased expression of NLA/PHO2 (UBC24), which keeps the ORE1 in check. Our conclusion is bolstered by the observation that ORE1 was unstable in WT as its levels were considerably increased by the 26S proteasome inhibitor MG132 (Supplementary Fig. 3f); by contrast, there were no changes in ORE1 levels with or without MG132 in *nla* and *pho2* (*ubc24*) (Fig. 4).

Recent papers have reported that *ANAC016*, *ANAC032* and *ANAC046* play important roles in both age-dependent and stress-induced leaf senescence in *Arabidopsis*[30,31]. In particular, ANAC046 overexpression remarkably impairs plant growth and promotes age-dependent leaf senescence. By contrast, ORE1 overexpression plants did not show these phenotypes, suggesting distinct functions between *ORE1* and *ANAC046*[5,8]. As shown here, *ore1* and ORE1 overexpression plants have clear delayed- and rapid-senescence phenotype under nitrogen deficiency, respectively. Therefore, these results argue that *ORE1* specifically regulates leaf senescence under nitrogen deficiency by a pathway different from that mediated by *ANAC016*, *ANAC032* and *ANAC046*. However, it is possible that these other NAC factors may play a minor role in nitrogen deficiency-induced senescence.

Nitrogen deficiency has been known to seriously affect growth but how plants adapt to this abiotic stress is largely unknown. Our studies highlight ORE1 as a major determinant of leaf senescence induced by nitrogen deficiency. The dynamic, post-translational fine-tuning of ORE1 levels by NLA/PHO2 ensures orderly progression of senescence, integrates other signalling inputs that may modulate this developmental event and facilitates rapid recovery when nitrogen becomes available (Supplementary Fig. 7). Our results contribute to a better understanding of the adaptability and survival strategies of plants when starved of nitrogen.

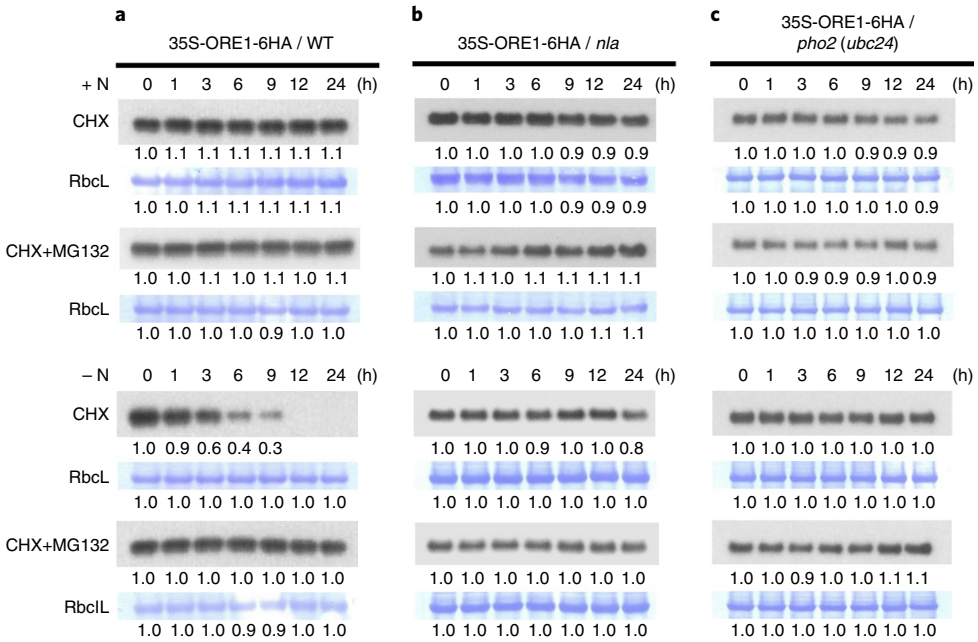

Fig. 4 | Nitrogen deficiency destabilizes ORE1 protein in *Arabidopsis*. **a**,**b**,**c**, WT (**a**), *nla* (**b**) and *pho2 (ubc24)* (**c**) transgenic plants expressing 35S:ORE1–6HA in 1 mM CHX or 50 μM MG132 + 1 mM CHX were transferred onto nitrogen-sufficient or -deficient medium. Samples were taken at various time points and ORE1–6HA protein levels were analysed by western blots using anti-HA antibody. Stained gel bands of the large subunit of Rubisco (RbcL) were used as a loading control. Intensity of the bands was measured using Adobe Photoshop CS3 Extended program. $n = 3$ (biologically independent experiments).

