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Arabidopsis NAP-related proteins (NRPs) are soluble nuclear proteins immobilized by heat

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Abstract

Nucleosome assembly protein-related proteins (NRPs) are multifunctional proteins having histone chaperone and phosphatase inhibitor properties. Although it is believed that these proteins are nuclear and bind the chromatin, they can be detected in the cytoplasmic but not in the nuclear protein fraction by immunoblotting analysis. It is shown here that under normal conditions NRPs are nuclear but soluble and leak out of the nuclei during their purification. However, under elevated temperatures (above 42°C), NRPs display significantly reduced mobility and are retained in the nuclei during purification probably due to binding other immobile macromolecules in the nucleus. Our observations highlight the necessity to use different techniques in parallel to unambiguously determine the intracellular localization of proteins. As heat adapted (38°C 2 h followed by 2 h recovery) and heat shocked (45°C, 1 h) *Arabidopsis* seedlings were found to have phenotypes similar to those observed in the NRP loss-of-function mutants *nrp1-1 nrp2-1* (short, branching roots, increased bleomycin sensitivity), it was also investigated whether the immobilization of NRPs by heat results in disturbed NRP functions. The results indicated, however, that heat affected the investigated traits independent of the presence of NRPs.

Keywords: *Arabidopsis thaliana (L.), cellular localization, heat shock, nuclear protein, nucleosome assembly protein-related protein, protein mobility*

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Author Contribution Statement

Ferhan Ayaydin: cell biology, microscopy, manuscript revision; Judit Bíró: protein purification, immunoblotting; Mónika Domoki: *Arabidopsis* culture, phenotype analysis; Györgyi Ferenc: RT-qPCR; Attila Fehér: research concept, experimental design, data analysis and evaluation, manuscript writing.

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1. Introduction

Even small environmental changes can induce gene expression or repression of hundreds of genes in plants. Regulation of such synchronized events requires chromatin remodeling – a process that involves histones, the core protein molecules of nucleosomes (Vermaak 2003). One group of proteins implicated in the regulation of histone modification patterns are the NAP (Nucleosome Assembly Protein) family of histone chaperones, which possess a central conserved NAP domain, and a highly acidic C-terminal domain (Park and Luger 2006). Histone chaperones play a crucial role in nucleosome assembly and are thought to be necessary for prevention of nonproductive aggregation between the highly positive charged histones and the highly negative charged DNA.

Plant NAP-related proteins (NRPs), form a distinct phylogenic group within the NAP family and are closely related to the animal SET/I_2^{PP2A} (patient SE translocation/inhibitor 2 of protein phosphatase 2A) protein (Dong et al. 2003). SET was first identified as the product of a translocated gene in acute undifferentiated leukemia (Adachi et al. 1994). In biochemical assays, SET/I_2^{PP2A} proteins stimulate replication of the adenovirus genome and inhibit protein phosphatase 2A (PP2A) (Haruki et al. 2006; Li et al. 1996). The SET/I_2^{PP2A} protein was identified in protein complexes with histone acetylation or methylation enzymes (Cervoni et al. 2002), B-type cyclins (Kellogg and Kikuchi 1995), a granzyme A–activated DNase (Fan et al. 2003) or transcription coactivators (Karetsou et al. 2005). SET/I_2^{PP2A} was also implicated in the heat shock response of fruit fly blocking histone H3 dephosphorylation at heat-shock loci (Nowak and Corces 2000). Concerning the plant NRPs, it has been shown that *Arabidopsis* NRP1 (UniProt accession: Q9CA59**)** and NRP2 (UniProt accession: Q8LC68**)** have important roles in cell cycle regulation and root meristem maintenance in addition to their potential histone chaperone and histone phosphatase inhibitor functions (Bíró et al. 2012; Zhu et al.

2006). Although the *Arabidopsis* single *nrp1-1* or *nrp2-1* mutants do not exhibit any observable phenotype under *in vitro* or greenhouse conditions (Zhu et al. 2006), *nrp1-1 nrp2-1* double mutants have short branched roots, increased sensitivity against genotoxic stress, and impaired somatic homologous recombination (Gao et al. 2012; Zhu et al. 2006).

In contrast to their above described nuclear functions, plant NRPs could be detected in cytoplasmic cell fractions by immunoblotting (Bíró et al. 2013). Here we report a series of experiments that was carried out in order to clarify the intracellular localization of NRPs. It was established that *Arabidopsis* NRPs are indeed nuclear proteins and their accumulation in the supernatant during the isolation of purified nuclei for immunoblotting analysis is only an experimental artefact due to their small size and high mobility. The obtained results underline the necessity to use various techniques to validate the intracellular localization of proteins since the isolation of nuclei, fixation, or the attachment of a large GFP-tag to small proteins all can strongly influence the obtained results as exemplified here by NRPs. The experimental results also indicate that the NRP proteins despite their known histone-binding capability are not strongly bound to the chromatin under normal conditions. However, NRPs become cross-linked to other proteins in plant nuclei subjected to heat shock (above 42°C for 30-60 min). The potential contribution of this phenomenon to the phenotype of heat-treated *Arabidopsis* plants similar to that of NRP-null mutants was also investigated. It was found that heat affected the phenotype of *Arabidopsis* plantlets independent of the presence of NRPs. The dissimilarity between plant and fruit fly NRP-type proteins in respect to their role in heat responses is discussed.

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2. Materials and methods

2.1. Plant cultures and heat treatment

Experiments were carried out with the wild-type Columbia ecotype of *Arabidopsis thaliana* (L.) Heynh or its double mutant *nrp1-1 nrp2-1*. Seeds of the *nrp1-1 nrp2-1* double mutant *Arabidopsis* plants deficient in At NRP1 and At NRP2 expression were kindly provided by Dr. Yan Zhu (State Key Laboratory of Genetic Engineering, Institute of Plant Biology, School of Sciences, Fudan University, Shanghai, China). Neither the transcripts (Zhu et al. 2006) nor the proteins (Bíró et al. 2012) of NRP1 or NRP2 could be detected in the mutant seedlings.

