The effects of heat induction and the siRNA biogenesis pathway on the transgenerational transposition of *ONSEN*, a *copia*-like retrotransposon in *Arabidopsis thaliana*

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Abstract

Environmental stress influences genetic and epigenetic regulation in plant genomes. We previously reported that heat stress activated a *copia*-like retrotransposon named *ONSEN* (Ito et al., 2011). To investigate the heat sensitivity and transgenerational activation of *ONSEN*, we analyzed the stress response by temperature shift and multi-treatments of heat stress. *ONSEN* was activated at 37°C, and the newly inserted *ONSEN* was transcriptionally activate and mobile to the next generation subjected to heat stress, indicating that the regulation of *ONSEN* is independent from positional effects on the chromosome. Reciprocal crosses with activated *ONSEN* revealed that the transgenerational transposition was inherited from both sexes, indicating that the transposition is suppressed independent of gametophytic regulation. We showed previously that *ONSEN* transposed in mutants deficient in siRNA biogenesis, including *nrpd2* and *rdr2*, but not *dcl3*. To define the functional redundancy of DCL proteins in *Arabidopsis*, we analyzed *ONSEN* activation in mutants deficient in Dicer-like proteins, including *dcl2*, *dcl3*, and *dcl4*. *ONSEN* was nearly immobile in a single Dicer mutant; however, some transgenerational transpositions were observed in *dcl2/dcl3/dcl4* triple mutants subjected to heat stress. This indicated that the Dicer family is redundant for *ONSEN* transposition. To examine the activation of *ONSEN* in undifferentiated cells, *ONSEN* transcripts and synthesized DNA were analyzed in heat-stressed callus tissue. In contrast with vegetative tissue, high accumulation of the transcripts and amplified DNA copies of *ONSEN* were detected in callus. This result indicated that *ONSEN* activation is controlled by cell-specific regulatory mechanisms.

Introduction

Transposable elements (TEs) are highly abundant in plant genomes and potentially become powerful mutagens (Feschotte et al., 2002). The activities of TEs are regulated by chromatin

modifications, including DNA methylation and histone modification (Jacobsen and Henderson, 2007, Scortecci et al., 1997, Slotkin and Martienssen, 2007, Zhang et al., 2006, Lisch, 2009, Chandler and Walbot, 1986, Bennetzen, 1987). DNA methylation is higher in transposon sequences than elsewhere in plant genomes (Rabinowicz et al., 2003, Tran et al., 2005). In plants, DNA cytosine methylation occurs in three sequence elements: CG, CHG, and CHH, where H stands for A, C, or T. *De novo* methylation is catalyzed by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) and maintained by three different pathways: CG methylation by DNA METHYLTRANSFRERASE 1 (MET1), CHG methylation by CHROMOMETHYLASE 3 (CMT3), and CHH methylation through persistent *de novo* methylation by DRM2 (Chan et al., 2005). Also in plants, the SWI2/SNF2-like chromatin-remodeling protein DECREASE IN DNA METHYLATION 1 (DDM1) is required to maintain DNA methylation (Vongs et al., 1993, Jeddeloh et al., 1999, Brzeski and Jerzmanowski, 2003). In *Arabidopsis*, the *ddm1* mutation induces transcriptional and transpositional activation of some transposons (Hirochika et al., 2000, Miura et al., 2001, Martienssen et al., 2001). The most diverse families of transposons in plants are the LTR-type retrotransposons (Vitte and Bennetzen, 2006). In self-pollinated *ddm1* mutant lines, various LTR-type retrotransposons in the *gypsy* and *copia* families increased in copy number (Tsukahara et al., 2009). The retrotranspositions occurred stochastically and independently for each element, suggesting an additional autocatalytic process.

In *Arabidopsis*, the *met1* and *cmt3* mutations induce transcription from retroelement-related repeats (Reinders et al., 2009, Martienssen and Colot, 2001, Tompa et al., 2002); however, mobilization of retrotransposons has not been observed (Richards and Rangwala, 2007, Zhang et al., 2006, Lister et al., 2008, Lippman et al., 2003). Recently the *copia*-type retrotransposon *Evade* (*EVD*) was found to be transpositionally activated during inbreeding of hybrid epigenomes consisting of *met1*- and wild-type-derived chromosomes (Mirouze et al., 2009). Furthermore, the retrotransposition was

inhibited by plant-specific RNA polymerase IV/V (PolIV/V) and the histone methyltransferase KRYPTONITE (KYP) through a mechanism that is independent of DNA methylation. The transposition was limited to *EVD*, whereas other potentially active elements remained immobile, suggesting selective epigenetic control that distinguishes different transposon families (Mirouze et al., 2009). The transpositional regulation of *EVD* indicated that DNA methylation-independent epigenetic regulation restrains post-transcriptional steps in retrotransposition.

Environmental stress activates transposons (Capy et al., 2000). The induction of activity has been described in response to UV exposure, temperature, radiation, wounding, cell culture, pathogen infection, and polyploidization (Martienssen and Slotkin, 2007). Epigenetic regulation is also affected by environmental stresses that activate transposons. For example, the *Antirrhinum majus* transposon *Tam3* undergoes low temperature-dependent transposition (Hashida et al., 2003). Growth at 15°C permits transposition, whereas growth at 25°C strongly suppresses it. The degree of *Tam3* DNA methylation is altered somatically and is positively correlated with the growth temperature. *Tam3* transposase (TPase) binds to a major repeats in subterminal regions of *Tam3*. Methylcytosines in the motif impair the binding ability of the TPase. TPase binding to *Tam3* occurs only during growth at low temperature and immediately after DNA replication, resulting in a *Tam3*-specific decrease in methylation of the transposon DNA (Hashida et al., 2006). *Ac/Ds*, another stress-activated transposon, is largely silent during normal growth, but is activated upon stress treatments that include wounding, pathogen attack, and cell culture (Takeda et al., 1998, Wessler, 1996). Activation of *Ac/Ds* has also been reported to be inversely correlated with methylation status (Brutnell et al., 1997). In maize, cold stress down-regulates DNA methyltransferase (*ZmMET1*) in root tissue, causing hypomethylation in the *Ac/Ds* transposon region (Steward et al., 2000). This demethylation was specific to this transposon sequence in root tissue, indicating that the methylation level was selectively reduced by cold stress.

We previously reported that a *copia*-type retrotransposon named *ONSEN* was activated by heat stress (Ito et al., 2011). Full-length *ONSEN* transcripts were detected in plants subjected to a temperature shift from 6°C for 24 h to 37°C for 24 h. *ONSEN* expression was much higher in mutants impaired in the biogenesis of small interfering RNAs (siRNAs): *nrpd1*, impaired in PolIV; *nrpd2*, impaired in the common subunit of PolIV and PolV; *rdr2*, impaired in RNA-dependent RNA polymerase 2; and *dcl3*, mutated in Dicer-like 3. This indicated that siRNA-mediated regulation was responsible for restriction of the *ONSEN* transcript level after heat stress. Active *ONSEN* not only produced full-length transcripts but also synthesized extrachromosomal DNA. The *ONSEN* transcripts and the extrachromosomal DNA decayed within several days after heat treatment, and no transposition was detected in the vegetative tissue. Surprisingly, a high frequency of new insertions was detected in the progeny of heat-stressed plants deficient in siRNAs (including *nrpd1*, *nrpd2*, and *rdr2*) although no transposition occurred in *dcl3* (Ito et al., 2011). Given the functional redundancy of Dicer-like proteins in *Arabidopsis* (Vaucheret, 2008, Gasciolli et al., 2005), another Dicer-like protein may substitute for DCL3 in suppressing transgenerational retrotransposition. The insertion pattern of *ONSEN* on the sibling revealed that transgenerational retrotransposition occurred during flower development and before gametogenesis. This observation indicated that *ONSEN* activation required an environmental trigger and was controlled both developmentally and by an epigenetic pathway. Here we show that *ONSEN* activity is regulated epigenetically and in a cell-specific manner.

