A regulatory mechanism of a heat-activated retrotransposon in *Arabidopsis*

（シロイヌナズナ熱活性型レトロトランスポゾンの制御機構）

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CONTENTS

4-4. Stress memory could be regulated in undifferentiated tissues during plant development

DISCUSSION

The activation of *ONSEN* was regulated by a stress-responsible transcriptional factor

ONSEN transcription was activated throughout the plant

ONSEN transcription was initially regulated by siRNA biogenesis pathway and re-silenced independently of RdDM

ONSEN was transposed in reproductive tissue caused by changing chromatin modification initiated by flowering

FIGURELEGENDS

EXPRERIMENTAL PROCEDURES

Plant material and growing conditions

Plasmid constructs

Plant transformation

Genotyping PCR

Sequencing

TAIL-PCR

Fluorescence microscopy

Stress treatments

Southern blot analysis

Real-time PCR

RT-PCR

Laser capture microdissection and RNA extraction

REFERENCES

ACKNOWLEDGMENTS

FIGURES AND TABLES

INTRODUCTION

Transposable elements (TEs) were discovered in maize by Barbara McClintock over a half century ago (McClintock, B. 1951). In plants, TEs constitute a large fraction of the genomes that can insert into new chromosomal locations (Feschotte *et al.* 2002). Eukaryotic TEs are divided into two classes according to their transposition intermediate; RNA (class 1) or DNA (class 2). Class 2 elements, known as DNA transposons, have terminal inverted repeats and do not require a reverse transcription step. Class 1 elements can be roughly divided into two groups, non-LTR and LTR retrotransposons, based on their transposition mechanisms and structures. Non-LTR retrotransposons include long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) (Schmidt. 1999).

LTR retrotransposons have long terminal repeats on each end of the sequence with the same orientation. Autonomous elements contain at least two genes called *Gag* and *Pol*. The *Gag* gene encodes a capsid-like protein and the *Pol* gene encodes a polyprotein that is responsible for protease, reverse transcriptase, RNase H, and integrase activities (Kumar and Bennetzen 1999, Feschotte *et al*. 2002). LTR retrotransposon is further divided into two elements according to the order of the coding genes. *Ty3/gypsy* retrotransposons are enriched on intergenic regions that includes centromeric heterochromatin in some plants (Jiang *et al.* 2003). On the other hand, *Ty1/copia* retrotransposons are conserved in euchromatic region of some plant species (Kumar 1996).

The activities of TEs are regulated by epigenetic modification including DNA methylation and histone modifications (Jacobsen and Henderson. 2007, Scortecci *et al*. 1997, Slotkin and Martienssen, 2007, Zhang *et al*. 2006, Lisch. 2009, Chandler and Walbot V. 1986, Bennetzen. 1987). The level of DNA methylation is higher in transposon-related sequences than it is elsewhere in the plant genome (Rabinowicz *et al*. 2003, Tran *et al*. 2005).

In plants, DNA cytosine methylation is found in three sequence contexts: 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 the DNA methylation status (Vongs *et al*. 1993, Jeddeloh *et al*. 1999, Brzeski and Jerzmanowski, 2003). In *Arabidopsis*, *ddm1* mutation induces transcriptional and transpositional activations of some transposons (Hirochika *et al*. 2000, Miura *et al*. 2001, Martienssen *et al*. 2001). The most abundant families of transposons in plants are LTR retrotransposons (Vitte and Bennetzen. 2006). In self-pollinated *ddm1* mutant lines, various LTR retrotransposons in a *gypsy* and *copia* families increased the copy number (Tsukahara *et al*. 2009).

One of the most well known mechanisms of *de novo* DNA methylation is RNA

directed DNA methylation (RdDM) that regulates TEs by small interfering RNA (siRNA)-mediated silencing (Gao *et al.* 2010, Wierzbicki *et al.* 2008). Higher plants have a specific DNA-dependent RNA polymerase named RNA polymerase IV (PolIV) and V (PolV) (Kanno *et al.* 2005, Onodera *et al.* 2005, Pontier *et al.* 2005) for siRNA inducing methylation. In *Arabidopsis*, an ectopic transcript produced from TEs is transcribed by PolIV, then the double-stranded RNA synthesized by RNA-dependent RNA polymerase 2 (RDR2) and subsequently processed on 24 to 26 nt siRNAs by DICER-LIKE 3 (DCL3) (Pontier et al. 2005, Mosher *et al.* 2008, Zhang *et al.* 2007). The siRNAs bind to an RNA-induced silencing complex containing ARGONAUTE 4 (AGO4) that interacts with PolV to recruit the DRM2 leading to *de novo* DNA methylation on the target TEs (Cao and Jacobsen 2002, Matzke and Birchler 2005). Although most of TEs are epigenetically silenced, some TEs were activated by abiotic stress accompanied with DNA demethylation. The *ZnMI 1* elements in maize was activated by cold stress (Steward *et al.* 2002). In contrast, the *Tnt1* and *Tto1* retrotransposon in tobacco were activated by biotic stress such as tissue culture, wounding, and pathogen infections and also by abiotic stress that include salicylic acid and jasmonate (Hirochika 1993, Peschke *et al.* 1987, Pouteau *et al.* 1994, Takeda *et al.* 1998).

Recent study showed that a *Ty1/copia* retrotransposon named *ONSEN* was found to be activated by heat stress in *Arabidopsis* (Ito *et al.* 2011). The LTR of *ONSEN* interacts with a heat response transcription factor, HsfA2 (Cavrak *et al.* 2014). HsfA2 has conserved N-terminal DNA binding domain (DBD) that binds a heat response

element (HRE) (Schramm *et al.* 2006). An electrophoretic mobility shift assay demonstrated that HsfA2 bound a HRE in the *ONSEN* LTR (Cavrak et al. 2014). *ONSEN* was not expressed in a hypomethylation mutant or a mutant lacking RdDM component without a heat stress (Ito *et al*. 2011).

The full-length transcripts of *ONSEN* were detected in the plants subjected to a temperature shift 6°C for 24 hours followed by 37°C for 24 hours (Ito *et al.* 2011). The expression of *ONSEN* was much higher in mutants impaired in the biogenesis of siRNAs: *nrpd1* impaired in plant specific POLIV, *nrpd2* impaired in the common subunit of PolIV and PolV, *rdr2* and *dcl3* (Ito *et al.* 2011). In addition to the transcriptional regulation, a high frequency of new *ONSEN* insertions was observed in the progeny of stressed plants that deficient of siRNA biogenesis. The results suggested that siRNA-mediated pathways regulated the transgenerational transposition (Ito *et al.* 2011). However, a molecular mechanism of *ONSEN* activation and retrotransposition were still unknown.

RESULTS

Chapter 1 *ONSEN* transcription was regulated by heat shock factor

1-1 Transcription of *ONSEN* was activated by heat stress

To verify a heat-stress sensitivity of *ONSEN*, a temperature shift assay was applied to 1 week-old seedling. The level of *ONSEN* transcript was analyzed by quantitative RT-PCR (qRT-PCR). The transcription of *ONSEN* was almost undetectable in seedlings subjected to 21°C, 27°C, or 32°C for 24h respectively (Fig.1a). However, the transcription of *ONSEN* was detected in seedlings subjected to 37ºC for 24h. The transcription of *ONSEN* was about 28-fold higher in *nrpd1* than in wild-type seedlings subjected to 37ºC for 24h (Fig.1a).

The relative copy number of *ONSEN* was increased in the plants subjected to a temperature shift from 6ºC for 24h to 37ºC for 24h in both wild type and *nrpd1* (Ito et al., 2011). The copy number of *ONSEN* was correlated with the transcript level of *ONSEN* and synthesized extrachromosomal DNA copies. Therefore, the DNA copy number of *ONSEN* was qualified by quantitative PCR (q-PCR). The copy number was increased in seedlings subjected to 37°C for 24h although it did not change in seedlings subjected to a temperature shift from 6°C for 24h to 21°C, 27°C, or 32°C for 24h (Fig.1b). The copy number of synthesized DNA was about 8.5-fold higher in *nrpd1* than that in wild type immediately after the heat stress treatment (Fig.1b). The result indicated that the activation of *ONSEN* might be regulated by a heat-sensitive factor

with a threshold at around 37 °C and an siRNA-mediated pathway.

1-2 New *ONSEN* copies were activated by multi-generational stress

A high frequency of transposition was observed in the progenies of heat-stressed *nrpd1* (Ito et al. 2011) To determine whether the newly inserted copies are able to activate, multi-generational stress (MS) was performed on seedlings that had been subjected to 37°C heat stress in the parent line in wild type and *nrpd1* respectively. As a control, *ONSEN* activation in a non-stressed progeny (NS) was analyzed. In addition, seedlings were analyzed in which only a parent line (S1) or only the progeny (S2) were subjected to heat stress (Fig.2a). Quantitative analyses showed that in wild type, the transcript level of *ONSEN* was not significantly higher in MS seedlings than that in S2 seedlings. (Fig.2b). On the other hand, in *nrpd1*, *ONSEN* transcription was about 14.6-fold higher in MS than that in the S2 *nrpd1*. *ONSEN* was not activated in NS and S1 seedlings both in wild type and *nrpd1*. Consistent with the transcriptional activation, the copy number of *ONSEN* was much higher in MS than that in S2 in *nrpd1* seedlings, although the copy number was almost the same in MS wild type and in S2 wild type (Fig.2c). These results indicated that a newly inserted copy of *ONSEN* was activated under heat stress and that they synthesized DNA copies in an *nrpd1* mutant that impaired in siRNA biogenesis. To confirm new *ONSEN* insertions in the progeny, Southern blot analysis was conducted to the offspring of MS plants in wild type and *nrpd1* mutant (Fig.3). A large number of new *ONSEN* copies were detected in the offspring of MS plants subjected to heat stress in *nrpd1* (Fig.3b). The new *ONSEN*

insertions in siblings derived from a single plant showed a common pattern indicating somatic transposition of *ONSEN* during flower development. The unique *ONSEN* insertions specific to a single progeny and a common insertion pattern in MS progeny indicated that the transposition occurred in each generation (Fig.3b).

Next, I compared a transgenerational activation of *ONSEN* in the progeny of multi-stressed plants in wild type and *nrpd1* by qRT-PCR. The transcriptional activation of *ONSEN* under non-stress was not detected in the progenies of heat-stressed wild type and *nrpd1* (Fig.3c). The result suggested that the newly inserted copies of *ONSEN* were re-silenced when the progeny was not subjected to heat stress.

1-3 *ONSEN* was activated by high light stress and oxidative stress

The promoter of *ONSEN* in the LTR contains a *cis*-regulatory sequence of HRE that bounds to a heat-induced transcriptional factor, HsfA2 (Cavrak, Lettner, Jamge, Kosarewicz, Bayer and Scheid 2014). HsfA2 was reported to have an important role to response to other environmental stress including high light stress (Nishizawa *et al.* 2006). To analyze the response of high light stress on *ONSEN*, young seedlings were exposed to high light (800 μmol photons $m^{-2}S^{-1}$) for 6 hours. The transcript level of *ONSEN* was gradually increased for 6 hours indicating that *ONSEN* was activated by high light stress (Fig.4a). The expression level of *ONSEN* was much higher in *nrpd1* compared with wild type suggesting that the transcriptional activation was controlled by siRNA-mediated mechanisms (Fig.4a).

Next I performed N,N'-dimethyl-4,4'-bipyridinium dichloride (Paraquat) treatment

on young seedlings. Paraquat produced hydrogen peroxide that becomes oxidative stress, which was also produced by high light stress on plant. Seven days old seedling was exposed to 50μM Paraquat for 6 hours. The expression level of *ONSEN* was activated and increased the transcript level 6 hours after the treatment (Fig.4b). In the *nrpd1* mutant, *ONSEN* was highly activated as the same response to high light stress (Fig.4b). Transcriptional activation was not detected on *hsfa2* mutant subjected to neither high light stress nor oxidative stress (Fig.4a and 4b). The results indicated that *ONSEN* was activated by oxidative stress via HsfA2 and that the activation was regulated by siRNA-mediated pathway.

Chapter 2 *ONSEN* transcription was activated throughout the plant

2-1 Heat activation of a transgene was regulated by an siRNA-mediated pathway

To gain further insight into the transcriptional regulation of *ONSEN*, I produced a transgenic plant that involved an intact LTR of *ONSEN* fused with a green fluorescent protein (GFP) gene (Fig.5a). A transgenic plant with a single transgene was analyzed for reporter gene expression. I performed the thermal asymmetric interlaced PCR (TAIL-PCR) assay for identify the insertion site. The insertion was mapped on an intergenic region between *AT1G26850* and *AT1G26860* on chromosome 1 (Fig.5b). To understand a role of siRNA for the transcriptional activation of *ONSEN*, the transgenic plant was crossed with *nrpd1* mutant. The produced transgenic line involved a LTR fused with a GFP impaired in PolIV. A heat treatment was conducted on 1-week-old

seedlings and the GFP signal was detected immediately after heat treatment and 3 days after the treatment respectively (Fig.6a). The GFP signal was detected on a whole plant that were subjected to the heat treatment, but the signal was gradually decayed and was below the detection limit at 3 days after heat treatment (Fig.6b). The intensity of the GFP signal was stronger in the *nrpd1* transgenic line than that in the wild-type line although the GFP signal was no longer detected by 3 days after the heat treatment (Fig.6b). The intensity of the GFP signals was consistent with the transcript level of GFP (Fig.6c) and the endogenous *ONSEN* (Fig.6d). The result indicated that the transcriptional regulation of the transgene was controlled by an siRNA-mediated pathway.

To clarify a mechanism of re-silencing on the transgene, I performed a methylation-sensitive Southern blot assay on the LTR sequence of the transgene (Fig.7a). The analysis was performed on seedlings of the wild-type and *nrpd1* transgenic lines that were exposed to 37 °C for 24 hours and 5 days after the treatment. Smaller bands corresponding to non-methylated cytosine were detected to the transgenic lines (Fig.7b). In addition, cytosine methylation site was only detected at 3' end of LTR (Fig.7a). These results indicated that a mechanism of re-silencing on the transgene was independent of DNA cytosine methylation. However, hypermethylation of the CG sites was detected to the transgenic line in *nrpd1* background indicating that loss of an NRPD1 might induce CG methylation activity independent of RdDM. Previous study showed that *ddm1* induces global decrease but a local increase of

cytosine methylation at a locus called *BONSAI* (Sasaki et al. 2012). In this analysis, *nrpd1* might induce local-specific methylation on the transgene.

