Heat-induced ribosome pausing triggers mRNA co-translational decay in *Arabidopsis thaliana*

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES LEGEND

Figure S1: Representation of the experimental procedure for seedlings treatment, RNA preparation and sequencing and RNA-seq bioinformatic analysis.

Figure S2: (A-H) Correlation of expression levels between replicates in input (A, B, E, F) or polysomes (C, D, G, H). X and Y axis correspond to read counts in rpkm (read per million per kilobases). (I-L) Correlation of fold (F=q38°/q20°) between replicates in input (I, K) and polysomes (J, L).

Figure S3: (A, B) Impact of XRN4 depletion on polysomal levels at 20°C (A) and 38°C (B) for mRNAs found to be downregulated more than twofold by heat in wild-type polysomes. We compared $q^{xrn4\text{-}5\text{pol}}$ to q^{WTpol} by calculation of the $\log_2$ value of their ratio and represented the results as a graph. (C) Venn diagram representation of the number of genes found to be downregulated at least twofold by heat in total or input RNA from wild-type seedlings by RNA-seq analyses reported in (1) (T1, in blue) and in the present study (T2, pink). To assess the significance of the overlap, a p value was calculated testing the hypergeometric distribution with the R package, ($p=\Sigma(m,i)/(N\text{-}m;n\text{-}i)/(N,n)$ N=19,200 (total number of genes), m=4433 (genes down in T1), n=3999 (genes down in T2) and i=2333 (gene in the intersection)). (D, E) Impact of XRN4 depletion on mRNA levels in input at 20°C (D) and 38°C (E) for transcripts found to be downregulated by heat in wild-type input. We compared $q^{xrn4\text{-}5\text{inp}}$ to q^{WTinp} by calculation of the $\log_2$ value of their ratio and represented the results as a graph. (F) qRT-PCR analyses of Fold ($q^{38°}$/ $q^{20°}$) variations for randomly chosen mRNAs from class I, II or III in wild-type (solid bars) or *xrn4-5* (hatched bars) in input (pink bars) or polysomes (green bars). qRT-PCR

values were normalized to ACTIN 7 (At5g09810) and calculated as $q=2^{(CtRef-CtGene)}$. Values reported are the mean values of the folds, respectively, calculated from three biologically independent experiments. Standard Deviations are shown.

Figure S4: Splint Ligation(SL)-RT-PCR assays. (A) Accumulation of decapped intermediates were tested for two class I (At3g44550 and At3g48410) and one class III (At3g62930) mRNAs. Assays were conducted on input (lanes 1 to 4) and polysomal (lanes 5 to 8) RNAs prepared from the same crude extract from seedlings (wild-type or *xrn4-5*) either control (20°C, odd lanes) or heat-treated for 30 min at 38°C (even lanes). From a same preparation, RNAs were either mock treated (Splint assay) (lanes 1 to 8) or TAP treated (TAP) (lanes 9 to 16) before SL-RT-PCR, or ligase was omitted during the splint ligation step (no ligase) (lanes 17 to 24). After SL, RTs were primed with Gene Specific Primers (GSP). Subsequently PCR were conducted with primers Pa and P2 or P1 and P2. (B) Accumulation of decapped intermediates for class III At5g49480 in Light (L) and heavy (H) *xrn4-5* polysomal fractions either at 20°C (lanes 1, 2) or in a time-course experiment at 38°C (lanes 3-8). Samples were identical to those used in Figure 3E. Red arrows mark the positions of the specific PCR products.

Figure S5: (A, B) Gene Ontology (Cellular Component category) analysis conducted on products of class III (A) and class I (B) genes. Analysis was conducted and represented with the AgriGO tool kit (http://bioinfo.cau.edu.cn/agriGO/index.php) (2) and performed with the list of genes detected in the present RNA-seq analysis as custom reference. (C) Alignment of the protein sequences between human HSP70 and HSC70 and Arabidopsis HSC/HSP70 proteins. HsHSC70 (NP_006588), HsHSP70 (NP_005337), AtHSP70 (At3g12580), AtHSP70b (At1g16030), AtHSC70-1 (At5g02500), AtHSC70-2

(At5g02490) and AtHSC70-3 (At3g09440). Sequences were aligned using the multiple sequence comparison by log-expectation (MUSCLE v3.7) software (3). Alignment shading was obtained with Jalview and represents percent identity. (D) Enlargement of the region corresponding to the ATPase domain (squared in red on C) of HSC/HSP70 proteins. Amino acids found to be involved in VER-155008 binding are highlighted in red. VER-155008 inserts into the ATP binding pocket between R272 and R342 and forms direct or indirect hydrogen bounding with S275, K271 and D234 (4). (E) Monitoring of Stress Granule formation induced by VER-155008 exposure. 5 day old seedlings stably expressing tRFP-PAB2 were either mock treated (60 min in DMSO and 60 min in MS), exposed to Ver-155008 (60 min in 100μM VER) or to 38°C (60 min @ 38°C). The tRFP-PAB2 subcellular distribution was observed in seedlings root tips by confocal microscopy.

