Cell Reports
Article

XRN4 and LARP1 Are Required for a Heat-Triggered mRNA Decay Pathway Involved in Plant Acclimation and Survival during Thermal Stress

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http://dx.doi.org/10.1016/j.celrep.2013.11.019

SUMMARY

To survive adverse and ever-changing environmental conditions, an organism must be able to adapt. It has long been established that the cellular reaction to stress includes the upregulation of genes coding for specific stress-responsive factors. In the present study, we demonstrate that during the early steps of the heat stress response, 25% of the *Arabidopsis* seedling transcriptome is targeted for rapid degradation. Our findings demonstrate that this process is catalyzed from 5′ to 3′ by the cytoplasmic exoribonuclease XRN4, whose function is seemingly reprogrammed by the heat-sensing pathway. The bulk of mRNAs subject to heat-dependent degradation are likely to include both the ribosome-released and polysome associated polyadenylated pools. The cotranslational decay process is facilitated at least in part by LARP1, a heat-specific cofactor of XRN4 required for its targeting to polysomes. Commensurate with their respective involvement at the molecular level, LARP1 and XRN4 are necessary for the thermotolerance of plants to long exposure to moderately high temperature, with *xrn4* null mutants being almost unable to survive. These findings provide mechanistic insights regarding a massive stress-induced posttranscriptional downregulation and outline a potentially crucial pathway for plant survival and acclimation to heat stress.

INTRODUCTION

Exposure to elevated temperatures triggers the heat stress response (HSR), a coordinate gene expression program that allows plants and most other organisms to become thermotolerant and survive heat stress (Kotak et al., 2007; Mittler et al., 2012; Yeh et al., 2012). At the center of the HSR is the quick and global reprogramming of gene expression patterns set up by a general inhibition of translation initiation (for review, see Holcik and Sonenberg, 2005) and a massive modification of transcriptome profiles (Castells-Roca et al., 2011a; Fan et al., 2002; Gasch et al., 2000; Grigull et al., 2004; Larkindale and Vierling, 2008). Although mRNAs can be either up- or downregulated following heat stress, in *Arabidopsis*, transcriptomic analyses showed that many more transcripts are downregulated rather than upregulated (Larkindale and Vierling, 2008). Also, in yeasts and mammals, global transcriptomic analyses comparing mRNA steady-state levels to modifications of transcription rates upon heat stress suggest not only that transcription is modified, but also that the stability of a large fraction of mRNAs is reprogrammed (Castells-Roca et al., 2011a; Fan et al., 2002; Grigull et al., 2004). These and similar approaches using other stress situations (such as oxidative or osmotic) suggest the existence of a global stress-induced mRNA decay pathway in eukaryotes (Castells-Roca et al., 2011b; Fan et al., 2002; Molin et al., 2009; Molina-Navarro et al., 2008). Numerous studies reported cases of a single transcript or a small group of transcripts destabilized by the binding of specific *trans*-acting factors to common *cis* boxes upon developmental or external stimuli (Parker and Song, 2004), but how several thousand mRNAs with no obvious common structural features are targeted to rapid decay in response to stress is unknown. It is also unclear what components of the general mRNA decay process are required and how their activity/specificity is modified by stress sensing.

In the cytoplasm, bulk mRNA decay can occur through an evolutionarily conserved deadenylation-dependent mechanism in which, after a rate-limiting poly(A) tail-shortening step, the transcript is either degraded from its 3′ end by the exosome or processed by the DCP2/DCP1/HEDLS decapping holoenzyme that excises the 5′-m^{7}Gpp protecting structure, allowing 5′-3′ degradation by the exoribonuclease XRN1 (Garneau et al., 2007; Parker, 2012). While this pathway is mostly prominent in budding yeast, this is likely not the case in other eukaryotes

where distinct pathways exist. In particular, transcripts can be 5′ unprotected through endonucleolytic cleavage (small RNA guided or not) or decapped in a deadenylation-independent way before being 5′ digested by XRN1 (Morozov et al., 2010; Nagarajan et al., 2013; Rissland and Norbury, 2009).

While it has long been assumed that ribosome dissociation was absolutely necessary before transcripts can be degraded, studies conducted in budding yeast demonstrated that this is not always the case, as transcripts decapping and subsequent 5′-3′ decay were shown to take place cotranslationally while polysomes are finishing elongation (Hu et al., 2009, 2010). Messenger RNA nucleoproteic particles (mRNPs) disengaged of polysomes are not always readily degraded and can aggregate in large complexes that are either processing bodies (p-bodies) or stress granules (SGs). P-bodies contain translation repressors, mRNA degradation enzymes, and cofactors such as decapping holoenzyme and XRN1 exoribonuclease (Franks and Lykke-Andersen, 2008; Parker and Sheth, 2007), while SGs contain translation initiation machinery components (Anderson and Kedersha, 2008). Contrary to SGs, a limited number of p-bodies can be detected in cells under normal conditions, but both granule types increase in size and number upon stress-limiting translation initiation, such as heat (Bruno and Wilkinson, 2006; Weber et al., 2008). Although the mRNA decay machinery concentrates in p-bodies, it is still debated whether they are actual sites of mRNA decapping and degradation. SGs and p-bodies are often proposed to be dynamic sites of mRNP storage and remodelling, with at least some transcripts moving from one to the other and/or disassembling from them to be decayed or return to translation (Kulkarni et al., 2010; Parker, 2012).

In plants, cytoplasmic mRNA decay processes mediated through either endonucleolytic or decapping steps exist and are likely regulated by developmental and/or environmental cues (Belostotsky and Sieburth, 2009; Chiba and Green, 2009; Xu and Chua, 2011). Homologs of the main enzymatic mRNA turnover activities (deadenylation, decapping, and exoribonuclease) have been identified, and some have been characterized (Chiba et al., 2004; Dupressoir et al., 2001; Goeres et al., 2007; Kastenmayer and Green, 2000; Kastenmayer et al., 2001; Walley et al., 2010; Xu et al., 2006). Some evidence supports the idea that mRNAs could also be decayed cotranslationally in *Arabidopsis* (Sement et al., 2013), and the existence of p-body and SG-like aggregates of mRNPs is now well accepted (Weber et al., 2008). XRN4, the *Arabidopsis* functional equivalent of XRN1, was shown to degrade 5′-monophosphate mRNAs produced by decapping or by small RNA-mediated endonucleolytic cleavages (Gregory et al., 2008; Gy et al., 2007; Kastenmayer and Green, 2000; Kastenmayer et al., 2001; Souret et al., 2004). XRN4 appears to target a very limited set of endogenous polyadenylated transcripts in standard growth conditions (Estavillo et al., 2011; Gregory et al., 2008; Rymarquis et al., 2011; Souret et al., 2004) and does not seem to play a crucial role in plant growth and development processes (Gy et al., 2007; Kastenmayer and Green, 2000). XRN4 is nevertheless an important component of the ethylene response system (Olmedo et al., 2006; Potuschak et al., 2006) and as such is likely involved in plant adaptation to stress.

The La-motif protein superfamily is composed of RNA binding factors conserved across eukaryotes that can be classified into five families (namely genuine LA and LARP1, 4, 6, and 7), with members of each family sharing structural, evolutionary, and functional features (Bayfield et al., 2010; Bousquet-Antonelli and Deragon, 2009). Animal LARP1 proteins are required for proper gametophyte development and mitotic progression (Blagden et al., 2009; Burrows et al., 2010; Nykamp et al., 2008). Human LARP1 is likely an mRNA binding protein (Castello et al., 2012), and its nematode homolog is apparently required for the cytoplasmic turnover of at least certain transcripts (Nykamp et al., 2008). The *Arabidopsis thaliana* genome codes for three members of the LARP1 family, but only one (the LARP1a protein) displays in addition to the La motif every highly conserved domain characteristic of members of this family (Bousquet-Antonelli and Deragon, 2009).

We show in this work that *Arabidopsis* LARP1a associates during heat stress (15 min at 38°C) with the 5′-3′ exonuclease XRN4 and that both are most likely required to set up a massive heat-induced mRNA decay process that targets more that 4,500 mRNAs in seedlings. LARP1a is the factor (or one of the factors) involved in addressing XRN4 to polysomes during heat stress, suggesting that part of the degradation could be directly initiated on mRNAs engaged in translation. We also show that *xrn4* mutant plants are hardly able to survive to prolonged periods at moderately high temperatures (35°C) and that *larp1* mutant plants are also affected, but to a much lesser extent. Overall, these results suggest that plants might regulate the HSR at the posttranscriptional level by inducing a global mRNA decay process.

RESULTS

Our first objective was to evaluate the extent of mRNA over- and underaccumulation in the early steps of the HSR and the molecular contribution of LARP1a (encoded by the At5g21160 locus and referred to as LARP1 from now on) to these processes.

To do so, we conducted a global quantitative analysis of the transcriptomes of wild-type (WT) and full knockout *larp1-1* (a mutant T-DNA insertion line from the Salk Institute, SALK_151251; see Figure S1) seedlings after a 15 min incubation at 20°C for nonstressed (NS) or at 38°C for heat stressed (HS) conditions. Four cDNA libraries prepared from polyadenylated mRNAs (corresponding to the WT-NS, WT-HS, *larp1-1*-NS, and *larp1-1*-HS conditions) were sequenced, analyzed, and filtered as described in Supplemental Experimental Procedures. After filtering, counts for 19,804 distinct loci representing 76% of the total estimated number of *Arabidopsis* genes remained for further analyses. To assess the impact of the heat treatment on mRNA accumulation levels, we divided the number of reads at 38°C ($q^{38°C}$) by the number of reads at 20°C ($q^{20°C}$), giving a fold (F) of $q^{38°C}/ q^{20°C}$, respectively, for each gene in each genotype. As a cutoff, we considered as significant variation of 2-fold or more ($F \geq 2$ and $F \leq 0.5$) and found 801 (4%) upregulated and 4,745 (25%) downregulated mRNAs in WT and 824 upregulated and 3,705 downregulated mRNAs in *larp1-1* plants (Figures 1A and S2A; Table S1). As expected in the WT background, upregulated transcripts mostly code for

proteins involved in heat and abiotic stress response (Figure S2C; Table S1). Transcripts upregulated in the *larp1-1* background fall in the same Gene Ontology (GO) categories as those upregulated in WT (Figure S2C), and 84% of the upregulated mRNAs following heat stress in WT are also upregulated to the same extent in *larp1-1* mutant plants (Figures S2A and S2B), suggesting that LARP1 has no global impact in the production/stabilization of heat-increased mRNAs.