2-2 Transcriptional suppressor were screened on a transgenic plant

Previous study showed that the expression of *ONSEN* under heat stress was much higher in mutants that include *nrpd1*, *nrpd2*, *rdr2*, and *dcl* (Ito et al., 2011). This indicated that siRNA-mediated regulation was responsible for a regulation of *ONSEN* transcription after heat stress. To clarify a mechanism of *ONSEN* suppression under non-stress conditions, I screened ethyl methanesulfonate (EMS) mutated transgenic plants by GFP positive signals to find a regulator for *ONSEN* transcriptional suppressor under non-stress conditions. Three independent mutants that activated GFP expression in seedlings were isolated named *GFP expression mutants* (*gem1, gem2, gem3*). The GFP intensity was analyzed on 1-week-old seedlings and the GFP signals were detected on a whole plant (Fig.8a-c). These mutants revealed phenotypes that hyponastic growth (*gem1*), large leaves (*gem2*) and no visible phenotypes (*gem3)* respectively（Fig.8a-c）. The GFP signals of the three mutants were stronger than *nrpd1* mutant after heat treatment (Fig.8d-f). To confirm whether the endogenous *ONSEN* expression was affected on these mutants, I performed qRT-PCR analysis after non-stress and heat stress treatment. No activation of *ONSEN* was detected on three mutants under non-stress condition (Fig. 8g). However, *ONSEN* transcription on each mutant was detected 10- to 14-fold higher than wild type and 1.6- to 2.3-fold higher than *nrpd1* mutant subjected to heat stress (Fig.8h). The results indicated that promoter activity of

the transgene in the three mutants under non-stress condition was independent of the activation of endogenous *ONSEN*, although the expression of endogenous *ONSEN* was enhanced in the mutants by heat stress.

2-3 *ONSEN* transcription was maintained in callus

Although the extrachromosomal DNA of *ONSEN* was gradually decreased during subsequent growth after heat stress, *ONSEN* transposition seemed to occur between flower development and before the differentiation of male and female gametophytes (Ito et al. 2011). In addition, the transcription of *ONSEN* was not detected 3 days after the heat treatment (Fig.6c) indicating that siRNAs suppressed *ONSEN* transcript during somatic growth. Therefore, in *nrpd1*, I presumed that the *ONSEN* transcription was specifically maintained in shoot apical meristem until transposition was occurred. To test the above hypothesis, *ONSEN* activation was analyzed in callus. Seeds in *nrpd1* and wild type were germinated and induced callus for 14 days on a callus-inducing medium. The callus was subjected to 37ºC for 24 hours. As a control, *nrpd1* or wild-type callus were subjected to 21ºC for 24 hours. Transcript level of *ONSEN* was analyzed by qRT–PCR 30 days after heat treatment. The transcript level in seedlings subjected to heat stress were about five times higher than that in non-stressed wild type and 60 times higher than that in *nrpd1* seedlings without heat stress, respectively (Fig. 9a). The expression level of *ONSEN* in callus was significantly higher than that in vegetative tissue in which *ONSEN* was re-silenced within 10 days after heat treatment, in both *nrpd1* and wild type (Fig. 9a). This indicated that a regulation of *ONSEN* activation

differed between callus and vegetative tissue. Consistent with the transcriptional activation, the number of DNA copies of *ONSEN* in stressed callus was 2-fold higher in the wild type and 15-fold higher in *nrpd1* compared with the non-stressed control (Fig. 9b). During the recovery phase (for 30 days after the heat treatment), the extrachromosomal DNA of *ONSEN* was gradually decreased, reaching the initial number in vegetative tissue in wild type and *nrpd1* (Ito et al. 2011). In contrast, in heat-stressed callus, the newly synthesized DNAs remained high in *nrpd1*. One hypothesis of the *ONSEN* accumulation in callus is that it is due to the extrachromosomal DNA synthesized through the continuous expression of *ONSEN* in callus. The other hypothesis is that it is due to the large number of *ONSEN* integration in callus. To clarify the two possibilities, a Southern blot analysis was conducted with *Eco*RV-digested DNA or non-digested DNA (Fig. 9c, d). Smear bands were detected in heat-stressed callus in the wild type and the *nrpd1* mutant indicating that a somatic transposition of *ONSEN* was occurred. In addition, extrachromosomal DNA was detected in the heat-stressed callus suggesting that the *ONSEN* expression lasting 30 days after the heat treatment was responsible for synthesizing the extrachromosomal DNA and a somatic transposition (Fig. 9c, d).

2-4 The transcription of *ONSEN* was not remained in undifferentiated tissues

The above results might be important if the mechanism involved is similar to undifferentiated cells such as shoot apical meristem (SAM). I presumed that transcriptional activity of *ONSEN* was retained in SAM. To verify the hypothesis, I

analyzed the transcriptional activity of *ONSEN* in the shoot apex. The shoot apex, including the apical meristem, was fixed in paraffin and isolated by laser capture microdissection (Fig.10). In *nrpd1* plants, *ONSEN* was highly activated in the shoot apex after heat treatment; however, the activity level returned to the baseline value 5 days after heat stress (R5). In the wild-type plant, *ONSEN* was expressed at relatively low levels even after heat treatment (Fig.10). The result indicated that an siRNA-mediated pathway regulated transcriptional activation of *ONSEN* in a shoot apex.

Next, I analyzed a transcript level of *ONSEN* in *ap1-1; cal1-1* (CS67157) that induced continuous formation of floral meristem. *ONSEN* transcripts were analyzed by qRT–PCR after heat treatment and subsequent growth under non-stressed recovery phase for 31 days after germination (R31) (Fig.11a). The meristem-specific *WUS* expression was detected in wild-type flower, *nrpd1* flower, and a cauliflower-like floral meristem on *ap1-1;cal1*-1 confirming that they contained SAM (Fig.11b). Transcript level of *ONSEN* in seedlings subjected to heat stress was not different between wild type and *ap1-1; cal1-1* (Fig.11a). In addition, *ONSEN* transcription was no longer detected on R31 in *ap1-1; cal1-1*. The result indicated that *ONSEN* transcription was not retained in meristematic tissues on reproductive stage.

Chapter 3 *ONSEN* transposition was regulated by an siRNA-mediated mechanism

3-1 A role of Dicer-like proteins in the *ONSEN* transposition

Previous study showed that *ONSEN* was activated in mutants deficient in siRNA biogenesis that include *nrpd2*, *rdr2* and *dcl3*. Transpositions were observed in *nrpd2* and *rdr2* indicating that siRNA biogenesis is crucial in preventing the transgenerational transposition 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*, and the other DCL proteins may compensate the role 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.12a, b).

Southern blot analysis showed no transposition in *dcl3* or *dcl4*, and a single new insertion was detected in one of six progeny in *dcl2* (Fig.12b). The transgenerational transposition was also analyzed in double and triple mutants for DCL proteins that include *dcl2/dcl3* and *dcl2/dcl3/dcl4* (Fig.12c, d). No transposition was detected in progeny of the double mutant (Fig.12c). However, new copies were detected in two of the six progenies from the heat-stressed triple mutant (Fig.12d). The result indicates that DCL proteins are redundant in the regulation of transgenerational transposition of *ONSEN* in *Arabidopsis*.

3-2 The expression level of *ONSEN* affected the transposition frequency

To verify the effect of transcriptional activity for the transposition frequency, 1-week-old seedlings were exposed to 37ºC for 6 hours (HS6) and 24 hours (HS24),

respectively. Quantitative analysis showed that the transcription level of HS24 was six times higher than in HS6 in wild type and in *nrpd1* (Fig. 13a). In addition, the number of synthesized extrachromosomal DNA copies of HS24 was six times higher than that of HS6 in wild type and 14 times in *nrpd1* (Fig. 13b). New insertions of *ONSEN* were detected in the progeny of *nrpd1* that were subjected to heat stress for 24 hours but not for 6 hours. A transposition was not detected in the progeny of wild-type plants subjected to heat stress (Fig. 13c). These results suggested that a high amount of *ONSEN* RNA and/or extrachromosomal DNA were important for transgenerational transposition in *nrpd1*.

Chapter 4 *ONSEN* transpositions occurred on reproductive tissue before gametogenesis

4-1 Transgenerational transposition was inherited from both sexes

To test whether the transgenerational transposition of *ONSEN* was inherited from male and female, reciprocal crosses were conducted and two independent factors were examined; heat stress and NRPD1-mediated regulation. 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 showed new *ONSEN* insertions in the offspring (Fig.14a). To test whether transgenerational transposition was regulated by parent of origin, reciprocal crosses that were heat-stressed in the male or the female parent were

performed. As a control, heat-stressed self-fertilized *nrpd1* and wild-type plants were analyzed. Southern blot analysis showed transpositions were occurred in the offspring generated by crosses in either direction (Fig.14b). These results indicated that the transgenerational transposition was inherited from both parents that were deficient in siRNA biogenesis.

4-2 Heat stress at a young seedling induced transgenerational transposition

To investigate whether the timing of the heat stress affected transgenerational transposition, *nrpd1* and wild-type plants were exposed to 37 °C for 24 hours at early developmental stage; from 1 to 6 days after germination (Fig. 15a). Transgenerational transposition was analyzed in the progenies of heat-stressed plants. The new copies were detected in the progeny of heat-stressed *nrpd1* although not detected in the progeny of wild type (Fig. 15b and 15c). Interestingly, transgenerational transposition was observed in seedlings whose parents were heat-stressed at a very early developmental stage, as young as 1 day old (Fig. 15c).

4-3 Transgenerational transpositions were independent of flowering time

To investigate whether the transposition frequency was affected by flowering time, we analyzed the transposition frequency on *nrpd1* grown under long-day and short-day conditions. In *Arabidopsis*, flowering is controlled by photoperiod and is promoted by day length (Mikael Johansson and Dorothee Staiger. 2014). Under long-day condition (18 hours light and 6 hours dark), bolting was observed 30 days after germination in

both wild type and *nrpd1* mutant. Under short-day conditions (6 hours light and 18 hours dark), bolting started approximately 50 days and 40 days after germination in wild type and *nrpd1*, respectively (Fig. 16).

Seedlings that were growing under long-day or short-day conditions were exposed to heat treatment 7, 10, 14, and 21 days after germination. The flowering time of the stressed *nrpd1* was slightly affected by the treatment (Fig. 16). No new copy of *ONSEN* was detected in the wild-type progeny, which were growing under neither long day nor short day (Fig. 17a). On the other hand, transgenerational transposition was observed in the progeny of *nrpd1* plants that were grown under either long-day or short-day conditions (Fig. 17b). These results suggested that transposition frequency is not affected by flowering time and that the new insertions were transmitted into the reproductive tissue even when 3-week-old *nrpd1* plants were exposed to heat stress.

4-4 Stress memory could be regulated in undifferentiated tissues during plant development

To better understand the somatic regulation of *ONSEN*, we analyzed the new insertions in the siblings of a single flower on each branch of a stressed *nrpd1* plant. New insertions were observed in the siblings of a single branch. The pattern of new insertions was similar to the progeny within a same flower although differ among flowers on the same branch (Fig. 18a, b). The new insertion was not detected on some branches of a single plant indicating that the transposition could occur after changing the fate of the cell type for branch differentiation. To understand the relationship

between the transcriptional activation and transposition events on branch development, I analyzed the transgenerational transposition on the secondary produced branches. Surprisingly, new insertions were detected on an *nrpd1* progeny originate from the side shoots that was produced after cutting the initial shoot subjected to heat stress (Fig. 19). The result indicated that the transpositional activity could be maintained long period of time after heat stress.

DISCUSSION

The activation of *ONSEN* was regulated by a stress-responsible transcriptional factor

I analyzed a transcriptional activation of *ONSEN* by a quantitative analysis and a reporter gene assay. Transcriptional activity of *ONSEN* was regulated by a transcription factor and the threshold was around 37ºC. In addition, transcriptional activation was transient and immediately recovery both in wild type and *nrpd1* indicated that siRNA mediated suppression functioned only at transcriptional initiation.

Heat-activated *ONSEN* was transposed to the next generation and increased copy number in *nrpd1* (Fig.3b). The higher level of *ONSEN* transcripts in multi-stressed plants indicates that the newly amplified *ONSEN* copies were transcriptional activated by heat stress. *ONSEN* transcription was not detected under non-stress condition in the progenies of stressed plant indicating that *ONSEN* was re-silenced and/or simply would not be activated without heat-stress.

According to the expression analysis with high light and chemical treatment, *ONSEN* was activated by oxidative stress via the HsfA2 and the activation was regulated by siRNA-mediated pathway. The result indicated that *ONSEN* might be activated by several stress condition in natural environment.

For *Arabidopsis*, heat can be classified as warm ambient temperature (about 22-27ºC), high temperature (27-30ºC), and extremely high temperature (37-42ºC, known as heat stress) (Liu et al. 2015). Heat stress results in an increased global

methylation that might be caused by the up-regulation DRM2, NRPD1, and NRPE1 (Boyko et al. 2010, Naydenov et al. 2015). However, *ONSEN* transcription was activated by heat stress at 37ºC for 24h that regulated by HsfA2. Furthermore, the expression of *ONSEN* could not be explained by the reduction of DNA methylation at the promoter under heat stress (Cavrak et al 2014). These results suggested that transcription of *ONSEN* under heat stress was regulated by heat-responsible transcription factor that independent of DNA methylation on a promoter region.

ONSEN transcription was activated throughout the plant

I assumed that transcriptional activation of *ONSEN* was much higher and/or retained longer after heat stress in SAM because transposition seemed to be occurred on reproductive stages. Although the somatic transposition of *ONSEN* and the extrachromosomal DNA were detected in heat-stressed callus on 30 days after heat stress (Fig.9), a regulatory mechanism of callus was not directly reflected a regulation on a SAM. According to the analysis of the heat-induced promoter of *ONSEN* using a GFP reporter gene, a transgene expressed on a whole plant and enhanced in *nrpd1*. Furthermore, the expression of *ONSEN* on shoot apex and cauliflower-like floral meristem was not different from the somatic tissues (Fig. 10 and 11). RdDM has been known to have a function specific in the shoot apical meristem and reinforces the silencing of TEs during early vegetative growth (Baubec et al. 2014). The finding was consistent with the results that the expression of *ONSEN* was re-silenced in SAM.