Figure S6: (A) Monitoring of decapped species accumulation by SL-RTPCR for the class III gene At5g49480 after VER155008 treatment. Samples are the same as those utilized for Figure 4D-G. (B) Negative "No ligase" control of the SL-RTPCR analyses presented in Figure 4D-G.

SUPPLEMENTAL METHODS

Genome wide RNA-sequencing analyses

Crude polysomal extracts were prepared from 21 day old whole seedlings either wild-type (WT) or *xrn4-5* treated as reported in the experimental procedure. After stress treatment seddlings were harvested, frozen and pulverized in liquid N_2. RNA were extracted from crude polysomal extract (Input) and pooled polysomal fractions 10 to 15 (Polysomal) as described in the experimental procedure section. RNAs were prepared

from two independent biological replicates, one replicate corresponding to: WT and *xrn4-5* seedlings sown, grown and stressed simultaneously. RNAs were DNase treated with Ambion Turbo DNase kit (Life technology) and their quantities and qualities assessed both by agarose gel electrophoresis and ethidium bromide staining, nanodrop absorbance measurement at 260 and 280 nm and Agilent 2100 bioanalyser.

Total RNA (15 µg) was enriched for poly(A) RNA using the Absolute mRNA purification kit (Agilent Technologies). RNA-Seq libraries were constructed as per manufacturer's instruction using the TruSeq RNA Sample Prep kit v2 (Illumina). All 16 libraries ($I^{20°WT}$, $P^{20°WT}$, $I^{38°WT}$, $P^{38°WT}$, $I^{20°xrn4}$, $P^{20°xrn4}$, $I^{38°xrn4}$, $P^{38°xrn4}$; each in duplicate) were multiplexed and sequenced in a same channel as single-end 50-nt reads with a HiSeq 2000 instrument.

Bioinformatic analyses

For each library, 10-20 $.10^{E6}$ reads were obtained with 85-90% of the bases displaying a Q-score ≥ 30, with a mean Q-score of 38 as assessed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). After filtering out reads corresponding to chloroplastic, mitochondrial, ribosomal and transfer RNAs with Bowtie 2 (5), reads were mapped against the TAIR 10_genome using gtf annotation file TAIR10_genes_transposon and default parameters of TopHat2 (6). Assembly and transcript quantification were conducted with Cufflinks (7). Finally, we filtered out transcripts which did not have at least 1 rpkm (read per kilobase per million) in wild-type or *xrn4-5* libraries at 20 or 38°C in Input and Polysomes.

Splint-Ligation RT-PCR assay

100 μg of polysomal or input RNA were DNAse treated with the Turbo DNAse (Ambion) according to manufacturer's instructions. For a complete assay with positive (TAP) and negative (no ligase) controls we used three times 20 μg of DNAse treated RNA from a same batch. Two 20μg samples were mock treated and one was TAP treated (Epubio) according to the manufacturer's instructions. Next 15 μg of each sample were added with 20 pmol of DNA splint and 30 pmol of RNA anchor and incubated for 15h at 16°C with 20 U of T4 DNA ligase (Promega) for TAP and one mock treated RNA and without ligase for the second mock treated RNA (no ligase control). DNA splints were removed by a second DNAse treatment. 4 μg of splint ligated and control RNAs were used to gene specific (2μM) reverse transcription using the Superscript III enzyme (Invitrogen). RT was performed with a GSP primer located at the 3' end of the transcript to produce only full length products (see Table S2). PCRs were performed with 1 μl of cDNA with the anchor primer (Pa) as forward and a transcript specific reverse primer (P2). A forward primer (P1) located upstream to P2 on the transcript was used to run a control PCR (P1+P2). We used the RNA anchor described in (8) (ordered as PAGE purified from IBA-GmBH). DNA splints were fully complementary to the anchor at their 5' ends and complementary to the 5' end region of the transcript studied at their 3' ends (ordered as PAGE purified from IBA-GmBH). The mRNA specific part of the splint DNAs were designed to have a Tm above 60°C. See Table S2 for primers sequences.