Results

ONSEN activity in plants subjected to heat stress

To verify a heat-stress response for *ONSEN*, a temperature-shift assay was applied to 1-week-old seedlings. The level of *ONSEN* transcript was analyzed by quantitative RT-PCR

(qRT-PCR). *ONSEN* transcription was almost undetectable in seedlings subjected to a temperature shift from 6°C for 24 h to 21°C, 27°C, or 32°C for 24 h, in wild-type and *nrpd1* mutant plants (Fig. 1A). Next, the relative DNA copy number of *ONSEN* was qualified by quantitative PCR (q-PCR). The copy number did not change in seedlings subjected to a temperature shift from 6°C for 24 h to 21°C, 27°C, or 32°C for 24 h. Increased copy number, evidenced by the activation of *ONSEN* transcripts and synthesized extrachromosomal DNA copies, occurred in seedlings subjected to a temperature shift from 6°C for 24 h to 37°C for 24 h (Fig. 1B). *ONSEN* transcription was about 28-fold higher in *nrpd1* than in the wild-type, and the copy number of synthesized DNA was about 85-fold higher in *nrpd1* than in the wild-type, immediately after the stress treatment. This result indicates that *ONSEN* activation might be regulated by a heat-sensitive factor with a threshold around 37°C, and that an siRNA-mediated pathway might regulate the transcriptional activation.

ONSEN activation in plants subjected to multi-generational stress

Heat-activated *ONSEN* transposed and was amplified in copy number in progeny. To examine the activity of newly amplified copies, multi-generational stress (MS) was performed on seedlings from the parental generation that had been subjected to 37°C heat stress. As a control, *ONSEN* activation in non-stressed progeny (NS) was also analyzed. In addition, seedlings were analyzed in which only the parental generation (S1) or only the progeny generation (S2) were subjected to heat stress (Fig. 2A). Quantitative analyses showed that in the wild-type, *ONSEN* transcription was not significantly higher in MS seedlings than in S2 seedlings. *ONSEN* was silent in NS and S1 seedlings in both the wild-type and *nrpd1* (Fig. 2B). In contrast, in *nrpd1*, *ONSEN* transcription was about 55-fold higher in MS *nrpd1* than in the S2 wild-type. Consistent with transcriptional activation, the copy number of *ONSEN* was much higher in MS *nrpd1* than in S2 *nrpd1* seedlings, although the copy number was almost same in

MS wild-type as in S2 wild-type (Fig. 2C). These results indicate that the newly inserted copies of *ONSEN* were activated under heat stress in regions to which they relocated, and that they again synthesized DNA copies in mutants impaired in siRNA biogenesis. To confirm the new *ONSEN* insertions in the progeny, Southern analysis was conducted on the offspring of MS plants in the wild-type and in *nrpd1* mutants (Fig. 3A, B). A high frequency of new transpositions was detected in the offspring of MS *nrpd1* plants subjected to heat stress (Fig. 3B). New *ONSEN* insertions in siblings derived from a single plant showed patterns in common, indicating somatic movement of *ONSEN* during flower development, as shown by a previous report (Ito et al., 2011). Unique *ONSEN* insertions specific to a single progeny individual and common insertion patterns in MS *nrpd1* progeny indicated that the transposition occurred each generation (Fig. 3B).

We next compared the transgenerational activation of *ONSEN* in the progeny of multi-stressed plants between wild-type and *nrpd1*. No activation of *ONSEN* was detected by qRT-PCR in the progeny of heat-stressed wild-type or *nrpd1* mutants (Fig. 3C). These results suggest that the newly inserted copies of *ONSEN* were again activated by the multi-heat stress, and that they were subsequently transmitted to the next generation and increased in copy number, although activated *ONSEN* was re-silenced when the progeny were without heat stress.

Transgenerational transposition is inherited from both sexes

To test the role of parental sex in transgenerational transposition, reciprocal crosses were conducted and two independent factors were examined, heat stress and NRPD1 function. To test whether the transgenerational retrotransposition was affected by heat activation, heat-stressed *nrpd1* was reciprocally crossed with non-stressed *nrpd1*, and transposition was analyzed in the progeny. As a control, self-fertilized, heat-stressed and non-stressed *nrpd1* were analyzed. A Southern blot analysis

detected new *ONSEN* insertions in offspring from the reciprocal crosses (Fig. 4A). The effect of *nrpd1* was analyzed with reciprocal crosses in which plants with or without *nrpd1* were heat-stressed in the male or the female parent (i.e., four treatment combinations). As a control, heat-stressed, self-fertilized *nrpd1* and the wild-type were analyzed. Southern blot analysis detected the transposition in offspring generated by crosses in either direction (Fig. 4B). These results indicate that the *ONSEN* transposition was derived from either parent and was activated by heat stress in the mutant deficient in siRNA biogenesis.

A role of Dicer-like proteins in *ONSEN* transposition

We showed previously that *ONSEN* was activated in mutants deficient in siRNA biogenesis, including *nrpd2*, *rdr2*, and *dcl3*. Transpositions were observed in *nrpd2* and *rdr2*, indicating that siRNA biogenesis is crucial in preventing the transgenerational mobility of *ONSEN*. In *dcl3*, no new insertions were detected, although DCL3 restricted the level of *ONSEN* transcripts after heat stress (Ito et al., 2011). This indicated that DCL3 is dispensable in the control of transgenerational transposition in *Arabidopsis*, or that other DCL proteins may substitute for DCL3 in transpositional regulation. To define the functional redundancy of DCL proteins, seedlings deficient in DCL2, DCL3, or DCL4 were subjected to heat stress, and the *ONSEN* transposition was analyzed in six progeny from each parental line (Fig. 5A, B). No transposition was detected by Southern blot analysis in *dcl3* or *dcl4*, and a single new insertion was detected in one of six progeny in *dcl2* (Fig. 5B). The transgenerational transposition was also analyzed in double and triple mutants for dicer-like proteins, including *dcl2/dcl3* and *dcl2/dcl3/dcl4* (Fig. 5C, D). No transposition was detected in progeny of the double mutant, with or without heat stress (Fig. 5C). However, new copies were detected in two of the six progeny from the heat-stressed triple mutant (Fig. 5D). This result indicates dicer-like proteins are redundant in the

regulation of transgenerational transposition of *ONSEN* in *Arabidopsis*.

Activation of *ONSEN* in undifferentiated cells

We previously reported that *ONSEN* transposition occurred in a late stage of plant development, before the differentiation of male and female gametophytes (Ito et al., 2011). This indicated an involvement of siRNAs in erasing “stress memory” during somatic growth and/or suppressing retrotransposition in flower tissues. To define the role of siRNA in dividing cells, *ONSEN* activation was analyzed in undifferentiated callus tissue. Seeds from *nrpd1* and the wild-type were germinated and grown for 14 days on a callus-inducing medium. The callus-formed seedlings were subjected to a temperature shift from 6°C for 24h to 37°C for 24 h. As a control, *nrpd1* or wild-type callus-formed seedlings were subjected to a temperature shift from 6°C for 24 h to 21°C for 24 h. *ONSEN* transcripts were analyzed by qRT-PCR 30 days after heat treatment. Interestingly, *ONSEN* transcripts in seedlings subjected to heat stress were about five- and 60-fold higher than in non-stressed wild-type and in *nrpd1* seedlings, respectively. The transcriptional level was significantly higher than in vegetative tissue in which *ONSEN* was re-silenced within 10 days after heat treatment, both in *nrpd1* and the wild-type (Fig. 6A and Ito et al., 2011). This indicates that *ONSEN* activation differs in regulation between callus and vegetative tissue. Consistent with the transcriptional activation, the number of DNA copies of *ONSEN* in stressed callus was two-fold higher in the wild-type and 15-fold higher in *nrpd1* than in the non-stressed control (Fig. 6B). During 30 days of subsequent growth of both the wild-type and *nrpd1* heat-stressed plants, *ONSEN* copy number gradually decreased, reaching the initial number in vegetative tissue (Ito et al., 2011). In contrast, in heat-stressed callus, the copy number remained high in both *nrpd1* and the wild-type compared to non-stressed callus. There are two possible explanations for this high copy number. One is that it is due to extrachromosomal DNA

synthesized through the continuous expression of *ONSEN* in callus; the other is that it is due to somatically inserted copies. To clarify the two possibilities, a Southern blot analysis was conducted with *Eco*RV-digested DNA or non-digested DNA (Fig. 6C, D). Smear bands were detected in heat-stressed callus in the wild-type and the *nrpd1* mutant, indicating somatic transpositions. In addition, extrachromosomal DNA was detected in heat-stressed callus, suggesting that the *ONSEN* expression lasting 30 days after the heat treatment was responsible for synthesizing the extrachromosomal DNA (Fig. 6C, D).