Seeds of *Arabidopsis thaliana* were surface-sterilized by soaking in 70% ethanol (v/v) for 1 min then in 30% (v/v) commercial bleach (with 4.8% w/w sodium hypochloride) for 10 min and then rinsed five times with sterile distilled water and plated on solid Murashige and Skoog (MS) medium (Duchefa Biochemie, B.V., Haarlem, The Netherlands) containing 3% (w/v) sucrose, 0.6% (w/v) agar, with pH adjusted to 5.8. The seeds were germinated at 24°C using an 8 h light/16 h dark regime. Experiments were carried out at least in triplicates with 7-8-days-old seedlings. Heat shock treatment was applied in a water bath at 45°C for 1 h with or without preceding heat acclimation (38°C for two hours followed by two hours recovery at 22°C). Survival rates were determined by scoring the percentage of healthy seedlings with green leaves that showed continuous growth during six weeks after the treatment.

2.2. Protein purification and detection

Total plant proteins were extracted by grinding samples with quartz sand in the homogenization buffer containing 25 mM Tris-HCl pH 7.6, 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM NaF, 60 mM β-glycero-phosphate, 1 mM PMSF, 2 mM DTT and 10 µl/ml plant-specific protease inhibitor cocktail (Sigma, St-Louis, USA). Nuclei and the nuclear protein fraction were purified according to (Bowler et al. 2004). The supernatant following the precipitation of nuclei

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by centrifugation was further cleared by an additional centrifugation at 10000 g for 10 min, at 4°C, and was used for the isolation of the cytosolic protein fraction. Detection of NRP proteins by immunoblotting was performed according to standard procedures as described elsewhere in more details (Bíró et al. 2012).

2.3. Immunohistochemistry on protoplasts and isolated nuclei

Protoplasts were prepared from *Arabidopsis* cell suspension with the enzyme solution of 3% Cellulase RS, 1% Cellulase R-10, 1% Macerozyme R-10, 0.5 M mannitol, 5 mM $CaCl_2$, pH 5.3. They were then fixed with 4% formaldehyde (Sigma). In experiments where isolated nuclei were used for immunofluorescence (Fig 2d and 2e), nuclei were isolated by chopping unfixed (heat-treated or control) cells on ice with a razor blade (Kotogány et al. 2010) then fixed with 1% formaldehyde on ice for 30 min. Following fixation, cells or nuclei were immobilized on poly-L-lysine coated slides and rehydrated with 0.05% Triton X-100. The polyclonal anti-NRP antibody (see Bíró et al. 2012) was diluted 1:200 in phosphate-buffered saline (PBS). After 1 h incubation with the diluted primary antibody, the slides were washed three times with PBS. Samples were then incubated with the secondary anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC) (Sigma). Following final washes with PBS, slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence was recorded using an Olympus FV1000 confocal laser scanning microscope.

2.4. Transient gene expression and the fluorescence loss in photobleaching (FLIP) assay

For the transient expression assay, the pK7FW62 vector (Karimi et al. 2002) was digested with *Hind*III and *Xba*I and the resulted fragment was subcloned into pBSK vector cut with the same restriction enzymes resulting in pBSK-GFP-GW. A PCR fragment carrying the coding

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sequence of At NRP1 was inserted into the vector by GATEWAY recombination using LR recombinase (Invitrogen, Carlsbad, USA). The constructs were sequence verified.

Isolation and transfection of *Arabidopsis* cell culture protoplasts were made according to (Mathur et al. 1995).

Fluorescence loss in photobleaching (FLIP) assay was performed using an Olympus FV1000 confocal laser scanning microscope as described in Ayaydin and Dasso (2004). After heat-treatment (1h at 45°C or 25°C) of one-day-old *Arabidopsis* protoplasts expressing the GFP:NRP1 protein, an intranuclear spot (1 µm x 1 µm square) was continuously photobleached using high intensity 488nm Argon laser excitation. During spot bleaching, images of the whole nucleus were also captured. On captured images, intranuclear GFP:NRP1 intensity changes were recorded at a fixed distance (5 µm) away from the bleaching spot. In case of immobile proteins only the tight bleaching spot is expected to be photobleached and depleted, while for highly mobile nuclear proteins whole nuclear fluorescence intensity is expected to drop considerably. Recorded values were plotted to compare the intranuclear mobility of GFP:NRP1 on heat-treated and untreated cells.

2.5. Gene expression analysis by real-time quantitative PCR (RT-qPCR)

RNA isolation, cDNA synthesis, and RT-qPCR analysis by an ABI Prism 7700 sequence detection system have been described earlier in details (Domoki et al. 2006). DNA damage and repair-associated (*PARP2*, At4g02390; *RAD51,* At5g20850; *AtMYB,* At5g03780; *RNR2*, At3g27060) as well as the control unresponsive (UBC18, At5g42990) genes were selected based on the work of Chen et al. (2003). Primer sequences are listed as supplementary material in Supplementary Table 1.

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3. Results and Discussion

3.1. NRPs are soluble nuclear proteins

The animal homologue of plant NRPs, the SET protein, has various functions ranging from the inhibition of different enzyme complexes (protein phosphatase 2A (PP2A), histone deacetylase, granzyme A-activated DNase) to the regulation of transcription and nucleosome assembly (Park and Luger 2006). The information regarding the function of plant NRPs is limited, but these proteins are most likely multifunctional, as well. They have been reported to inhibit PP2A activity *in vitro* (Bíró et al. 2012) and to possess histone chaperone-like properties (Zhu et al. 2006). Their possible roles in cell division control, gene silencing, homologous recombination, and sensitivity against DNA damaging agents have also been shown (Gao et al. 2012, Zhu et al. 2006) Most of the above mentioned functions require the nuclear localization of the NRP proteins. Fluorescent protein-tagged NRPs localize to the nuclei of *Arabidopsis* cells (Fig 1a) in accord with their possible functions (Zhu et al. 2006). However, the detection of NRP proteins in cytoplasmic cell fractions by immunoblotting contradicts these results (Fig. 1c; Bíró et al. 2013).