Confocal microscopy

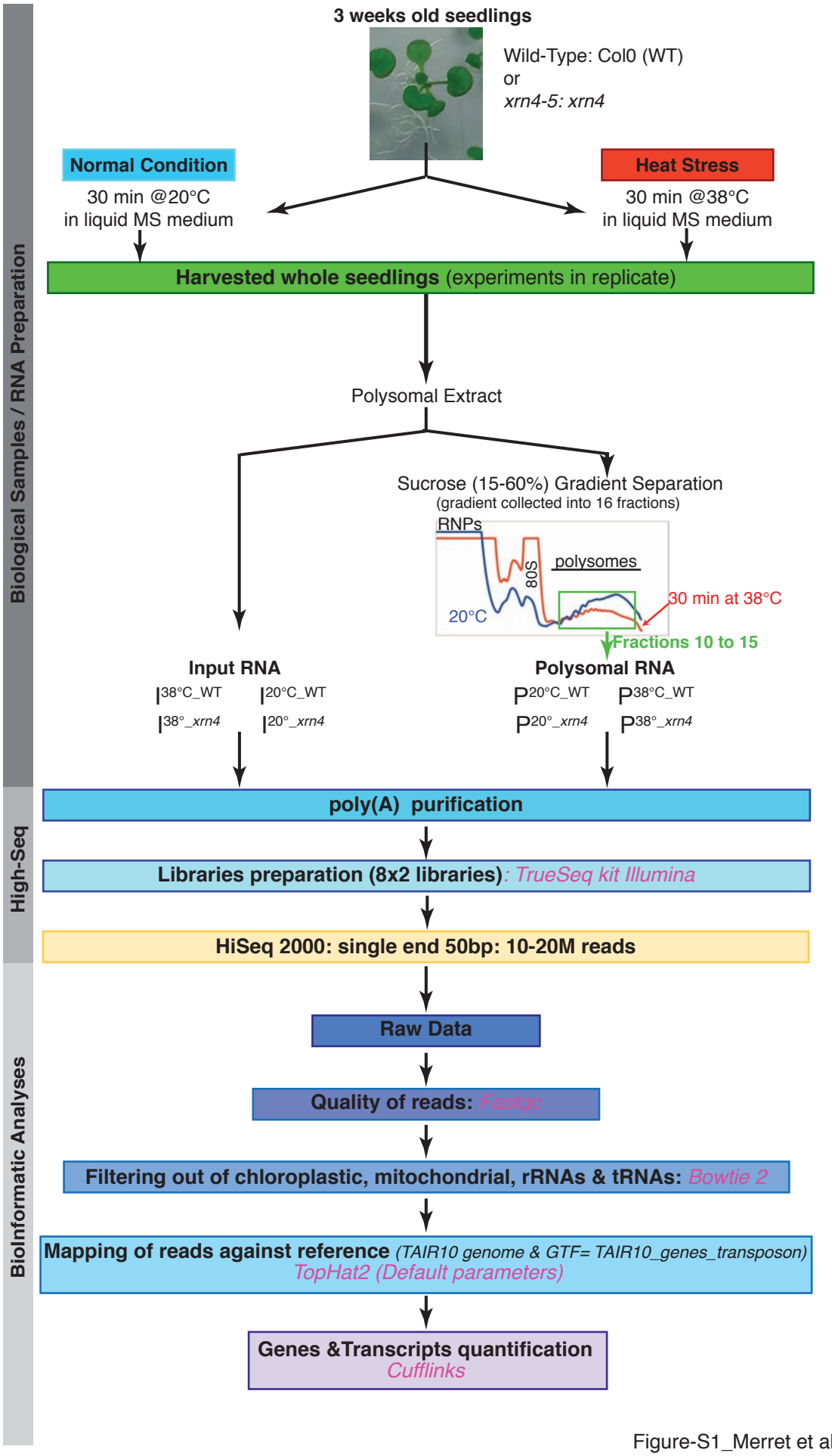
We used 5 day old Arabidopsis seedlings stably expressing the tRFP-PAB2 fusion stress granule marker. This fusion is expressed at endogenous levels from a transgene containing the natural upstream and downstream sequences of the gene, as well as the genomic copy of the coding region (containing both intronic and exonic regions) (JJ.