Discussion

ONSEN was silent before the temperature shift up to 37°C. The transcriptional activation ceased, and the synthesized extrachromosomal form of *ONSEN* DNA in seedlings degraded during development. Interestingly, new *ONSEN* insertions were detected in the progeny of heat-stressed plants deficient in siRNAs, indicating two independent steps in *ONSEN* regulation. The first step is transcriptional regulation. The transcriptional activation of *ONSEN* might be controlled by a heat-response promoter. The level of *ONSEN* transcripts was much higher in mutants deficient in siRNA than in the wild-type, indicating that transcriptional regulation directly or indirectly involves a siRNA-mediated mechanism. Heat-activated *ONSEN* was transposed to the next generation and increased in copy number in the host genome. The higher level of *ONSEN* transcripts in multi-stressed plants than in single-stressed plants indicates that the activation of amplified *ONSEN* depended on heat stress in the relocated host genome. Ito et al. (2011) previously reported that new *ONSEN* insertions confer heat responsiveness to nearby genes, which also supports the view that active *ONSEN* includes a heat response promoter. Therefore, the transcriptional activation of *ONSEN* could be regulated both by a heat-sensitive promoter and epigenetically by siRNAs. *ONSEN* was re-silenced

in the progeny after heat activation, even in *nrpd1* mutants, indicating that *nrpd1* plays an important role in maintaining silencing but is dispensable in initiating silencing.

The second step is transpositional regulation. An siRNA pathway suppresses the transgenerational transposition, although the mechanism remains to be elucidated. Here we report that the *dcl2* single mutant and the *dcl2/dcl3/dcl4* triple mutant released *ONSEN* transposition in the progeny of heat-stressed plants. These observations indicate that DCL2 might play the primary role in silencing *ONSEN* transposition, but with overlapping help from DCL4. *Arabidopsis* encodes several DICER-like (DCL) proteins. DCL1 produces miRNAs, DCL2 produces virus-derived siRNAs, DCL3 produces endogenous RDR2-dependent siRNAs, and DCL4 plays a role in producing trans-acting siRNAs (Gasciolli et al., 2005). Molecular and phenotypic analyses of *dcl* double mutants revealed partially compensatory functions among DCL proteins. The frequency of transposition was still much lower than in the *nrpd1* mutant background, indicating the involvement of other factors.

Epigenetic reprogramming occurs during plant development and in reproductive stages (Feng et al., 2010). In *Arabidopsis*, certain transposons are up-regulated in expression and are mobile in pollen (Slotkin et al., 2009). Demethylation of transposons occurs in the vegetative nucleus, causing transposons to be reactivated, although their movement is not inherited by the next generation. This hypomethylation of the vegetative cell is thought to reactivate transposons that could serve to reinforce transposon silencing in sperm cells by RNA-directed DNA methylation (RdDM) pathway (Slotkin et al., 2009, Mosher et al., 2009). To examine whether *ONSEN* transposition is regulated by reproductive stage, reciprocal crosses were conducted between heat-stressed parents. New insertions were detected in the progeny, showing that active *ONSEN* was inherited through male and female gametogenesis and that *ONSEN* transposition was regulated independent of gametogenesis. The insertion pattern of *ONSEN* also supported the conclusion that the transgenerational retrotransposition

occurred during flower development and before gametogenesis.

Some plant retrotransposons are activated during tissue/cell culture (Hirochika, 1993, Pouteau et al., 1991), such as the tobacco retrotransposons *Tto1* and *Tnt1*, which have been observed to be activated in protoplast and tissue culture. A high level of transcripts of *Tto1* were also detected in transgenic *Arabidopsis* callus with a *ddm1* mutant background (Takeda et al., 2001). Here we demonstrated the heat-induced activation of *ONSEN* in callus. Interestingly, this activation was retained for 30 days after the heat treatment. Specific regulation of *ONSEN* activation in callus might be important if the mechanism involved is common to undifferentiated cells, including shoot apical meristem (SAM), because the stress memory of *ONSEN* was inherited up to a late stage of development, and retrotransposition occurs at flowering time. This suggests that an active signal for *ONSEN* is retained during development. It remains unknown, however, whether some undifferentiated tissue such as SAM is important in regulating *ONSEN* activation.

Material and methods

Plant material and stress treatments

Plants were grown on MS (Murashige and Skoog) plates with continuous light at 21°C. Heat stress or the control treatment was applied to seven-day seedlings subjected to a temperature shift from 6°C for 24 h to 21°C, 27°C, 32°C, or 37°C for 24 h. After heat treatment, plants were transplanted to soil for further growth at 21°C. All mutants used in this study (*nrpd1a-3*, *dcl2-3*, *dcl3-1*, *dcl4-2*, *dcl2-1/dcl3-1*, *dcl2-1/dcl3-1/dcl4-2*t) and wild-type plants were of the *Arabidopsis thaliana* ecotype *Columbia* background.

Southern blot analysis

Arabidopsis genomic DNA was isolated by using a Nucleon PhytoPure DNA extraction kit (GE Healthcare Life Science). Blotting of genomic DNA was performed as described by Miura et al. (2004). Hybridization signals were detected by using a radiolabeled *ONSEN*-specific probe generated with the Megaprime DNA Labeling System (GE Healthcare Life Science) in high-SDS hybridization buffer (Church and Gilbert, 1984).

Real-time PCR

For expression analysis, total RNA was extracted from seedlings or leaves by using TRI Reagent (Sigma T9424) according to the supplier's recommendations. Around 3-5 ug of total RNA were treated with RQ1 RNase-free DNase (Promega) and reverse-transcribed by using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa 6210A) with an oligo dT primer. For quantification of *ONSEN* DNA, genomic DNA was extracted from seedlings or leaves with the DNeasy Plant Mini Kit (QIAGEN 69104) according to the supplier's recommendations. Real-time PCR was performed with the Applied Biosystems 7300 Real Time PCR System using Power SYBR Green PCR Master Mix (Applied Biosystems). Quantities were determined from a standard curve and were normalized to the amount of 18s rDNA. At least three experimental replicates were done and standard errors were determined.

Callus induction and stress treatment.

Seeds were incubated on plates with callus-inducing medium (2,4-D, 0.5 mg/ml; kinetin, 0.1 mg/ml). Callus induced from seedlings was maintained on this medium for 14 days, and the callus-formed seedlings were subjected to a temperature shift from 6°C for 24 h to 37°C for 24 h. After the stress treatment, calluses were continuously incubated for 30 days at 21°C with continuous light.

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Figure legends

Figure 1. Transcriptional activity and synthesized *ONSEN* DNA in seedlings subjected to temperature shifts. **A**. Relative *ONSEN* transcript levels after temperature shifts, analyzed by qRT-PCR (mean±s.e.m., n=3, values relative to the 37°C wild-type). **B**. Relative copy number of *ONSEN* after temperature shifts, analyzed by q-PCR (mean±s.e.m., n=3, values relative to 21°C wild-type).