However, GFP could be move cell to cell and it might affect the detection of tissue specific transgene activation. It is worth to study more detail of tissue specific transcriptional activation of *ONSEN*.

ONSEN transcription was initially regulated by siRNA biogenesis pathway and re-silenced independently of RdDM

I showed that the transcript level of the transgene and the endogenous *ONSEN* were enhanced in *nrpd1*. However, it was gradually decreased and was no longer detected both in wild type and *nrpd1* (Fig.6, 10, 11). This indicated that the activation of *ONSEN* was initially regulated by heat shock transcriptional factors and that the re-silencing of *ONSEN* was controlled independently of RdDM. According to the methylation analysis, the LTR of the transgene was less methylated at CHG or CHH sites. However, CG methylation was detected in an *nrpd1* line indicating that a lack of NRPD1 function might induce CG methylation activity independent of RdDM (Fig.7b). PolIV might regulate *ONSEN* expression not only through RdDM but also through other regulatory processes. Recent study showed that a Calmodulin-like 41 (CML41) protein whose promoter contains a TE insertion increased the transcription level accompanied with hypomethylation by heat stress (Naydenov et al. 2015). Interestingly, in *nrpd1 nrpe1* double mutant that lacks the largest subunits of PolIV and PolV, transcriptional levels of *CML41* was not different from that of wild type. These results indicated an independent role for PolIV and/or PolV in addition to the RdDM (M. Naydenov et al. 2015).

The transposition of *ONSEN* during flower differentiation might require an epigenetic mark in addition to the transient activation by heat stress. Heat could change the chromatin state as "active" that induces *ONSEN* activation and subsequence chromatin remodeling as "silence" state that re-silences the activation. A transgenerational transposition of heat-activated *ONSEN* was not observed in a mutant deficient in histone H3K9 methyltransferases (SUVH4/KRYPTONITE) (Ito et al. 2011) that indicates the regulation of transposition was independent of histone H3K9 dimethylation.

Histone H3K27 trimethylation (H3K27me3) is associated with a large number of gene in *Arabidopsis*. H3K27me3 is correlated with gene repression, and its release preferentially results in tissue-specific gene expression. H3K27me3-targeted genes are expressed in a very tissue-specific manner during differentiation processes and induced by abiotic or biotic stresses in *Arabidopsis* (Zhang et al. 2007). H3K27me3 dynamically regulated the gene expression during plant differentiation. H3K27me3 targets were a high number of TEs in a meristem-specific manner. The result showed that stem cells must be protected from transposable element activation to form germline (Lafos et al. 2011). *ONSEN* was a target of H3K27me3 however the methylation level was not significantly different in meristem and leaves in wild type (Lafos et al. 2011). The transcriptional activation of *ONSEN* on heat-stressed *nrpd1* might be caused by active chromatin state including loss of H3K27me3 (Fig.20). In contrast, it might be difficult to bind active HsfA2 to *ONSEN* promoter due to repressive chromatin state (Fig.20). It

is a great interest to see whether H3K27 modification was changed on *nrpd1* with heat stress.

In *Arabidopsis*, several repetitive elements that are regulated by an epigenetic silencing are activated by heat stress without loss of DNA methylation (Pecinka et al. 2010). The activations are re-silenced upon recovery from heat stress, but are delayed in mutants with CAF-1 that loads nucleosomes onto replicated DNA. The result provides evidence that environmental stress can activate override epigenetic regulation and CAF1 restore the silencing state (Pecinka et al. 2010). Recently it was reported that Decreased DNA methylation 1 (DDM1) and Morpheus Molecule 1 (MOM1) act redundantly to restore silencing state after heat stress (Iwasaki and Paszkowski. 2014). The heat-induced activation of some genes was transiently and was not transmitted to the progeny. However in *ddm1 mom1* double mutant, transcriptional activated state were able to inherit to the progeny (Iwasaki and Paszkowski. 2014). The finding supports my study that heat-induced transcription of *ONSEN* was transiently and chromatin changes might play for resetting expression state on recovery phase (Fig.20).

I identified *gem1, gem2, gem3* mutants that release an expression of the transgene without stress (Fig.8). The endogenous *ONSEN* was not activated in the mutants without heat stress (Fig.8g). It is possible that RNA degradation was affected the amount of RNA of endogenous *ONSEN*. I presumed that a mRNA of endogenous *ONSEN* was degraded and mRNA derived from *GFP* was only detected under non-stress condition. The transcriptional activation of *ONSEN* and *GFP* subjected to heat stress on *gem1, 2 and 3* were stronger than wild type and *nrpd1* (Fig.8) that

indicated the *ONSEN* promoter in the mutants was more activated than that of wild type and *nrpd1*. I presumed that the increased *ONSEN* transcription might be caused by the active chromatin state in these mutants under heat stress. It is worth to study more detail of the *ONSEN* regulatory factors.

ONSEN was transposed in reproductive tissue caused by changing chromatin modification initiated by flowering

Here I reported that the *dcl2* single mutant and *dcl2/dcl3/dcl4* triple mutant released *ONSEN* transposition in the progeny of heat-stressed plants (Fig.12). *Arabidopsis* encodes several DCL proteins. DCL1 produces microRNAs, 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). Previous study showed that 21-nt siRNA class was mainly detected after heat stress both in wild type and *nrpd1* (Ito et al. 2011). DCL2 and DCL4 act redundantly on post-transcriptional gene silencing (PTGS) via 21-nt siRNA (Parent JS et al. 2015) indicated that *ONSEN* transposition might be suppressed by RNA degradation. According to my result, these DCL proteins might function compensatory to suppress *ONSEN* transposition.

A substantial *ONSEN* RNA and/or extrachromosomal DNA might be necessary for transgenerational transposition. However, transcriptional activity of *ONSEN* was no longer detected in the shoot apex and a cauliflower-like floral meristem on *ap1-1; cal1-1* after heat stress (Fig.6, 10. 11). Furthermore, *ONSEN* extrachromosomal DNA

was gradually decreased during plant development after heat stress (Ito et al. 2011). In addition, I showed that *ONSEN* was inherited through male and female gametogenesis. And the transposition occurred in seedlings in which the inflorescence meristem has not been formed and *ONSEN* transposition activity was maintained independent of recovery length after heat stress (Fig14-17).

These results suggested that some stress memory might be maintained during plant development although the transcript of *ONSEN* gradually decreased. As a result, *ONSEN* transposition occurred during flower development before gametogenesis in *nrpd1*. Although epigenetic modification is generally stable in somatic tissue, global epigenetic reprogramming occurs in germ cells and early embryos. (Feng *et al*. 2010). In *Arabidopsis*, the gene expression of certain transposons are up-regulated in pollen vegetative nucleus that does not provide DNA to the fertilized zygote (Slotkin *et al*. 2009). Transient TE reactivation potentially signals TE silencing in the neighboring sperm *via* siRNAs. (Slotkin *et al*. 2009). These findings suggested that the transcription of *ONSEN* was activated again in reproductive tissues caused by epigenetic reprogramming. It might be worthwhile to investigate tissue specific reactivation of *ONSEN*.

In addition, in the heat-stressed progeny, *HsfA2* and *ONSEN* were activated with decreasing of global DNA methylation under non-stress condition (Migicovsky *et al*. 2014). The transgenerational epigenetic changes were partially deficient in the Dicer-like mutant however *ONSEN* expression increased in the progeny of heat-stressed plants regardless of mutant type (Migicovsky *et al*. 2014). A mutant of *DCL3* showed

hypermathylation in the progeny of heat stress plants while hypomethylation was observed in a mutant of *DCL2* (Migicovsky *et al*. 2014). These results indicated that heat stress memory could inherit to the next generation. It will be worthwhile to investigate the mechanism by which the stress memory is maintained during plant development in *nrpd1*.

I analyzed transposition pattern on offspring from each branches (Fig.18). The same transposition pattern on offspring within single branch flower indicated that some transposition occurred prior to flower branch differentiation. In contrast, the different transposition pattern in the offspring from adjacent flowers indicated that transposition was occurred only one side of homologous chromosome and the different transposition pattern was appeared via meiosis. The new insertions were detected on an *nrpd1* progeny originate from the side shoots (Fig. 19) indicated that *ONSEN* transposition was not occurred on SAM immediately after heat stress. Some transposition occurred just before the flower bud initiation. According to these results, transposition activation might be maintained over a long period of time, although the transcriptional activation was transient. After that, *ONSEN* transposition might be occurred on inflorescence meristem triggered by changing vegetative to reproductive stage.

Previous study showed that a large-scale reorganization of chromatin was observed during floral transition in *Arabidopsis* (Tessadori *et al*. 2007). The pericentric heterochromatin reduced prior to bolting and recovered after elongation of the floral stem. Also decondensation of chromatin in gene-rich regions coincided with the floral transition. After the global decondensation of chromatin, sequential condensation was

occurred on pericentromeric repeat. As a result, *ONSEN* transposition might be suppressed in wild type. In contrast in *nrpd1*, global chromatin decondensation might be occurred on larger scale than wild type and/or could not restore the condensation state (Fig.21).

In this study, I showed that *ONSEN* transcription was regulated by heat shock factor and siRNA-mediated pathway and re-silenced independent of NRPD1 function. A transient expression of *ONSEN* might be regulated independent of DNA methylation. It is worth to study the chromatin modification state in *nrpd1* and find another regulatory component by using transcriptional suppressor mutants. In addition, I presumed that tissue specific reactivation of *ONSEN* might be initiated by global epigenetic reprogramming and/or changing vegetative to reproductive phase. *ONSEN* transposition was occurred on some siRNA biogenesis deficient mutants. These results indicated that PolIV-related pathway might suppress *ONSEN* transposition via RdDM and/or chromatin modification. In *nrpd1*, transposition was occurred during flower development before gametogenesis because of global change of chromatin modification. PolIV might restore the chromatin decondensation on flower tissues and inhibit *ONSEN* transposition. However, *ONSEN* transcription was transient and I still do not know the maintenance mechanism of heat stress memory (Fig.21). It is interesting to study the stress memory of *ONSEN*. Recent study showed that heat stress-induced splice variant of *HsfA2* acts as a functional Hsf and could bind to the HSE in the HsfA2 promoter to activate its own expression (Liu *et al*. 2013). It might be also act as heat stress memory on tissue specific reactivation of *ONSEN*. Furthermore, a truncated form of the

Saccharomyces Ty1 retrotransposon capsid protein inhibits virus-like particle assembly and function (Garfinkel et al. 2015). It is worth to study the *ONSEN* VLP stability during the plant development. Plant has evolved the regulation mechanisms that were independent of suppression by constitutive heterochromatin. The regulation was important during the process of plant development and may have a function in undifferentiated cells.

FIGURE LEGENDS

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

Figure 2 *ONSEN* activation subjected to multi-generational heat stress. **(a)** A scheme of multi-generational treatment of heat stress. Seedlings were subjected to 21°C for 24 hours (black frame) or 37°C (red frame) for each generations. **(b)** Relative *ONSEN* transcript levels after single- or multi-stress treatment were analyzed at progeny 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 treatments were analyzed at progeny by q-PCR (mean±s.e.m., n=3, values relative to 21°C wild-type). NS; non-stress, S1; heat stress in the first parent generation, S2; heat stress in the progeny. MS; heat stress in the first and the second generations.

Figure.3 Southern blots of *Eco*RV digested DNA isolated with the progenies of multi-stressed plants (MS-1 and MS-2) and a non-stressed plant (NS). **(a)** In wild type, no new insertion was detected in the MS progenies. **(b)** In *nrpd1,* high frequency of new transpositions was detected in the MS progenies. An ethidium bromide (EtBr)-stained

gel was shown as loading control. **(c)** Relative *ONSEN* transcript levels in the progenies of non-stressed (PNC), single-stressed on the second generation (PS2) or multi-stressed (PMS) plant were analyzed by qRT-PCR (mean±s.e.m., n=3, values relative to the transcript directly after 37°C (HS) in wild-type).

Figure 4 Activation of *ONSEN* by high light stress and oxidative stress. Plants before stress treatment, after continuous treatment for 1 h and after continuous treatment for 6 h were analyzed. **(a)** Relative transcription level of *ONSEN* immediately after high light stress (HL). **(b)** Relative transcription level of *ONSEN* immediately after Paraquat treatment (PQ). Error bars represent SDs (n = 3).

Figure 5 Structure of the genes used to produce transgenic plants, and the insertion position of the transgene in the genome. **(a)** The structure of the DNA sequence between the left border (LB) and the right border (RB) of the T-DNA. Intact long terminal repeat (LTR) of *ONSEN* (*AT5G13205*) was fused with the green fluorescent protein (*GFP*) gene. **(b)** The transgene was inserted in the intergenic region between *At1g26850* and*At1g26860*, in the same direction. Numerals indicate the nucleotide number according to the AGI map.

Figure 6 Expression analysis of *GFP* driven by the *ONSEN* promoter. **(a)** Scheme of the heat stress (HS) experiment. **(b)** Photographs of bright field and GFP fluorescence images. Wild-type plants (WT) and *nrpd1* plants (*nrpd1*) were observed immediately

after HS and after the recovery phase (Recovery). **(c)** Relative transcription level of *GFP*. WT and *nrpd1* plants (*nrpd1*) were analyzed immediately after HS and after the recovery phase (R3). **(d)** Relative transcription level of endogenous *ONSEN*. The same samples as those used in c were used. NS, non-stress control samples. Error bars represent the mean ± SEM, $n = 3$; values are relative to heat-stressed wild type (WT HS).

Figure 7 Methylation sensitive Southern blot analysis of a transgene. **(a)** Scheme of the restriction enzyme assay and the position of specific GFP probe. **(b)** Southern blot analysis was performed on seedlings of the wild type and *nrpd1* transgenic lines were exposed to 37 °C for 24 hours and 5 days after the treatment respectively. To detect cytosine methylation, *Sac*I and *Hpa*II, *Msp*I, and *Alu*I double-digested DNA was used respectively. WT: wild-type transgenic line, nrpd1: *nrpd1* transgenic line, NS: non stress, HS: heat stress, R5: 5 days recovery phase after NS or HS.