Favory, J. Descombin, JM Deragon and C. Bousquet-Antonelli, manuscript in preparation). For chemical treatments, seedlings were transfered to MS-1%sucrose liquid medium either containing 0.1% of DMSO (as mock) or 100 μM of VER-155008 for 60 min at 20°C. For heat treatment, seedlings were transfered to MS-1%sucrose liquid medium and incubated for 60 min either at 20°C (as control) or 38°C (as heat stress). After treatment seedlings were fixed in 4% paraformaldehyde in MTSB (50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$ (pH7, KOH), 0.2% Triton X-100 (9) for 10 min in a vacuum, followed by 5 min additional incubation. Samples were washed in MTSB (2 x 5 min) and kept in MTSB at 4°C until observation. Root tips were observed on the AxioObsever. Z1 microscope with the LSM 700 scanning module (Zeiss).

SUPPLEMENTAL REFERENCES

1. Merret, R., Descombin, J., Juan, Y., Favory, J., Carpentier, M., Chaparro, C., Charng, Y., Deragon, J. and Bousquet-Antonelli, C. (2013) XRN4 and LARP1 are required for a heat-triggered mRNA decay pathway involved in plant acclimation and survival during thermal stress. *Cell reports*, **5**, 1279-1293.
2. Du, Z., Zhou, X., Ling, Y., Zhang, Z. and Su, Z. (2010) agriGO: a GO analysis toolkit for the agricultural community. *Nucleic acids research*, **38**, W64-70.
3. Edgar, R.C. (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics*, **5**, 113.
4. Schlecht, R., Scholz, S.R., Dahmen, H., Wegener, A., Sirrenberg, C., Musil, D., Bomke, J., Eggenweiler, H.M., Mayer, M.P. and Bukau, B. (2013) Functional analysis of Hsp70 inhibitors. *PloS one*, **8**, e78443.
5. Langmead, B. and Salzberg, S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nature methods*, **9**, 357-359.
6. Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R. and Salzberg, S.L. (2013) TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome biology*, **14**, R36.
7. Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J. and Pachter, L. (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature biotechnology*, **28**, 511-515.
8. Blewett, N., Coller, J. and Goldstrohm, A. (2011) A quantitative assay for measuring mRNA decapping by splinted ligation reverse transcription polymerase chain reaction: qSL-RT-PCR. *RNA*, **17**, 535-543.

9. Friml, J., Benkova, E., Mayer, U., Palme, K. and Muster, G. (2003) Automated whole mount localisation techniques for plant seedlings. *The Plant journal : for cell and molecular biology*, **34**, 115-124.