In order to clarify this discrepancy regarding the localization of NRPs, we carried out further experiments. In transfected protoplasts, the ectopically expressed GFP-fused At NRP1 could exclusively be detected by immunoblotting in the nuclear fraction, while the endogenous untagged NRPs were accumulating in the cytoplasmic (supernatant) fraction (Fig. 1d). This result suggested that the increased size due to the GFP-tag may be responsible for the nuclear retention of the protein fusion. We tested this hypothesis by determining the localization of endogenous NRP proteins in intact cells by indirect immunofluorescence using the same At

NRP1 antibody as in immunoblotting experiments. The strong nuclear fluorescence indicated that the NRP proteins are predominantly nuclear in formaldehyde-fixed intact cells (Fig. 1b, asterisks indicate two NRP-containing nuclei). The GFP-tag, therefore, is not required for the nuclear localization of NRPs. In a subsequent experiment, we investigated the effect of cell fixation on NRP detection in nuclear and cytoplasmic fractions. Formaldehyde-fixed and unfixed cells were fractionated, and the fractions were tested for the presence of NRPs by immunoblotting. The NRP proteins could only be detected in the nuclei of fixed but not that of the unfixed cells (Fig. 1e). Moreover, the anti-NRP antibodies in immunolabeling experiments did not label the mitotic chromosomes in dividing cells, indicating that the protein is not chromatin-bound in these cells, either (Fig. 1b, arrow indicates a mitotic cell devoid of NRP labeling).

These data altogether can be explained if we suppose that the relatively small (<30 kD), nuclear NRP proteins leak out from the nuclei into the supernatant during cell fractionation. GFP-tagged NRP proteins having a size of approximately 60 kD are most likely large enough to be retained within the isolated nuclei, while formaldehyde fixation may crosslink NRPs to other macromolecules and prevents leakage. YFP-NRP fusions were reported to be bound to the chromatin (Zhu et al. 2006). Our observations using an anti-NRP1 antibody and cell fractionation are against a strong chromatin binding and support a view that NRPs are soluble and mobile within unfixed nuclei. Further experimental data obtained during the investigation of the effect of heat on NRP solubility supported this hypothesis.

3.2 Heat shock immobilizes NRPs within the nucleus

In previous publications, we have reported the analysis of the potential role of NRPs in heat stress response of plants (Bíró et al. 2012, 2013). During our experiments we have realized that

strong heat-treatment (45°C, 1 h) of *Arabidopsis* cells resulted in the retention of NRPs in isolated nuclei in contrast to the untreated cells (Bíró et al. 2013). In our follow up experiments presented here, it was established that lower temperature (37°C) was not but shorter time (half an hour) at 45°C heat treatment was sufficient to achieve this retention (Fig. 2a,b). Moreover, it was found that the retention was sustained. Nuclei which were isolated 42 h after the one-hour heat treatment still contained NRPs (Fig. 2b). Nuclear retention of NRPs in response to heat was also verified in heat-treated *Arabidopsis* seedlings (Fig. 2c). Preconditioning of the plantlets at 37°C prior to heat-treatment (45°C, 1 h) did not prevent this nuclear retention phenomenon (Fig. 2c). In order to verify the results obtained by immunoblotting, immunofluorescence-based microscopy assays were performed using nuclei isolated from unfixed control and heat-treated cells (Fig. 2d,e). Only the nuclei derived from heat-treated cells exhibited immunofluorescence signal indicating the presence of NRPs. Other stress treatments, including, heavy metal, salt, genotoxic and UV stress, did not result in the retention of NRPs during the isolation of nuclei (Supplementary Fig. 1) indicating that the phenomenon is heat specific.

Fluorescence loss in photobleaching (FLIP) assay using laser scanning confocal microscope on live cells provided further proof that NRPs are mobile soluble nuclear proteins, but are immobilized in response to heat stress. In this experiment, *Arabidopsis* protoplasts were transfected with a GFP-At NRP construct. GFP fluorescence was bleached repetitively only in a restricted, 1 square micron area within selected fluorescent nuclei, and the depletion of emission was measured at a fixed position away from the depletion zone (Ayaydin and Dasso 2004). In control protoplasts (no heat treatment), successive spot-bleaching resulted in the gradual loss of fluorescence in the whole nucleus due to the mobility (solubility) of NRPs (Fig. 3a). In contrast, when the protoplasts were treated by heat (45°C, 1 h), only the excited area lost the fluorescence signal indicating that the NRP proteins got immobilized and could not diffuse

to the site of bleaching. Quantitative analyses corroborated these microscopy observations in that the kinetics of loss of fluorescence displayed a dramatic drop of nuclear fluorescence intensity in untreated samples as compared to heat-treated samples (Fig. 3b).

DNase or RNase treatment of the heat-treated nuclei prior to immunoblotting, did not affect nuclear retention (Fig. 3c), suggesting that the heat treatment resulted in crosslinking of NRPs to other nuclear proteins rather than nucleic acid polymers.

The animal SET/I_2^{PP2A} protein, the homologue of plant NRPs, is considered to be a scaffold protein being present in several large protein complexes. The same may hold true for plant NRPs. However, until now, *Arabidopsis* NRPs were shown to interact only with histones and the catalytic subunit of the plant PP2A phosphatase (Bíró et al. 2012; Zhu et al. 2006). It has long been known that heat causes protein aggregation also in the nuclei of animal cells (Borrelli et al. 1996; Laszlo et al. 1992; Littlewood et al. 1987; Roti Roti and Turkel 1994 a,b; Warters et al. 1986). Although the degree of heat-induced protein aggregation could be correlated with the thermotolerance of HeLa cells (Roti Roti and Turkel 1994 a,b) and several oncogenes have also been found to be associated with these complexes (Littlewood et al. 1987), the significance of the phenomenon is unclear.

3.3. NRP immobilization does not result in an NRP loss-of-function phenotype

Although acquired thermotolerance can ensure survival, extreme heat alters the development of even acclimatized plants. *Arabidopsis* seedlings exposed to a short but strong heat treatment (45°C, 1 h) following an acclimation period (38°C, 2 h) exhibit an almost 100% survival rate but show reduced hypocotyl and root length as compared to untreated controls (see e.g. Larkindale and Knight 2002). This indicates that the thermotolerance mechanisms do not prevent certain sublethal developmental changes caused by heat.

In order to determine whether the heat-mediated crosslinking of NRPs to the chromatin contributes to the phenotype of heat-treated seedlings, we compared the NRP loss-of-function *nrp1-1 nrp2-1* double mutants (Zhu et al. 2006) to seedlings acclimatized at 38°C for two hours followed by one hour heat shock (45°C) (see Materials and methods for details). Hundred percent of acclimatized plants could survive the applied, otherwise lethal, heat shock treatment that resulted in the crosslinking of NRPs to the chromatin (Fig 4a, see also Fig 2c). Therefore, these plants were suitable for comparison to the *nrp1-1 nrp2-1* mutants.