Methods

Plant materials, growth conditions and preparation of plant samples. *Arabidopsis thaliana* WT (Col-0) was used. Seeds were germinated on 1% agar medium containing Murashige and Skoog salts, 0.5 g l^{-1} MES and 10 g l^{-1} sucrose. WT and mutants were grown at 22 °C with 60% relative humidity in long-day conditions (16 h light/8 h dark) under white light at 100 μmol m^{-2} s^{-1} in a growth chamber[8]. Plants were grown in either a nitrogen-sufficient or -deficient medium by modified Hoagland's solution containing 2 mM $CaCl_2$ and 3 mM KCl (pH 5.8) instead of 2 mM $Ca(NO_3)_2$ and 3 mM KNO_3 (pH 5.8) for 2 weeks[32].

Transfer DNA insertion mutants used in this study were *nla* (CS858095), *ore1* (SALK_090154C) and *pho2 (ubc24)* (CS802267). The *nla/ore1* and *pho2 (ubc24)/ore1* double mutants were generated by genetic crossings.

Preparation of constructs. *Arabidopsis NLA* complementary DNA encoding the full-length protein amplified by PCR was inserted into *pMAL C2* (Amersham Biosciences) and *pGEX4T-1* (GE Healthcare) to produce *maltose binding protein (MBP)-NLA* and *glutathione S-transferase (GST)-NLA* fusion genes. Fragments encoding full-length NLA, SPX domain (SPX; amino acids 1–167) and RING domain (RING; amino acids 220–335) were cloned into pGEX4T-1 to give *GST-SPX* and *GST-RING*, respectively. cDNAs encoding full-length *Arabidopsis ORE1, NAC1* and *PHO2 (UBC24)* were inserted into *pMAL C2* and *pGEX4T-1* to give *MBP-ORE1, GST-NAC1-HA* and *GST-PHO2 (UBC24)*.

cDNAs encoding *NLA* and *ORE1* were inserted into *pBA002* and *pKO* to produce *35S:NLA-6MYC* and *35S:ORE1–6HA*, respectively. Transgenic plants expressing combinations of these transgenes were used for co-immunoprecipitation and ORE1 protein stability experiments. For yeast two-hybrid assays full-length cDNA of *NLA* and *ORE1* was inserted into *pGAD424* as bait and *pGBT9* as a prey, respectively. To investigate localization and in vivo interaction, cDNAs encoding *NLA* and *ORE1* were inserted into *pBA002-cYFP* and *pBA002-nYFP* to produce 35S:*NLA-cYFP* and 35S:*ORE1-nYFP* for BiFC, respectively[18,19].

In vitro and in vivo pull-down of ORE1 with NLA. Constructs *GST-NLA*, *GST-SPX domain*, *GST-RING domain, GST-SINAT5, MBP-NAC1-MYC* and *MBP-ORE1-MYC* were transformed into *Escherichia coli* BL21/DE3(pLysS) cells. Transformed cells were treated with isopropyl-β-D-thiogalactoside for 12 h at 22 °C to induce expression of fusion proteins. To determine possible interactions of NLA, SPX domain, RING domain and SINAT5 with ORE1 or NAC1, 1 μg of *GST-NLA*, *GST-SPX domain*, *GST-RING domain, GST-SINAT5, MBP-NAC1-MYC* and *MBP-ORE1-MYC* was added to 1 ml binding buffer (50 mM Tris–HCl (pH 7.5), 200 mM NaCl, 1% Triton X-100, 0.2% glycerol, 0.5 mM beta-mercaptoethanol). After incubation at 25 °C for 2 h, the reaction mixtures were incubated with glutathione resin for 2 h before being washed 4 times for 10 min each time with binding buffer. Absorbed proteins were analysed by SDS–PAGE and detected by western blot using an anti-MYC antibody (Santa Cruz Biotechnology). To determine in vivo interactions between NLA and ORE1, plants of WT (Col-0), *nla* and *35S:ORE1–3HA* were used. Constructs for transgenic plants were transformed into *Agrobacterium* strain GV3101 and infiltrated into WT using the floral dip method[33].

In vitro ubiquitination assays. The reaction mix of 30 μl contained 50 mM Tris (pH 7.5), 50 mM $MgCl_2$, 20 μM $ZnCl_2$, 20 mM ATP, 100 ng purified MBP-ORE1-HA or GST-NAC1-HA, 500 ng purified MBP–NLA-MYC or MBP-SINAT5 (E3), 25 ng UBE1 (E1, BostonBiochem), 100 ng HIS-UBCH5b (BostonBiochem), HIS-UBC8 or GST-PHO2 (UBC24) (E2) and 8 μg HIS-ubiquitin or GST-ubiquitin (BostonBiochem).