Several Thousand mRNAs Underaccumulate in a LARP1-Dependent Manner after a Short Heat Stress

Following heat stress, five to six times more loci have their expression downregulated rather than upregulated (Figures 1A and S2A; Table S1). A GO analysis shows that underaccumulating mRNAs encode factors involved in postembryonic development (lowest p value in the GO analysis and highest fraction of affected mRNAs), transcription, and biological and cellular regulations (Figure S2D), suggesting that downregulated genes are mostly involved in housekeeping functions necessary for plant growth and development at the plant stage we used (3-week-old seedlings; postembryonic stage). A northern blot analysis conducted on three randomly chosen mRNAs predicted to be downregulated by the transcriptomic data confirmed a decrease in steady-state levels of these full-length transcripts upon heat stress (Figure 1C; compare lanes 1 and 2), while transcripts At3g58730 and At3g14420 remained consistently stable with the RNA sequencing (RNA-seq) data.

To assess the involvement of LARP1 in this massive heat-induced phenomenon, we analyzed in the *larp1-1* background the fold of the 4,745 mRNAs found to be downregulated more than 2-fold in WT. Strikingly, 98.9% of them are also downregulated by heat stress in the absence of LARP1, with 3,005 mRNAs being downregulated more than 2-fold (Figures 1A and 1B; Table S1). This suggests that globally, the qualitative heat-triggered downregulation response is unaffected by LARP1 loss of function. We then analyzed, from a quantitative point of view, the role of LARP1 in this downregulation phenomenon by comparing the F_{WT} to $F_{larp1\text{-}1}$ for the 4,745 mRNAs targeted by the heat stress (Figures 1A and 1B). Calculation of their log2 ($F_{WT}/F_{larp1\text{-}1}$) shows a deviation toward negative values (Figure 1A, left panel) with a total significant number of transcripts (3772: 80%) with a log2 value below zero (Figure 1A, right panel). Since levels of the transcripts downregulated by heat stress are not globally affected in the *larp1-1* mutant at 20°C (Figure S2E), this suggests that the heat-induced fold reduction in the absence of LARP1 is less severe than in WT. To further confirm this, we compared by quantitative RT-PCR (qRT-PCR) assays the fold ($q^{38°C}/q^{20°C}$) decrease for the transcripts previously analyzed by northern blot from WT and *larp1-1* plants. Since RNA-seq analyses were conducted on polyadenylated mRNAs, cDNAs for quantitative PCR (qPCR) assays were reverse transcribed by oligo(dT_{18}) priming. After a 15 min period at 38°C, levels of all three transcripts from WT plants are significantly decreased by at least 2-fold and at least to the same extent as what has been observed by RNA sequencing (Figure 1D; Table S1). We also confirm that in *larp1-1*, the fold decrease in mRNA levels is less important than in WT plants. When *larp1-1* plants express a stable transgenic LARP1 protein under the control of its own promoter (*pro$_{LARP1}$-LARP1*), heat-induced mRNA downregulation is restored to that of WT, showing that LARP1 loss of function is directly responsible for the observed molecular phenotype.

Overall, these data suggest that in the early steps of the HSR, the LARP1 protein is involved in a massive downregulation of the *Arabidopsis* seedling transcriptome affecting around 25% of the plant mRNAs.

XRN4 Forms a Heat-Triggered Complex with LARP1 In Vivo

To get a better understanding of LARP1 molecular functions, we sought for partners by yeast two-hybrid (Y2H) system and identified nine putative interactants with scores ranking from very high to good confidence, including the cytoplasmic 5′-3′ exoribonuclease XRN4. Four independent clones containing XRN4 cDNA fragments in frame with the GAL4 activation domain were retrieved. The region shared by these fragments (or selected interacting domain [SID] in Figure 2A), which likely represents the minimal domain of XRN4 sufficient to interact with LARP1 in a Y2H assay, is highly conserved among XRN proteins (either of the XRN1 or XRN2/RAT1 type) and maps to the so-called CR2 subdomain of their catalytic core (Figures 2A and S3) (Jinek et al., 2011; Xiang et al., 2009).

We tested by in vivo pull-down assays the existence of a LARP1-XRN4 complex in either unstressed (20°C) or heat-stressed (15 min at 38°C) plants. Crude extracts prepared from either control or heat-stressed seedlings stably expressing a GFP-LARP1 fusion were used for immunoprecipitations with anti-GFP. We found that XRN4 can be detected in the eluate fractions of stressed extracts while the amount of XRN4 in the eluate fraction of 20°C extracts, if any, is beyond detectable levels. On the other hand, the amounts of the GFP-LARP1 fusion in both eluates were similar or slightly lower at 38°C (Figures 2B and 2C). This enrichment of XRN4 in the eluate fraction of the heat-stressed extract is not due to a modification of the steady-state levels of XRN4 as shown by the input lanes of Figure 2 and steady-state western blot analyses conducted on total protein extracts (Figure S1C, lanes 1 and 2). We deem it unlikely that the 20°C LARP1-XRN4 binding is disrupted upon crude extract preparation or the immunoprecipitation procedure, because we did not find an enrichment of XRN4 in the 20°C eluate fraction when pull-downs were conducted under milder conditions (0.1 instead of 1% of detergent in the lysis/binding buffer) (data not shown). Finally, XRN4 coimmunoprecipitation is specific to the LARP1 moiety of the GFP-LARP1 fusion, because it is not detected in the 20°C or 38°C bound fractions of pull-downs performed with extracts prepared from plants expressing solely GFP or from WT plants expressing an untagged LARP1 protein (Figure 2B, lanes 5, 6, 11, and 12). To determine whether LARP1-XRN4 complex formation is dependent upon the presence of RNA, we challenged the LARP1-XRN4 interaction by mock or RNase treatments of the crude extracts (Figure 2C). To degrade RNAs, we used a mix of RNase A/T1 (which hydrolyzes RNAs at C, U, or G) (lanes 4 and 7), RNase I (which hydrolyzes single-stranded RNAs) (lanes 5 and 8), or a mix of all three (lanes 11) and monitored the digestion efficiencies on the unbound fractions (Figure 2C, right). Structured RNAs (rRNAs, tRNA, sn/snoRNAs) were cut into discrete bands in RNase-I-treated

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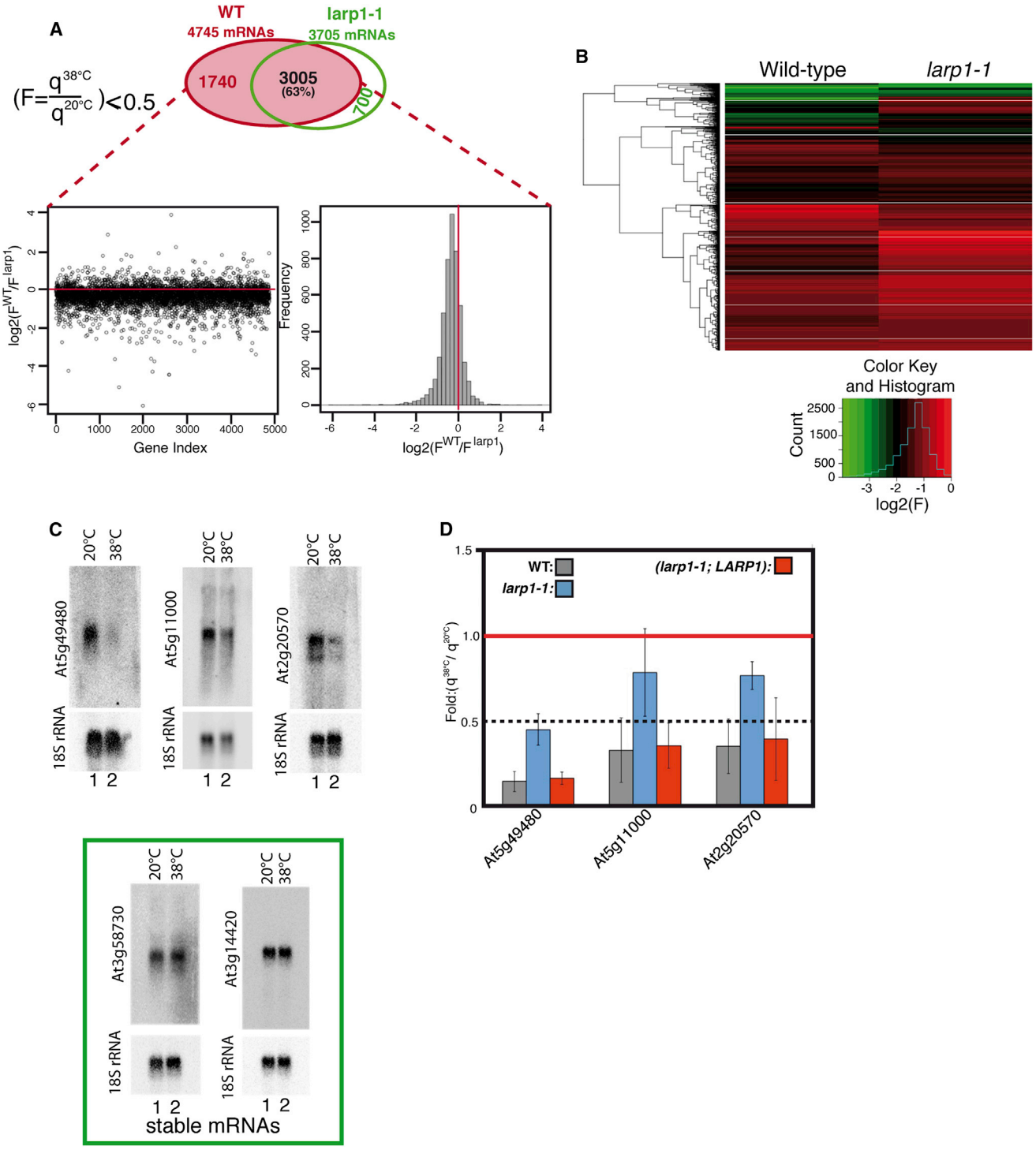