The *nrp1-1 nrp2-1* mutants have been characterized by Zhu et al. (2006). One of the characteristic phenotypes of *nrp1-1 nrp2-1* seedlings is altered root growth resulting in a small, branched root system, very similar to that of heat-treated *Arabidopsis* plantlets (Fig. 4a). However, heat-treatment of the *nrp1-1 nrp2-1* plantlets resulted in further root growth retardation (Fig. 4b) indicating that the effects of heat and NRP loss-of-function are additive and therefore it is unlikely that they affect the same pathway.

Another distinctive phenotype of the *nrp1-1 nrp2-1* mutants is their sensitivity towards bleomycin, a DNA damaging agent (Zhu et al. 2006). The survival rate of bleomycin-treated *nrp1-1 nrp2-1* mutant seedlings (~50%) was similar to wild type ones subjected to combined genotoxic and heat stresses (~39%) (Fig. 5a). Heat and *nrp1-1 nrp2-1* mutation together decreased the survival rate of seedlings on bleomycin plates down to ~23% indicating again that heat and NRP loss-of-function do not affect the same pathway. This sensitivity was not due to the altered expression of genes implicated in DNA repair as these genes exhibited similar induction under the investigated conditions (Fig. 5b).

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3.4. NRPs and the heat response of *Arabidopsis*

The NRP homolog animal SET/I_2^{PP2A} protein regulates the expression of heat shock protein (HSP) genes through the inhibition of histone H3 dephosphorylation at heat-shock gene loci (Nowak et al. 2003). We have previously shown that NRPs are dispensable for the heat-induced expression of heat shock protein genes in *Arabidopsis*, despite the fact that they act as potent histone H3 phosphatase inhibitors *in vitro* (Bíró et al. 2012). Here, we provide further evidence that NRPs are not responsible for heat-induced phenotypes of high temperature acclimated *Arabidopsis* plants; although there are remarkable phenotypic similarities between NRP-deficient mutants and heat-treated acclimated wild types (Figs. 4-5). Therefore, NRPs seem not to be involved in acquired thermotolerance in a way described for the orthologous Drosophila SET protein. Nevertheless, the overexpression of the At NRP1 protein slightly increased the survival of non-acclimated *Arabidopsis* cells/seedlings exposed to high temperature (45°C for 1 h; Bíró et al. 2013). In contrast, the *nrp1-1 nrp2-1 Arabidopsis* mutants did not exhibit increased heat sensitivity according to our investigations (data not shown). We hypothesize that the improved survival of NRP-overexpressing *Arabidopsis* cells exposed to heat might be associated with the crosslinking of NRPs to other nuclear proteins in response to heat (see 3.2). This hypothesis, however, still needs to be experimentally validated.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 Fractionation and microscopy analyses of intracellular compartmentalization of *Arabidopsis* NRP (At NRP) proteins

(a) Intracellular localization analysis on protoplasts shows nuclear accumulation of GFP:NRP1. Nuclei and nucleoli (arrowheads) of GFP:NRP1-transfected protoplasts are located using differential interference contrast (DIC) imaging. (b) In formaldehyde-fixed cultured *Arabidopsis* cells, NRP immunodetection (green) shows nuclear accumulation of NRP in interphase cells (asterisks) but not in mitotic cells (arrow). Nuclei are counterstained using DAPI (pseudocolored red). Scale bars are 5 µm. (c) Immunoblotting and detection of At NRP after fractionation of nuclei (N) and supernatant (S) of unfixed cells. NRP is detected in supernatant fraction. Immunodetection of a nucleocytoplasmic protein PP2Ac (Protein phosphatase 2A catalytic subunit) is used as loading control on the lower panel. (d) Immunodetection of NRP after fractionation of nuclei (N) and supernatant (S) of GFP:NRP1 expressing unfixed cells. Note that GFP-tagged NRP1 is retained in the nuclear fraction. (e) Nuclear and cytoplasmic fractionation and immunodetection of NRP using fixed and unfixed cells. Note that fixed cells' nuclear fractions and unfixed cells' cytoplasmic fractions contain NRP.

Fig. 2 The effect of heat shock on nuclear retention of Arabidopsis NRP (At NRP) proteins. (a) Immunoblotting of At NRP on nuclear (N) and supernatant (S) fractions after 1 h heat treatment at various temperatures. NRP of heat-treated cells (45°C and 50°C) remains nuclear during fractionation procedure. (b) Immunodetection analysis of induction and longevity of temperature-induced nuclear NRP retention using suspension cells. Left panel shows the nuclear detection of NRP in the nuclei after half an hour of heat treatment at 45°C. Right

panel shows persistence of nuclear retention after 30 h and 42 h following a 1 h, 45°C treatment. (c) Immunodetection of At NRP in nuclear protein fractions isolated from heat-treated Arabidopsis seedlings. Treatment temperature and durations are indicated below the image. (d), (e) Immunolocalization of NRP (green) in isolated nuclei of control (25°C) and heat treated (45°C) cells. Unfixed cells were used for isolation of nuclei which were then fixed with formaldehyde (see Methods). DAPI as nuclear counterstain is shown in red pseudocolor. Merged images with differential interference contrast (DIC) overlay are shown at the last panel. Scale bar is 5 μm.

Fig. 3 Immobilization of soluble NRPs in heat-shocked nuclei.
(a) Fluorescence loss in photobleaching (FLIP) assay. Live cell images of heat-shocked (45°C, upper panel) and control (25°C, lower panel) GFP:NRP1-transfected Arabidopsis protoplasts were collected during successive laser bleaching of a localized intranuclear spot. Black squares of 1 μm width indicate bleached region. Yellow triangles represent repetitive activation of laser bleaching. White circles are 5 μm away from the bleaching spot and were used to measure the depletion of fluorescence to assess the mobility of intranuclear GFP:NRP1. Prebleach images indicate the initial fluorescence intensity of nuclei and the position of nucleoli (dark circular regions). Representative timepoints are shown. Scale bar is 5 μm. (b) Kinetics of loss of fluorescence during FLIP assay of heat-treated (45°C) and control (25°C) GFP:NRP1-transfected samples. Intensity values were recorded at the intranuclear region which was 5 μm away from the bleached spot as shown on part (a). Averages and standard deviations of five measurements on different nuclei are shown. (c) Heat-treatment-induced nuclear retention (immobilization) of NRPs in isolated nuclei cannot be reverted by DNase and/or RNase treatment. Electrophoretic analysis of nucleic acids in an ethidium bromide-stained agarose gel (left) and the detection of NRPs in isolated nuclei of

heat-treated cells by an anti-NRP antibody (right). M: molecular weight marker; NT: Not treated, D: DNase-treated, D+R: DNase and RNase-treated.

