

RESEARCH PAPER

Role of HSP101 in the stimulation of nodal root development from the coleoptilar node by light and temperature in maize (*Zea mays* L.) seedlings

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Abstract

Nodal roots (NRs) constitute the prevalent root system of adult maize plants. NRs emerge from stem nodes located below or above ground, and little is known about their inducing factors. Here, it is shown that precocious development of NRs at the coleoptilar node (NRCNs) occurred in maize seedlings when: (i) dark grown and stimulated by the concurrent action of a single light shock of low intensity white light ($2 \mu\text{mol m}^{-2} \text{s}^{-1}$) and a single heat shock; (ii) grown under a photoperiod of low intensity light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$); or (iii) grown in the dark under a thermoperiod ($28 \text{ }^\circ\text{C}/34 \text{ }^\circ\text{C}$). The light shock effects were synergistic with heat shock and with the photoperiod, whereas the thermoperiodical and photoperiodical effects were additive. Dissection of the primary root or the root cap, to mimic the fatal consequences of severe heat shock, caused negligible effects on NRCN formation, indicating that the shoot is directly involved in perception of the heat shock-inducible signal that triggered NRCN formation. A comparison between *hsp101-m5::Mu1/hsp101-m5::Mu1* and *Hsp101/Hsp101* seedlings indicated that the heat shock protein 101 (HSP101) chaperone inhibited NRCN formation in the light and in the dark. Stimulation of precocious NRCN formation by light and heat shocks was affected by genetic background and by the stage of seedling development. HSP101 protein levels increased in the coleoptilar node of induced wild-type plants, particularly in the procambial region, where NRCN formation originated. The adaptive relevance of development of NRCNs in response to these environmental cues and hypothetical mechanisms of regulation by HSP101 are discussed.

Key words: Adventitious root, coleoptile, crown root, heat shock, Hsp, mesocotyl, photoperiod, root cap, seminal root.

Introduction

Roots help plants to absorb minerals and water and provide a firm anchorage to the soil. The architecture of the root system is the result of both a developmental programme and a response to environmental signals (Hochholdinger *et al.*, 2004). According to their origin, during development, maize, like other herbaceous monocotyledons, displays two types of roots: the seminal roots, of embryonic origin, and the nodal roots that have a post-embryonic origin and arise from stem tissues (Kiesselbach, 1999). The primary and seminal roots are formed during embryo development and become visible after the second or third day of germination,

the latter emerging from the scutellar node. Stem or shoot-borne roots are formed a few weeks after germination and arise from both below-ground and above-ground nodes of the stem (Feldman, 1994). These roots are usually called adventitious or nodal. When they are below-ground they are also called crown roots, and when they are above-ground they are called brace roots. Thus, during the first few days after germination, the embryonic root system is functional and the most important. A few weeks after germination, the post-embryonic root system becomes the major root system of the plant. Throughout this work the

term nodal root (NR) is used when referring to the so-called ‘adventitious roots’ from grasses, whereas the term adventitious root (AR) is used when referring to such roots from *Arabidopsis* or other dicotyledonous plants. This preferred nomenclature is because in monocotyledons NRs are part of their normal development and are not strictly ‘adventitious’, whereas in dicotyledons ARs are the result of wounding or cutting of the stem.

Very little is known about the factors that trigger the initiation of NRs or ARs. Light, nutrients, temperature, and plant growth regulators influence NR and AR formation. In intact seedlings of *Arabidopsis* induced by exogenous application of sugars, light acts as an inhibitor of ARs, whereas in the dark, and in the presence of low concentrations of sucrose and other sugars in the medium, AR development is promoted (Takahashi *et al.*, 2003). In stem cuttings of *Arabidopsis* and other dicotyledonous plants, light can be both an inhibitor and a promoter of ARs according to the fluence rate (Eliasson, 1980; Konishi and Sugiyama, 2003). In rice, light enhances the growth of NRs (Shimizu *et al.*, 2009). Auxin and light control AR formation in *Arabidopsis* through the *ARGONAUTE1* gene (Sorin *et al.*, 2005) and the auxin response factor 17 (*ARF17*) (Sorin *et al.*, 2005). As in dicotyledons, in grasses plant growth regulators influence the emergence of NRs. Auxins such as indole acetic acid (IAA) and indole butyric acid (IBA) induce nodal root development (NRD) in maize (Ludwig-Müller, 2000; Hochholdinger and Zimmermann, 2008; McSteen, 2010), whereas in rice and maize ethylene triggers NRD at submerged nodes under flooded conditions (Drew *et al.*, 1979; Lorbiecke and Sauter, 1999).

The genetic analysis of NRD in grasses is still at a very early stage. Two mutants deficient in NR formation are known in maize. The *rtcs* mutant lacks all NRs and the embryonic seminal roots (Hetz *et al.*, 1996). The *rt1* mutant shows reduced NR formation (Jenkins, 1930). As a consequence, knowledge about the identity of genes associated with NRD in the grass family is very limited. Nonetheless, notorious similarities are found. The *crl1* and *ar11* genes from rice and the *rtcs* gene from maize are involved on NRD and encode transcription factors containing a LOB domain (Hochholdinger and Zimmermann, 2008). Their promoters contain auxin-responsive elements and their expression is in fact auxin inducible, indicating that these genes most probably perform early in the auxin signalling pathway. Thus, the involvement of plant growth regulators, nutrients, light, and LOB domain proteins on NRD suggests that a cross-talk exists between different signalling pathways and that, most probably, numerous genes participate in this developmental response.

As explained below it is shown in this work that HSP101, a heat shock protein of 101 kDa, regulates NRD in maize. Maize HSP101 is classified in the Hsp100/ClpB subfamily of molecular chaperones whose members are encoded by bacterial, fungal, protozoan, and plant genomes, and display the capacity to disaggregate and reactivate protein aggregates that accumulate during heat shock (Schirmer *et al.*, 1996; Nieto-Sotelo *et al.*, 1999; Agarwal *et al.*, 2002;

Lee *et al.*, 2006; Liberek *et al.*, 2008). In yeast, Hsp104, the Hsp100 homologue, forms a disaggregase machinery in conjunction with Hsp70 and Hsp40 (Glover and Lindquist, 1998; Liberek *et al.*, 2008). Hsp104 also catalyses the elimination and formation of autoreplicating prions such as Sup35 (Shorter and Lindquist, 2004). HSP100 proteins are essential for basal and induced thermotolerance in yeast, *Arabidopsis*, and maize (Sánchez and Lindquist, 1990; Hong and Vierling, 2001; Nieto-Sotelo *et al.*, 2002). It has also been suggested that HSP101 functions as a translational regulator under specific conditions (Ling *et al.*, 2001) and negatively influences primary root growth (Nieto-Sotelo *et al.*, 2002). The *Hsp101* transcript and HSP101 protein levels are enhanced under heat stress and during development (Young *et al.*, 2001; Nieto-Sotelo *et al.*, 2002). Dehydration or abscisic acid (ABA) treatment also induces *Hsp101* transcripts in wheat (Campbell *et al.*, 2001).

During comparative studies aimed to evaluate the response to heat shock in maize using the wild type (wt) and mutants in the *Hsp101* gene, differences in the development of NRs were observed. The results presented here showed that environmental signals such as light and heat shocks, thermoperiods, or photoperiods induced the formation of NRs in the coleoptilar node (CN) and that the emergence of NRs in response to these signals was inhibited by HSP101.