Figure 1. Heat Stress Induces a Massive mRNA- and LARP1-Dependent Downregulation

(A) Venn diagram representations of the number of mRNAs that are downregulated by at least 2-fold in wild-type (WT) (red) and/or *larp1-1* mutant plants (green). Graphic representation of the comparison of the WT (F^{WT}) with the *larp1-1* ($F^{larp1-1}$) folds for mRNAs that are significantly downregulated upon heat stress. In the left panel, the log2 (F^{WT}/ $F^{larp1-1}$) values are reported according to 4,745 gene indexes. The right panel is a graphic representation of the frequency distributions of the log2 (F^{WT}/ $F^{larp1-1}$) values.

(B) Heatmaps of the folds ($F = q^{38°C}/q^{20°C}$) of mRNA downregulated at least 2-fold in WT or *larp1-1* plants. The log2 values of the fold are color coded according to the scale reported beneath the heatmaps. RNA-seq analyses were conducted on poly(A)$^+$ samples as explained in the Supplemental Experimental Procedures.

(legend continued on next page)

samples consistently with its substrate specificity, while no RNA remained to be detected in the other conditions, likely because RNase A, which we used in large excess, also cut double-stranded RNAs. Degradation of RNAs (either structured or single stranded) present in the native extracts does not appear to destabilize the heat-triggered LARP1-XRN4 complex as the amounts of XRN4 detected in the eluates from RNase and mock-treated samples are comparable (Figure 2C; compare lane 6 to lanes 7 and 8 and lane 10 to lane 11).

These data suggest that the LARP1 and XRN4 proteins exist in a complex in plants and that their association is strikingly triggered by heat sensing. Because LARP1 and XRN4 are able to bind to each other when expressed in a yeast heterologous system and given that their interaction is not dependent upon the presence of RNA or poly(A) tails (as suggested by RNase I treatment), we postulate that they are direct interactants in vivo.

Heat-Triggered mRNA Downregulation Is the Consequence of an Accelerated 5′-3′ Cytoplasmic Degradation Process Requiring XRN4 and LARP1

The heat-dependent association of LARP1 with XRN4 suggests that the heat-mediated mRNA downregulation could require XRN4 function. We hence compared by qRT-PCR the levels at 20°C and 38°C of 23 randomly chosen polyadenylated transcripts in WT, *larp1-1*, and *xrn4-5* full-deletion mutant plants (Figures 3A and S4A). Again, this assay backs up our transcriptomic data, because the newly analyzed mRNAs are decreased by at least 2-fold in WT but are always less affected in *larp1-1* plants. In the *xrn4-5* mutant, we can observe that the fold of every tested transcript is higher than that of WT (Figures 3A and S4A), supporting the idea that XRN4 is required for the heat-mediated downregulation. In most cases, LARP1 and XRN4 loss of function gives similar folds that are always above WT levels, but not restored completely to the 20°C situation (i.e., still below a fold of 1). However, for 5 of the 23 transcripts tested (At2g40750, At2g19880, and At4g14400 [Figure 3A] and At5g26340 and At5g49480 [Figure S4A]), the *xrn4-5* fold is higher than the *larp1-1* fold and exceeds the 20°C situation (i.e., fold above one). Finally, the At1g47128 transcript, whose levels were shown by RNA-seq to remain constant upon heat stress, was always unaffected, regardless of the background by qRT-PCR assay.

These data suggest that, as for LARP1, the loss of XRN4 function impairs the heat-induced mRNA decrease, at least for the 23 tested messengers.

Given that XRN4 degrades 5′ unprotected mRNAs in the cytoplasmic turnover process, we postulate that the observed transcript downregulation after a short heat stress is linked to their accelerated cytoplasmic decay. In order to discriminate between transcriptional and posttranscriptional causes, we assessed mRNA half-lives using actinomycin D (Johnson et al., 2000) or cordycepin as a transcriptional inhibitor (Gutierrez et al., 2002). Three-week-old plantlets were transferred in transcriptional inhibitor containing liquid medium and further incubated at 20°C or 38°C for up to 60 min. Transcript abundances were assessed at different time points and the remaining percentage (with the 0 time point set at 100%) plotted against time. We followed eight heat-targeted mRNAs plus the stable At1g47128 (Figures 3B and S4B). In the WT background, the quantity of remaining transcript decreases faster at 38°C than at 20°C. Transcripts appear to be differentially affected by heat, with some decaying very rapidly at 38°C (At1g75900, At5g11000, and At5g49480) and some less affected but still decaying faster than at 20°C. As expected, the At1g47128 messenger decay is unaffected by heat. In all cases, the 38°C regression curves do not fit straight lines but rather two curves of different slopes: an initial steep one and a later flatter one. This suggests that the heat-induced decay could be biphasic, with a rapid phase at the very early time points of heat stress followed by a slower one at later time points.

We ran identical analyses with *larp1-1* and *xrn4-5* mutant plants and found that, in all cases, depletion of any of the two proteins significantly slows down the mRNA decrease over time at 38°C as compared to WT plants (compare the colored curves to the light gray ones at 38°C on Figures 3B and S4B). For every mRNA in *xrn4-5* and for some of them in *larp1-1* mutants, the 38°C regression curves are restored to the 20°C ones.

This piece of evidence supports the idea that XRN4 and LARP1 are required for the accelerated heat-induced decay process, which is fully mediated by XRN4, at least for the tested transcripts. In some cases, LARP1 commitment to this process is only partial.

A Fraction of LARP1 Localizes to XRN4-Containing P-Bodies

Using the stable transgenic GFP-LARP1 line, we analyzed LARP1 subcellular distribution in *Arabidopsis* root tips and found that, while it displays a completely diffuse signal in the cytosol under normal conditions, it forms aggregates after 15 min incubation at 38°C. These LARP1 stress-induced foci are dynamic and their formation is inhibited by cycloheximide-mediated translation elongation freeze, suggesting that they might be p-bodies or SGs (Figure S5A). To further analyze the nature of the stress-induced LARP1 foci, we used transient assays in onion epidermal cells as previously reported (Merret et al.,

(C) Northern blot analyses conducted on total RNA extracted from WT plants either unstressed (lane 1) or heat stressed (15 min at 38°C) (lane 2). Three randomly chosen heat-downregulated mRNAs (At5g49480, At5g11000, and At2g40750) and two heat-stable transcripts (At3g58730 and At3g14420) were analyzed. The 18S rRNA was used as loading control. This experiment was performed on a batch of RNAs different from that used for RNA-seq.

(D) qRT-PCR analyses of the fold variations between 38°C and 20°C ($q^{38°C}/q^{20°C}$) from WT (gray bars), *larp1-1* (blue bars), or (*larp1-1; LARP1*) (complemented) (red bars) plants. qPCRs were conducted on cDNA oligo(dT_{18}) reverse transcribed from total RNAs. Values were normalized to Actin7 (At5g09810) and calculated as $q = 2^{(CtRef-CtGene)}$.

Data are mean ± SD of three independent biological replicates plus two qPCR technical replicates for each biological replicate. Folds ($q^{38°C}/q^{20°C}$) were respectively calculated for each biological replicate and the mean fold values and SD calculated out of these three values. This experiment was performed on batches of plants different from those used for transcriptomic and northern blots assays. See also Figure S2.