Fig. 4 Effect of heat shock on root growth of wild type and NRP-deficient *nrp1-1 nrp 2-1* mutant seedlings.

(a) Two-weeks-old wild type (WT) and NRP-deficient (-NRP) seedlings have been subjected to heat adaptation and heat shock (38°C, 2h►22°C, 2h ►45°C, 1h) or left untreated as control. Root images were taken on the 10th day post-treatment. (b) The length of the main roots was measured daily during the investigated period. Average main root length is shown for each treatment with standard deviation (n>25).

Fig. 5 Effect of heat shock on bleomycin sensitivity of wild type and *nrp1-1 nrp 2-1* mutant seedlings.

(a) One week old wild type (WT) and NRP-deficient *nrp1-1 nrp 2-1* mutant (-NRP) seedlings, untreated control (CTRL) or heat adapted then heat shocked (HS) (38°C, 2h►22°C, 2h ►45°C, 1h) have been transferred to fresh media supplemented with 1 mg/L bleomycin and their survival was scored after six weeks. Averages and standard deviations from three experiments are shown. (b) The expression of the indicated genes, known to have a role in genotoxic stress response (Chen et al. 2003), was determined by real-time quantitative PCR using whole seedlings one hour after being transferred to bleomycin (BLEO) plates. The expression of the UBC18 (At5g42990) gene was used as reference for normalization and expression of untreated seedlings was used as a reference value (value = 1; a.u.: arbitrary unit).

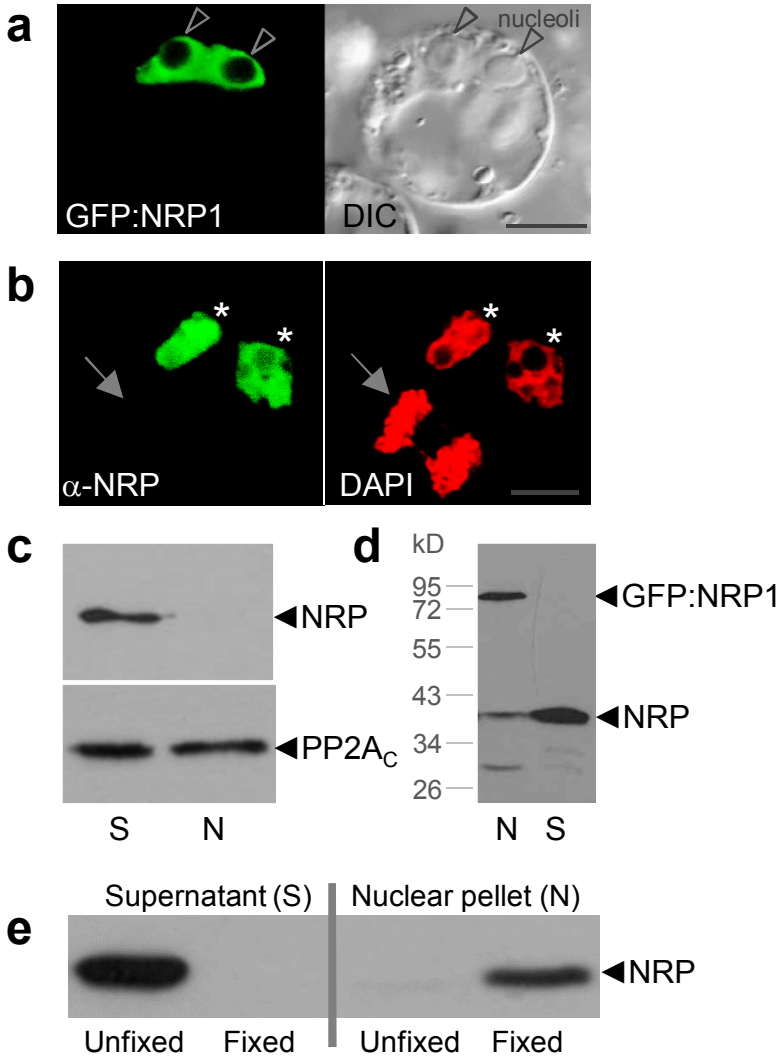

a
nucleoli
GFP:NRP1
DIC
b
α-NRP
DAPI
c
NRP
PP2A$_C$
S
N
d
kD
95
72
55
43
34
26
GFP:NRP1
NRP
N
S
e
Supernatant (S)
Nuclear pellet (N)
NRP
Unfixed
Fixed
Unfixed
Fixed