Materials and methods

Plant materials and growth conditions

The maize caryopses of the Merit line were from a commercial harvest (Merit N+ yellow *su1*., Asgrow Seed Company, Kalamazoo, MI, USA). The A63 cultivar was obtained from the Maize Genetics Cooperation Stock Center (University of Illinois, Urbana/Champaign, IL, USA). The L4 mutant (*hsp101-m2::Mu1 hsp101-m2::Mu1*), L4 wt (*Hsp101/Hsp101*), L10 mutant (*hsp101-m5::Mu1 hsp101-m5::Mu1*), and the L10 wt (*Hsp101/Hsp101*) inbred lines were the same as described by Nieto-Sotelo *et al.* (2002). The L4 wt, L4 mutant, L10 wt, L10 mutant, and A63 maize plants were cultivated during the autumn–winter season in Tapachula, Nayarit, Mexico, to avoid high temperatures during their growth cycle, thus minimizing any heat-related phenotypic effects. Daily temperatures at this site never exceeded 30 °C. Genotyping of L4 wt and L4 mutant lines was carried out by PCR using primers P17 (5′ CGGCACCTGC CTGACAAAGCCATAGAC 3′), P18 (5′ CCCGCCTTTTACTCC TCGTCCATGCC 3′), and 9242 (5′ AGAGAAGCCAACGC-CA[AT]CGCCTC[CT]ATTTCGTC 3′) as described (Nieto-Sotelo *et al.*, 2002). Genotyping of L10 wt and L10 mutant lines was done by PCR using primers 27238 (5′ AGGCCGAGGGGAA GGTCATTCTCT 3′), 27241 (5′ GCACGCTGCACGACTACTC-TACTAGGAGAC 3′), and 9242 as described (Nieto-Sotelo *et al.*, 2002).

Heat shock experiments

Maize (var. Merit) caryopses were germinated under aseptic conditions. Caryopses were selected at random and surface sterilized with a solution of household bleach (7% Clorox) for 10 min followed by three washes with sterile distilled water. Caryopses were placed equidistantly embryo side-down in enamel trays containing three layers of Whatman 3 MM Chr paper saturated with 0.1 mM CaCl₂ and covered with aluminium foil. Caryopses were incubated in growth chambers in the dark at 28 °C or in the light under a 12 h light/12 h dark photoperiod

[80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetic photon flow (400–700 nm)]. When the growth chamber lights were turned on, the light intensity in the interior of the germination trays, once covered with aluminium foil, was 0.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$. At the indicated times (36, 60, or 84 h after the start of imbibition) seedlings were transferred, in a sterile laminar flow hood, to sterile glass beakers containing 0.1 mM CaCl_2 . Beakers were maintained at 28 °C or exposed to a heat shock for 1 h, under the normal illumination of the laboratory (2 $\mu\text{mol m}^{-2} \text{s}^{-1}$), in a water bath with gentle shaking. After heat shock treatments, seedlings were returned to trays and the lengths of the primary root and shoot were recorded. Trays were wrapped in aluminium foil and incubated for recovery during 96 h at 28 °C in the dark or in the light, as described above. The percentage of seedlings showing different organs, their number, and final length were evaluated at the end of the fourth day of recovery. Only living organs were scored. Organs were considered dead when their tissues looked necrotic (i.e. displaying a brown-black colouration) and showed no further growth during the recovery period following the heat shock treatments. Only seedlings that developed NRs at the CNs (NRCNs) were considered to estimate the number of NRCNs per plant or their growth, removing from the calculation seedlings where NRCNs were absent. To compare the degree of damage among the different seedling organs after heat shock, the LT_{50} was estimated. LT_{50} is defined as the temperature at which 50% of the seedlings showed total loss of the corresponding organ. For a description of how LT_{50} was calculated, see 'Statistical analysis'. All experiments were repeated at least three times on different dates. For each time point or treatment, 10 seedlings were used.

Dissection of the primary root or the root cap

All manipulations were carried in a laminar flow hood under aseptic conditions. The primary root or the root cap was carefully removed by microsurgery with a scalpel and the aid of a dissecting microscope as described (Feldman, 1976). To evaluate if the dissection was successful, intact seedlings were incubated in parallel and their growth compared with that of the dissected seedlings. Growth of the shoot diminished, but continued after removal of the primary root or the root cap. Similarly, the primary root remained viable after removal of the root cap, although its final length was reduced. Once a new root cap was reformed from distal quiescent centre cells 72 h after dissection, growth was resumed (Barlow, 1974; Ponce *et al.*, 2000). Dissected seedlings whose shoot or primary root lost viability after microsurgery were excluded from the experiment.

Total protein extraction and quantification

Frozen tissues were ground with a mortar and pestle under liquid nitrogen. Powder plant material was resuspended in 2× Laemmli buffer (Laemmli, 1970) containing 2 mM phenylmethylsulphonyl fluoride (PMSF). Extracts were mixed in a thermomixer (Eppendorf) at 95 °C at 700 rpm for 10 min. At 2 min intervals extracts were agitated at full speed in a vortex apparatus for 10 s. Extracts were spun at 14 000 rpm at room temperature in an Eppendorf microfuge for 5 min. Supernatants were spun a second time for 30 min. Final supernatants were frozen under liquid nitrogen and stored at –20 °C. Total protein was estimated with a modified procedure of the Lowry method that was calibrated against bovine serum albumin (BSA) standards (Schleif and Wensink, 1981).

Protein immunoblotting

Separation of proteins was performed by SDS-PAGE on 10% polyacrylamide gels. Each lane was loaded with 20 μg of total protein. After electrophoresis, proteins were transferred to Hybond-C Extra membranes (Amersham Biosciences) and processed according to standard protocols (Gallagher *et al.*, 1993). Membranes were blocked and incubated with primary and

secondary antibodies as described (Luján *et al.*, 2009). Blots were developed with ECL reagent (Amersham cat. RPN2109) and exposed to an X-ray film (Kodak cat. 6040331). To detect HSP101, primary antibody HSP101-P15C (Nieto-Sotelo *et al.*, 2002) was used at a 1:250 dilution. To detect HSP70, anti-HSP70 primary antibody (Stressgen, cat. SPA-812) was used at a 1:2500 dilution. HSP90 was detected with anti-*Arabidopsis* HSP90 (at-115) antibody (Santa Cruz Biotechnology, Inc. cat. sc-33755) at a 1:100 dilution. A secondary antibody conjugated with horseradish peroxidase was used at a 1:5000 dilution.

Histochemistry and immunocytochemistry

Coleoptiles were allowed to imbibe for 16 h in distilled water at 28 °C in the dark and then fixed in 0.5% glutaraldehyde and 2.5% paraformaldehyde in buffer A (10 mM phosphate buffer, pH 7) overnight at 4 °C. The tissue was dehydrated in a graded ethanol/H₂O series followed by an ethanol/xylene series and a xylene/paraffin series, then embedded in paraffin blocks for sectioning, and 10 μm slices were mounted on Probe-on Plus slides (Fisher Scientific, Pittsburgh, PA, USA). After removal of paraffin, sections were rehydrated in an ethanol/H₂O series finishing with buffer A and then blocked at room temperature with 2.5% low-fat milk in buffer A and 150 mM NaCl (buffer B) for 1 h. Slides were washed with buffer A, 0.1% Tween-20, and 8 mg ml^{-1} BSA (buffer C) for 10 min and rinsed briefly with buffer B.

Thereafter, sectioned tissues were incubated overnight at 4 °C with anti-HSP101-P15C antibody at a dilution of 1:20 in buffer B. Sections then were washed twice for 10 min with buffer A and with 500 mM NaCl, 0.1% Tween-20, and 1 mg ml^{-1} BSA, and rinsed briefly with buffer B. Finally, slides were incubated with secondary goat anti-rabbit antibody conjugated with alkaline phosphatase (AP) at a dilution of 1:500 in buffer B for 1 h at 37 °C in a humidity chamber in the dark. Subsequently, sections were washed twice for 10 min with buffer C and with water for 15 min. Immunodetection was performed by adding the AP substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium in 100 mM TRIS-HCl buffer, pH 9.5, 100 mM NaCl, and 5 mM MgCl_2 . To reduce endogenous AP, 2.4 mg ml^{-1} levamisole was added to the AP reaction. The colour reaction was stopped with 10 mM TRIS-HCl and 1 mM EDTA buffer. Sections were mounted for microscopic observation. Controls without primary antibody, without secondary antibody, and without both primary and secondary antibodies were analysed.

Safranin O/Fast green staining was performed by immersing slides in 1% aqueous Safranin O for 2 min and washing several times until no more dye came out; specimens were then dehydrated in a graded ethanol/H₂O series. Sections were dipped for 30 s in 95% ethanol:Fast green (0.1% w/v), then washed twice for 2 min in 100% ethanol, and finally cleared in 100% xylene and mounted for microscopic observation.