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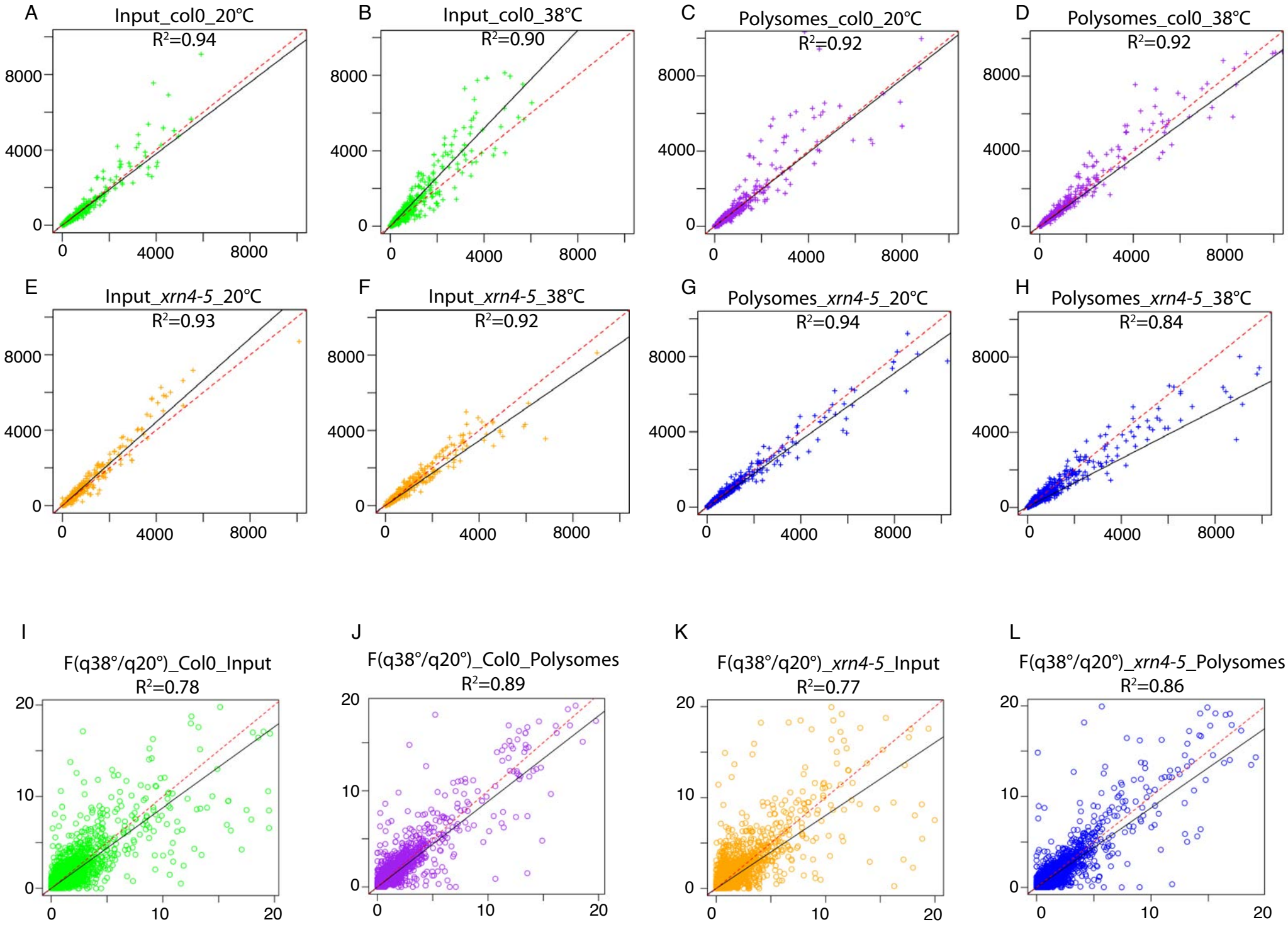

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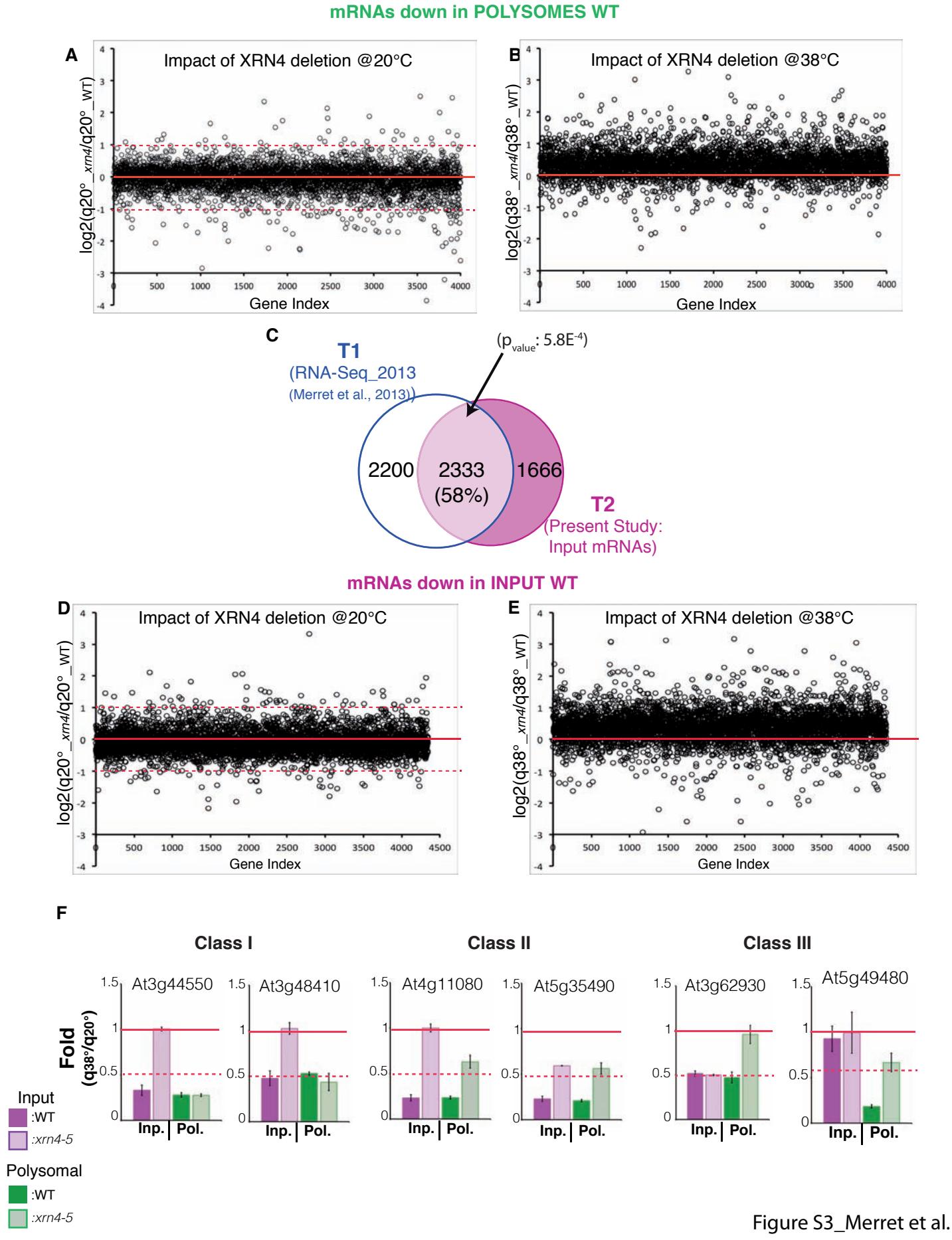