Fig. 1

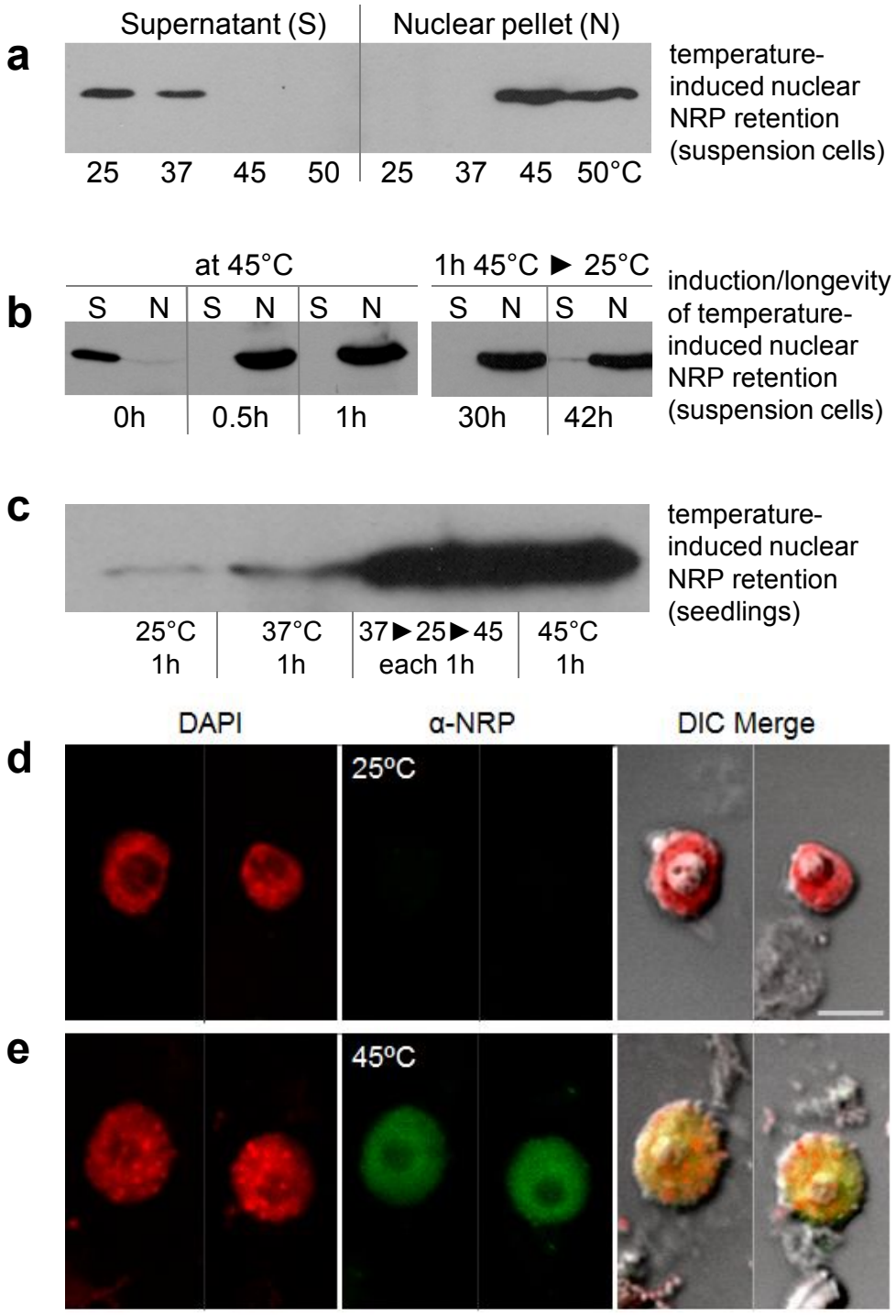

a
Supernatant (S)
Nuclear pellet (N)
25 37 45 50 25 37 45 50°C
temperature-induced nuclear NRP retention (suspension cells)
b
at 45°C
1h 45°C ► 25°C
S N S N S N S N S N
0h 0.5h 1h 30h 42h