Statistical analysis

Unless otherwise described, each experiment reported in this work represents the results obtained in at least three independent biological replications. Data were transferred to Microsoft Excel spreadsheets, and their mean value and standard deviation (SD) were calculated and plotted. Student's *t*-test (two sample, unpaired, with two tails) was used to evaluate if statistically significant differences existed between a given treatment and the control sample. For multiple comparisons, data were subjected to analysis of variance (ANOVA), and differences between the means compared by Tukey (one-way) or Bonferroni (two-way) post-tests. TL_{50} were calculated by non-linear regression analysis of the percentage of survival versus temperature plots. An inhibitory dose–response curve-fitting model was used to calculate the temperature for 50% survival. Treatments were considered as

statistically different from the control when $P \leq 0.05$. The software package used was Prism 5.0.

Results

Differences in heat tolerance in organs of embryonic origin during seedling development

The effect of heat shock on the viability of organs was studied in seedlings that were incubated in the dark at 28 °C, except that during the time of heat shock (36, 60, or 84 h after the start of caryopses imbibition), they were exposed to the light of the laboratory [‘light shock’ (LS), $2 \mu\text{mol m}^{-2} \text{s}^{-1}$]. This treatment is referred to as D-LS (darkness plus a single light shock). In seedlings of the Merit hybrid, LT_{50} values of the primary root, scutellar node, mesocotyl, and coleoptile were >49 °C when the heat shock was imposed at 36 h (Fig. 1). In contrast, seminal roots were slightly less tolerant, with an LT_{50} of 47.6 °C. Without exception, tolerance to heat shock decreased in all organs when given at 60 h or 84 h. The LT_{50} s of primary and seminal roots at 84 h were 45.7 °C and 45.5 °C, respectively, both significantly lower than those observed in the scutellar node, mesocotyl, and coleoptile, that remained at ~ 47 °C. In the L10 wt inbred line the LT_{50} values at 36 h in all organs were lower than in Merit, ranging from 47.0 °C to 47.8 °C. In contrast to Merit, no statistically significant differences were found between organs (Fig. 1 and data not shown). At 60 h the primary and seminal roots in the L10 wt inbred were significantly more sensitive to heat shock (LT_{50} of 44.9 °C and 44.7 °C, respectively) than shoot organs (LT_{50} s from 46.3 °C to 47.8 °C). As in Merit, all organs in L10 wt showed their lowest heat tolerance at 84 h, but no differences in sensitivity were

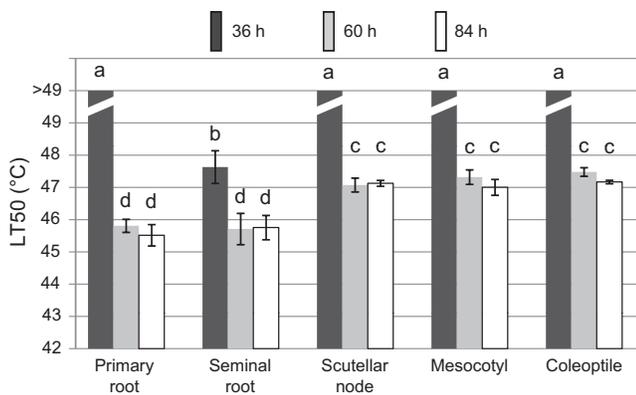


Fig. 1. Survival of different organs of a maize seedling after heat shock. Maize seedlings (Merit) were grown in the dark at 28 °C and heat shocked in the light ($2 \mu\text{mol m}^{-2} \text{s}^{-1}$) as described in the Materials and methods (D-LS treatment). Seedlings were exposed to the heat and light shocks at 36, 60, or 84 h after kernel imbibition as indicated. Heat tolerance after 4 d of recovery in the dark at 28 °C was evaluated as the LT_{50} as described in the Materials and methods. Data represent average \pm SD values. Bars with different letters differ significantly ($P < 0.05$).

found between them at this time point (data not shown). In the A63 inbred, the primary and seminal roots were also more sensitive to heat shock than shoot organs when heat shocked at 60 h (see Supplementary Fig. S1A available at *JXB* online). Thus, organ sensitivity to heat shock was dependent on genotype, although the primary and seminal roots lost thermotolerance more rapidly than shoot organs during development of the maize seedling.

Emergence of NRs in maize seedlings in response to single light and heat shocks

NRCN development was enhanced by heat shock in Merit, A63, and L10 wt seedlings grown under D-LS conditions. As seen in Fig. 2, the percentage of seedlings that developed NRCNs at 28 °C was negligible at all time points in Merit

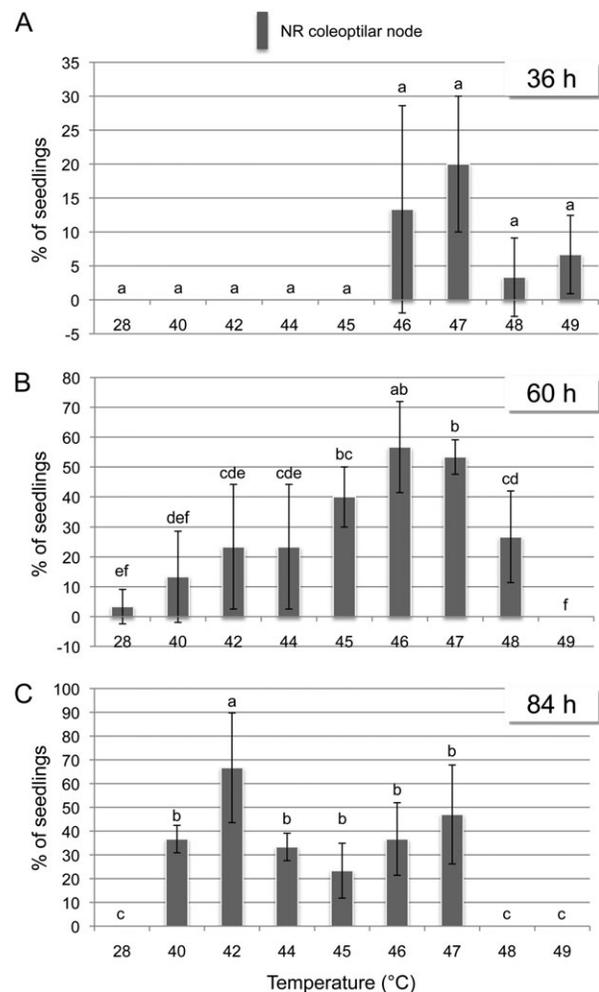


Fig. 2. Production of NRCNs in seedlings exposed to heat and light shocks. Maize seedlings (Merit) were grown in the dark at 28 °C and heat shocked in the light ($2 \mu\text{mol m}^{-2} \text{s}^{-1}$) as described in the Materials and methods (D-LS treatment). Seedlings were exposed to the heat and light shocks at 36 h (A), 60 h (B), or 84 h (C) after kernel imbibition. Data represent average \pm SD values of the percentage of seedlings showing NRCNs 4 d after temperature and light treatments. Bars that do not share at least a common letter differ significantly ($P < 0.05$).

(36, 60, or 84 h). Similarly, the percentage of A63 seedlings producing NRCNs was very low (3%) at 60 h if kept at 28 °C (see Supplementary Fig. S1B at *JXB* online). In contrast, seedlings of the L10 wt inbred triggered NRCN formation at 28 °C when 'light shocked' at 60 h and 84 h since $7\pm 11\%$ and $43\pm 29\%$ of the seedlings contained NRCNs, respectively, whereas, at 36 h, the percentage was zero when treatments were carried out at 28 °C (see Supplementary Fig. S2). At 36 h, the highest percentage of seedlings forming NRCNs in Merit was after 47 °C shocks, while in L10 wt the highest percentage was following shocks at 45 °C (Fig. 2A; see Supplementary Fig. S2A). At 60 h, the percentage of seedlings with NRCNs peaked at 46 °C and 44 °C in Merit and A63, respectively, whereas in L10 wt the response was bimodal with a peak at 42 °C and a second peak at 46 °C (Fig. 2B; see Supplementary Figs S1B, S2B). At 84 h, the response in Merit was bimodal, with a first peak at 42 °C and a second at 47 °C (see images in Fig. 7A, B), whereas in L10 wt maximal production was at 42 °C (Fig. 2C; see Supplementary Fig. S2C). Clearly, there was a tendency to show a maximal response at lower temperatures when seedlings were exposed to heat and light shock treatments at late stages of development.