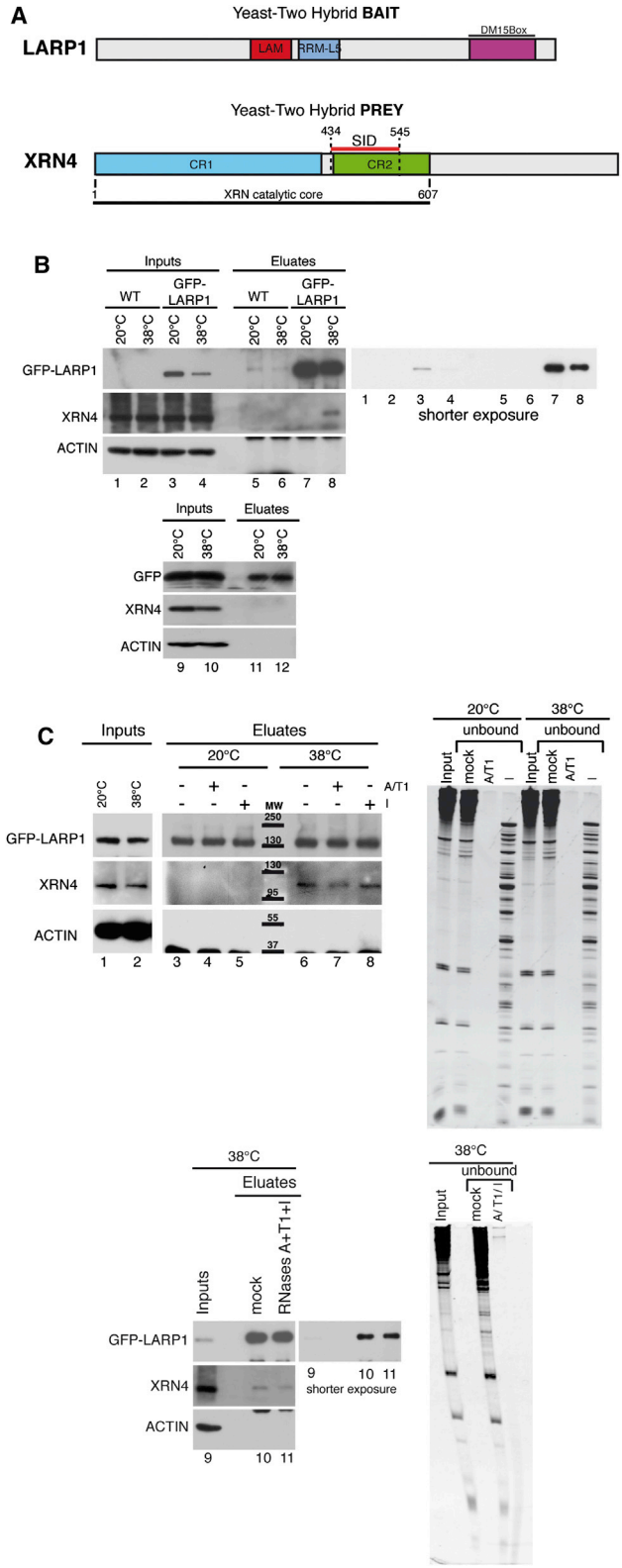


Figure 2. XRN4 Is a Heat-Stress-Dependent LARP1 Interactant

(A) XRN4 was identified as a putative LARP1 interactant by yeast two-hybrid (Y2H) screen. Schematic representation of the LARP1 (used as Y2H bait) and XRN4 (prey) proteins. Conserved domains are depicted as colored boxes with the La motif (LAM) in red, the RRM-like (RRM-L5) in blue, and the DM15 box in purple for LARP1. The two subdomains (CR1 in light blue and CR2 in green) of the XRN4 catalytic core are depicted. The minimal region from XRN4 sufficient to interact with LARP1 in a Y2H assay (selected interaction domain [SID]) is shown as a red line.

(B) Coimmunoprecipitation experiments with anti-GFP on crude lysates of WT plants (untagged LARP1; lanes 1, 2, 5, and 6), plants expressing GFP-LARP1 (lanes 3, 4, 7, and 8), or plants expressing GFP alone (lanes 9–12). Crude extracts were prepared from 21-day-old whole plantlets incubated for 15 min at 20°C (lanes 1, 3, 5, 7, 9, and 11) or 38°C (lanes 2, 4, 6, 8, 10, and 12). For western blots, 2% of the input and 40% of the eluate fractions were analyzed with anti-GFP, anti-XRN4, or anti-Actin.

(C) Pull-down experiments with RNase-treated crude extracts. Samples were either mock treated (lanes 3, 6, and 10) or treated with RNase A/T1 (lanes 4 and 7), RNase I (lanes 5 and 8), or a mix of RNase A, T1, and I (lane 11). Western blot analyses (left panels) were conducted on 2% of input and 25% of eluate fractions. RNA degradation was followed by ethidium bromide staining of a polyacrylamide gel (right panels) loaded with 10% of input or 10% of mock- or RNase-treated unbound fractions.

See also Figure S3.

2013) (Figures 4, S5B, and S5C). We found that while under normal conditions LARP1 shows a diffuse cytosolic pattern, it forms aggregates upon stress (hypoxia) (Figure 4A). Using GFP-tagged RBP47 (an homolog of TIA-1) or yellow fluorescent protein (YFP)-tagged DCP1, respectively, as SG and p-body markers (Weber et al., 2008), we set out to determine the nature of the stress-induced LARP1 aggregates (Figures 4B, 4C, and S5B). We observed that most LARP1 foci contain the RBP47 protein, suggesting the LARP1 colocalizes with SGs (Figure 4B). This is further backed up by the observation that over a prolonged period of stress, the LARP1-RBP47 foci tend to form larger, irregular aggregates (Figure 4B; compare subpanels c and f), as compared to p-bodies, which were reported to display a regular rounded shape (Erickson and Lykke-Andersen, 2011). Nevertheless, we noticed that a subfraction of LARP1 foci does not contain RBP47 (Figure 4B). We then performed colocalization experiments between LARP1 and DCP1 (Figures 4C and S5B) and found that indeed a subset of LARP1 aggregates, although limited, contain DCP1, suggesting that a portion of this protein could localize in p-bodies. Since XRN4 was shown to be a p-body component (Weber et al., 2008) and is a LARP1 interactant, we wondered whether they could be found in the same p-body structures. We hence performed colocalization experiments with tagRFP-LARP1, YFP-DCP1, and cer-XRN4 fusions (Figure 4C). LARP1 does appear to colocalize with XRN4, but only with a small subfraction, as suggested by the fact that only a subset of XRN4 foci contain the LARP1 protein (Figure 4C, subpanel e, and Figure S5C) while, consistent with previously reported data, a much larger fraction of XRN4 foci also contain DCP1 (Figure 4C, subpanel f). Nevertheless, we observed that LARP1-XRN4 foci also contain DCP1, suggesting that XRN4 and LARP1 could interact in some p-body structures (Figure 4C, subpanel g).

These data suggest that LARP1 colocalizes with stress-induced (heat stress and hypoxia) mRNP aggregates, but unexpectedly these structures largely seem to be SGs, whereas only a small fraction are p-bodies. In support of our immunoprecipitation data (Figure 2B), a small fraction of LARP1 was found to

colocalize with XRN4-containing p-bodies, a result that does not exclude the possibility that these proteins also interact at a different cytoplasmic location.

LARP1 Can Address XRN4, but Not the Decapping Complex, to Polysomes

Studies conducted in yeast and animals led to a model suggesting that once transcripts have been 5′ unprotected, their XRN1-mediated degradation takes place in a ribosome-free state (after the last ribosome has completed elongation). In budding yeast, it has been shown that at least a subset of the 5′-3′ decay can also take place on polysomes while mRNAs are finishing elongation (Hu et al., 2009, 2010). We wondered whether LARP1 and XRN4 function in the heat-triggered decay could take place on ribosome-associated transcripts. Crude polysomal extracts were prepared from 20°C unstressed or 38°C heat-stressed plants and ribosomal complexes separated by centrifugation on a sucrose gradient and collected as fractions. Western blot analyses of each fraction show that a portion of LARP1, XRN4, and the decapping complex DCP1/DCP2 sedimented down to the heavy fractions of the gradient either from unstressed or heat-stressed plants (Figures 5A and S6A). When the crude extracts were treated with polysome-dissociating compounds (EDTA [not shown] or puromycin; Figure S6B and data not shown), all four proteins disappeared from fractions 7–11, where polysomes are usually found. This strongly suggests that LARP1, XRN4, DCP1, and DCP2 are detected in the gradient heavy fractions because they are associated with translating ribosomes. Strikingly, we observe that despite the strong decrease of translating ribosomes at 38°C, there is a higher proportion of LARP1, XRN4, DCP1, and DCP2 sedimented to heavy fractions of the gradient (Figures 5A and S6A; compare lanes 7–11 from 20°C to 38°C and quantitation of Figure 5D), regardless of the fact that their steady-state levels are unaffected by heat stress (Figure S1C).

Because we found that LARP1 binding to XRN4 is triggered by heat stress, we tested whether the 5′-3′ exoribonuclease remains associated to polysomes in its absence. The same sucrose gradient analyses were conducted as previously described but using *larp1-1* full-knockout seedlings (Figure 5B). Full deletion of the LARP1 protein does not appear to globally impact translation at 20°C or 38°C, because both polysome profiles are identical to the WT ones. The western blot analyses of the ribosome-containing fractions show that while the accumulation of DCP1 appears unaffected by LARP1 depletion, the XRN4 signal is significantly diminished in 20°C and 38°C extracts (Figures 5B and 5D), although its steady-state amounts remain unchanged (Figure S1C). To readily prove that XRN4 strong underaccumulation is directly linked to the loss of LARP1, we analyzed its polysomal association in mutant seedlings expressing a transgenic copy of the LARP1 cDNA under the control of its own upstream genomic sequences (Figure 5C). Complementation of the *larp1-1* mutant allows XRN4 to be detected back in polysomal fractions both at 20 and 38°C (compare lanes 7–11 from Figure 5B with lanes 7–11 in Figure 5C).

To rule out that the observed differences in protein levels in polysomal fractions between temperature conditions or plant genotypes are due to technical discrepancies, we repeated the sucrose gradient analyses from biological samples, prepared western blots loaded with input and polysomal fractions 7–11, and quantified LARP1 and XRN4 signals (Figure 5D). Blots from different conditions (three genetic backgrounds in two temperature conditions) were prepared and hybridized with relevant antibodies in parallel and placed in the chemiluminescence quantification device (ChemiDoc imaging system; Bio-Rad) simultaneously. LARP1 and XRN4 signals values normalized to the RPL13 signal are reported as histograms in Figure 5D. We can observe a significant increase of XRN4 and LARP1 at 38°C as compared to 20°C and a strong drop in the XRN4 signal in the polysomal fractions from *larp1-1* seedlings, especially at 38°C. Consistently with Figure 5C, expression of a transgenic LARP1 protein in the *larp1-1* background allows reaccumulation of XRN4 in polysomal fractions, showing that this latter molecular phenotype is directly linked to *LARP1* gene inactivation and is not the consequence of otherwise undetected genomic alterations that would have arisen upon creation of the T-DNA insertion line.