Figure 8 Phenotype and endogenous *ONSEN* expression of GFP expression mutant. **(a-c)** GFP intensity of 7 day-old seedlings under non stress. **(d-f)** GFP intensity of 7 day-old under non stress (NS) and after heat stress (HS). (g) Expression analysis of endogenous *ONSEN*. WT HS: heat stress treated wild type. *nrpd1* HS: heat stress treated *nrpd1*. *gem*: GFP expression mutants under non-stress (NS) (h) Expression analysis of endogenous *ONSEN* after heat stress (HS). WT HS: heat stress treated wild

type. *nrpd1* HS: heat stress treated *nrpd1*. *gem*: GFP expression mutants after heat stress.

Figure 9 (a) Relative transcription level of *ONSEN* in heat-stressed callus after 30 d of recovery (HS + 30) or in non-stressed callus (NS +30). *ONSEN* was active in callus for 30 d after the heat treatment. (b) Relative copy number of *ONSEN* in non-stressed callus (NS+ 30) and in heat-stressed callus after 30 d of recovery (HS + 30). (c) Southern blot of *EcoR*V-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*.

Figure 10 A tissue specific regulation of *ONSEN*. **(a)** A paraffin section of young seedling of *Arabidopsis*. **(b)** A paraffin section of young seedling after isolating a leaf primordium by laser capture microdissection. **(c)** A relative transcript level of *ONSEN* in young leaf tissue and leaf primordium subject to heat stress (HS) and 5 days after heat stress (HS+R5). WT: young leaf tissue in wilt type. *nrpd1*: young leaf tissue in *nrpd1*. WT SA: the shoot apex in wild type. *nrpd1* SA: the shoot apex in *nrpd1*.

Figure 11 Expression analyses of *ONSEN* and *WUS.* (a) A relative transcript level of *ONSEN* in 8 day-old seedlings of wild type, *nrpd1* and *ap1-1; cal1-1*, 31 day-old flower of wild type and nrpd1, cauliflower-like floral meristem of *ap1-1; cal1-1*. (b) A

transcript level of *Wus* and *Act2* in 8 day-old seedlings of wild type, *nrpd1* and *ap1-1; cal1-1*, 31 day-old flower of wild type and nrpd1, cauliflower-like floral meristem of *ap1-1; cal1-1*. HS: heat stress, NS: non stress, R31: 31day-old plant at recovery phase.

Figure 12 Southern blots of *Eco*RV digested DNA isolated with the six progenies of each mutants; *dcl2*, *dcl3*, *dcl4*, *dcl2dcl3*, and *dcl2dcl3dcl4*. **(a)** No transposition was observed on the progenies of non-stressed *dcl2*, *dcl3*, and *dcl4*. **(b)** One new copy was detected on a heat-stressed progeny of *dcl2* (arrowhead) although no transposition was detected on *dcl3* and *dcl4* single mutant. **(c)** *ONSEN* was silent in *dcl2dcl3* double mutant and *dcl2dcl3dcl4* triple mutant under non-stressed condition. **(d)** Transgenerational transpositions were detected in the two progenies of a triple mutant subjected to heat stress (arrowheads) although no transposition was detected in the progenies of a stressed double mutant. HS: heat stress.

Figure 13 The quantitative analyses of transcripts, extrachromosomal DNAs, and transposed copies in the progeny. WT and *nrpd1* plants were exposed to HS for 6 h and for 24 h at 7 days after germination. NS: non-stressed samples. **(a)** Relative transcription level of *ONSEN*. Error bar represents the mean ± SEM, $n = 3$, values relative to 24 h heat-stressed WT. **(b)** Relative number of copies of extrachromosomal DNA of *ONSEN*. Error bar represents the mean ± SEM, $n = 3$; values are relative to the NS WT seedlings. **(c)** Southern blot analysis of *ONSEN* in progeny plants.

Figure 14 Southern blot of *Eco*RV digested DNA isolated with the progenies of three independent reciprocal crosses showed the transgenerative transpositions of *ONSEN*. **(a)** Heat-stressed *nrpd1* was crossed with non-stressed *nrpd1* reciprocally. As a control, heat-stressed or non-stressed *nrpd1* was self-fertilized and analyzed the next generation. **(b)** Heat-stressed *nrpd1* was crossed with wild type reciprocally. As a control, heat-stressed *nrpd1* or wild type was self-fertilized and the progenies were analyzed on the next generation. An ethidium bromide-stained gel is shown as loading control.

Figure 15 Transgenerational transposition of *ONSEN* in young seedling. (a) Scheme of the HS experiment. On each day after germination, seedlings were exposed to HS for 24 h. (b,c) Southern blot analysis of *ONSEN* using the progeny of WT (b) and *nrpd1* (c)plants that were exposed to HS, as indicated in (a). Durations of HS are indicated over the lanes. NS plants were used as controls. Arrowheads indicate the transposed copies of *ONSEN*.

Figure 16 The average of the day on which bolting occurred was measured in 3 to 8 plants that grew under long-day (LD) or short-day (SD) conditions. Plants were exposed to HS for 24 h at 7, 10, 14, and 21 days after germination. The WT and *nrpd1* plants that grew under LD and SD were compared, respectively. An asterisk indicates significantly different, $p < 0.05$. NS: control plants.

Figure17 Transgenerational transpositions in wild-type (a) and *nrpd1* (b) plants. Data for plants grown under long-day (LD) and short-day (SD) conditions are shown in the left panels and right panels, respectively. 7, 10, 14, and 21 denote plants that were exposed to HS for 24 h at 7, 10, 14, and 21 days after germination, respectively. NS: non-stressed plants.

Figure 18 Southern hybridization of *ONSEN* using the progeny of heat-stressed plants. (a) DNAs were extracted from the progeny of an *nrpd1* plant. Numbers over lanes indicate the parent lines. Four progenies from the same seedpod were analyzed. The location of each seedpod on the parent plant is shown on the illustrated plant. Arrowheads indicate the new copies of *ONSEN* and the same color denotes that they transposed to the same loci. (b) DNAs was extracted from the progeny of another *nrpd1* plant.

Figure 19 Transgenerational transpositions in late-differentiated branches. (a) A scheme to obtain seeds for progeny. Parent *nrpd1* plants were exposed to HS for 24 h at 7 days after germination, then the primary flower stalk was cut immediately after bolting, and seeds were harvested from secondary branches. (b) Southern hybridization of *ONSEN*. DNAs of progeny from two NS parents and five HS parents were analyzed. Numbers over the lanes indicate that DNA was extracted from the progeny of different parents. Arrowheads indicate the new copies of *ONSEN*.

Figure 20 A model of transcriptional activation of *ONSEN* after heat stress and on recovery phase in wild type and *nrpd1*.

Figure 21 A model of tissue specific transpositional activation of *ONSEN*. White triangle represents endogenous *ONSEN* and red triangle represents newly inserted *ONSEN*. Red arrowhead represents a chromosomal integration event.

EXPERIMENTAL PROCEDURES

Plant material and growing conditions

All plants of *Arabidopsis thaliana* wild type, *nrpd1*, (Herr *et al.* 2005) and transgenic plant that involved a full length LTR of *ONSEN* fused with a GFP gene were grown on MS (Murashige and Skoog) plates with continuous light at 21°C. For the analysis of high light and oxidative stress, wild type, *nrpd1*, and *HsfA2* T-DNA insertion mutant (Nishizawa et al. 2006) were grown on MS at 25˚C under continuous light (irradiance 100 μmol m^{-2} s^{-1}). For short-day and long-day analysis, plants were grown on a MS pot for 7 days with continuous light at 21°C and move to a long-day chamber (16 hours light and 8 hours dark) or short-day chamber (8 hours light and 16 hours dark) respectively. All mutants used in this study (*nrpd1a-3* SALK_128428*, dcl2-3* SALK_095069*, dcl3-1* SALK_005512*, dcl4-2* CS66075*, dcl2-1/dcl3-1* CS16393, *dcl2-1/dcl3-1/dcl4-2* CS16391 and *ap1-1;cal1-1* CS67157) and wild-type plants were of the *Arabidopsis thaliana* ecotype *Columbia* background.

Stress treatments

Heat treatment was applied to wild type and all mutants in this study subjected to a temperature shift from 21°C to 37°C. After heat treatment, plants were transplanted to soil for further growth at 21°C. High light and oxidative stress were performed at 7-day-old wild type, *nrpd1,* and *hsfa2* plants that were exposed to high light (HL) at 800 μmol m^{-2} s^{-1} at 25˚C or sprayed with 50 μM methylviologen (MV).

Callus induction and stress treatment

Seeds were incubated on plates with callus-inducing medium (2,4-D, 0.5 mg ml^{-1}; kinetin, 0.1 mg ml^{-1}). 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 ºC for 24 h to 37ºC for 24 h. After the stress treatment, calluses were continuously incubated for 30 days at 21ºC with continuous light.

Plasmid constructs

All experiments were performed by Gateway technology (Life Technology). An artificial chromosome (T31B5 BAC clone; ARBC stock center) containing full length of LTR on *At5g13205* was amplified using PrimeStar GXL DNA polymerase (TaKaRa R050A) and oligonucleotide primers AT5G13205-GFP-F1, and AT5G13205-GFP-R1 (see Fig. 3. and Table1). PCR condition was 5 min at 94ºC for first denature, followed by 30 sec at 94ºC and 30 sec at 55ºC and 1 min at 72ºC for 30 cycles, and finishing with 7 min incubation at 72°C. Blunt-end PCR products were sub-cloned into pENTR D TOPO vector (Invitorogen K2400-20) subsequently cloned into pMDC107 vector (Curtis and Grossniklaus, 2003) using Gateway LR Clonase Enzyme mix (Invitrogen 11791-019) according to the supplier's recommendations.

Plant transformation

All experiments were performed on contract service by Inplanta Innovations (http://www.inplanta.jp/traits_conversion.html). Transformed pMDC107 vector contained plasmid DNA was transformed to *Agrobacterium tumefaciens* GV3101, transfected into Col-0. Transformant were selected 50ug/ml hygromycin containing 0.5 X Murashige and Skoog basal medium (SIGMA).

Genotyping PCR

Total DNA was extracted from seedlings by using EDM buffer (200mM Tris pH7.5, 250mM NaCl, 25mM EDTA,0.5% SDS) according to the K.Edwards et al. (1991). PCR was performed with the Applied Biosystems 2720 Thermal Cycler by using TaKaRa Ex Taq (TaKaRa RR001A).

Sequencing

Cycle sequencing was performed by using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems 4337455) according to the supplier's recommendations. Nucleotide sequence determination was performed by using ABI3130 Genetic Analyzers (Applied Biosystems).

TAIL-PCR

Total DNA was extracted from seedlings by using EDM buffer (200mM Tris pH7.5, 250mM NaCl, 25mM EDTA,0.5% SDS) according to the K.Edwards et al. (1991). PCR

was performed with the Applied Biosystems 2720 Thermal Cycler by using TaKaRa Ex Taq (TaKaRa RR001A). Cycle PCR condition was described at Table 2.

Fluorescence microscopy

Transgenic seedlings were visualized by using Zeiss AXIO Zoom. V16 microscopy. For observation of GFP signals, fluorescence was excited with a 470 nm metal halide laser, and emission images were collected in the 525 nm range. And transgenic seedlings were visualized by using FUJIFILM LAS3000. For observation of GFP signals, fluorescence was excited with a 460 nm blue LED, and emission images were collected in the 510 nm range by using 510DF10 filter.

Southern blot analysis

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 was carried out using a radio labeled *ONSEN* and *GFP*-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 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 ReverTraAce qPCR RT Kit (TOYOBO FSQ-101) with an oligo dT primer and a random 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 THUNDERBIRD SYBR qPCR Mix (TOYOBO QPS-201). For high light and oxidative stress experiments, RNA was extracted using the Plant RNA Reagent (Life technologies). Two μg of total RNA were subjected to RNase-free DNaseI (Thermo scientific) and reverse-transcribed by using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa 6210A). Quantitative Real-Time PCR was performed with a LightCycler 96 System (Roche), using the FastStart Universal SYBR Green Master (ROX) (Roche). 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.

RT-PCR

Total RNA was extracted from seedlings 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 ReverTraAce qPCR RT Kit (TOYOBO FSQ-101) with an oligo dT primer and a random primer. RT-PCR was performed with the Applied Biosystems 2720 Thermal Cycler by using TaKaRa Taq (TaKaRa R001A).

Laser capture microdissection and RNA extraction

Arabidopsis seedlings were fixed in Farmer's fixative (Ethanol : Acetic acid = 3 : 1) overnight at 4ºC. Subsequently fixation, dehydration, and paraffin infiltration were performed using microwave processor (H2850 EBS). Paraffin-embedded sections were cut to a thickness of 12um and mounted on PEN membrane glass slide (Applied Biosystems LCM0522). To remove paraffin, slides were immersed in Histo-Clear II (National Diagnostics HS-202) for 5 min twice and air-dried at room temperature. Laser capture microdissection was performed using the ArcturusXT LCM Instrument (Applied Biosystems). Selected areas were captured by an infrared (IR) laser onto Arcturus CapSure Macro LCM Cap (LCM0211 Applied Biosystems) and subsequently cut by a UV laser. Total RNAs were extracted using PicoPure RNA Isolation Kit (Applied Biosystems KIT0204), quantified with Agilent RNA 6000 Pico Kit (Agilent 5067-1513).