Figure 2. *ONSEN* activation in plants subjected to multi-generational heat stress. **A**. Diagram showing the experimental design for the multi-generational heat stress treatments. Seedlings were subjected to a temperature shift from 6°C for 24 h to 21°C or 37°C for 24 h in each generation. **B.** Relative *ONSEN* transcript levels after a single- or multi-stress treatment, analyzed in the second (progeny) generation by qRT-PCR (mean±s.e.m., n=3, values relative to 37°C wild-type). **C**. Relative copy number of *ONSEN* after a single- or multi-stress treatment, analyzed in the second (progeny) generation by q-PCR (mean±s.e.m., n=3, values relative to 21°C wild-type). NS, no stress; S1, heat stress in the first (parental) generation; S2, heat stress in the second (progeny) generation; MS, heat stress in both the first and the second generations.

Figure 3. A, **B**. Southern blot of *Eco*RV-digested DNA isolated from the progeny of multi-stressed plants (MS-1 and MS-2) and a non-stressed plant (NS). In the wild-type, no new insertion was detected in the MS progeny. (**A**) In *nrpd1*, a high frequency of new transpositions was detected in the MS progeny. (**B**) Unique *ONSEN* insertions specific to a single progeny individual and common transposition patterns in the MS progeny indicated that the transposition had occurred in each

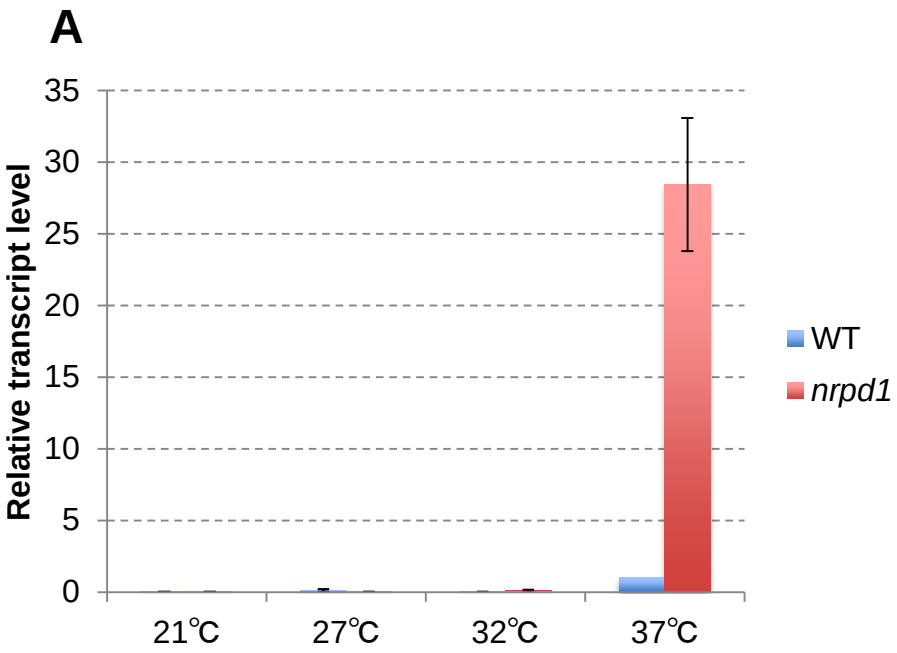
generation. A gel stained with ethidium bromide (EtBr) is shown as a loading control. **C**. Relative *ONSEN* transcript levels in the progeny of plants non-stressed (PNC), single-stressed in the second generation (PS2), or multi-stressed (PMS) were analyzed by qRT-PCR (mean±s.e.m., n=3, values relative to the transcript level directly after 37°C [HS] in the wild-type).

Figure 4. Southern blot of *Eco*RV-digested DNA isolated from the progeny of three independent reciprocal crosses, showing transgenerational transpositions of *ONSEN*. **A**. Heat-stressed *nrpd1* was reciprocally crossed with non-stressed *nrpd1*. As controls, heat-stressed and non-stressed *nrpd1* were self-fertilized and the progeny were analyzed. **B**. Heat-stressed *nrpd1* was reciprocally crossed with the wild-type. As controls, heat-stressed *nrpd1* and wild-type were self-fertilized and the progeny were analyzed. A gel stained with ethidium bromide (EtBr) is shown as loading control.

Figure 5. Southern blot of *Eco*RV-digested DNA isolated from the six progeny from each mutant (*dcl2*, *dcl3*, *dcl4*, *dcl/2dcl3*, and *dcl2/dcl3/dcl4*). **A**. No transposition was observed in progeny from non-stressed *dcl2*, *dcl3*, or *dcl4*. **B**. One new copy (arrowhead) was detected in a heat-stressed progeny from *dcl2*, although no transpositions were detected in the *dcl3* or *dcl4* single mutants. **C**. *ONSEN* was silent in the *dcl2/dcl3* double mutant and *dcl2/dcl3/dcl4* triple mutant under the non-stress condition. **D**. Transgenerational transpositions (arrowheads) were detected in two progeny from the triple mutant subjected to heat stress, although no transposition was detected in progeny from the stressed double mutant. HS, heat stress.

Figure 6. **A**. Transcripts of *ONSEN* in heat-stressed callus after 30 days of recovery (HS+30) or in non-stressed callus (NS+30). *ONSEN* was active in callus for 30 days after the heat treatment. **B**. DNA

copy number of *ONSEN* in non-stressed callus (NS+30) and in heat-stressed callus after 30 days of recovery (HS+30). **C**. Southern blot of *Eco*RV-digested DNA isolated from wild-type and *nrpd1* plants subjected to the HS+30 and NS+30 treatments. **D**. Southern blot of non-digested DNA isolated from wild-type and *nrpd1* plants subjected to the HS+30 and NS+30 treatments. Arrowheads indicate the extrachromosomal form of *ONSEN*.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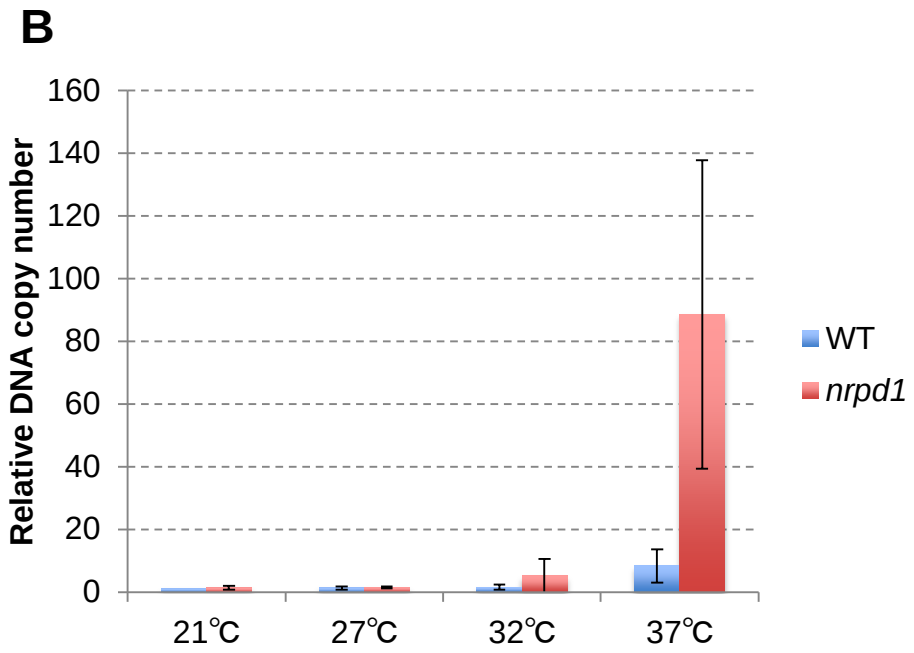
37℃
WT
nrpd1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Relative DNA copy number
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20
0
21℃
27℃
32℃
37℃
WT
nrpd1