Number and length of NRCNs in heat-shocked seedlings

The number and final length of NRCNs in D-LS-treated Merit seedlings were nearly similar at all temperatures at the 36, 60, or 84 h time points. The only exceptions were observed at 60 h, when the number of NRCNs was higher at 47 °C and the final length larger at 46 °C. At 84 h, both the number of NRCNs and their final length were higher after a 42 °C shock (see Supplementary Fig. S3C at *JXB* online; and data not shown). In the L10 wt inbred, the number of NRCNs per seedling was similar at all temperatures at 36 h and maximal after heat shocks at 42 °C at 60 h and 84 h (see Supplementary Fig. S4). The final length of NRCNs in L10 wt seedlings was similar at all temperatures at 36 h and 60 h, whereas at 84 h it was greater in response to shocks at 42 °C (data not shown).

The role of the root cap and the primary root in the induction of NRCNs

A considerable percentage of seedlings showing NRCNs in response to heat shock showed necrotic primary roots. To assess whether a possible connection existed between the integrity of the primary root and the formation of NRCNs, surgical experiments were carried out to remove the whole primary root. Moreover, because the root cap is known to be the site of perception of a multitude of stimuli that trigger plant tropisms (Cassab, 2008), the root cap was removed to assess its role in the perception of light and/or high temperature during NRCN induction. Because the primary root stops growing if care is not taken to avoid the excision of the root apical meristem or the quiescent centre after this operation (Feldman, 1976; Ponce et al.,

2000), seedling growth was evaluated. The percentage of seedlings with NRCNs and their final length were not affected after the root cap or the whole primary root was excised (Fig. 3A; and data not shown). On the other hand, removal of the root cap increased the number of NRCNs ($P < 0.01$) from 1.8 ± 0.86 in the control, to 2.95 ± 1.0 after a shock at 46 °C. However, removal of the whole primary root had no effect (Fig. 3B). So, the loss of the primary root or the root cap was not the primary trigger of the emergence of NRCNs.

Effect of light on the emergence of NRs

Because the emergence of NRCNs in seedlings grown under D-LS treatments was affected by a single light shock, seedlings were additionally exposed to a daily photoperiod to evaluate the combined effects of these two light treatments and temperature. The light fluence during the photoperiod was $0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$. A daily photoperiod

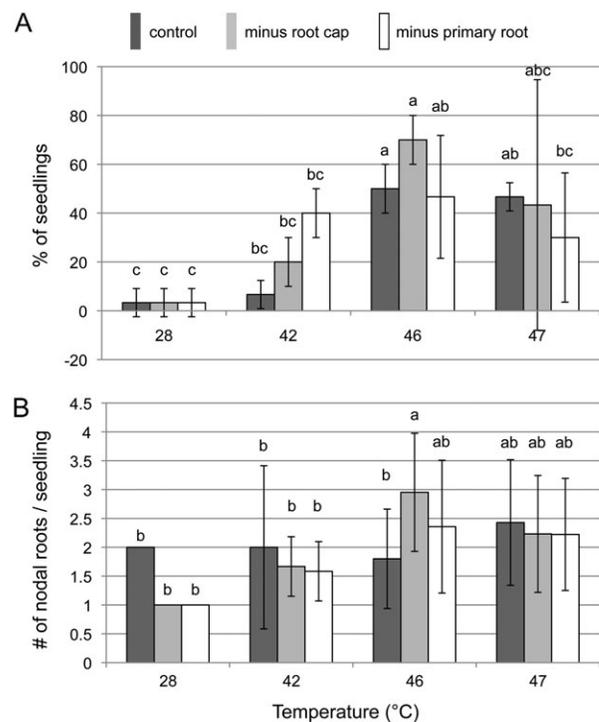


Fig. 3. Effect of dissection of the whole primary root or the root cap on the emergence of NRCNs in control and in heat-shocked seedlings. Maize seedlings (Merit) were grown in the dark at 28 °C. After 60 h of imbibition of kernels, the whole primary root or the root cap were surgically removed or left intact in the controls as described in the Materials and methods. Thereafter, seedlings were exposed to the indicated temperatures for 1 h and returned to the dark for 4 d at 28 °C before evaluation (D-LS treatment). During dissection and temperature treatments seedlings were handled under standard laboratory illumination ($2 \mu\text{mol m}^{-2} \text{s}^{-1}$). Data shown in the graphs are the average \pm SD values of the percentage of seedlings showing NRCNs (A) and the number of NRCNs per seedling (B). Bars that do not share at least a common letter differ significantly ($P < 0.05$).

supplemented by a single light shock (P-LS) triggered NRCNs emergence at all temperatures in Merit seedlings, when treatments were made at 36 h. At every temperature tested, all differences were significant relative to dark-grown seedlings receiving only a single light shock (D-LS) (Fig. 4A). At 60 h, the percentage of seedlings with NRCNs was significantly higher in the P-LS than in the D-LS group

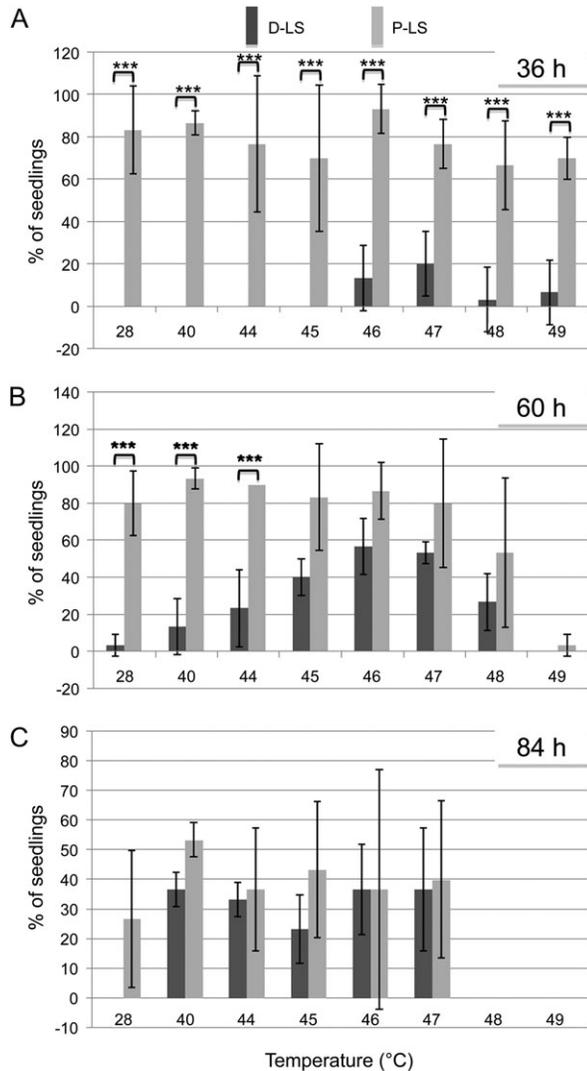


Fig. 4. Effect of a daily photoperiod and/or a single heat shock on the production of NRCNs. Maize caryopses (Merit) were germinated and grown at 28 °C in the dark (D-LS) or under a daily photoperiod of 12 h light:12 h dark at a light intensity of $0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ (P-LS) as described in the Materials and methods. At 36 h (A), 60 h (B), or 84 h (C) after imbibition, all seedlings were transferred to the light of the laboratory ($2 \mu\text{mol m}^{-2} \text{s}^{-1}$) and exposed to a heat shock or left at 28 °C for 1 h. After temperature treatments seedlings were returned to their initial incubation conditions for recovery during 4 d. Graphs show the average \pm SD of the percentage of seedlings with NRCNs observed at the end of the experiment. Asterisks on top of a given pair of bars indicate a statistically significant difference between the D-LS and the P-LS treatment: *** $P < 0.001$.