After incubation in a water bath at 30 °C for 2 h, reaction mixtures were separated on 8% SDS–polyacrylamide gel and substrates (MBP-ORE1-HA) were detected by western blot analysis using anti-HA antibody (Santa Cruz Biotechnology).

Effects of NLA and nitrogen starvation on ORE1 protein stability. Plants overexpressing *35S:ORE1–6HA* in WT (Col-0), *nla* and *pho2* (*ubc24*) mutant background were generated. Seedlings (14 d old) were treated with 1 mM CHX (Sigma-Aldrich) to block new protein synthesis and with or without 50 μM MG132 under full nutrient or nitrogen-starvation condition. Protein samples at different time points were analysed by western blots using anti-HA (Santa Cruz Biotechnology) antibody for the tagged proteins.

RNA extraction and RT–qPCR analysis. Total RNA was isolated from WT and plants of various genotypes using QIAGEN RNeasy Mini Kits (QIAGEN) according to the manufacturer's instructions. Reverse transcriptional reaction was performed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using the CFS96 Real-Time System (BioRad) with three sets of gene primers. *ACT2* was used as a reference gene in qPCR and the gene expression level was analysed by Genex Program (BioRad). Primers used for qPCR are listed in Supplementary Table 1.

***Agrobacterium*-mediated infiltration and fluorescence microscopy in *N. benthamiana* leaves.** For BiFC assays, 35S:*NLA-cYFP*, 35S:*ORE1-nYFP* and empty vectors as negative control were transformed into *Agrobacterium* strain *GV3101* by the freeze and thaw method. *Agrobacterium* cells were harvested and resuspended in 10 mM MgCl2 plus 150 μM acetosyringone (Sigma-Aldrich) and then kept at room temperature for at least 3 h without shaking. Transformed *Agrobacterium* were infiltrated into leaves of *N. benthamiana* according to a previous study[34]. Samples were

analysed using an LSM 510 laser scanning confocal microscope (Zeiss) after 2 d and excitation (emission) wavelengths were 500–535 nm (20–30 nm) for YFP[19].

Yeast two-hybrid assays. Yeast two-hybrid assays were performed using the GAL4-based two-hybrid system (Clontech). All constructs for yeast two-hybrid assays were transformed into yeast strain *AH109* by the lithium acetate method. Transformed yeast cells were grown on Leu^-/Trp^- minimal medium and were plated onto Leu^-/Trp^-/, $Leu^-/Trp^-/His^-$ minimal medium and $Leu^-/Trp^-/His^-$ minimal medium containing 20 mM 3-amino-1,2,4-triazole to test for possible interactions between NLA and ORE1.

Total chlorophyll content measurement. After growth on nitrogen-deficient medium for 2 weeks, leaves of seedlings were divided into 3 groups (leaves 1–3, 4–6 and 7–10). Leaves were extracted with 80% acetone at 4 °C for 24 h in darkness. Total chlorophyll per fresh weight of leaves was calculated as described previously[35].

Accession numbers. *NLA* (AT1G02860), *ORE1* (AT5G39610), *NAC1* (AT1G56010), *PHO2* (*UBC24*) (AT2G33770), *UBC8* (AT5G41700), *RNS3* (AT1G26820), *SINA1* (AT3G13672), *BFN1* (AT1G11190), *SAG29* (AT5G13170), *VNI2* (AT5G13180), *miR827* (AT3G59884), *miR399d* (AT2G34202), *miR164a* (AT3G47585), *miR164b* (AT5G01747), *miR164c* (AT5G27807) and *ACT2* (AT3G18780). For *Arabidopsis* genes, accession numbers given by the *Arabidopsis* Information Resource (http://www.arabidopsis.org/) are indicated.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated or analysed in this study are included in this article and Supplementary Information files. The data are available from the corresponding author upon request.