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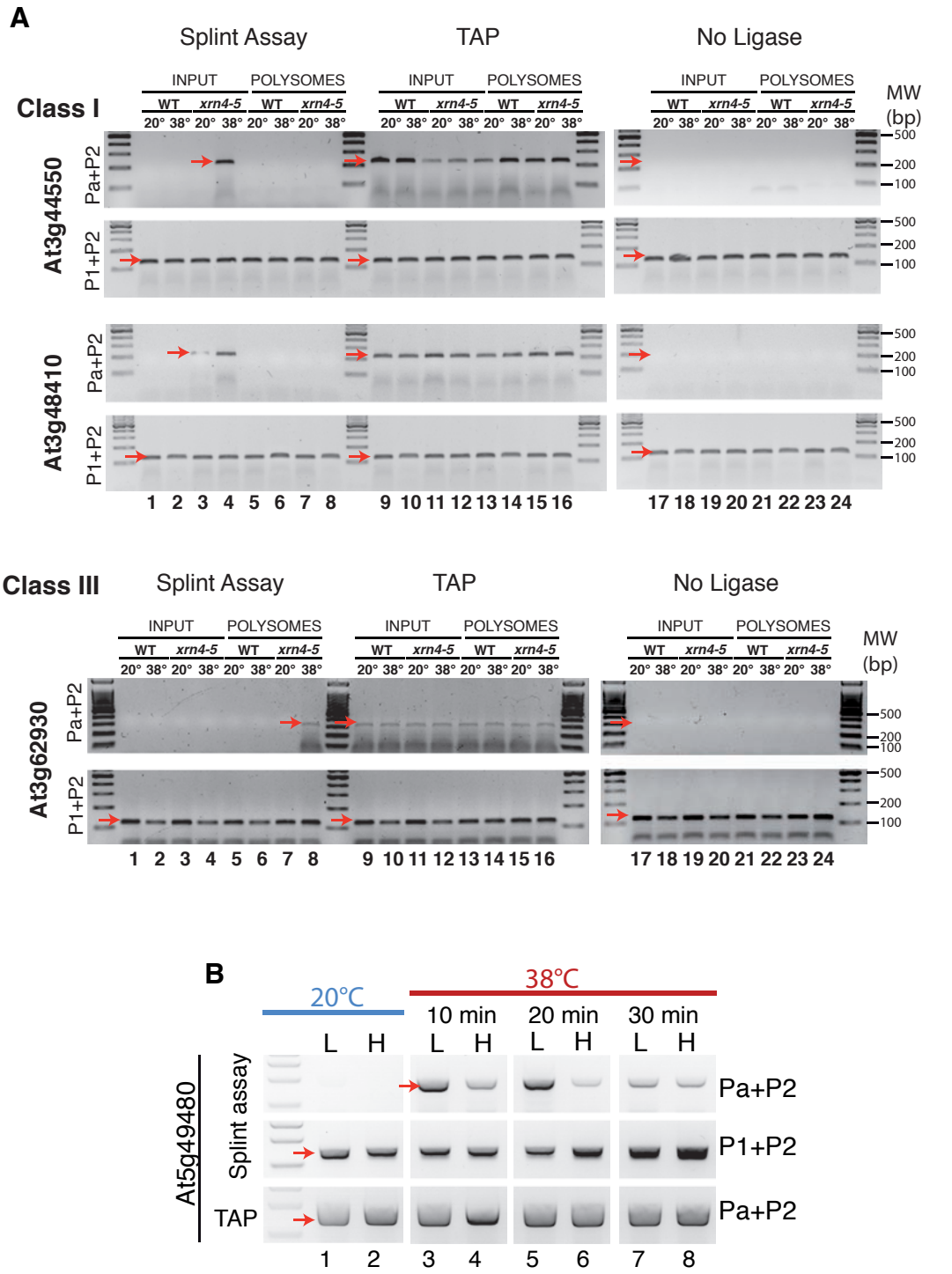