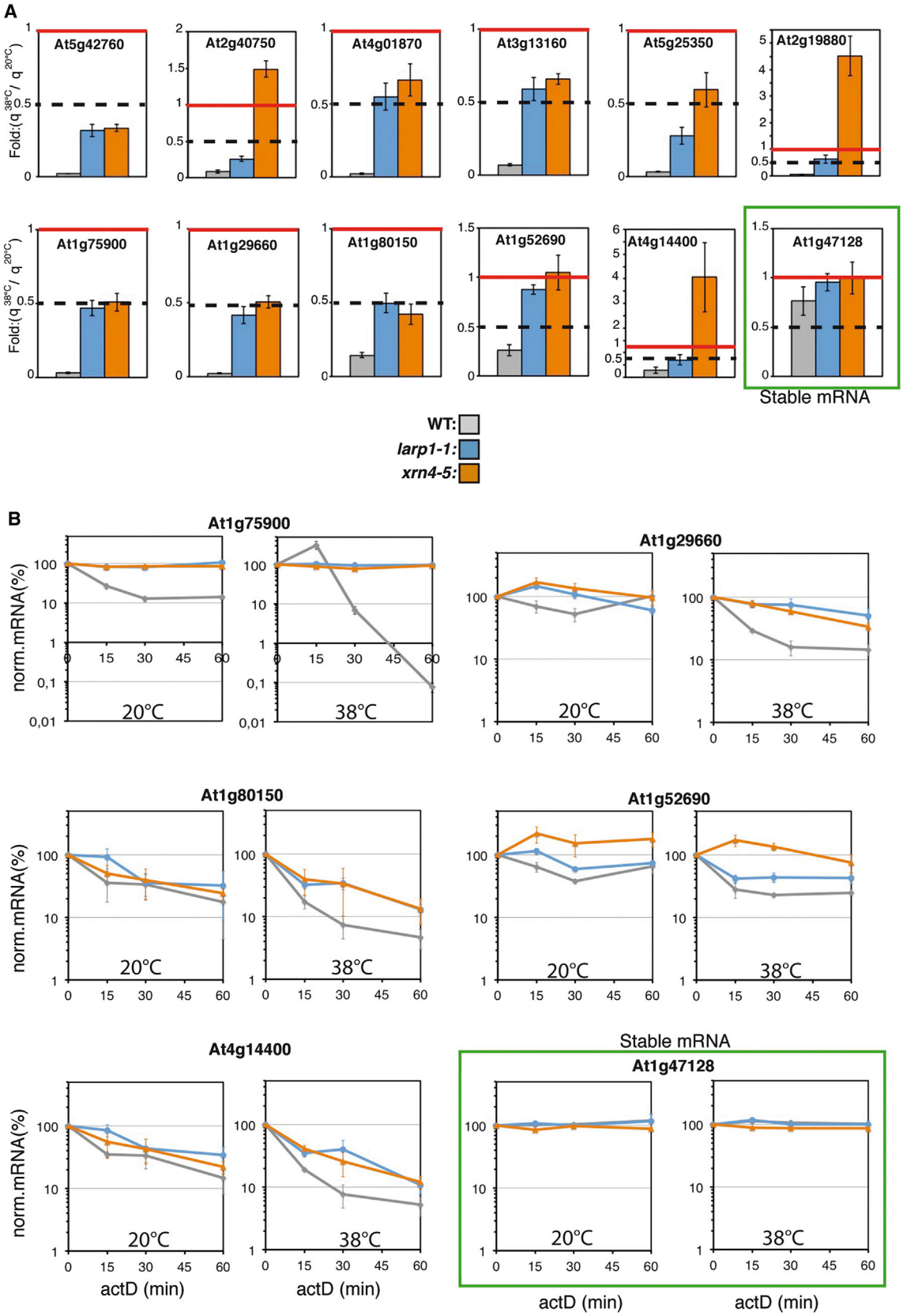
The data here show that major actors of the cytoplasmic 5′-3′ mRNA turnover associate with plant polysomes and that more of them are found on polysomes in a heat stress situation. Moreover, XRN4 targeting to polysomes, in particular following heat stress, is at least in part LARP1 dependent.

xrn4-Deficient Plants Lose Their Ability to Adapt to and Survive Heat Stress Situations

The above-presented results suggest that XRN4 and LARP1 are stress-responsive factors. To further test this hypothesis, we analyzed the ability of *xrn4* and *larp1* null plants to survive after exposure to heat stress. In their natural habitats, plants are exposed to a wide variety of heat stress conditions and have hence evolved a diversity of thermotolerance responses. To properly phenotype thermotolerance defects of plants deleted of XRN4 or LARP1 function, we analyzed the ability of *xrn4* and *larp1* null alleles to survive several heat stress regimens as recently described (Yeh et al., 2012). *xrn4-5*, *xrn4-6*, and *larp1-1* 5-day-old seedlings were tested and compared to WT plants for their basal thermotolerance (BT; abrupt exposure to 44°C), short- and long-term acquired thermotolerance (SAT and LAT; acclimation at 37°C and recovery at 20°C for 2 hr or 2 days before exposure to 44°C), and thermotolerance to moderately high temperature (TMHT; exposure to 35°C for 7 days). Following exposure to heat stress, the plant survival rates were assessed after 8 days of recovery (Figure 6 and data not shown). We found no significant difference in survival rates for any of the mutant plants when challenged for BT, SAT, or LAT (data not shown). Conversely, we respectively observed significant and very light survival rate defects when plants deleted of XRN4 or LARP1 were surveyed for their TMHT. Only 5%–10% of *xrn4-5* and *xrn4-6* and 40%–50% of *larp1-1* plants survived a long exposure at 35°C, while 50%–60% of WT plants survived this heat stress regimen (Figure 6). These results suggest that XRN4 and, to a much lesser extent, LARP1 functions are necessary for the plant to acclimate to a prolonged exposure at elevated temperature.

DISCUSSION

We show in the present work that one of the early responses of *Arabidopsis* seedlings to high temperature is to downregulate



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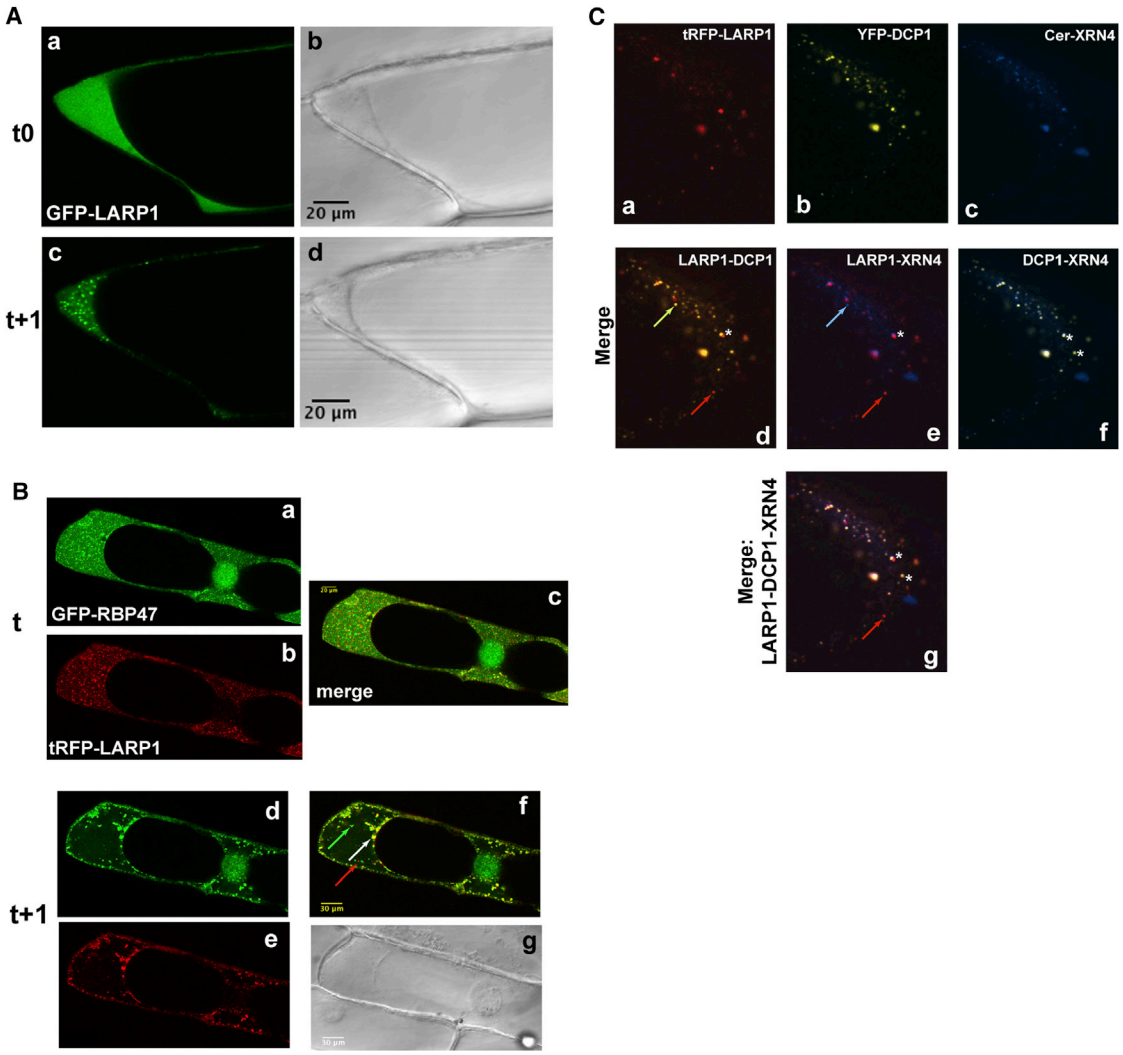


Figure 4. Subcellular Localization of LARP1 upon Stress

(A–C) Fluorescently tagged versions of LARP1 were transiently expressed in onion epidermal cells and their subcellular localizations analyzed by confocal microscopy. (A) GFP-LARP1 was expressed and analyzed alone under unstressed (t0) or stressed (t+1) conditions. The unstressed and stressed states are shown for the same cell. (B) tagRFP-LARP1 (tRFP-LARP1) was coexpressed with GFP-RBP47 and analyzed at two time points during stress (t and t+1) in the same cell. (C) tagRFP-LARP1, YFP-DCP1, and Cer-XRN4 were coexpressed in the same cells and their subcellular localizations analyzed under stressed conditions. Colored arrows point to foci showing no colocalization and white arrows or stars point to foci where colocalization is observed. See also Figure S5.