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References

1. Kant, S. et al. Genetic regulation by NLA and microRNA827 for maintaining nitrate-dependent phosphate homeostasis in *Arabidopsis*. *PLoS Genet.* **7**, e1002021 (2011).
2. Kim, J. H. et al. Trifurcate feed-forward regulation of age-dependent cell death involving *miR164* in *Arabidopsis*. *Science* **323**, 1053–1057 (2009).
3. Gan, S. et al. Making sense of senescence (molecular genetic regulation and manipulation of leaf senescence). *Plant Physiol.* **113**, 313–319 (1997).
4. Qiu, K. et al. EIN3 and ORE1 accelerate degreening during ethylene-mediated leaf senescence by directly activating chlorophyll catabolic genes in *Arabidopsis*. *PLoS Genet.* **11**, e1005399 (2015).
5. Rauf, M. et al. ORE1 balances leaf senescence against maintenance by antagonizing G2-like-mediated transcription. *EMBO Rep.* **14**, 382–388 (2013).
6. Woo, H. R. et al. The delayed leaf senescence mutants of *Arabidopsis*, *ore1*, *ore3*, and *ore9* are tolerant to oxidative stress. *Plant Cell Physiol.* **45**, 923–932 (2004).
7. Balazadeh, S. et al. Salt-triggered expression of the ANAC092-dependent senescence regulon in *Arabidopsis thaliana*. *Plant Signal. Behav.* **5**, 733–735 (2010).
8. Balazadeh, S. et al. A gene regulatory network controlled by the NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence. *Plant J.* **62**, 250–264 (2010).
9. Meng, S. et al. *Arabidopsis* NRT1.5 mediates the suppression of nitrate starvation-induced leaf senescence by modulating foliar potassium level. *Mol. Plant* **9**, 461–470 (2016).
10. Agüera, E. et al. Induction of leaf senescence by low nitrogen nutrition in sunflower (*Helianthus annuus*) plants. *Physiol. Plant.* **138**, 256–267 (2010).
11. Schulte auf'm Erley, G. et al. Leaf senescence induced by nitrogen deficiency as indicator of genotypic differences in nitrogen efficiency in tropical maize. *J. Plant Nutr. Soil Sci.* **170**, 106–114 (2007).
12. Matallana-Ramirez, L. P. et al. NAC transcription factor ORE1 and senescence-induced BIFUNCTIONAL NUCLEASE1 (BFN1) constitute a regulatory cascade in *Arabidopsis*. *Mol. Plant* **6**, 1438–1452 (2013).
13. Yaeno, T. & Iba, K. BAH1/NLA, a RING-type ubiquitin E3 ligase, regulates the accumulation of salicylic acid and immune responses to *Pseudomonas syringae* DC3000. *Plant Physiol.* **148**, 1032–1041 (2008).
14. Park, B. S. et al. NITROGEN LIMITATION ADAPTATION recruits PHOSPHATE2 to target the phosphate transporter PT2 for degradation during the regulation of *Arabidopsis* phosphate homeostasis. *Plant Cell* **26**, 454–464 (2014).
15. Peng, M. et al. A mutation in NLA, which encodes a RING-type ubiquitin ligase, disrupts the adaptability of *Arabidopsis* to nitrogen limitation. *Plant J.* **50**, 320–337 (2007).
16. Xie, Q. et al. SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. *Nature* **419**, 167–170 (2002).
17. Lin, W. Y. et al. NITROGEN LIMITATION ADAPTATION, a target of microRNA827, mediates degradation of plasma membrane-localized phosphate transporters to maintain phosphate homeostasis in *Arabidopsis*. *Plant Cell* **25**, 4061–4074 (2013).
18. Hu, C. D. et al. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* **9**, 789–798 (2002).
19. Kerppola, T. K. et al. Bimolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells. *Annu. Rev. Biophys.* **37**, 465–487 (2008).
20. Zhao, M. et al. Involvement of miR169 in the nitrogen-starvation responses in *Arabidopsis*. *New Phyt.* **190**, 906–915 (2011).
21. He, H. et al. Two young microRNAs originating from target duplication mediate nitrogen starvation adaptation via regulation of glucosinolate synthesis in *Arabidopsis thaliana*. *Plant Physiol.* **164**, 853–865 (2014).
22. Hsieh, L. C. et al. Uncovering small RNA-mediated responses to phosphate deficiency in *Arabidopsis* by deep sequencing. *Plant Physiol.* **151**, 2120–2132 (2009).
23. Pant, B. D. et al. Identification of nutrient-responsive *Arabidopsis* and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing. *Plant Physiol.* **150**, 1541–1555 (2009).
24. Liang, G. et al. Uncovering miRNAs involved in crosstalk between nutrient deficiencies in *Arabidopsis*. *Sci. Rep.* **5**, 11813 (2015).
25. Bari, R. et al. PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiol.* **141**, 988–999 (2006).
26. Kuo, H. F. et al. The role of microRNAs in phosphorus deficiency signalling. *Plant Physiol.* **156**, 1016–1024 (2011).
27. Fujii, H. et al. A miRNA involved in phosphate-starvation response in *Arabidopsis*. *Curr. Biol.* **15**, 2038–2043 (2005).
28. Aung, K. et al. pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. *Plant Physiol.* **141**, 1000–1011 (2006).
29. Chiou, T. J. et al. Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. *Plant Cell* **18**, 412–421 (2006).
30. Sakuraba, Y. et al. The *Arabidopsis* transcription factor NAC016 promotes drought stress responses by repressing AREB1 transcription through a trifurcate feed-forward regulatory loop Involving NAP. *Plant Cell* **27**, 1771–1787 (2015).
31. Mahmood, K. et al. ANAC032 positively regulates age-dependent and stress-induced senescence in *Arabidopsis thaliana*. *Plant Cell Physiol.* **57**, 2029–2046 (2016).
32. Millner, P. D. et al. The Beltsville method for soilless production of vesicular-arbuscular mycorrhizal fungi. *Mycorrhiza* **2**, 9–15 (1992).
33. Zhang, X. et al. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat. Protoc.* **1**, 641–646 (2006).
34. Liu, T. Y. et al. PHO2-dependent degradation of PHO1 modulates phosphate homeostasis in *Arabidopsis*. *Plant Cell* **24**, 2168–2183 (2012).
35. Porra, R. J. et al. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectrometry. *Biochim. Biophys. Acta* **975**, 384–394 (1989).