at 28, 40, and 44 °C (Fig. 4B). At 84 h, the percentage of seedlings with NRCNs was similar in P-LS and D-LS samples (Fig. 4C). The percentage of P-LS seedlings displaying NRCNs decreased as temperature and light shock treatments were made late in development (Fig. 4A–C). P-LS treatments influenced NRCN emergence in L10 wt seedlings with a different developmental pattern. At 36 h, no significant differences between the percentage of seedlings with NRCNs between the P-LS and D-LS groups were found at any temperature (see Supplementary Fig. S5A at *JXB* online). In contrast, at 60 h, the percentage was higher in the P-LS group following 28, 40, or 45 °C treatments, whereas at 48 °C the opposite was found (see Supplementary Fig. S5B). In Merit, the number of NRCNs per seedling and their final length in the P-LS and D-LS group were similar at 36 h or 84 h (see Supplementary Fig. S6A, C). However, the number of NRCNs per seedling was higher in the P-LS group after shocks at 45, 46, and 47 °C at 60 h (see Supplementary Fig. S6B). The final length of NRCNs was also greater in P-LS-treated Merit seedlings only after 40 °C and 45 °C shocks at 60 h (data not shown). In L10 wt seedlings, P-LS treatments had no effect on the number of NRCNs per seedling when treatments were performed at 36 h (see Supplementary Fig. S7A). However, at 60 h, the number of NRCNs per seedling was higher after 40 °C and 45 °C shocks in P-LS seedlings (see Supplementary Fig. S7B). The final length of NRCNs was greater in P-LS seedlings after 46 °C shocks at 36 h and after 40 °C and 44 °C shocks at 60 h (data not shown). The previous results showed that the emergence, number, and final length of NRCNs in response to P-LS treatments were developmentally regulated, as seedlings showed differences in their sensitivity to the combined heat and light shock (H-LS) treatments during their development. Merit seedlings were more sensitive when H-LS treatments were given at earlier (36 h) than at later (84 h) time points. In contrast, L10 wt seedlings were more sensitive at later than at earlier time points. Thus, the genetic background seemed to influence the sensitivity of photoperiodically grown seedlings to H-LS.

To separate the light effect from the temperature effect on NRCN induction, Merit seedlings were grown under complete darkness for 6.5 d at 28 °C (D) or at 28 °C in complete darkness plus a 46 °C shock treatment in the dark at 60 h (D-HS). The percentage of seedlings with NRCNs grown under D was $0 \pm 0\%$, while the percentage after D-HS was $6 \pm 1\%$, which is lower than in D-LS seedlings shocked at 46 °C at 60 h ($57 \pm 15\%$, see Fig. 2B), and similar to seedlings grown under D-LS at 28 °C ($3.3 \pm 5.8\%$, see Fig. 2B). When seedlings were grown without interruptions under a photoperiod (P), at 28 °C for 6.5 d, $38 \pm 20\%$ of seedlings showed NRCNs, a smaller percentage compared with seedlings grown at 28 °C under P-LS at 60 h ($89 \pm 1.4\%$). In the previous experiments, heat was transferred from the incubator ballasts to the trays containing the seedlings when lamps were turned on, increasing the tray temperature to 34 °C. So a thermoperiod was unintentionally provoked with nights at 28 °C and days at

34 °C. Therefore, the distance between trays and ballasts was increased to reduce the temperature effect during illumination. The percentage of Merit seedlings showing NRCNs, that were grown at 28 °C under P-LS at 60 h, decreased from 77±8% when the tray temperature during illumination was 34 °C to 51±2% when the tray temperature during illumination was 28 °C (see Supplementary Fig. S8 at *JXB* online). To evaluate the effect of a thermoperiod in the absence of the light stimulus, Merit seedlings were grown either under a thermoperiod of 28 °C/34 °C during 6.5 d in complete darkness (D-28/34), in complete darkness at 28 °C (D-28), or in complete darkness at 34 °C (D-34). NRCNs were observed in 76±7% of seedlings in the D-28/34 group. Meanwhile, 2.5±5% of seedlings developed NRCNs in the D-34 group and 0% of seedlings in the D-28 groups. Together, the previous experiments demonstrated that photoperiods, or thermoperiods given in the dark, separately induced the emergence of NRCNs in maize seedlings and, when combined, their effects were additive. Moreover, synergistic effects were observed when light shock and heat shock acted simultaneously as well as when photoperiod and light shock at 28 °C were combined.

Role of HSP101 in the emergence of NRCNs

To evaluate the role of HSP101 on NRCN emergence, homozygous *hsp101-m5::Mu1* (L10 mutant) seedlings were compared with near-isogenic homozygous *Hsp101* (L10 wt) seedlings. Under D-LS, the percentage of seedlings with NRCNs was significantly higher in the L10 mutant than in the L10 wt group at 28, 42, 44, and 45 °C at 36 h (Fig. 5A). This occurred in spite of L10 mutant coleoptiles having a lower LT_{50} (45.5±0.1 °C versus 47.8±0.7 °C for L10 wt coleoptiles). The highest percentage of L10 mutant seedlings producing NRCNs was 90±10% at 44 °C, whereas under similar conditions it was 3±6% in the L10 wt (Fig. 5A). At 60 h, the percentage of seedlings with NRCNs at 28 °C was higher in L10 mutants than in L10 wt, but lower in the mutants than in L10 wt at 42 °C, whereas at other temperatures values were similar in L10 mutant and L10 wt lines. Following shocks at ≥46 °C, L10 mutants no longer produced NRCNs because the viability of the coleoptile was completely lost (LT_{50} =44.8±0.3 °C versus 48.4±0.5 °C for L10 wt coleoptiles). This observation agreed with the known higher sensitivity of *hsp101* maize mutants to heat shock (Nieto-Sotelo et al., 2002). At 84 h, the percentage of seedlings with NRCNs in L10 mutant and L10 wt lines was similar at all temperatures tested. This effect was due mostly to the increase in NRCN formation in L10 wt seedlings at lower temperature, as discussed earlier (see Supplementary Fig. S2B at *JXB* online). Accordingly, at 84 h the LT_{50} s of mutant and wt coleoptiles were similar (44.4±0.7 °C and 44.9±0.6 °C, respectively).

The number of NRCNs per seedling was higher in the L10 mutant than in L10 wt after 45 °C at 36 h (see Supplementary Fig. S4A at *JXB* online). At 60 h, the number of NRCNs per plant after 40, 44, and 45 °C shocks was also higher in the L10 mutant (see Supplementary