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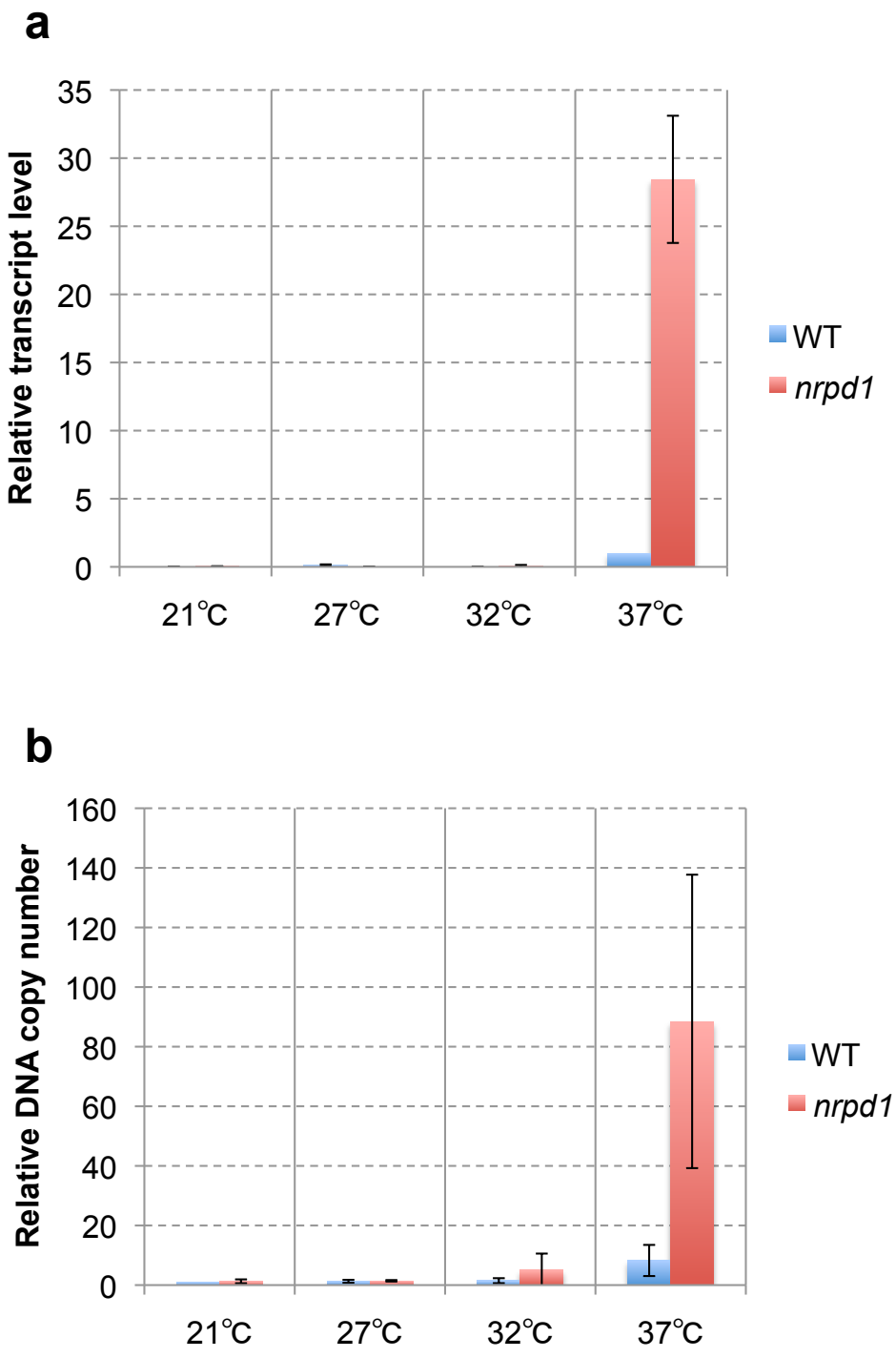
Fig.1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27℃
32℃
37℃
WT
nrpd1
b
Relative DNA copy number
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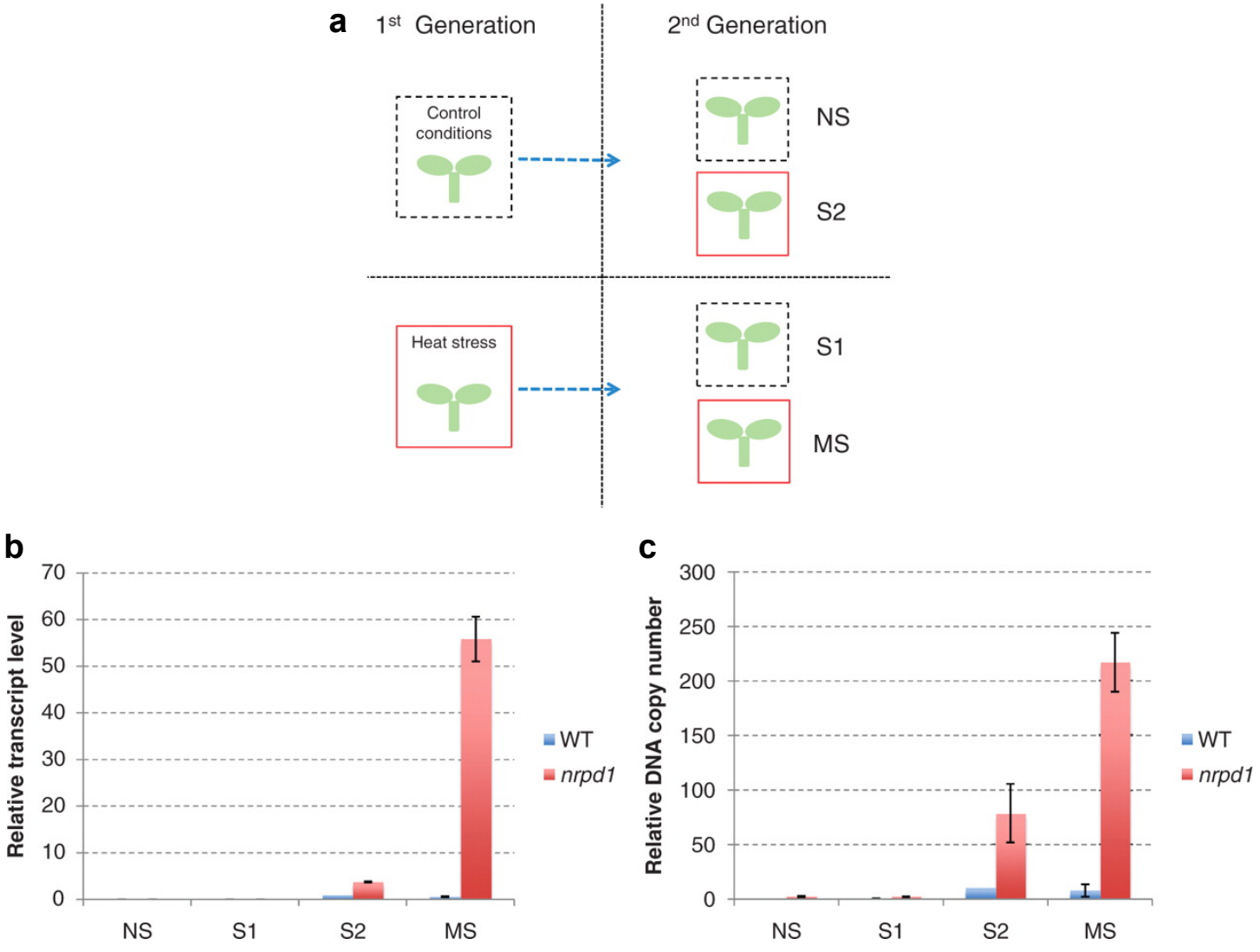
20
0
21℃
27℃
32℃
37℃
WT
nrpd1

Fig.2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1st Generation
2nd Generation
Control conditions
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S2
Heat stress
S1
MS
b
Relative transcript level
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50
40
30
20
10
0
NS
S1
S2
MS
WT
nrpd1
c
Relative DNA copy number
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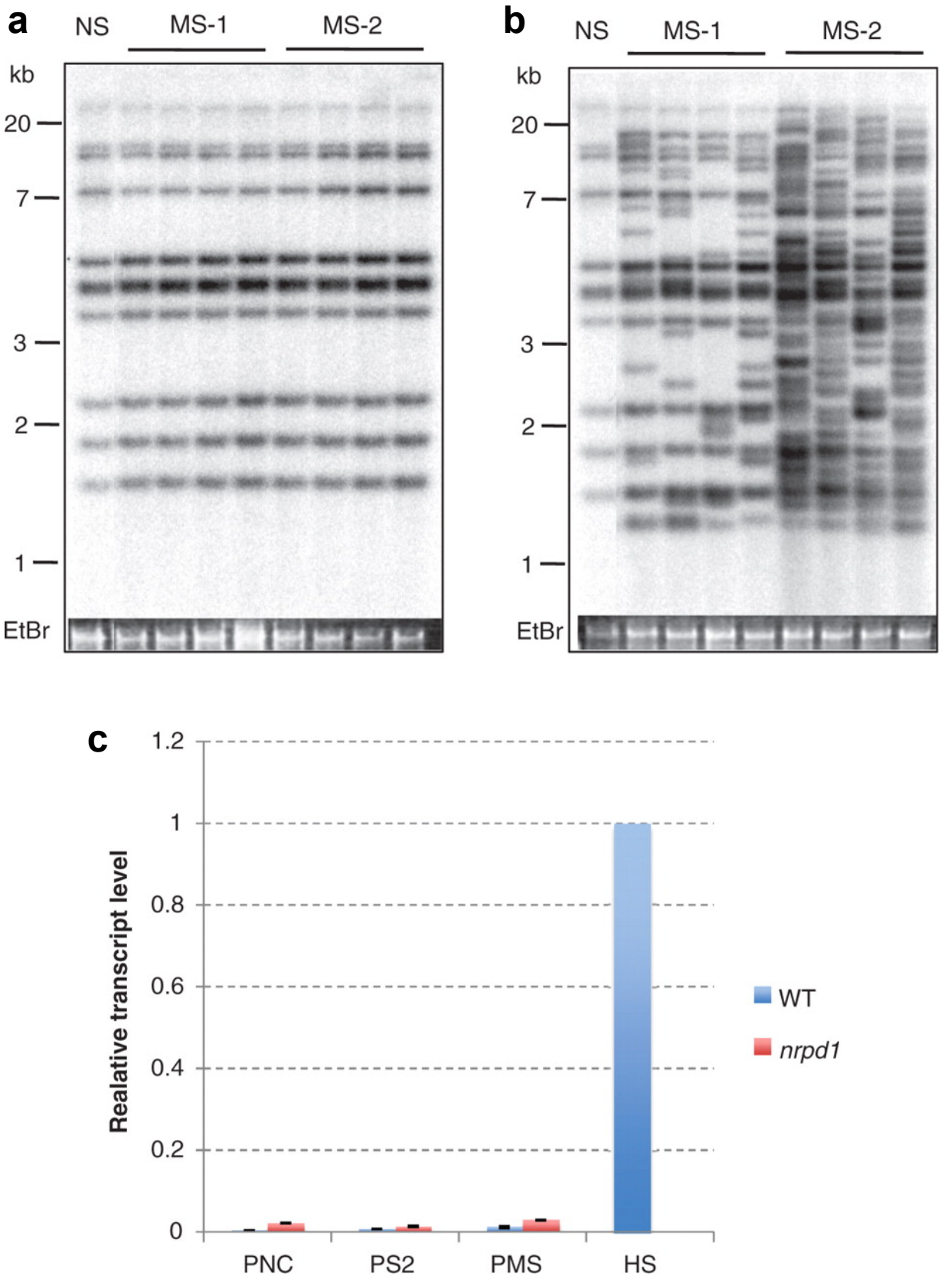
200
150
100
50
0
NS
S1
S2
MS
WT
nrpd1

Fig.3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MS-1
MS-2
kb
20
7
3
2
1
EtBr
b
NS
MS-1
MS-2
kb
20
7
3
2
1
EtBr
c
Realative transcript level
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0.6
0.4
0.2
0
PNC
PS2
PMS
HS
WT
nrpd1

Fig.4

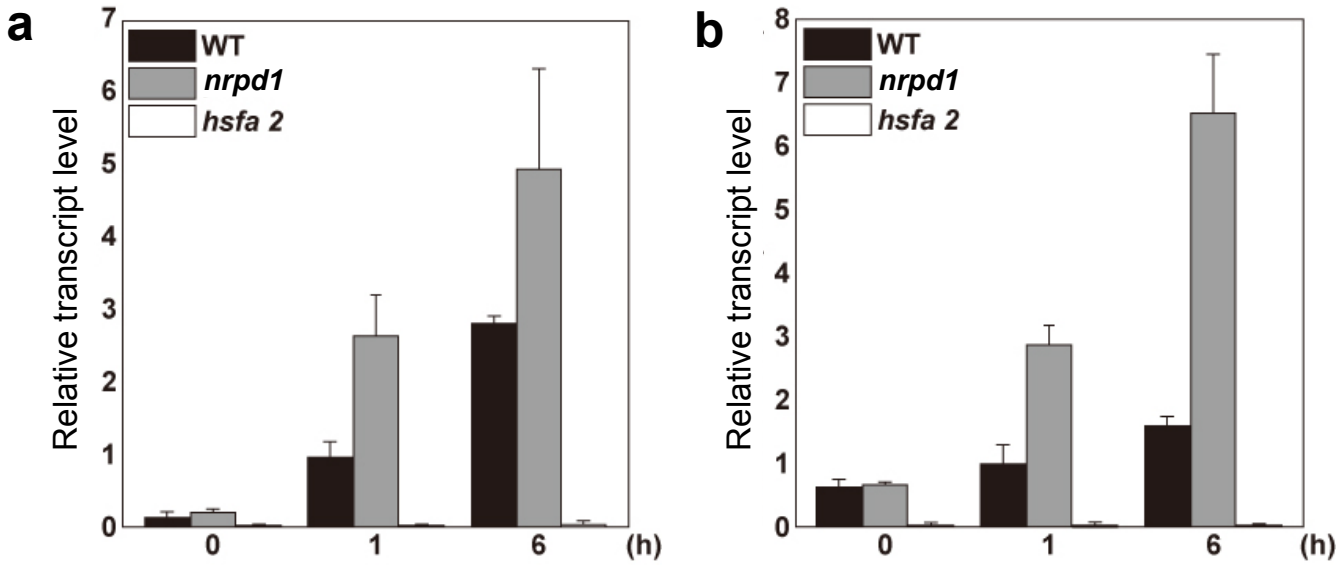
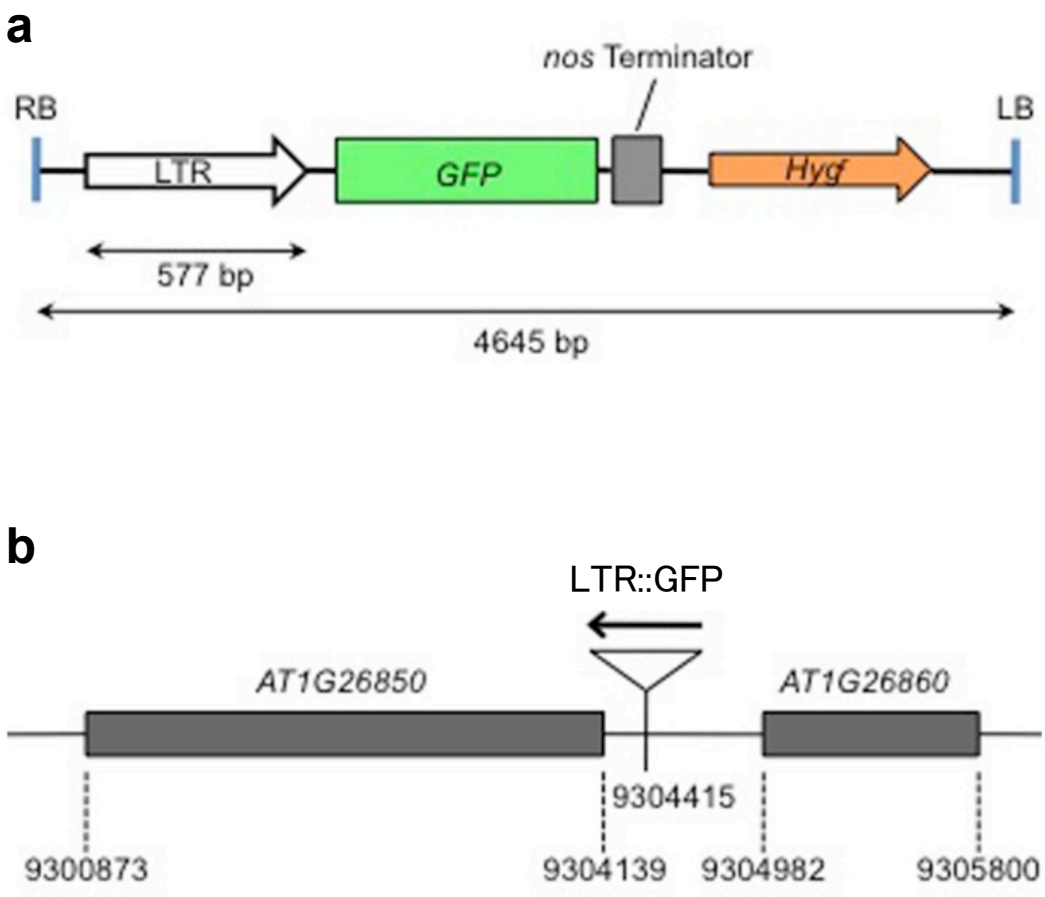