induction/longevity of temperature-induced nuclear NRP retention (suspension cells)
c
25°C 1h
37°C 1h
37►25►45 each 1h
45°C 1h
temperature-induced nuclear NRP retention (seedlings)
d
DAPI
α-NRP
DIC Merge
25°C
e
45°C

Fig. 2

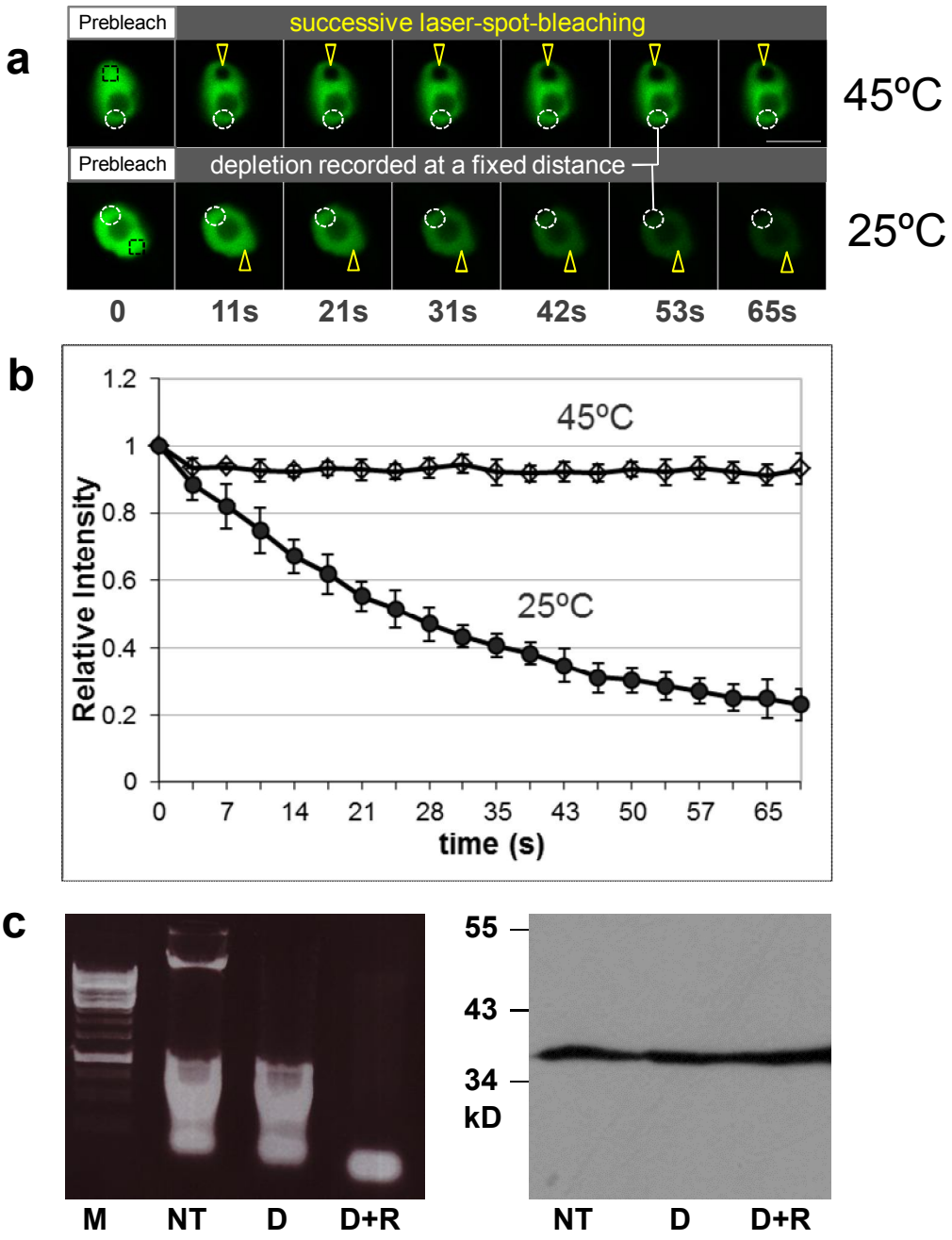
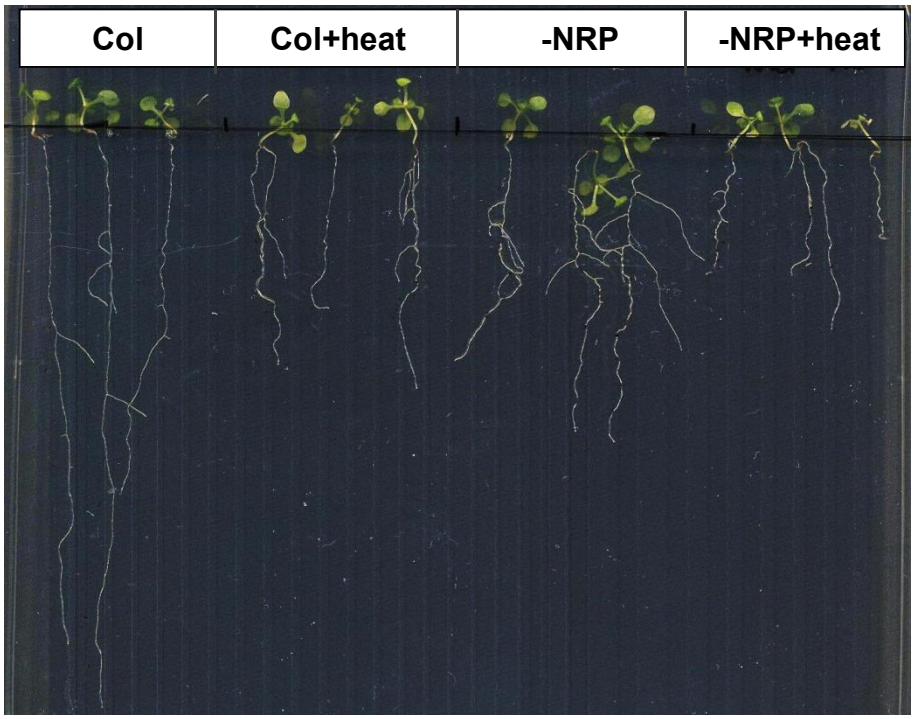