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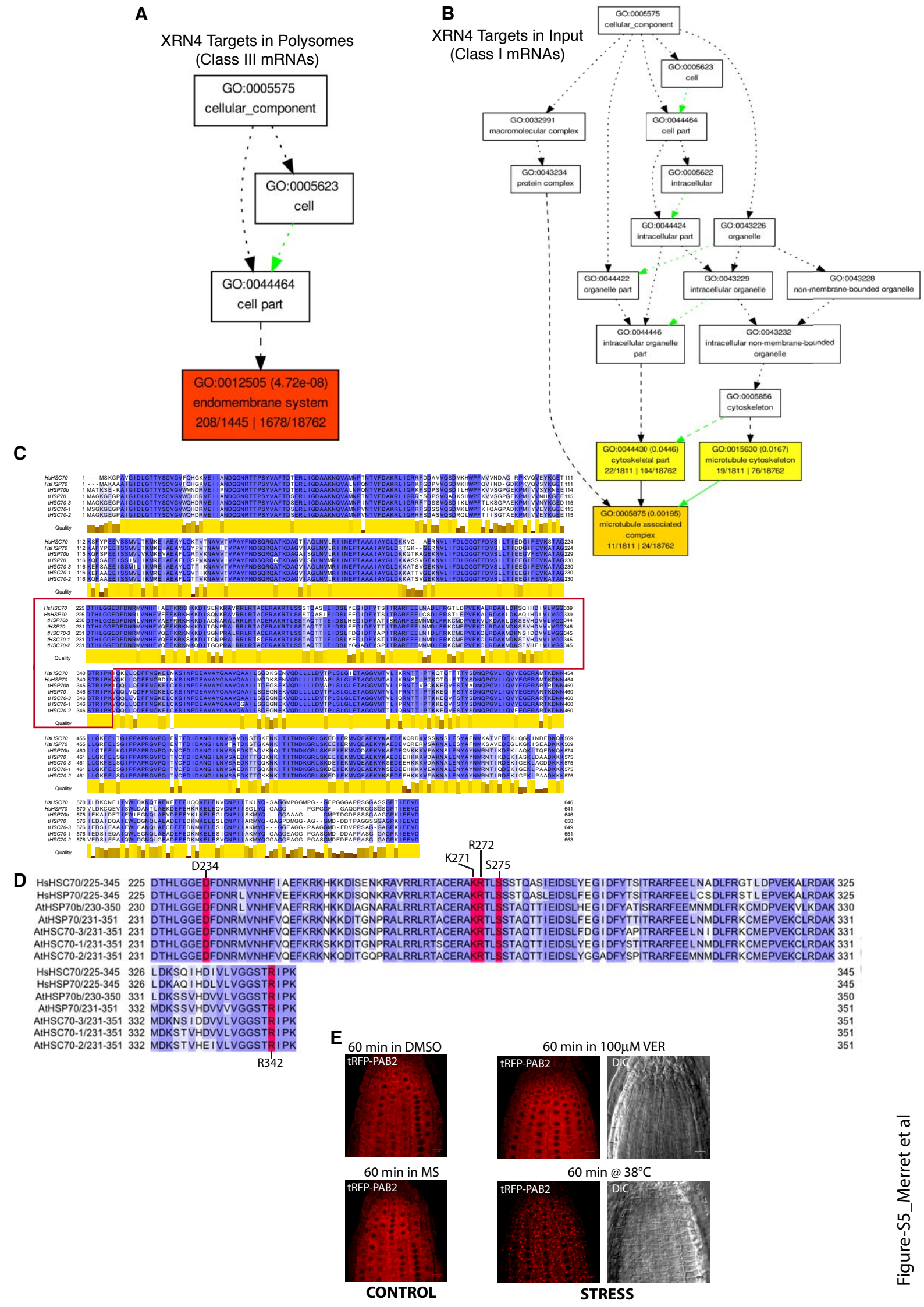