Fig.5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nos Terminator
RB
LB
LTR
GFP
Hyg
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4645 bp
b
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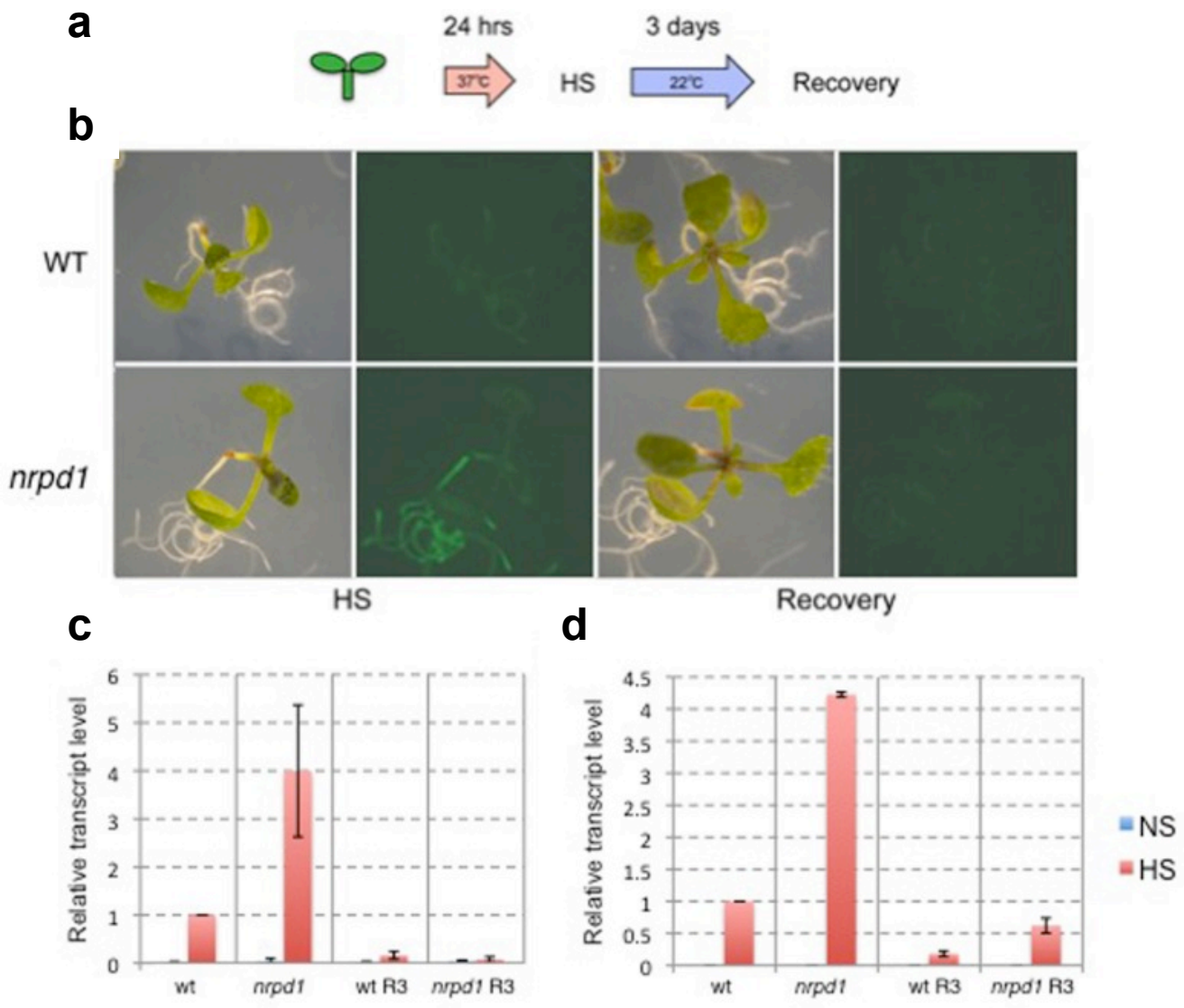
9304139
9304982
9305800

Fig.6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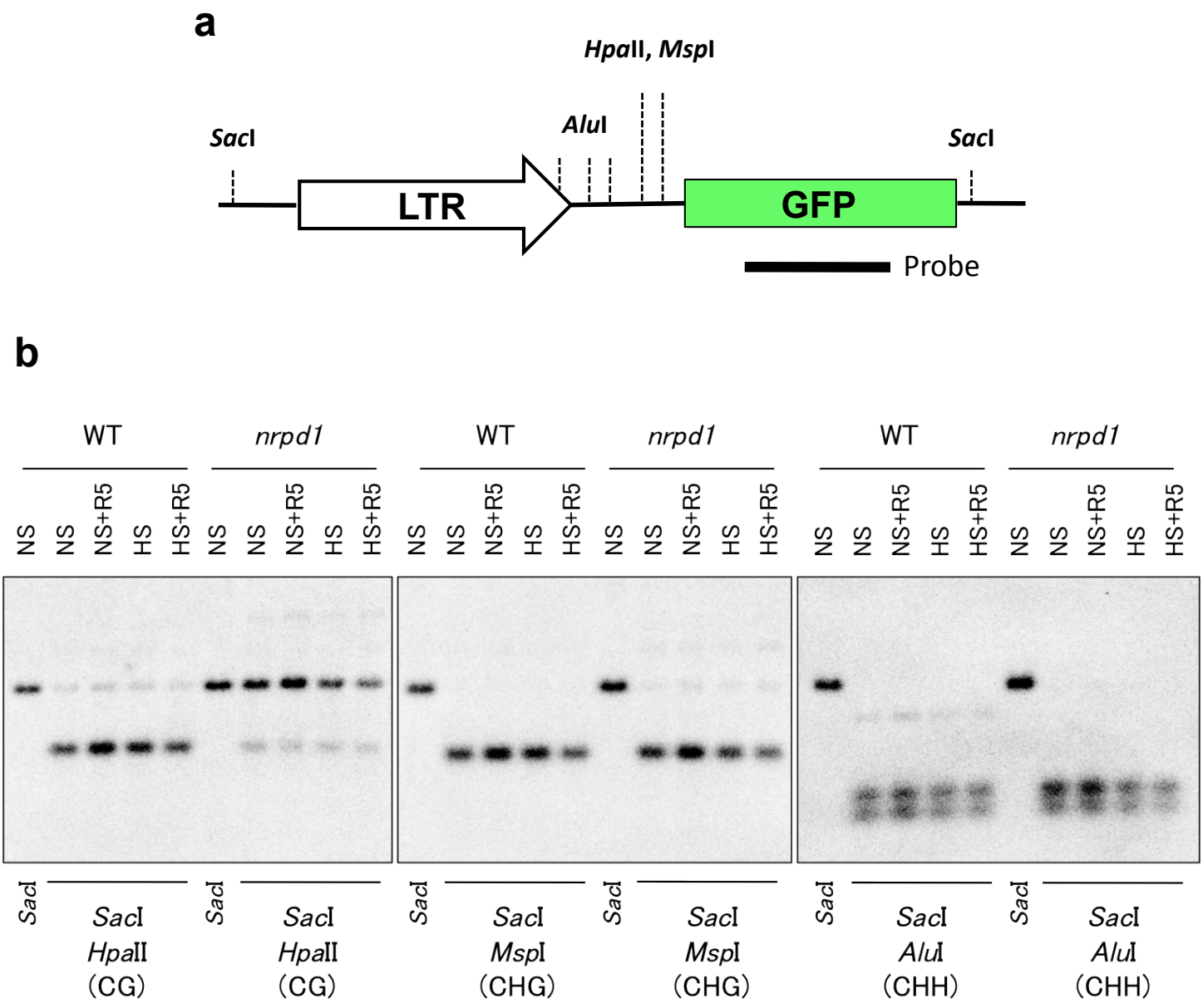
24 hrs
37°C
HS
3 days
22°C
Recovery
b
WT
nrpd1
HS
Recovery
c
Relative transcript level
6
5
4
3
2
1
0
wt
nrpd1
wt R3
nrpd1 R3
d
Relative transcript level
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
wt
nrpd1
wt R3
nrpd1 R3
NS
HS

Fig.7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HpaII, MspI
AluI
SacI
SacI
LTR
GFP
Probe
b
WT
nrpd1
WT
nrpd1
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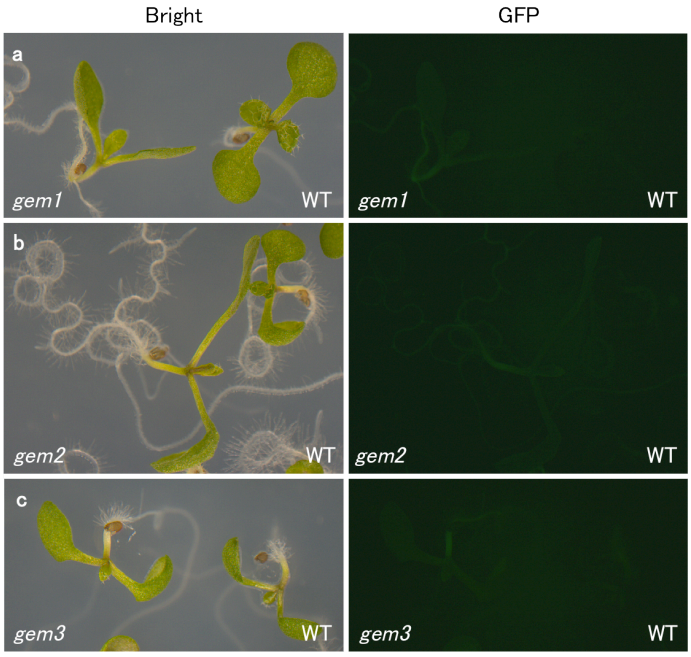
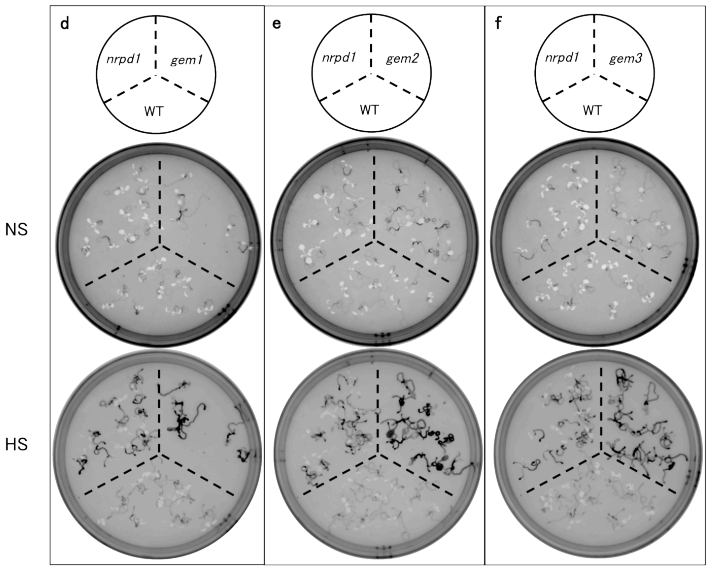
nrpd1
NS
NS
NS+R5
HS
HS+R5
SacI
SacI
HpaII
(CG)
SacI
MspI
(CHG)
SacI
AluI
(CHH)