a
Prebleach
successive laser-spot-bleaching
45ºC
Prebleach
depletion recorded at a fixed distance
25ºC
0
11s
21s
31s
42s
53s
65s
b
Relative Intensity
1.2
1
0.8
0.6
0.4
0.2
0
45ºC
25ºC
0
7
14
21
28
35
43
50
57
65
time (s)
c
M
NT
D
D+R
55
43
34
kD
NT
D
D+R

Fig. 3

a


Col
Col+heat
-NRP
-NRP+heat

b

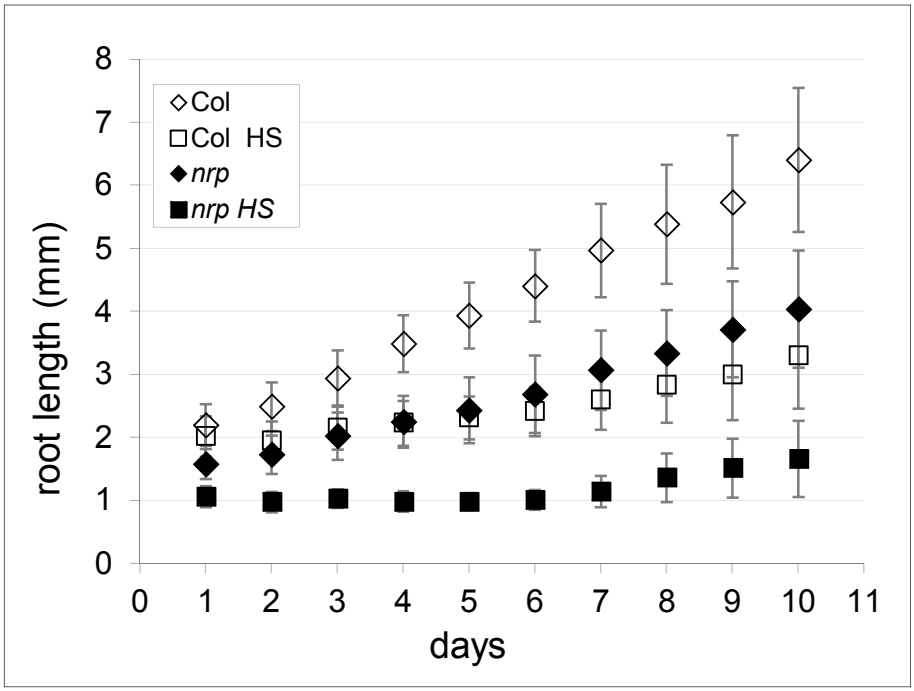

root length (mm)
days
Col
Col HS
nrp
nrp HS
0
1
2
3
4
5
6
7
8
9
10
11

Fig. 4

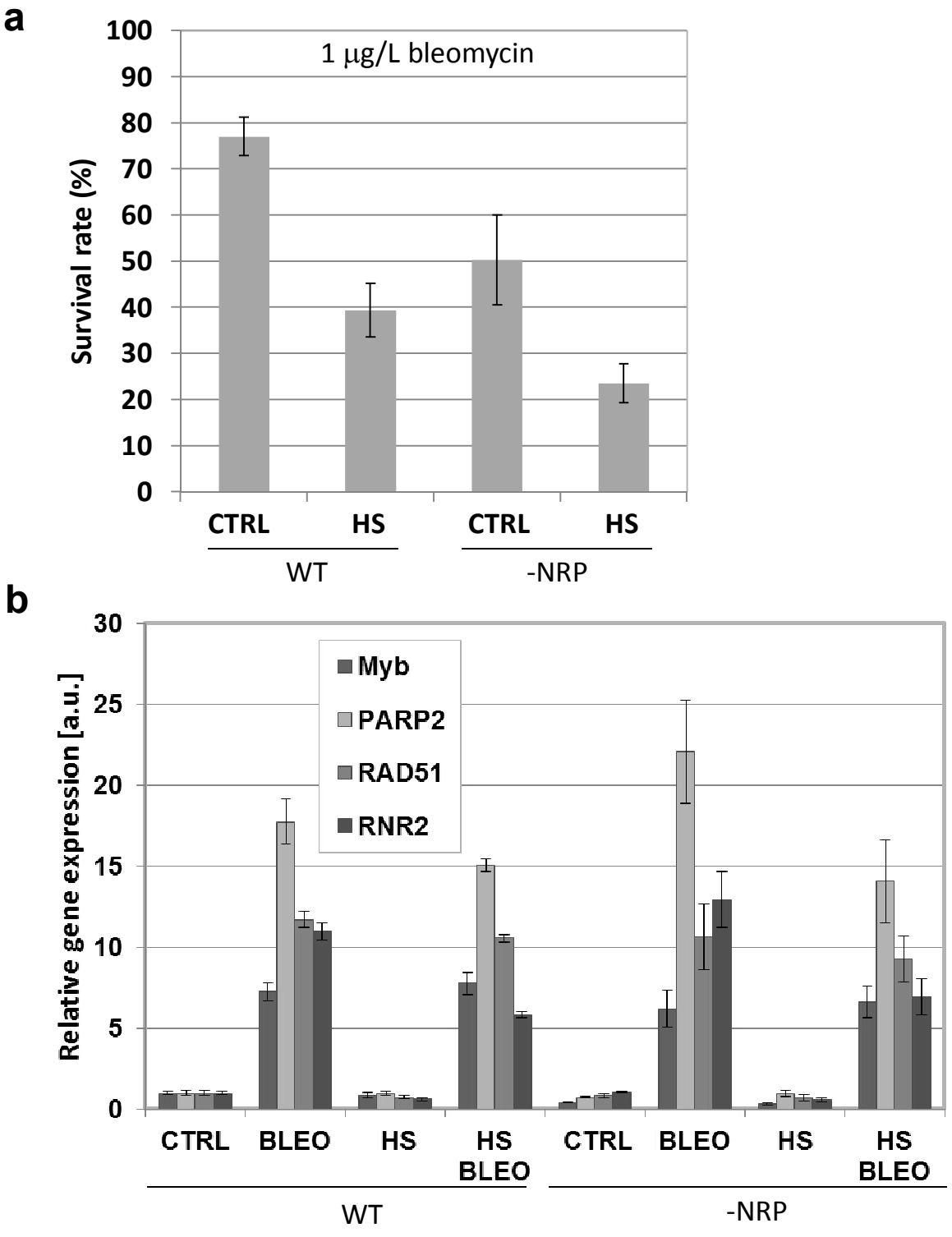
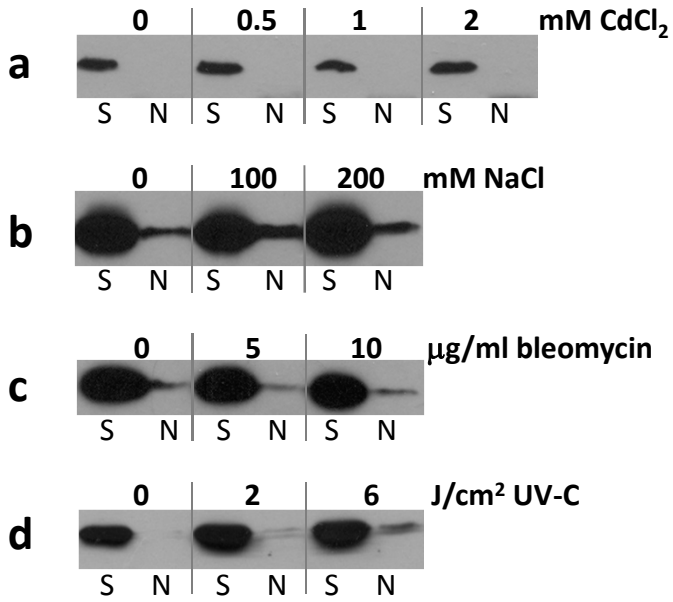

a
1 µg/L bleomycin
Survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
CTRL
HS
CTRL
HS
WT
-NRP
b
Relative gene expression [a.u.]
30
25
20
15
10
5
0
Myb
PARP2
RAD51
RNR2
CTRL
BLEO
HS
HS BLEO
CTRL
BLEO
HS
HS BLEO
WT
-NRP

Fig. 5



Supplementary Figure 1. NRP retainment in the nucleus is not a general stress response.
Heavy metal stress ($CdCl_2$, **a**), salt stress (NaCl, **b**) genotoxic stress (bleomycin, **c**) or UV-C irradiation (**d**) did not result in nuclear NRP retention in cultured Arabidopsis cells. N: nuclear pellet, S: supernatant

AGI code	Primer name	Sequence (5'-3')
At5g42990	UBC18 FW	ACAGCAATGGACATATTTGTTTAGA
	UBC18 REV	TGATGCAGACTGAACTCACTGTC
At4g02390	PARP2 FW	GCGAACTATTGCTATGCCAAC
	PARP2 REV	ATGTCTCCCAAAGCAACCTC
At5g20850	RAD51 FW	GATCACGGGAGCTCGATAAA
	RAD51 REV	GCGGAACTCACCATATAACTCTG
At3g27060	RNR2 FW	GAATCATCGCTTTCGCTTG
	RNR2 REV	TTCAGCCAGAAGATTGAACAAA
At5g03780	Myb FW	TGTGGCTAAGTCAAAGACAGTGA
	Myb REV	TCCACTCCCACCTTTAGCAT
At2g26760	ATCYCB1FW	CCTTAAGAGCTCGAAGCAAGG
	ATCYCB1REV	AGCATCCACAGCATCAATGT

Supplementary Table 1. Primers used in the study