Fig.1

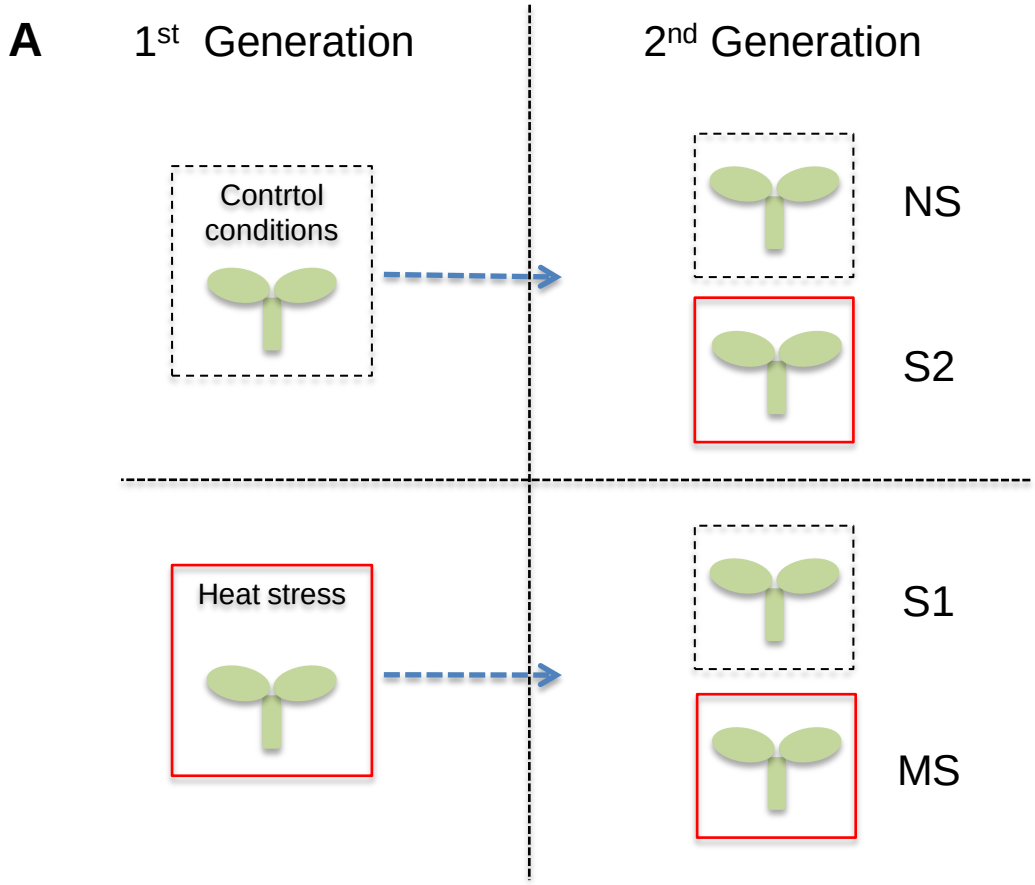
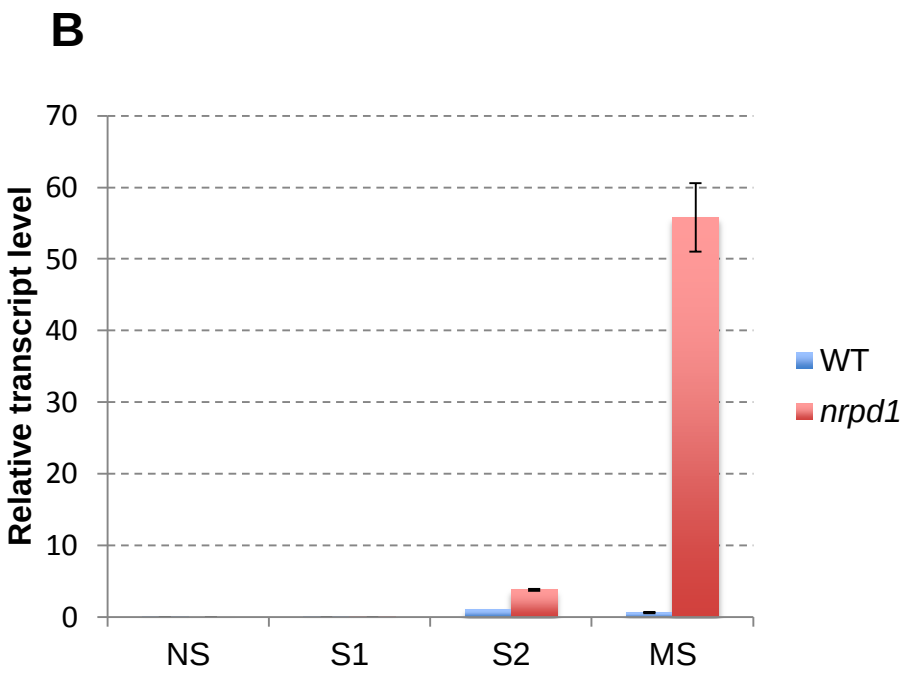