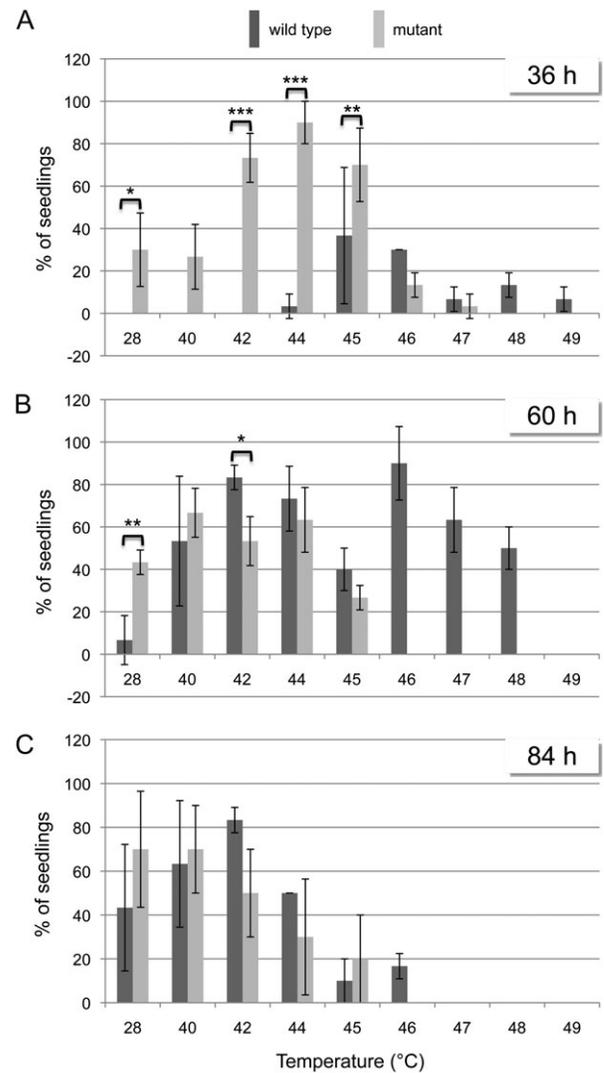


Fig. 5. HSP101 inhibits the emergence of NRCNs in seedlings exposed to heat and light shocks. L10 wt (*HSP101/HSP101*) and L10 mutant (*hsp101-m5::Mu1/hsp101-m5::Mu1*) seedlings were grown in the dark at 28 °C and heat shocked in the light ($2 \mu\text{mol m}^{-2} \text{s}^{-1}$) as described in the Materials and methods (D-LS treatment). Seedlings were exposed to the heat and light shocks at 36 h (A), 60 h (B), or 84 h (C) after kernel imbibition. Data represent average ±SD values of the percentage of seedlings showing NRCNs after 4 d of recovery at 28 °C in the dark following the heat and light shocks. Asterisks on top of a given pair of bars indicate a statistically significant difference between the wt and the mutant: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. S4B). At 84 h, L10 mutants showed more NRCNs at 28 °C but fewer NRCNs than L10 wt after 42 °C shocks (see Supplementary Fig. S4C). No differences in the final length of NRCNs were observed at 36 h between L10 mutant and L10 wt plants (data not shown). However, at 60 h significant differences in final length were observed after 40 °C (larger in L10 mutant) and after 42 °C (larger in L10 wt) (data not shown). At 84 h, the final length of NRCNs was larger in L10 wt after 42 °C shocks (data not shown). Thus, at certain developmental stages and

when seedlings were grown under D-LS, the combination of light and heat shock treatments phenocopied the *hsp101-m5::Mu1* mutation in terms of NRCN induction.

When seedlings were incubated under P-LS, the percentage of seedlings with NRCNs was higher in the L10 mutant than in the L10 wt group at 28, 40, and 44 °C at 36 h (see Supplementary Fig. S9A at *JXB* online). At 60 h, no differences were observed between mutant and wt plants other than a lower percentage of seedlings with NRCNs in the L10 mutant at 46 °C (see Supplementary Fig. S9B). These differences reflected the higher sensitivity to heat shock of L10 mutant relative to L10 wt seedlings when grown under P-LS (see Supplementary Fig. S10) or D-LS (see the results mentioned above) conditions. The number of NRCNs per seedling was also higher in the L10 mutant line at 36 h in response to 44 °C and 45 °C shocks (see Supplementary Fig. S11A). At 60 h, the L10 mutant also showed an increased number of NRCNs at 28 °C and after 44 °C shocks (see Supplementary Fig. S11B). No differences in the final length of NRCNs were observed at 36 h between L10 mutant and wt seedlings (data not shown). At 60 h, only a minor increase in final length was observed after 40 °C in the L10 mutant line (data not shown). The percentage of seedlings with NRCNs in the L10 mutant line was higher under P-LS than under D-LS treatment after exposure to 28 °C and 40 °C, at 36 h, and after exposure to 28 °C, at 60 h (see Supplementary Fig. S12). The number of NRCNs per seedling was higher in P-LS only at 60 h after 28 °C and 44 °C shocks (see Supplementary Fig. S13). The final length of NRCNs was also enhanced by the P-LS treatment at 84 h after a 40 °C heat shock but was not different from D-LS treatments at 36 h (data not shown).

Likewise, the percentage of seedlings with NRCNs was higher in the L10 mutant than in the L10 wt when grown under P at 28 °C, when grown under D-46 (complete darkness and heat shocked at 46 °C at the 60 h time point), and when grown in the dark under a D-28/34 thermoperiod (data not shown). Finally, when seedlings were grown in complete darkness at 28 °C (D-28) for 6.5 d, NRCNs were observed in 15±6% of L10 mutant seedlings in contrast to 0% in L10 wt seedlings. Hence, light induced the emergence of NRCNs in both *Hsp101* and *hsp101* plants. The higher levels of NRCNs in *hsp101* plants suggested that HSP101 antagonized the light stimuli that promote NRCN induction and strongly repressed NRCN induction under complete darkness.

To test further the involvement of HSP101 on NRCN formation, an independent mutant allele of *Hsp101* was tested. Similar to results obtained by using L10 mutant and L10 wt lines, L4 mutant seedlings (*hsp101-m2::Mu1/hsp101-m2::Mu1*) displayed a higher percentage of NRCN than L4 wt (*Hsp101/Hsp101*) when grown under P-LS conditions or under a thermoperiod (28 °C/34 °C) in the dark (data not shown).

Levels of HSP101 protein in dark- and photoperiod-grown seedlings

Because the percentage of seedlings with NRCNs in *hsp101* lines was higher under D-LS and P-LS conditions the

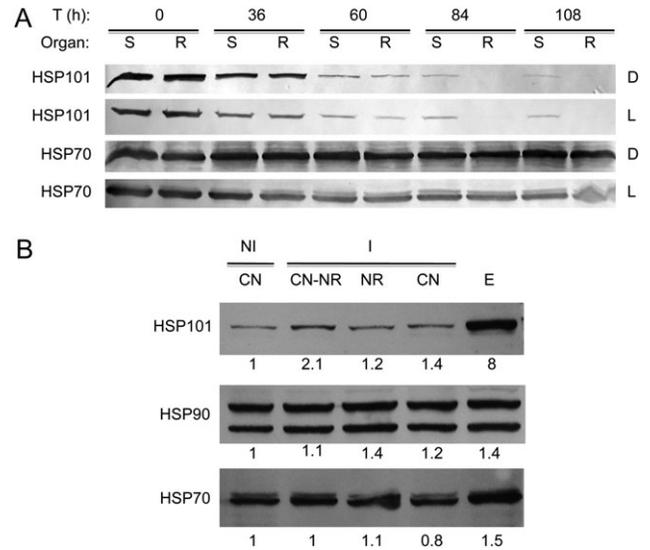


Fig. 6. Protein levels of HSPs during germination and growth of maize seedlings grown in the dark or in the light. (A) Maize caryopses (Merit) were germinated and grown at 28 °C in the dark (D) or under a daily photoperiod of 12 h light:12 h dark at a light intensity of 0.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (L) as described in the Materials and methods. At the indicated times after imbibition seedlings were transferred to the light of the laboratory (2 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and the primary root and shoot tissues were dissected and frozen. (B) Maize caryopses (Merit) were grown in the dark at 28 °C for 5.5 d (NI) or at 28 °C under a daily photoperiod of 12 h light:12 h dark (0.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 5.5 d with a light shock of 2 h (2 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 60 h after imbibition (I). Tissues around the coleoptilar node were dissected and frozen. Equal amounts of total protein were separated by SDS-PAGE and levels of HSP101, HSP90, and HSP70 proteins evaluated by western blotting as described in the Materials and methods. S, shoot; R, primary root; CN, coleoptilar node; NR, nodal root from the coleoptilar node; CN-NR, coleoptilar node after nodal roots were removed; E, positive control extract from mature embryos.

hypothesis that light may reduce the levels of HSP101 protein in wt seedlings was tested. HSP101 levels decreased constantly during the first 108 h after the start of imbibition under both D and P conditions (Fig. 6A). HSP101 protein levels decreased faster in the primary root than in the shoot, becoming undetectable in roots at 84 h, while levels in the shoot remained above background. No differences in HSP101 levels were observed between D and P seedlings, suggesting that an overall drop of HSP101 protein levels in the shoot is not the signal for NRCN induction. These results were in agreement with the observed decrease in basal thermotolerance as seedlings aged (Nieto-Sotelo *et al.*, 2002). In contrast to HSP101, no differences were observed in HSP70 levels between root and shoot, across all time points, either in D or in P conditions, although HSP70 levels decreased at late time points under P, while they remained constant under D conditions.