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Author contributions

B.S.P. and N.H.C. designed the experiments. B.S.P., T.Y., J.S.S., E.C.C.W., C.H.H. and N.M. executed the experiments. All of the authors interpreted and discussed the data. B.S.P. and N.H.C. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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Experimental design

1. Sample size

   Describe how sample size was determined.

   In figure1, we grow 8 plants per genotype and used 3 plates of plants as 3 biological repeats. This experiment was repeated 3 times (3x24 plants) using different biological samples. We consider this to be 3 biological repeats. Therefore, total 72 plants per genotype were used for plant phenotype (24 plants per genotype) and total chlorophyll measurement assay (48 plants per genotype).

2. Data exclusions

   Describe any data exclusions.

   In the figure 1, we show a photo of a representative series of leaves on one plant of a genotype because the sample size for phenotype test was 24 plants per genotype.

3. Replication

   Describe the measures taken to verify the reproducibility of the experimental findings.

   Each analysis was repeated multiple times to check consistency of results. All attempts at replication of in vitro and in vivo experiments were successful. Our experimental data were confirmed by technical and biological repeats.

4. Randomization

   Describe how samples/organisms/participants were allocated into experimental groups.

   In the plant phenotype assay, all plants were allocated randomly on the growth medium from seeds of each genotype.

5. Blinding

   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   Investigators collecting the data and samples were not blinded because all analyses depended on equivalent stage of plant and growth chamber conditions.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ A statement indicating how many times each experiment was replicated
☐	☒ The statistical test(s) used and whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒ Test values indicating whether an effect is present *Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.*
☐	☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒ Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Adobe Photoshop CS3 Extended(Adobe Photoshop version 10.0) was used for the measurement of band intensity on Western blot results and gene expression analysis by qPCR was conducted by GENEX program version 1.0 (Bio-Rad). All graph about qPCR results were made by Sigma Plot (Systat Software Inc.).

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

No restriction on the distribution of biological material used in this work.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Anti-NLA rabbit antibody was produced by Cocalico Biologicals using antigen, HIS-NLA C-terminal(150 amino acid) provided by us. ORE1(NAC6) antibody was provided by ABMART (Cat. No. X3-Q9FKA0). NLA and ORE1 antibody were validated by nla and ore1 mutant plants. Each product listed below is a combination of mouse monoclonal antibodies (mAbs) against a panel of synthetic peptide antigens from the corresponding region of the target protein. Each combination of mAbs is recommended to be used directly. HA tag rabbit polyclonal IgG (Y-11, sc-805) was provided and validated by Santa Cruz Biotechnology. MYC tag mouse monoclonal IgG (9E10, sc-40) was also provided and validated by Santa Cruz Biotechnology.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

AH109 is a strain of yeast (Saccharomyces cerevisiae) used in biological research for two-hybrid screening. The strain is sold commercially by Clontech and is used as a partner with strain Y187 in mating assays.

b. Describe the method of cell line authentication used.

The plasmid was transformed into the yeast strain AH109 using the lithium acetate method as described in the Clontech manual.

c. Report whether the cell lines were tested for mycoplasma contamination.

AH109 cell line was negative for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No cell lines used were listed in the database of commonly misidentified cell lines.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

No animals were used in this study.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not involve human research participants.