A
1st Generation
2nd Generation
Contrtol conditions
NS
S2
Heat stress
S1
MS

Fig.2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Relative transcript level
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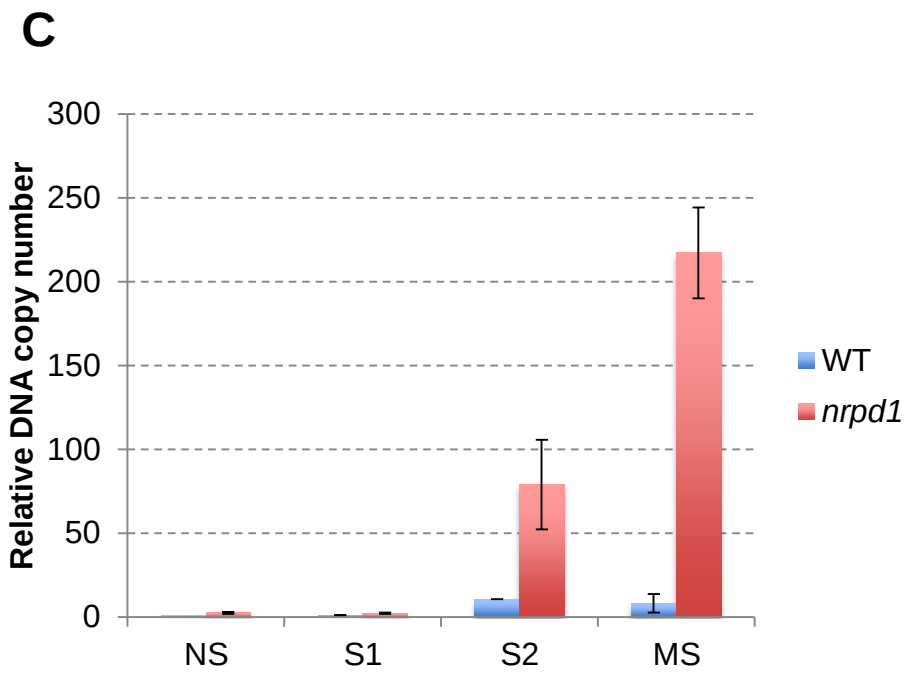
40
30
20
10
0
NS
S1
S2
MS
WT
nrpd1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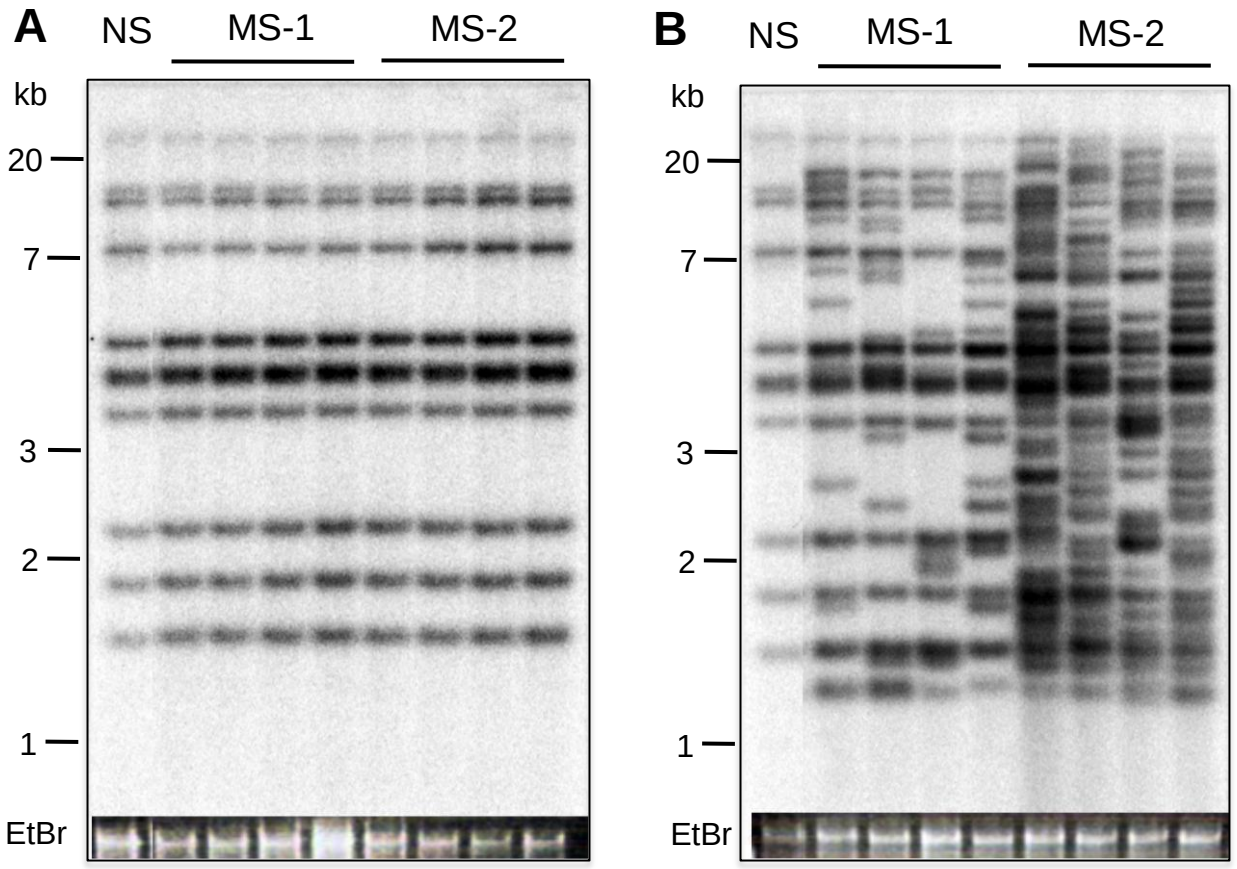
200
150
100
50
0
NS
S1
S2
MS
WT
nrpd1

Fig.2



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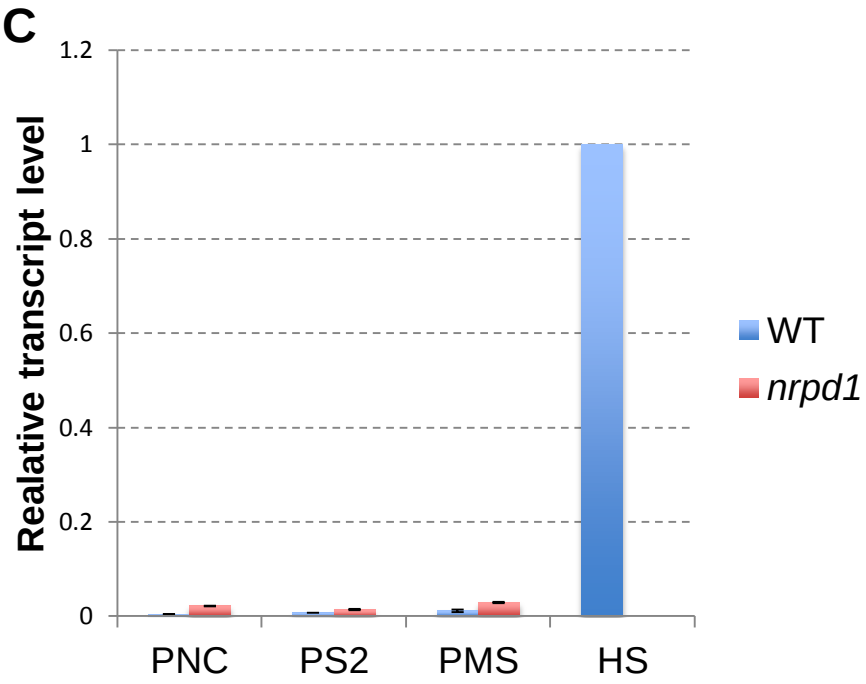
C
Realative transcript level
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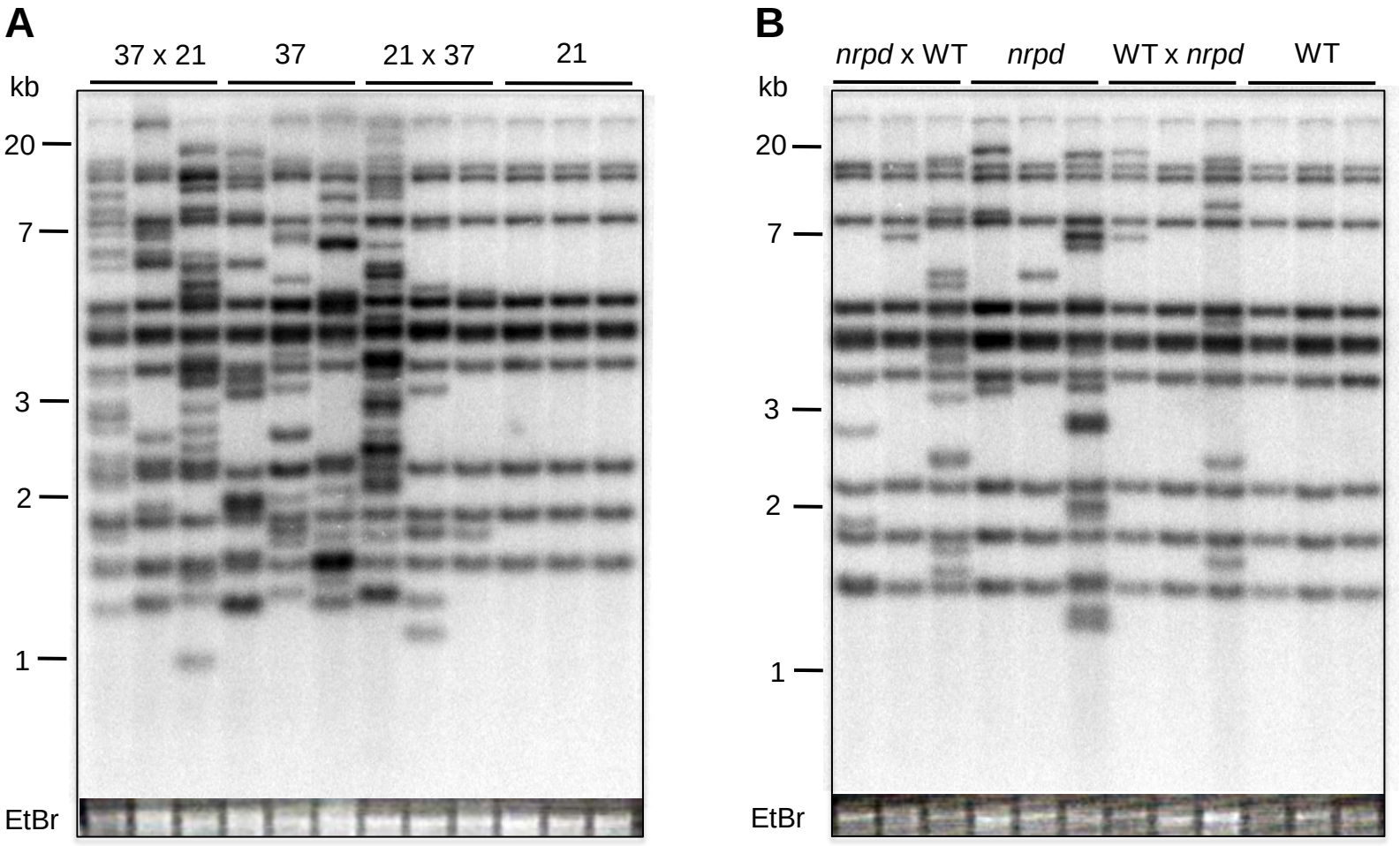
0.4
0.2
0
PNC
PS2
PMS
HS
WT
nrpd1