To assess whether changes in HSP101 levels could be more localized, CNs were dissected from non-induced (D)

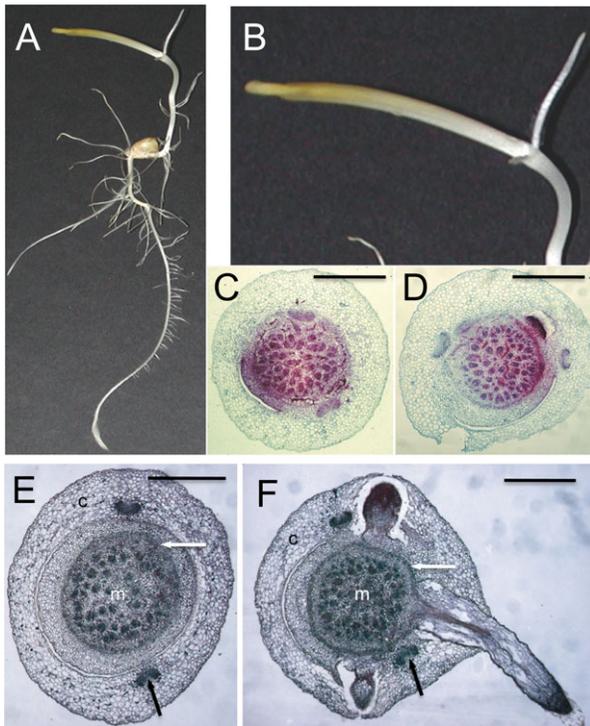


Fig. 7. Macroscopic, histochemical, and immunochemical analyses of NRCN formation. Germination and growth of maize caryopses (Merit) in (A) and (B) were under D-LS conditions with a heat shock of 40 °C at 84 h, as described in the Materials and methods. (A) Typical aspect of a 6.5-day-old seedling after growth under D-LS conditions and heat shock treatment. (B) Detail of the seedling in (A) showing two nodal roots protruding from the coleoptilar node. Germination and growth of maize caryopses in (C), (D), (E), and (F) was similar to the experiments described in Fig. 6B. At the end of the experiments, segments of tissue around the coleoptilar node were dissected for histochemical (C, D) or immunochemical (E, F) analysis as described in the Materials and methods. Images show transverse sections from: (C) a typical non-induced seedling lacking NR primordia in the region just above the coleoptilar node; (D) an induced seedling displaying one root primordium in the region just above the coleoptilar node; (E) a non-induced seedling showing the distribution of HSP101 and lacking any signs of NR primordia in the region just above the coleoptilar node; (F) an induced seedling showing the distribution of HSP101 and displaying two NR primordia and one NR protruding from the coleoptile in the region just above the coleoptilar node. Notice that the entire NR that is protruding from the coleoptile is in the same plane of the tissue section allowing the appreciation of its procambial origin. Black arrows point to one of the vascular bundles of the coleoptile; white arrows show procambium of the second internode; c, coleoptile; m, medullar region. The scale bar in (C), (D), (E), and (F) represents 1 mm. For a more complete description of the tissue sections, see text in the Results section.

and from induced (P-LS) seedlings, and levels of HSP101, HSP90, and HSP70 were determined at day 5.5, when NRCNs began to emerge from the coleoptile of P-LS plants. HSP101 and HSP90 levels increased slightly in P-LS

relative to D seedlings (Fig. 6B), whereas HSP70 levels decreased slightly. Because the CNs of the P-LS seedlings contained visible NRs, and HSP101 is known to be quite highly expressed in maize primary root tips (Young et al., 2001), NRs were removed from the CNs and extracts were made separately from the NRs and from the coleoptilar tissue that remained after NR removal (CN-NR). Levels of HSP101 were even higher in CN-NR samples than in NR or CN samples, and all three contained more HSP101 than non-induced CNs. HSP90 levels were higher in induced NR samples than in CN-NR and CN samples, the levels of CN-NR being similar between induced and non-induced CNs. HSP70 levels in CN-NRs were similar between induced and non-induced seedlings.

Because HSP101 levels were not reduced under P-LS conditions, an immunocytochemical analysis of HSP101 was made to look at its distribution at the cellular level. Under the experimental growth conditions, no NRCN primordia were observed in non-induced CNs (D conditions) as these were observed only under P-LS growth (Fig. 7C–F). Consistent with the immunoblot analyses of HSP101, the immunocytochemical detection of HSP101 in transverse sections just above the CN [second internode, according to Avery (1930)] of D and P-LS seedlings showed strong staining in the medullar region containing large numbers of poorly defined bundles, as well as in the pair of vascular bundles of the coleoptile, the external ring in this tissue section (Fig. 7E, F). However, the procambium of the second internode, from which NRCNs emerged (Martin and Harris, 1976), showed a stronger HSP101 signal in P-LS seedlings. This strong signal continued through the epidermis and root cap of the emerging NRCNs. The parenchymatous tissue of the coleoptile and the second internode in D and P-LS seedlings stained with less intensity for HSP101. The above results indicated that HSP101 levels did not decay in general in the CN and, specifically, in the procambium of the second internode under the conditions that triggered the emergence of NRCNs. These results were inconsistent with the precocious emergence of NRCNs under D-LS and D conditions in L10 mutants that lack HSP101.

Discussion

When young maize seedlings were grown under photoperiodic conditions, or under thermoperiodic conditions in the dark, NRCNs appeared precociously, whereas when both signals were provided simultaneously, the production of NRCNs was enhanced in an additive manner. In contrast, the effect of a single heat or light shock was not sufficient to promote NRCNs, but their concurrent action was synergistic since it caused a strong induction of NRCNs. Likewise, a single light shock supplemented with a daily photoperiod at 28 °C acted synergistically in the production of NRCNs (for examples see Figs. 2, 4, 5; and Supplementary Figs. S1B, S2, S5, S8, S9 at *JXB* online). Under D-LS conditions NRCN induction by heat shock showed a bimodal pattern

at certain time points (Fig. 2B; see Supplementary Figs S1B, S2B). The meaning of the bimodal response is currently unknown, but it could be a manifestation of two elements within the NRCN pathway whose optimal temperature for action is different, or two allelic states of the same component segregating within the populations under study. How is it that NRCN production by these physical signals, given under an artificial laboratory set-up, relates to plant biology? In nature, soil conditions vary in terms of temperature, moisture, pCO₂, pO₂, and nutrients influencing root growth, development, and action (Cooper, 1973; Zobel, 1996). Interestingly, NRCN emergence was promoted by thermoperiods of 28 °C and 34 °C in constant darkness (night/day) and not by either of the two temperatures if they were kept constant. The adaptive importance of heat shock and thermoperiods on root growth and development can be interpreted in terms of the well known variation of soil temperature, particularly in the upper 1 cm layer that can show daily variations of >20 °C (Nielsen, 1974). Whether this developmental response increases plant fitness under field conditions remains to be studied. It can be proposed that a plant's ability to sense soil temperature fluctuations during early growth represents an additional advantage to its capacity to sense light to ensure a successful establishment. NRCN formation in maize seedlings was quite sensitive to the low light intensities used during the light shocks (2 μmol m⁻² s⁻¹) or the photoperiod (0.1 μmol m⁻² s⁻¹) treatments. These levels represent <0.002 and 0.0001, respectively, of the PAR intensity [photosynthetically active radiation (between 400 nm and 700 nm)] received from the sun under a clear day at noon.

The dissection of the root cap or the whole primary root had no effect on the percentage of seedlings with NRCNs, or the number or final length of NRCNs in seedlings incubated at optimal temperature or after a single heat shock (Fig. 3). These results prove that the root cap or the primary root play no inhibitory role in the development of NRCNs and the signal that triggers the initiation of the NRCN primordia must be perceived and translated by the shoot itself.