Fig.8


Bright
GFP
a
gem1
WT
gem1
WT
b
gem2
WT
gem2
WT
c
gem3
WT
gem3
WT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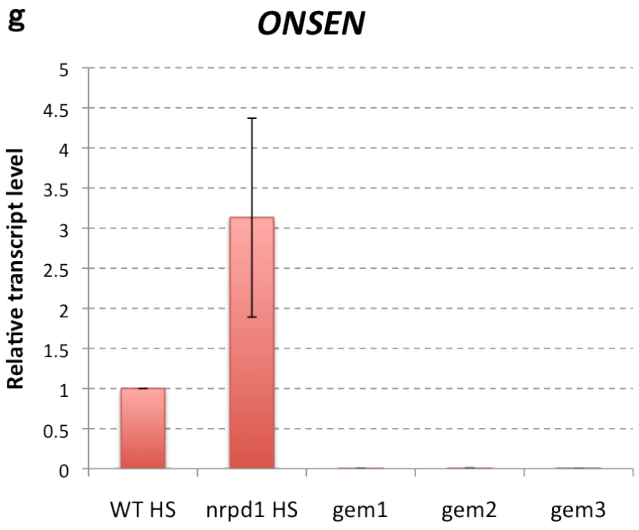
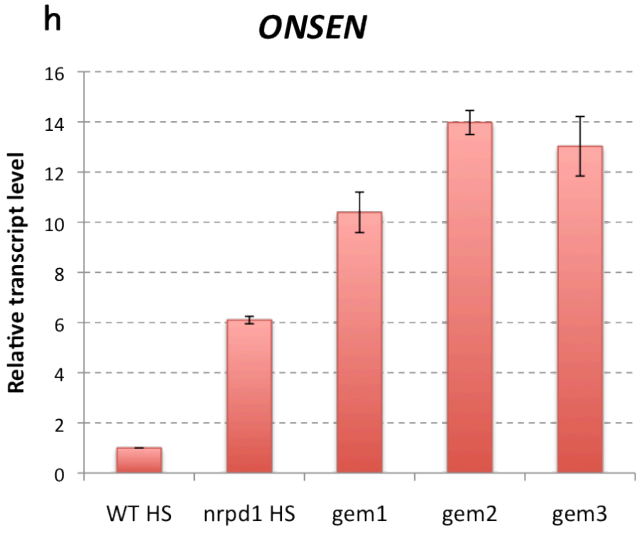
nrpd1
gem1
WT
e
nrpd1
gem2
WT
f
nrpd1
gem3
WT
NS
HS


g
ONSEN
Relative transcript level
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT HS
nrpd1 HS
gem1
gem2
gem3


h
ONSEN
Relative transcript level
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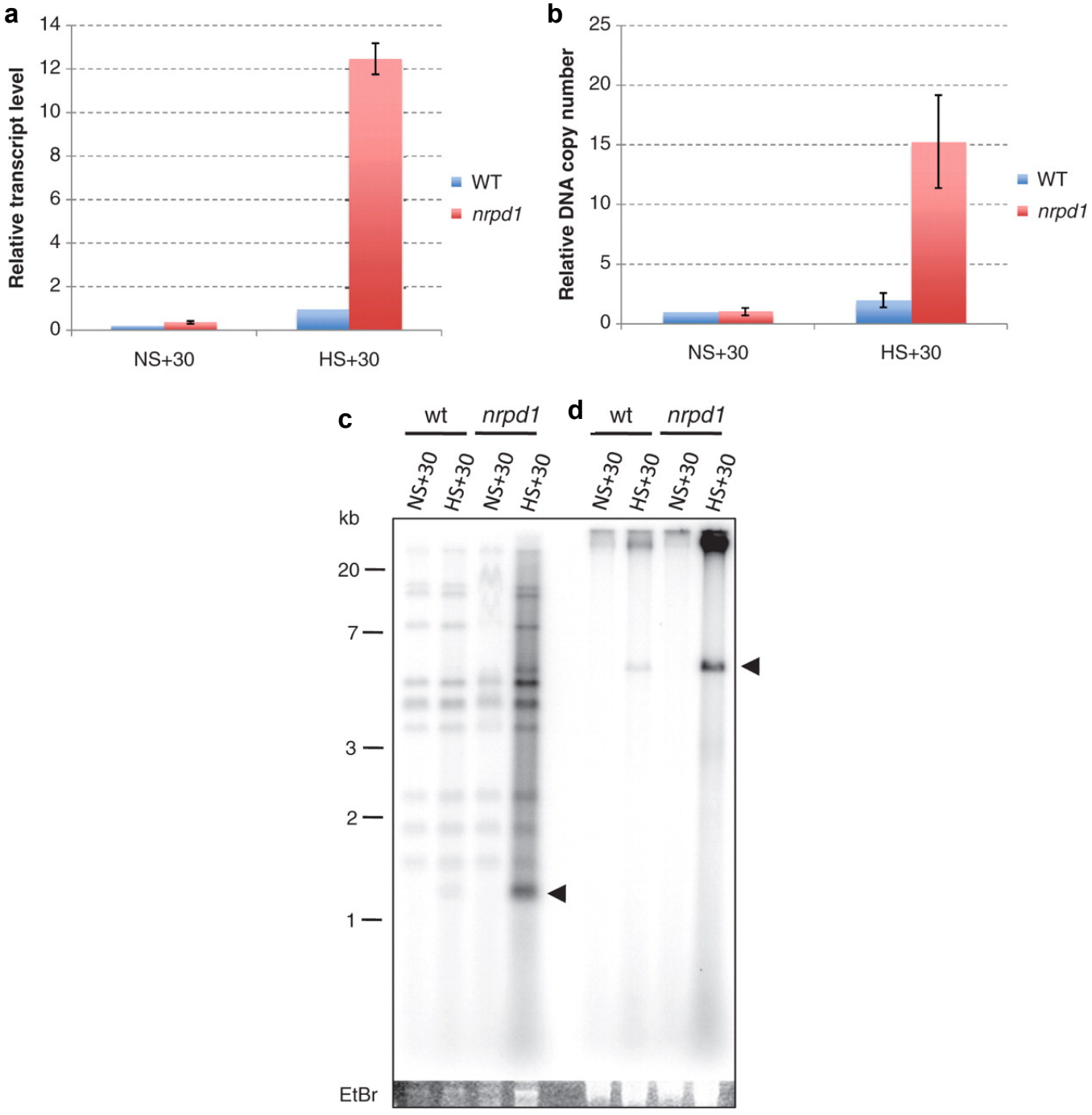
14
12
10
8
6
4
2
0
WT HS
nrpd1 HS
gem1
gem2
gem3

Fig.9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Relative transcript level
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12
10
8
6
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2
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HS+30
WT
nrpd1
b
Relative DNA copy number
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20
15
10
5
0
NS+30
HS+30
WT
nrpd1
c
wt
nrpd1
NS+30
HS+30
NS+30
HS+30
kb
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3
2
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d
wt
nrpd1
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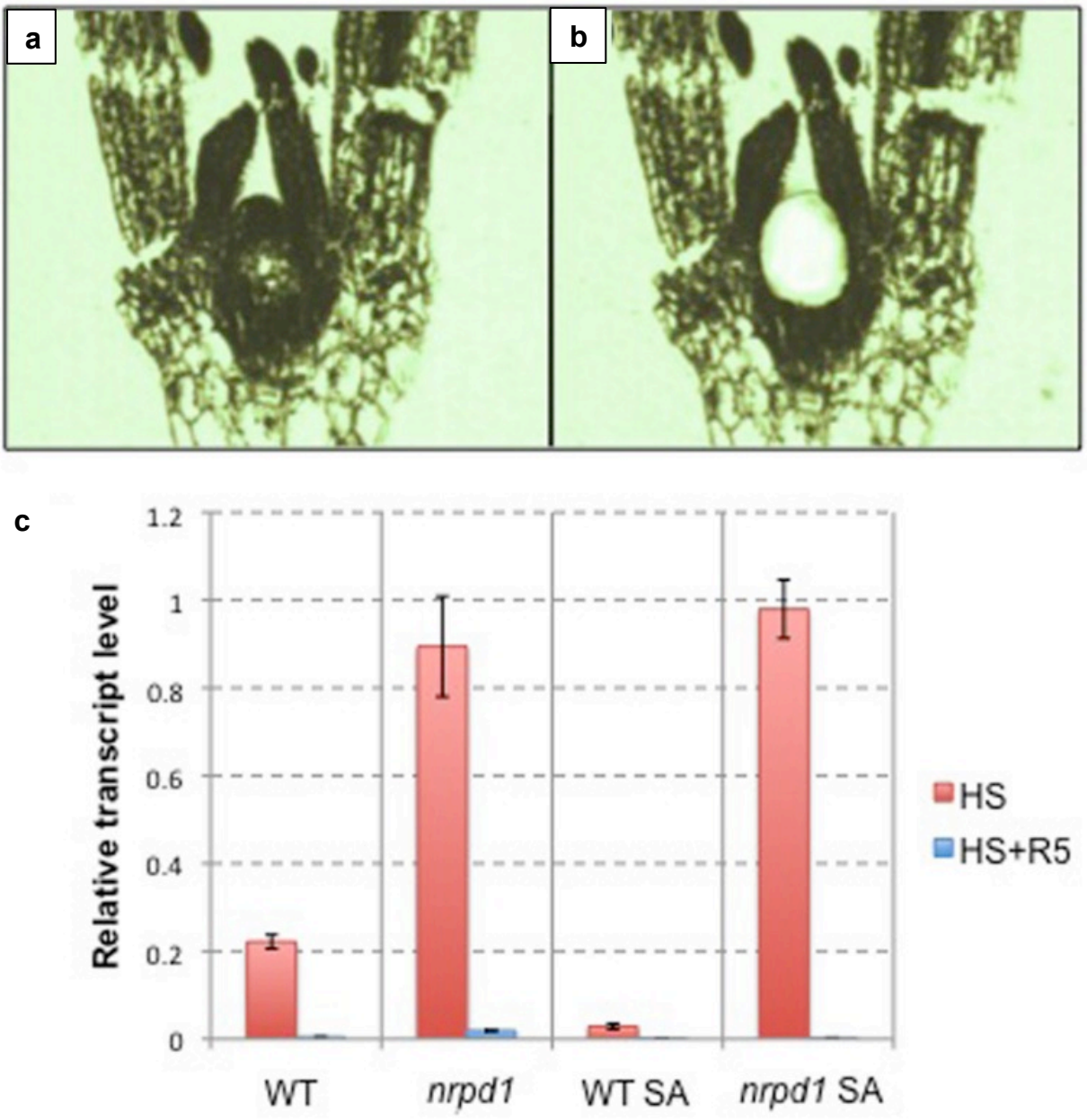
HS+30
NS+30
HS+30

Fig.10


a
b
c
Relative transcript level
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0.4
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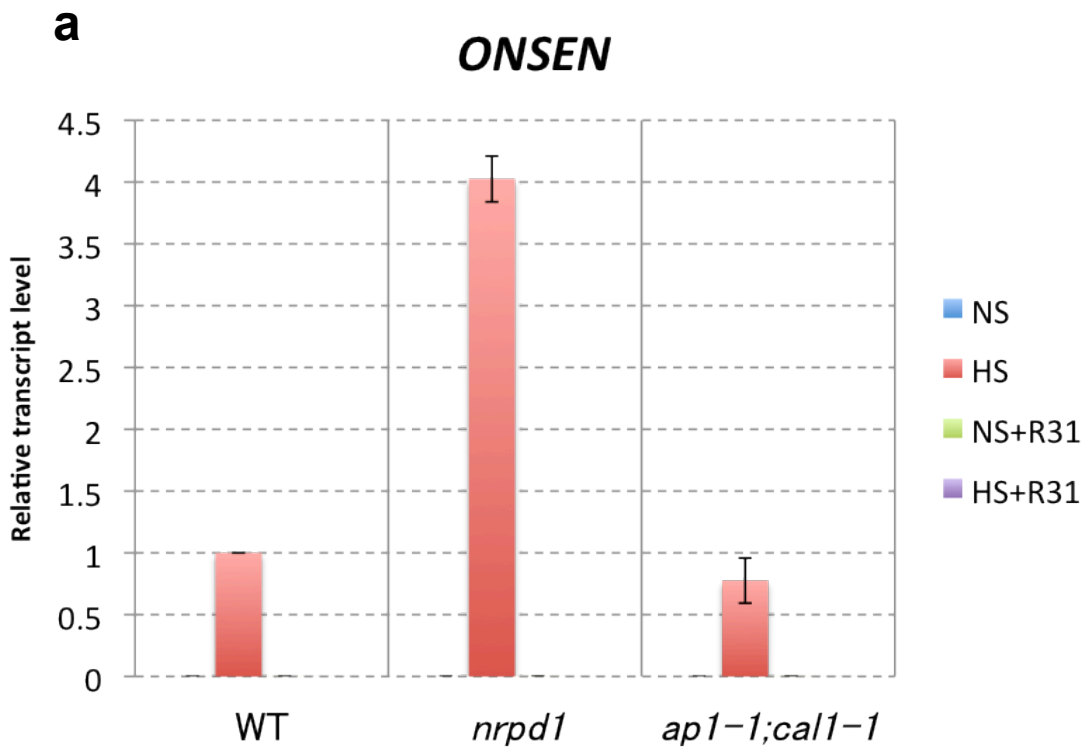
nrpd1
WT SA
nrpd1 SA
HS
HS+R5

Fig.11


a
ONSEN
Relative transcript level
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
nrpd1
ap1−1;cal1−1
NS
HS
NS+R31
HS+R31