Fig.3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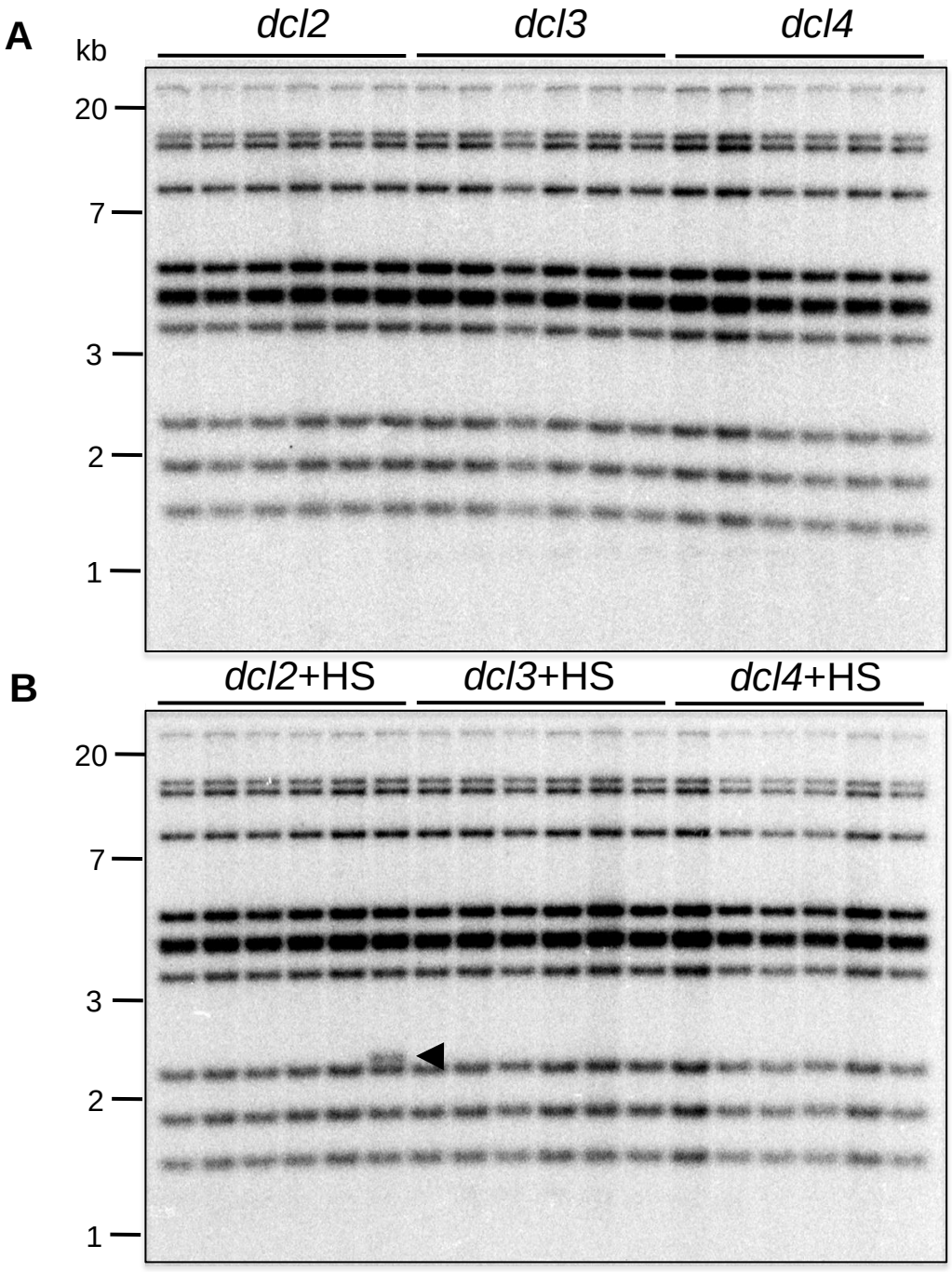
21 x 37
21
kb
20
7
3
2
1
EtBr
B
nrpd x WT
nrpd
WT x nrpd
WT
kb
20
7
3
2
1
EtBr