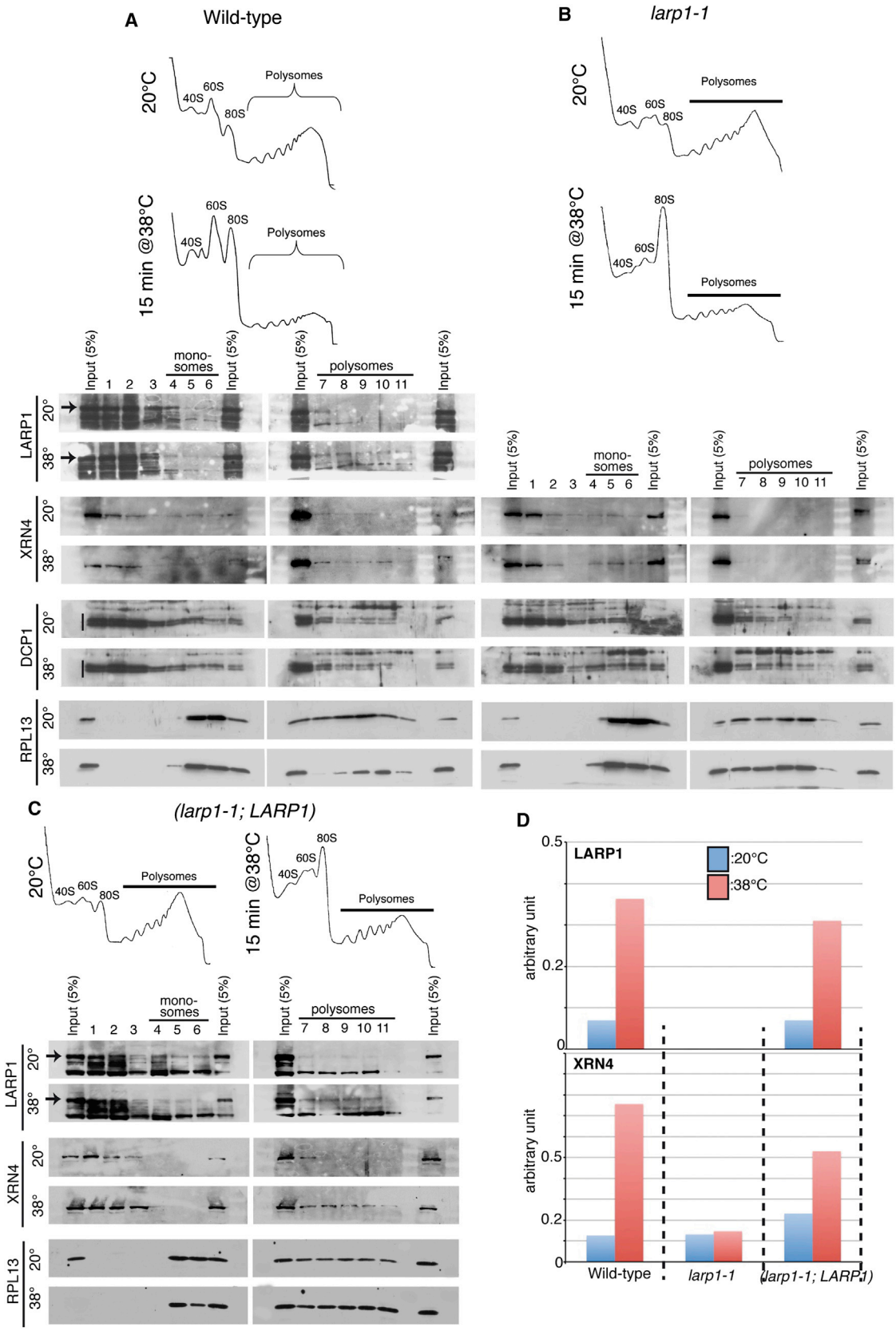
the levels of 25% of their transcripts. We observed that, after a short heat stress, more than 4,500 mRNAs are downregulated between 2- and 125-fold (mean of 3-fold), with 0.34% of them affected 2-fold and 87.7% affected between 2- and 5-fold (Table S1). We readily demonstrate that for all of the eight randomly chosen mRNAs tested, this is partly due to an increase in their decay rates at 38°C. Previous studies conducted in cell suspension (Narsai et al., 2007) and seedlings (Gutierrez et al., 2002) suggest that in *Arabidopsis*, the most unstable transcripts have half-lives of 12 to 30 min at 20°C. Therefore, even for these

Figure 3. Heat Stress Affects Steady-State Levels and mRNA Half-Lives in a LARP1- and XRN4-Dependent Manner

(A) qRT-PCR analyses of the fold variations between 38°C and 20°C ($F = q^{38°C}/q^{20°C}$) for 11 (distinct from those shown in Figure 1) transcripts targeted by heat stress and one heat-stable mRNA (At1g47128). Transcripts randomly chosen are of different size and abundance and code for factors of diverse molecular functions (such as transcription factors, kinase, protease, and sugar-modifying enzyme) involved in various biological processes (such as cell differentiation, organ growth, auxin response, organelle biogenesis, and cell death). Analyses were conducted as in Figure 1 but on different biological samples.

(B) mRNA decay under heat stress. WT (gray curves), *larp1-1* (blue curves), or *xrn4-5* (orange curves) plants were transferred to liquid medium containing the transcriptional inhibitor actinomycin D (actD) and incubated at 20°C or 38°C for 0, 15, 30, 45, and 60 min. Percentages of remaining mRNAs (relative to the zero time point) are plotted against time on a semilogarithmic scale. mRNA levels were assessed by qRT-PCR using Actin 7 as a reference.

Data are mean ± SD of three independent biological replicates and two qPCR technical replicates. See also Figure S4 for the analysis of other transcripts.



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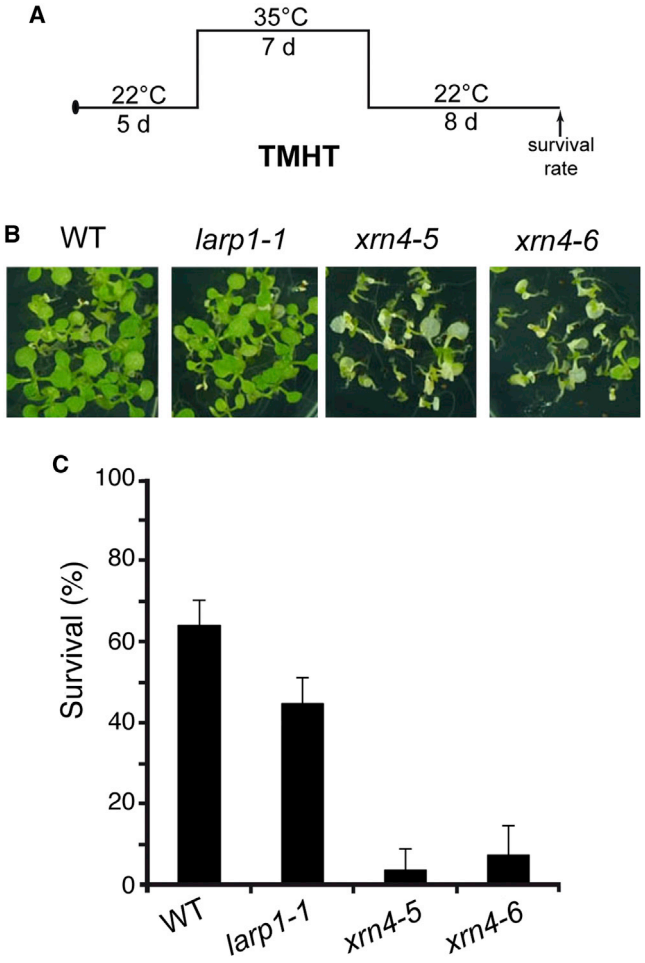

Figure 6. XRN4- and LARP1-Depleted Plants Are Impaired in Their Thermotolerance to Moderately High Temperature

(A–C) *xrn4* (*xrn4-5*, *xrn4-6*), *larp1* (*larp1-1*) *null*, and WT plants were challenged for their ability to engage their TMHT by exposing them to temperature variations as pictured in (A). After 8 days of recovery, plants were observed (B) and numbers of surviving plants reported as percentage of the total number of seedlings (C). The presented histograms display mean values calculated from four independent experiments, each conducted with 30 plantlets.

rare unstable transcripts, a complete transcriptional shutdown followed by decay could not explain a more than 2-fold reduction after 15 min at 38°C unless decay rates are increased by the heat stress. Hence, we propose the existence of a heat-mediated mRNA decay mechanism in plants affecting thousands of mRNAs. To date, such a phenomenon has been suggested to exist in yeast and mammalian cells (Castells-Roca et al., 2011a; Fan et al., 2002; Grigull et al., 2004), but the molecular mechanism by which transcripts are degraded and its physiological importance for stress acclimation remained unaddressed until now.