How are light and temperature perceived under field growth conditions by the second node which normally is underneath the soil surface? In etiolated young seedlings of oat and maize, the highest concentrations of phytochrome are found at the tip of the coleoptile, the CN, and the differentiating mesocotyl just below the node (Pratt and Coleman, 1971; Schwarz and Schneider, 1987). Within the CN of oat seedlings, phytochrome is more abundant in the epidermal cells and in a cell layer in a position corresponding to the procambium (Pratt and Coleman, 1971). Moreover, maize and other herbaceous plants conduct light captured by the aerial parts of the stem and direct it toward the roots using fibres and vessels as axial light conductors. This light is mostly enriched in the far-red and near infrared spectral region (Sun *et al.*, 2005). Further, maize coleoptiles respond to direct phototropic stimulation by blue light, which inhibits NRCN emergence on the illuminated side (Nick, 1997). Thus, in a young seedling whose coleoptilar

tip is emerging from the soil surface, light can be captured and transmitted to the endodermal cells of the CN where photoreceptors could relay a signal for the initiation of NRCN primordia. Additional studies are needed to evaluate whether light of high irradiance stimulates NRCN development as it is known that ARs in *Arabidopsis* are affected by light in totally opposite ways depending on the fluence rate (Eliasson, 1980; Konishi and Sugiyama, 2003). No sensors are known in plants for high temperature. However, it can be postulated that the accumulation of denatured and aggregated proteins, as a result of a heat shock or a sustained increase in soil temperature during the day, could divert HSP101 away from its role as an NRCN inhibitor, thus releasing the inhibition of this morphogenetic pathway. The synergistic effects of light shock with heat shock or photoperiod upon NRCN emergence indicate that a cross-talk exists between the light and heat shock pathways suggesting that: (i) light modulates the activity or the steady-state levels of HSP101 and/or other partners of the heat shock response; (ii) high temperatures affect the activity of photoreceptors and its signal transduction cascade, thus modulating the light pathway; or (iii) both pathways are required to activate a common switch that triggers NRCN formation. The additive effects of thermoperiod and photoperiod suggest that both pathways do not interact at all and instead act in parallel during the regulation of NRCN formation.

The promotion of NRCN by high temperature and light conditions was clearly affected by genetic background and by the developmental stage of the seedlings (Figs 1 and 2; Supplementary Figs S1, S2, S5, S9, S12 at *JXB* online), indicating that developmental factors subjected to genetic variation control the formation of NRCNs. Consistent with these observations, large variations in root growth and development due to genotype by environment interactions were reported (Zobel, 1996). Additionally, some *Arabidopsis* lines show high rooting, whereas others show low rooting when auxins are applied in the light (King and Stimart, 1998).

In plants, and particularly in monocots, very few mutations affecting NRCN development have been described. In this work, it is shown that HSP101 modulates the emergence of NRCNs and that HSP101 regulatory function is in turn influenced by high temperature and light. It is paradoxical that the production of NRCNs occurred either in the presence of high levels of HSP101 protein, as in the procambium of wt plants, or when the HSP101 protein was absent, as in the *hsp101* mutant (Figs 5–7). How can these contradictory results support the role of HSP101 in the initiation of NRCNs in maize? HSP101 belongs to a class of chaperones that promotes the disaggregation of protein aggregates (Nieto-Sotelo *et al.*, 1999, 2002). In *Saccharomyces cerevisiae* the HSP101 homologue, Hsp104, plays an important role in [PSI⁺] prion propagation (Chernoff *et al.*, 1995; Shorter and Lindquist, 2004; Grimminger-Marquardt and Lashuel, 2009). Overproduction or inactivation of Hsp104 by deletion leads to the loss of the [PSI⁺] prion state *in vivo*. Moreover, *in vitro* experiments indicate that, at low

concentrations, Hsp104 catalyses the assembly of soluble amyloidogenic peptides into a critical oligomeric intermediate capable of nucleating fibrillization. In contrast, at high Hsp104 concentrations, fibres are completely disassembled. Thus, the effects of Hsp104 on prion propagation are dependent on Hsp104 protein levels. Changes in the amyloid:Hsp104 concentration ratio have drastic effects on the balance between the assembly and the disassembly of fibres. At intermediate concentrations Hsp104 more efficiently catalyses the assembly of amyloidogenic oligomers that nucleate fibril formation, thus promoting prion replication. Similar to Hsp104, HSP101 could be involved in the disaggregation of an intrinsically unstable NRCN inhibitor with prion-like properties. In the absence of HSP101, as in the *hsp101* mutant, NRCN primordia could be induced because the NRCN inhibitor would not be maintained or propagated. In response to inducers (i.e. light or heat) the increase in HSP101 levels in the procambium of wt plants could completely solubilize the NRCN inhibitor causing its inactivation. At intermediate levels of HSP101, as in an uninduced wt plant, the proportion of the critical oligomeric intermediate of the NRCN inhibitor could be quite high and of the optimal size to prevent NRCN emergence. In an alternative hypothesis the activity of the NRCN inhibitor could be modulated, in addition to HSP101, by other chaperones. Disaggregation of protein aggregates requires the cooperation between Hsp100 proteins, Hsp70, Hsp40, and sHsps (Cashikar *et al.*, 2005; Lee *et al.*, 2005). The loss of HSP101 in *hsp101* mutants would result in the inactivation of the disaggregation machinery with a concomitant loss of NRCN inhibitor activity, thus promoting NRCN formation. It is proposed that, in response to the natural inducers of NRCNs, a wt plant decreases the levels of HSP70, HSP40, and/or sHSPs in the procambium. Therefore, other HSPs are good candidates for the modulation of NRCN development. Accordingly, transcripts related to two different sHSPs are down-regulated during early AR formation in pine (Brinker *et al.*, 2004). However, it is unclear whether the protein levels of a specific sHSP diminish during NRCN development. In another reasonable hypothesis, the translation of transcripts encoding the putative inhibitor of NRCN formation is HSP101 dependent. In support of this, HSP101 acts as a positive translational regulator of both tobacco mosaic virus and *Fed-1* transcripts (Wells *et al.*, 1998; Ling *et al.*, 2000). However, the high levels of HSP101 in the procambium during NRCN development complicate this model. Similarly to the previous hypothesis, a translational regulatory factor different from HSP101 could be the limiting factor during NRCN emergence in a wt plant. In support of this hypothesis, the *rtcs* maize mutant, unable to produce NRCNs and seminal roots, showed high levels of the translational elongation factor eEF1A relative to a wt plant producing NRCNs (Sauer *et al.*, 2006). Hence, the identification of other factors that cooperate with HSP101 during the regulation of NRCN development will be important in order to understand how light and high temperatures promote NRCN formation.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Effects of light and heat shock on survival and NRCN production in A63 seedlings.

Figure S2. Percentage of L10 wt seedlings developing NRCNs after exposure to high temperature shocks during a D-LS treatment.

Figure S3. Number of NRCNs per seedling in Merit plantlets exposed to heat shock during a D-LS treatment.

Figure S4. Number of NRCNs per seedling in L10 wt and L10 mutant lines after exposure to heat shock during a D-LS treatment.

Figure S5. Percentage of L10 wt seedlings developing NRCNs when exposed to heat shock during D-LS or P-LS treatments.

Figure S6. Number of NRCNs per seedling in Merit plantlets exposed to heat shock during D-LS or P-LS treatments.

Figure S7. Number of NRCNs per seedling in the L10 wt line exposed to heat shock during D-LS or P-LS treatments.

Figure S8. Percentage of Merit seedlings developing NRCNs when exposed to thermal periods during P-LS treatments.

Figure S9. Percentage of L10 wt and L10 mutant seedlings developing NRCNs when exposed to heat shock during P-LS treatments.

Figure S10. Survival of organs of L10 wt and L10 mutant seedlings to high temperature shocks during a P-LS treatment.

Figure S11. Number of NRCNs per seedling in L10 wt and L10 mutant lines when exposed to heat shock during P-LS treatments.

Figure S12. Percentage of L10 mutant seedlings developing NRCNs when exposed to heat shock during D-LS or P-LS treatments.

Figure S13. Number of NRCNs per seedling in L10 mutant line when exposed to heat shock during D-LS or P-LS treatments.

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