Fig.4



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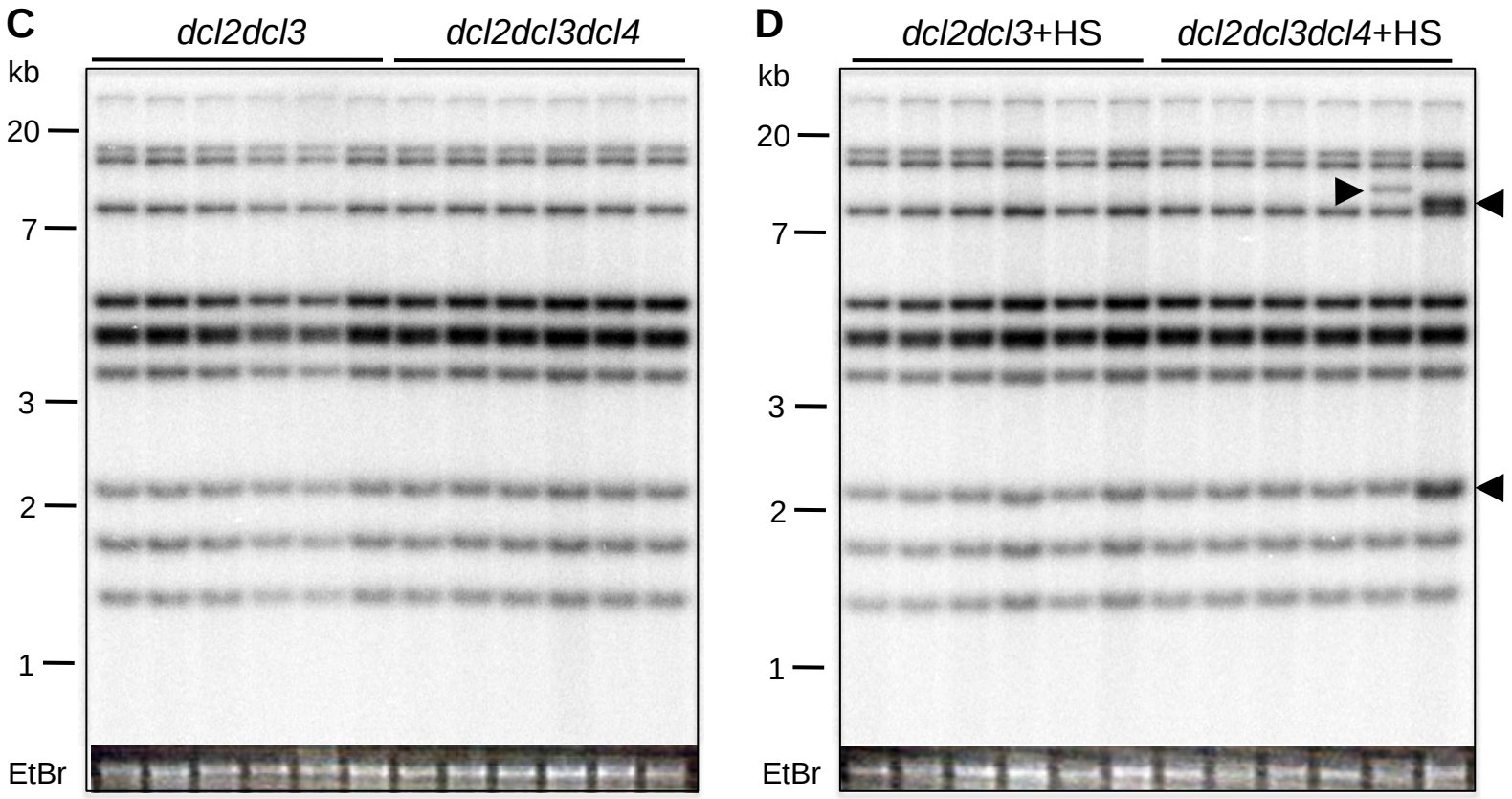
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kb
20
7
3
2
1
EtBr
D
dcl2dcl3+HS
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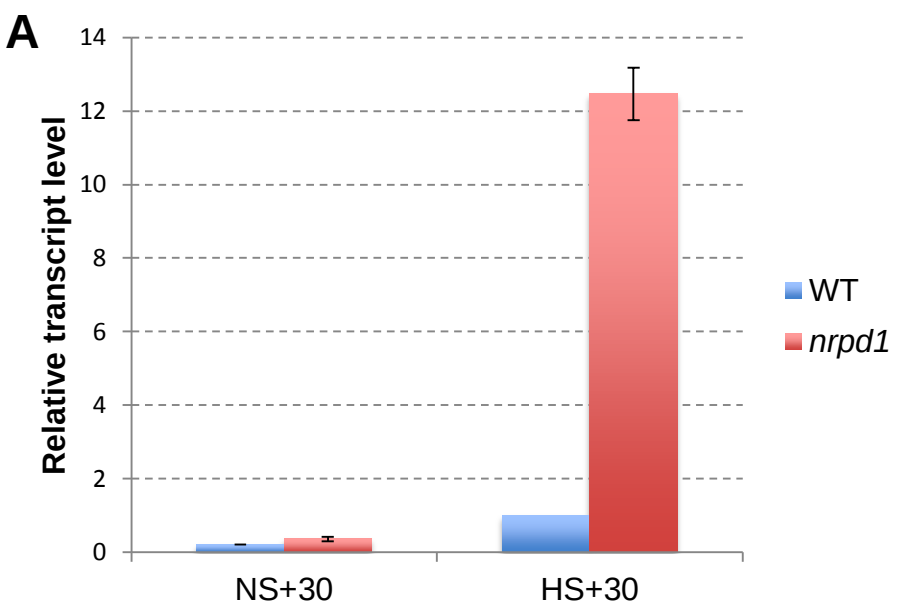
kb
20
7
3
2
1
EtBr

Fig.5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NS+30
HS+30
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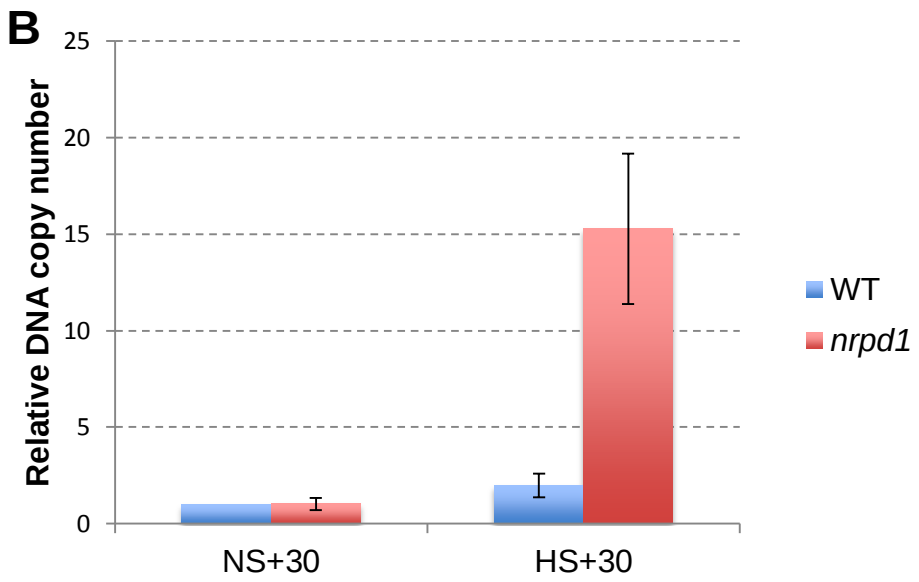
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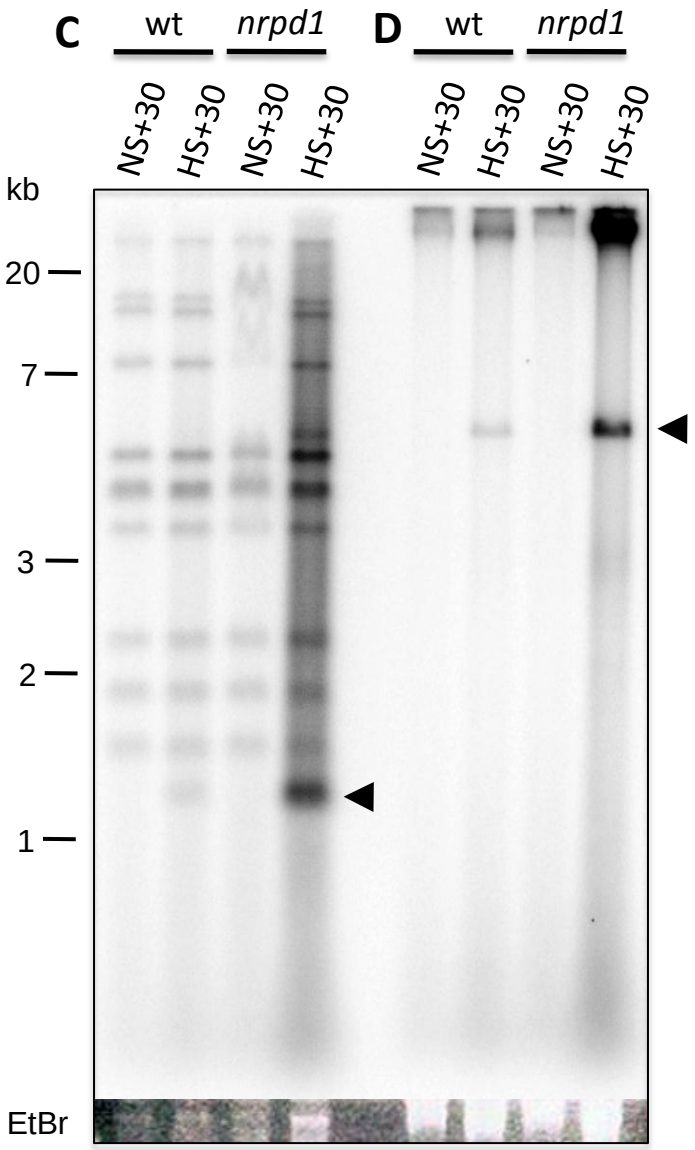
NS+30
HS+30
WT
nrpd1

Fig.6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nrpd1
D
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nrpd1
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HS+30
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HS+30
NS+30
HS+30
NS+30
HS+30
kb
20
7
3
2
1
EtBr

Fig.6