The animal LARP1 homolog is an mRNA binding protein (Castello et al., 2012) that could function in the stability control of at least some worm transcripts (Nykamp et al., 2008). LARP1 proteins are very highly conserved among eukaryotes and were previously suggested to share evolutionary conserved functions (Bousquet-Antonelli and Deragon, 2009). Consistent with a direct role of the *Arabidopsis* LARP1 in mRNA turnover, we found that its loss of function allows an increase of the fold ($q^{38°C}/q^{20°C}$) of every heat-targeted transcript and induces a decrease in mRNA decay rates at 38°C. Nevertheless, although we found LARP1 to be a general factor involved in the downregulation of several thousand transcripts in response to environmental variations, it is more likely to act as a cofactor in the heat-mediated mRNA turnover rather than as a catalytic *trans*-acting factor, because it carries no enzymatic domain (Bousquet-Antonelli and Deragon, 2009). We found that LARP1 forms an RNA-independent, heat-triggered complex in vivo with the 5′-3′ cytoplasmic exoribonuclease XRN4, which, together with the fact that they are Y2H partners, strongly suggests that they are directly interacting. While previous genome-wide analyses identified less than 100 transcripts as XRN4 baits (Estavillo et al., 2011; Rymarquis et al., 2011; Souret et al., 2004), we readily show that each of the 23 randomly chosen baits of LARP1 are also targeted by XRN4 in a heat situation. LARP1 is found in the cytoplasm, where it colocalizes with XRN4 in some p-bodies and in polysomes. LARP1 is required at least in part to address XRN4 to polysomes, a putative site of the heat-induced mRNA decay. For all these reasons, we postulate that LARP1 is a cofactor of XRN4 involved in the heat-dependent mRNA decay process.

XRN4 loss of function allows an increase of the fold ($q^{38°C}/q^{20°C}$) of heat-targeted transcripts and induces a decrease in mRNA decay rates at 38°C. Transcript stability at 38°C is restored to that at 20°C for every tested transcript in the absence of XRN4. Conversely, while LARP1 is likely involved at 38°C in the decay of most if not all of the heat-targeted mRNAs, its inactivation does not always fully restore their 20°C decay rates. We propose that the heat-mediated decay is largely accomplished by a 5′-3′ degradation process catalyzed by XRN4 and that it systematically involves various levels of LARP1 function. The mRNA-specific, fluctuating importance of LARP1 could indicate that two (or more) distinct heat-mediated 5′-3′ decay pathways exist, with both requiring XRN4 but only one requiring LARP1.

Figure 5. LARP1 Is Found on Polysomes and Is Involved in XRN4 Polysomal Targeting

(A–C) Polysomal extracts prepared from (A) WT plants, (B) *larp1-1* mutants, or (C) *larp1-1* mutant expressing a transgenic LARP1 (*larp1-1; LARP1*) incubated for 15 min at 20°C or 38°C were fractionated on a sucrose gradient into 11 fractions. Total proteins extracted from each fraction were analyzed by western blot with 5% of input, 10% of fractions 1–3 (free mRNAs), and 100% of fraction 4–6 (monosomes) and 7–11 (polysomes) loaded on gel. Blots were probed with antibodies against LARP1, XRN4, DCP1, and RPL13 (ribosomal proteins of the large subunit). Polysome profiles obtained by continuous UV_{254nm} absorption measurement during gradient collection are shown above western blot analyses.

(D) Quantitation of the chemiluminescence of LARP1 and XRN4 western blot signals relative to RPL13 signal in polysomal fractions (fractions 7–11) under unstressed (blue bars) and heat-stressed (red bars) conditions in WT, *larp1-1*, or (*larp1-1; LARP1*) plants.

See also Figure S6.

Although, in *xrn4* null plants, 38°C mRNA turnover rates are restored back to the ones at 20°C, this is only rarely the case for steady-state levels. This discrepancy suggests that the downregulation process does not solely depend upon a post-transcriptional mechanism, but rather involves a transcriptional activity decrease upon heat stress, which is not unexpected. The reason why the fold of a few transcripts (5 out of the 23 tested) in the *xrn4-5* mutant is above 1 (i.e., higher mRNA levels are observed at 38°C compared to 20°C) is not clear, but one possibility could be that XRN4 is not only involved in decaying these targets at 38°C (as directly proven for At4g14400 and At5g49480; Figures 3 and S4) but also indirectly participates in their heat-specific transcriptional shutdown.

Modulation of mRNPs subcytoplasmic localization is believed to play a role in their posttranscriptional regulation. mRNPs move between a productive translationally active polysomal state and a ribosome-free state, as such or in aggregates, where they are either degraded or stored. Recently, studies in budding yeast showed that the 5′-3′ decay can also take place while mRNPs are still engaged with elongating ribosomes (Hu et al., 2010, 2009), and several actors of the decapping and 5′-decay processes were consistently found to be associated with polysomes (Mangus and Jacobson, 1999). We report here that some key players of the *Arabidopsis* decapping holoenzyme (DCP1 and DCP2), XRN4, and its cofactor LARP1 associate with polyribosomes, suggesting that at least a subset of the plant mRNPs could also be degraded while elongating in translation as previously postulated (Sement et al., 2013). Strikingly, while upon stress the level of polyribosomes dramatically decreased, the detected amounts of these proteins in the polysomal fractions increased, consistent with the idea that cotranslational decay upon heat sensing could be more efficient and affect a wider range and/or a larger pool of elongating transcripts. Our analyses suggest that XRN4's association with mRNPs engaged in translation is LARP1 dependent and that part of LARP1 function as a cofactor would therefore be to target XRN4 to one of its mRNA degradation sites. LARP1 subcytoplasmic redistribution upon stress is complex, because in addition to being more associated with polysomes and colocalizing in some p-bodies with XRN4, it is mostly addressed to SGs. This points to a dual role for LARP1 in response to stress and opens the possibility (which we are currently exploring) that it could also target mRNAs or/and proteins to SGs. In any case, our results indicate that LARP1's contribution to the heat-mediated decay could be mostly cotranslational. We estimate, however, that the global contribution of the cotranslational decay following heat stress is not globally prominent, because the amount of decapping holoenzyme (DCP1/DCP2) and XRN4 exoribonuclease detected in polysomes, even at a higher temperature, is only a small fraction of their total cellular pools, suggesting that these enzymes mostly act on ribosome-free mRNPs. Moreover, under heat stress, LARP1 loss of function only allows the partial restoration of mRNA stability, indicating that only a portion of the destabilized pool is degraded cotranslationally.

Because the XRN nucleases are intrinsically unable to digest 5′-protected transcripts (Jinek et al., 2011), the 5′-3′ heat-mediated mRNA decay has to be preceded by a cap-excision event. In eukaryotes, cytoplasmic mRNAs can be 5′ degraded by various pathways, which are initiated either by deadenylation-dependent or deadenylation-independent decapping (mostly catalyzed by DCP1/DCP2/HEDLS) or by an endonucleolytic (small RNA guided or not) cleavage (Nagarajan et al., 2013). We found that as it is the case for LARP1 and XRN4, more of the DCP1/DCP2 decapping complex is found associated with polysomes in a heat stress situation, suggesting that the heat-mediated 5′-mRNA decay could involve decapping. We focused our studies on poly(A) plus transcripts (RNA-seq experiments were conducted on oligo[dT] affinity-purified messengers and qPCRs on oligo[dT_{18}] reversed-transcribed cDNAs) and yet found that they are targeted by heat stress to XRN4-mediated digestion. Previously published genome-wide studies that were conducted on oligo(dT)-purified transcripts identified less than 100 mRNAs upregulated by *xrn4* loss of function (Estavillo et al., 2011; Rymarquis et al., 2011; Souret et al., 2004). This suggests that, under normal conditions, only a very limited set of polyadenylated mRNAs are XRN4 decayed. Conversely, our data support the idea that upon heat stress, polyadenylated mRNAs could be much more widely addressed to the 5′ degradation mechanism following decapping and/or endonucleolytic cleavage. Although the deadenylation-dependent decapping process is the main 5′-decay pathway in budding yeast (Parker, 2012), the contribution of distinct pathways to the decay of a given pool of transcripts is variable in other organisms (Rissland and Norbury, 2009). The level of contribution of each pathway to mRNA decay in plants is not known at present, but our work suggests that the deadenylation-independent pathway could be largely enhanced in the early phase of the HSR.

Commensurate with their respective involvement at the molecular level, LARP1 and XRN4 are necessary for the thermotolerance of plants to long exposure to moderately high temperature (TMHT response); *larp1* null plants are only very slightly impaired in their survival after heat stress, whereas *xrn4* mutant plants almost completely die. The consistency between the molecular requirement of these two proteins in the heat-mediated decay and their physiological importance in a heat stress situation makes it tempting to speculate that plants might need to be able to degrade polyadenylated transcripts upon temperature increase to be able to properly set a TMHT response. It is, however, not clear why the accumulation of mRNAs devoid of a cap structure and hence translationally incompetent in *xrn4* null plants would dampen the necessary cellular metabolism alterations en route to plant acclimation to heat. Some recent studies suggest that 5′-monophosphorylated transcripts could be turned back to translationally competent forms by cytosolic recapping (Mukherjee et al., 2012; Otsuka et al., 2009; Schoenberg and Maquat, 2009). Recapping is likely to have a physiological importance across development or in stress recovery processes, as for example in mammals, where impediment of cytoplasmic capping alters the ability of the cells to survive an arsenite stress, perhaps because cap readdition is necessary to allow specific transcripts that were stored to re-enter translation and participate in the stress-recovery processes (Otsuka et al., 2009). We found that in the early stages of heat stress, around 25% of the transcriptome is downregulated, in part via an accelerated decay process. This finding is not mutually exclusive from the fact that upon heat, some of the mRNAs that exit

translation can be stored either in SGs or in p-bodies. One possibility to explain the TMHT phenotype would be that upon XRN4 inactivation, transcripts that are not degraded upon heat stress could enter a recapping process that would allow them to turn back to translation and compete with those mRNAs (either stored or heat induced) needed for the thermotolerance response.