Figure-S5_Merret et al

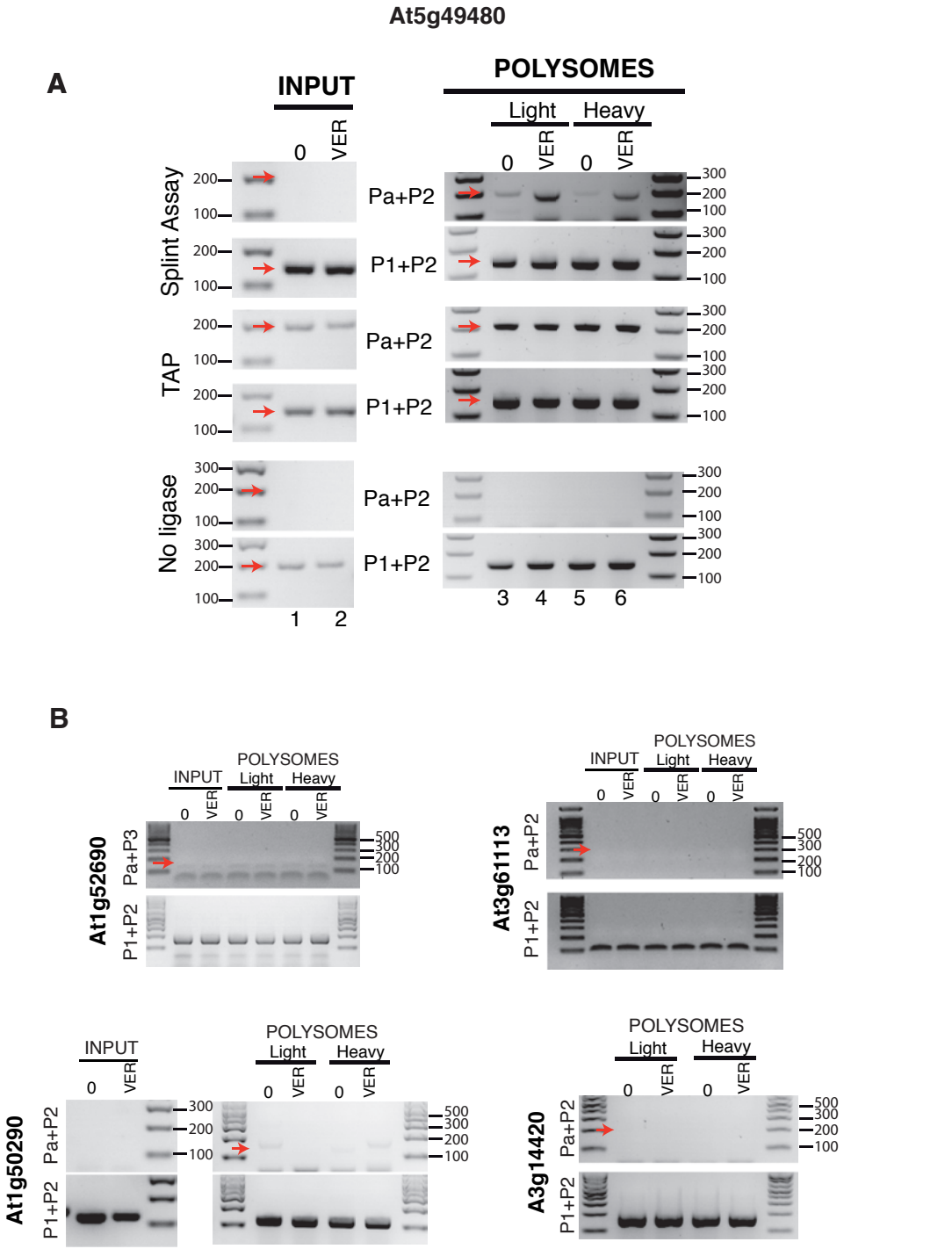

Figure S6_Merret et al.