EXPERIMENTAL PROCEDURES

Vectors, *Arabidopsis* Lines, and Growth Conditions

Transient assays and stable transgenic lines were done with the coding regions of LARP1 (At5g21160.1), XRN4 (At1g54490.1), or DCP1 (At1g08370.1) obtained by PCR on total cDNAs. The LARP1 promoter region was PCR amplified from total genomic DNA and begins at position −1,682 from the start codon. For onion transient assays, LARP1 and XRN4 were fused 3′ to the fluorescent tag in pSITEII-6C1 or pSITEII-2C1 (Martin et al., 2009), DCP1 in plasmid pEarleyGate-104N (Earley et al., 2006), and the 35S-GFP-RBP47 cassette (Weber et al., 2008) was subcloned in pPZP221 (Hajdukiewicz et al., 1994). Table S2 shows the list of primers used. The *larp1-1* (SALK_151251) mutant was obtained from the NASC Stock Center (see Figure S1), and the *xrn4-5* and *xrn4-6* lines were from Souret et al. (2004). The GFP-LARP1 fusion is stably expressed from a CaMV 35S promoter in a WT (Col-0) background and LARP1 is stably expressed from its own promoter region in the *larp1-1* mutant background.

Analyses were carried out with 21-day-old whole plantlets grown on synthetic Murashige and Skoog (MS) medium (Duchefa) containing 1% sucrose and 0.8% plant agar at 20°C under continuous light. Heat stress was carried as followed: six to eight plantlets were transferred to 5 ml liquid MS medium-1% sucrose prewarmed at 38°C (heat stressed plants) or at 20°C (control plants). Heat-stressed plants were incubated 15 min at 38°C while control plants were kept at 20°C for 15 min. Plants were harvested and immediately frozen in liquid nitrogen. BT, SAT, LAT, and TMHT tests were performed according to Hu et al. (2012). For TMHT, 7-day-old plants were shifted at 35°C for 7 days (photoperiod of 18 hr light/6 hr dark) and their survival rates assessed after an 8-day recovery at 22°C with the same photoperiod.

Total RNA Extraction, RNA-Seq, qRT-PCR Analyses, and mRNA Half-Life Determination

Total RNA was isolated from liquid nitrogen pulverized tissues as described in Logemann et al. (1987). cDNA library preparation on poly(A) plus transcripts, high-throughput sequencing, quality control, and bioinformatic analyses of the RNA-seq were performed by Fasteris Life Sciences (http://www.fasteris.com). For further details, see Supplemental Experimental Procedures.

For northern blot analysis, 10 μg of total RNA was resolved on a 1.2% agarose denaturing gel and transferred to a Hybond NX membrane (GE Healthcare). Hybridization was performed with random-primed ^{32}P-labeled (MegaPrime Kit, GE Healthcare) specific probes (amplified by PCR with primers described in Table S2) in Ambion ULTRAhyb (Life Technologies) buffer. For qRT-PCR analyses, total RNA was treated for 3 hr at 37°C with RQ1 DNase (Promega), which was subsequently removed by phenol-chloroform extraction. Reverse transcription was conducted on 3 μg DNase-treated RNA using the GoScript RT-system and an 18-nt-long oligo(dT) (Promega). Real-time PCR was performed in a LC 480 384-well thermocycler (with the cycling program 5 min 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C) followed by the generation of a dissociation curve to check for amplification specificity. The PCR mix contained Takyon qPCR master mix (Eurogentec), 500 nM gene-specific primers, and 1.5 μl cDNA in a total volume of 9 μl. Primer efficiencies were close to 100% and calculated on standard curves generated using a 4-fold dilution series of all cDNAs over at least five dilution points measured in duplicate. See Table S2 for primer sequences, amplicon sizes, and primer efficiencies. To assess mRNA half-lives, 3-week-old plantlets were transferred to MS medium-1% sucrose-0.6 mM cordycepin (Sigma-Aldrich) or 200 μg/ml actinomycin D (Sigma-Aldrich) medium and incubated at either 20°C or 38°C for up to 60 min. Plantlets were collected at various time points following the shift in transcriptional inhibitor-containing medium and quickly harvested in N2. Total RNA was extracted and mRNA levels were assessed by qRT-PCR analyses. The percentages of remaining mRNAs calculated relative to the zero time point value were plotted against time and a regression curve obtained.

Western Blotting

After electrophoretic separation by SDS-PAGE gels, proteins were electrotransferred on polyvinylidene fluoride membranes. Immunoblottings were performed in TBS-5% skimmed milk-Tween with horseradish peroxidase -coupled secondary antibodies and revealed with the Immobilon-P kit from Millipore. Antibodies against ACTIN (Thermo Fischer) and GFP (Clontech) were purchased and utilized according to the manufacturers' instructions. The anti-DCP1 (Weber et al., 2008) and RPL13 antibodies (Saez-Vasquez et al., 2000) were utilized at 1/2,000th and 1/100,000th, respectively. Antibodies against XRN4 and LARP1 were produced in rabbits using the Eurogentec double X immunization program. The XRN4 protein was detected using the serum affinity-purified against peptide 1 (H_2N-IYQAKTQPQHRGAN) at 1/500th dilution; the LARP1 protein was detected either with serum purified against the C-terminal peptide (pep1: H_2N -RAKTITNQKENKSH) or with serum purified against the N-terminal peptide (pep2: H_2N -MAETEGSVADDRELI) at 1/250th dilution.

Subcellular Localization Experiments

Onion epidermal cells were transiently transformed using a Biolistic PDS-1000/He particle delivery system (Bio-Rad) according to the manufacturer's instructions. Onion cells were transformed or cotransformed with vectors as indicated in the figure legends. Then 16 to 24 hr after bombardment, the onion epidermis was peeled off and analyzed with a LSM710 Zeiss confocal microscope. A total of 2 to 4 hr after placing the onion epidermis on slide, a hypoxia-derived stress state was monitored as previously reported (Merret et al., 2013; Weber et al., 2008).

Polysome Extraction and Analysis

Polysome extraction was performed as described previously (Mustroph et al., 2009). A total of 1 g N2 pulverized tissue was incubated on ice with 3 vol polysome extraction buffer. After clarification by centrifugation and miracloth filtering, 1.2 ml of polysome extract (400 mg equivalent tissues) was loaded on a 15%–60% sucrose gradient (9 ml) and centrifuged for 2.5 hr at 18,400 rpm with rotor SW41. Polysome profile analyses were performed with an ISCO absorbance detector at 254 nm and sucrose gradient collected into 11 fractions of 600 μl each. To extract proteins, each fraction was added with 2 vol 100% ethanol and incubated 6 hr at 4°C before centrifugation. Proteins were subsequently resuspended in 4× Laemmli buffer, incubated 5 min at 95°C, resolved on SDS-PAGE gel, and analyzed by western blotting. To quantify LARP1 and XRN4 signal intensities, blots loaded with 5% of input and 100% of each polysomal fraction (fractions 7–11) were prepared from the three genetic backgrounds (WT, *larp1-1*, *[larp1-1; LARP1]*) cultivated in two temperature conditions (six blots) and hybridized with the LARP1, XRN4, and RPL13 antibodies. All blots were prepared and immunoblotted in parallel and exposed simultaneously in a ChemiDoc (Bio-Rad) device to quantify chemiluminescence. Pictures were acquired every 1 s until appearance of the first saturating signal. Quantitation was subsequently performed on the last picture taken before the saturating point in each polysomal fraction. On each blot and respectively for LARP1, XRN4, and RPL13, polysomal signals (from fractions 7–11) were summed up and corrected to their respective input signal. Finally, for each temperature-genetic condition, LARP1 and XRN4 input-normalized values were divided by the RPL13 input-normalized value from the same blot.

Y2H Screen

A Y2H screen was performed on a 7-day-old *Arabidopsis* seedling cDNA library with the full-length LARP1 protein encoded by the At5g21160.1 cDNA as bait. The screens, preys recovery, and subsequent bioinformatics analyses were subcontracted at Hybrigenics (http://www.hybrigenics.com/).

Immunoprecipitations

After grinding in liquid nitrogen, tissues were homogenized in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 5 mM $MgCl_2$, 1% NP40,

10% glycerol, 1 mM phenylmethanesulfonylfluoride, 40 μM MG132, 1% of protease inhibitor cocktail [P9599]; Sigma-Aldrich). The ratio (ground tissues)/(volume of lysis buffer) was always 300 μl of buffer per 100 mg of tissue. Extracts were clarified by two successive centrifugations at 4°C and 14,000 rpm for 15 min. The immunoprecipitation was conducted with the μMACS GFP isolation kit (Miltenyi Biotec) with 600 μl of crude extract treated with 50 μl of anti-GFP beads according to the manufacturer's protocol. For RNase treatment, 750 μl of crude extract treated with 50 μl of anti-GFP beads for 30 min at 4°C was divided into three 250 μλ samples that were respectively mock, RNase A/T1 (2.5 mg of A, 7,000 U of T1), or RNase I (100 U) (Thermo Scientific) treated for 30 min at 4°C. A total of 2% of the input and 40% of the bound (eluate) fractions were analyzed by western blotting.

ACCESSION NUMBERS

The NCBI Gene Expression Omnibus accession number for the RNA-seq data reported in this paper is GSE51879.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.11.019.

ACKNOWLEDGMENTS

We would like to thank the following people for generous gifts of plasmids, antibodies, and plant lines: M. Fauth (JW Goethe University, Frankfurt), M. Goodin (University of Kentucky), P. Green (University of Delaware), H. Vaucheret (INRA, Versailles), J. Saez-Vasquez (LGDP, University of Perpignan-CNRS), and L. Sieburth (University of Utah). We also thank Thierry Lagrange for critical reading of the manuscript and the Montpellier RIO Imaging platform for access to confocal microscope. R.M. was the recipient of an Université de Perpignan (UPVD) short-term fellowship followed by an ANR fellowship. This work was supported by l'Agence Nationale pour la Recherche (ANR-2010 BLANC 1707 01), the CNRS, and the UPVD through utilization of the confocal microscope, qPCR, and Chemidoc devices at the Technoviv platform.

Received: March 26, 2013
Revised: October 8, 2013
Accepted: November 9, 2013
Published: December 12, 2013

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