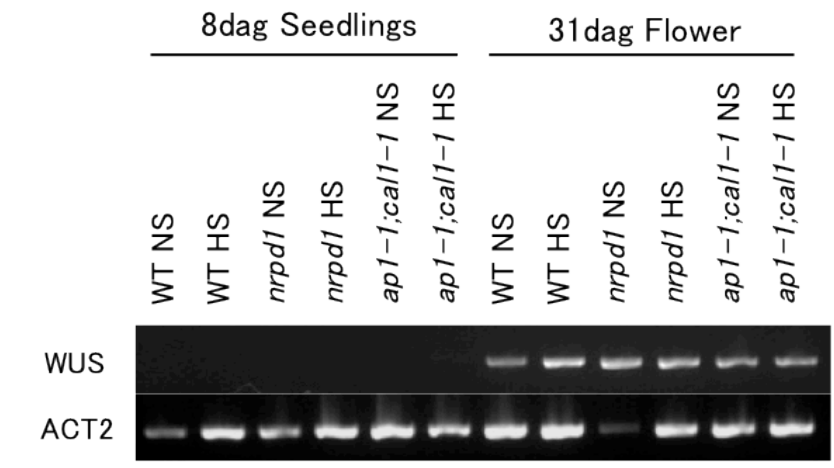
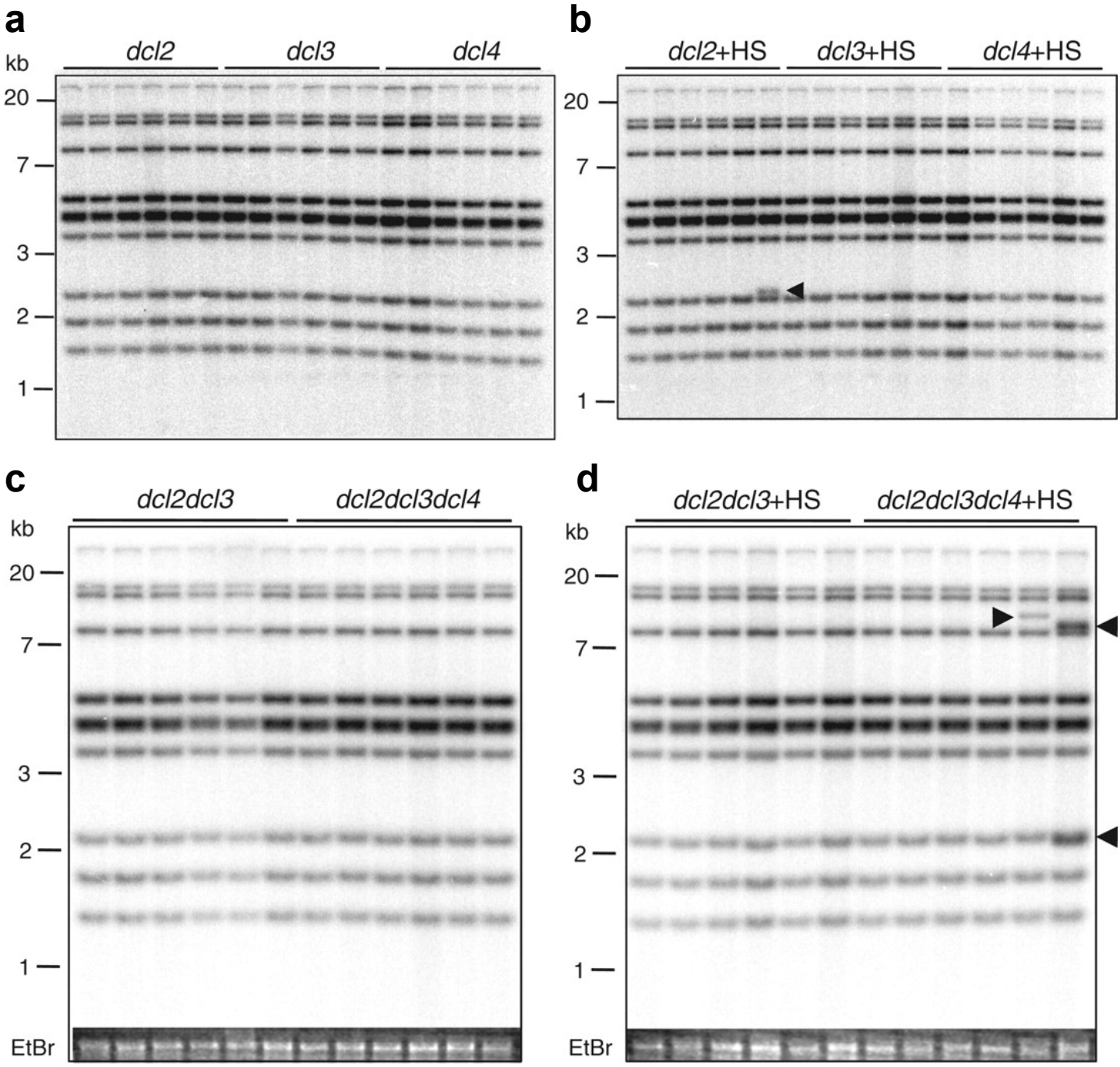

b
8dag Seedlings
31dag Flower
WT NS
WT HS
nrpd1 NS
nrpd1 HS
ap1−1;cal1−1 NS
ap1−1;cal1−1 HS
WT NS
WT HS
nrpd1 NS
nrpd1 HS
ap1−1;cal1−1 NS
ap1−1;cal1−1 HS
WUS
ACT2

Fig.12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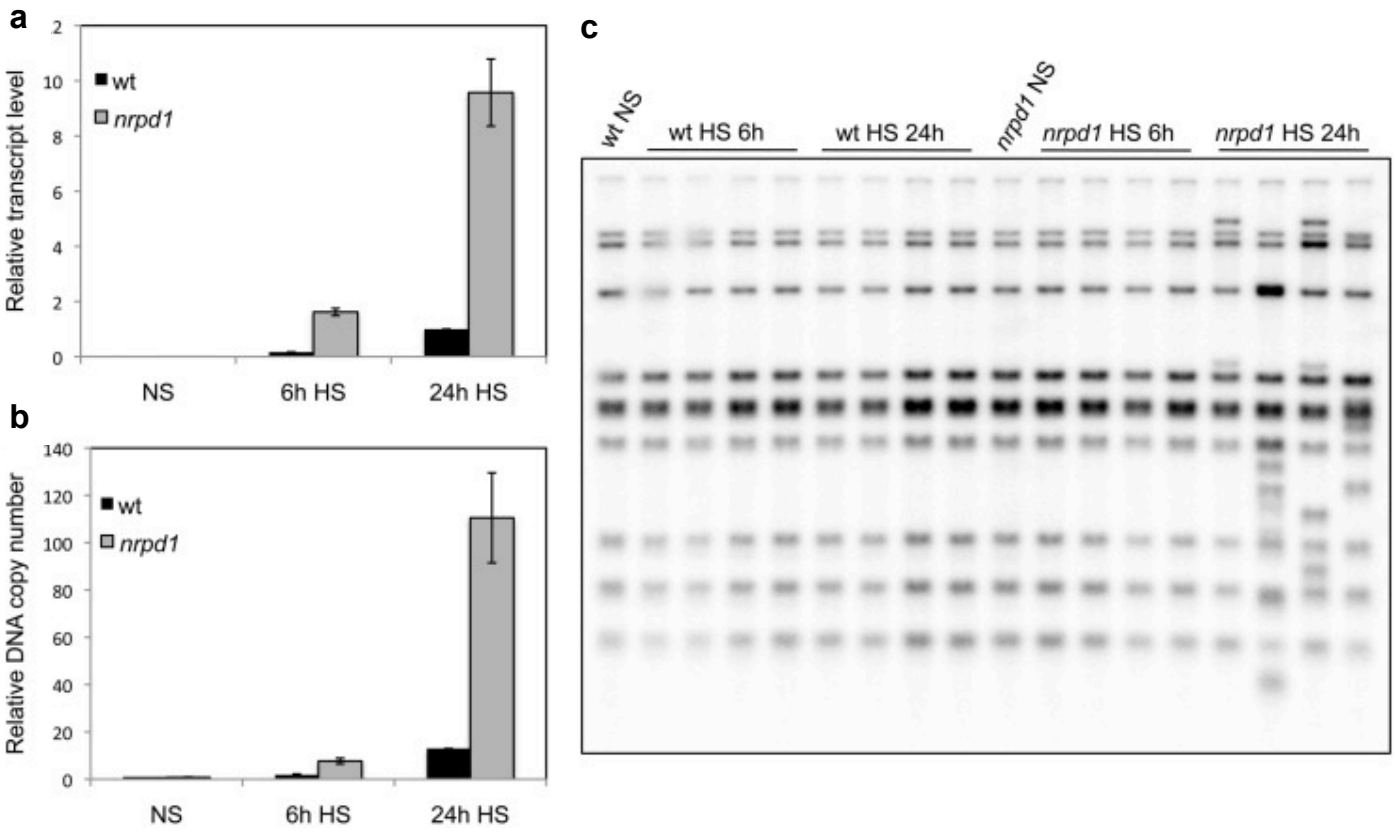
dcl2
dcl3
dcl4
kb
20
7
3
2
1
b
dcl2+HS
dcl3+HS
dcl4+HS
20
7
3
2
1
c
dcl2dcl3
dcl2dcl3dcl4
kb
20
7
3
2
1
EtBr
d
dcl2dcl3+HS
dcl2dcl3dcl4+HS
kb
20
7
3
2
1
EtBr

Fig.13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NS
6h HS
24h HS
b
Relative DNA copy number
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nrpd1
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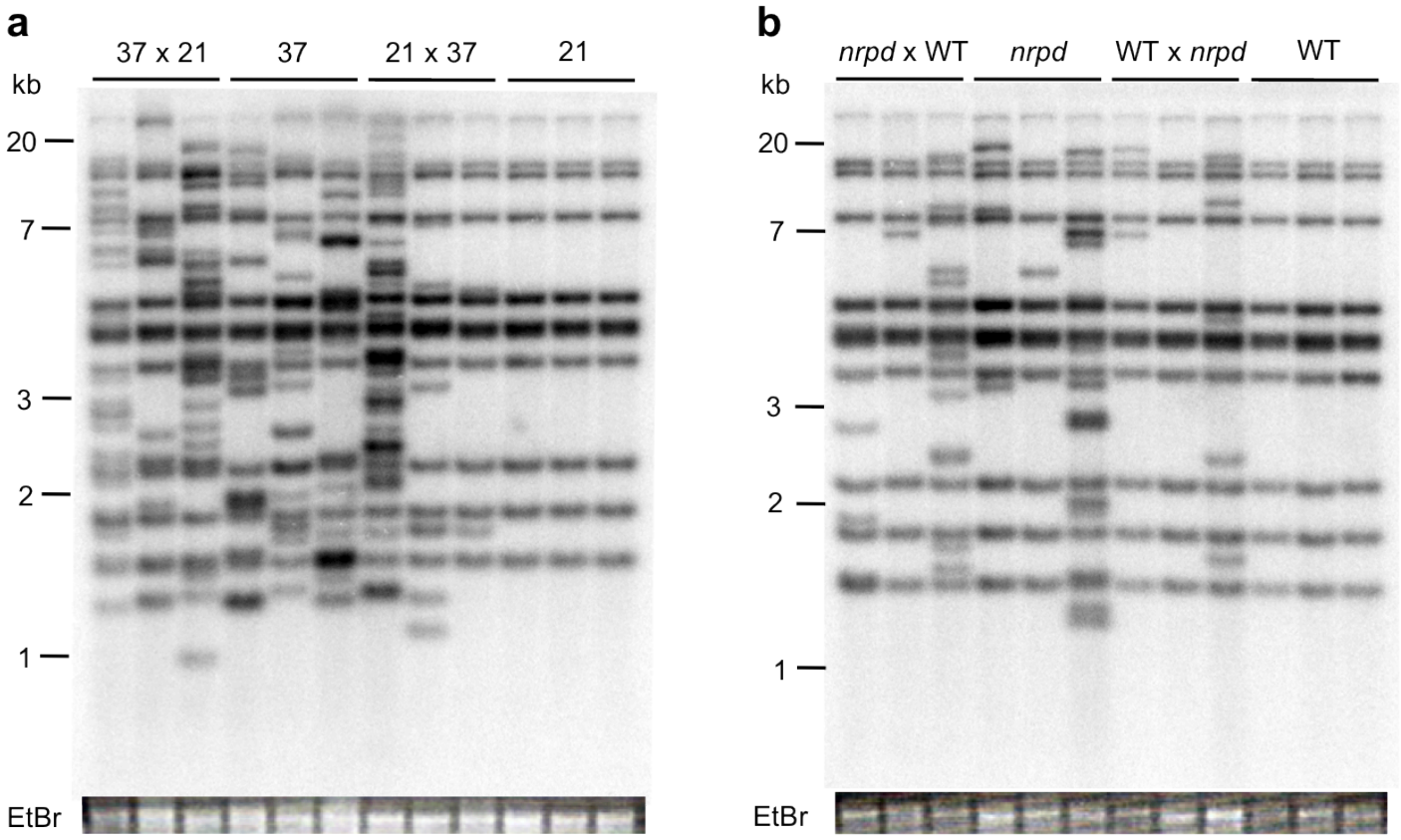
c
wt NS
wt HS 6h
wt HS 24h
nrpd1 NS
nrpd1 HS 6h
nrpd1 HS 24h

Fig.14


a
37 x 21
37
21 x 37
21
kb
20
7
3
2
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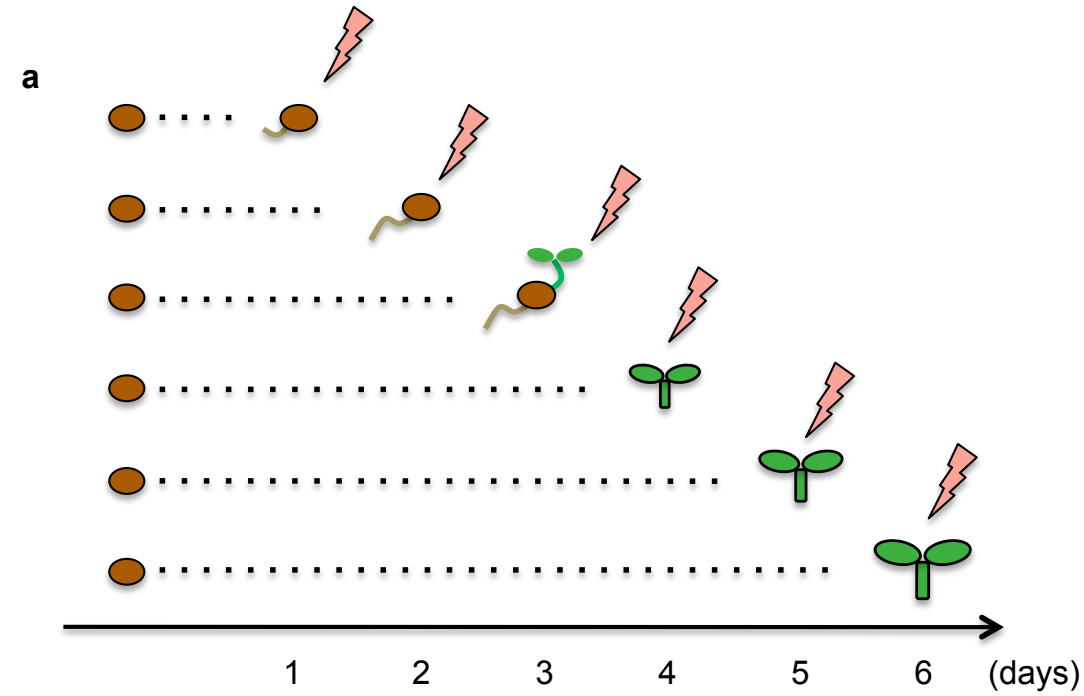
EtBr
b
nrpd x WT
nrpd
WT x nrpd
WT
kb
20
7
3
2
1
EtBr

Fig.15


a
1
2
3
4
5
6
(days)

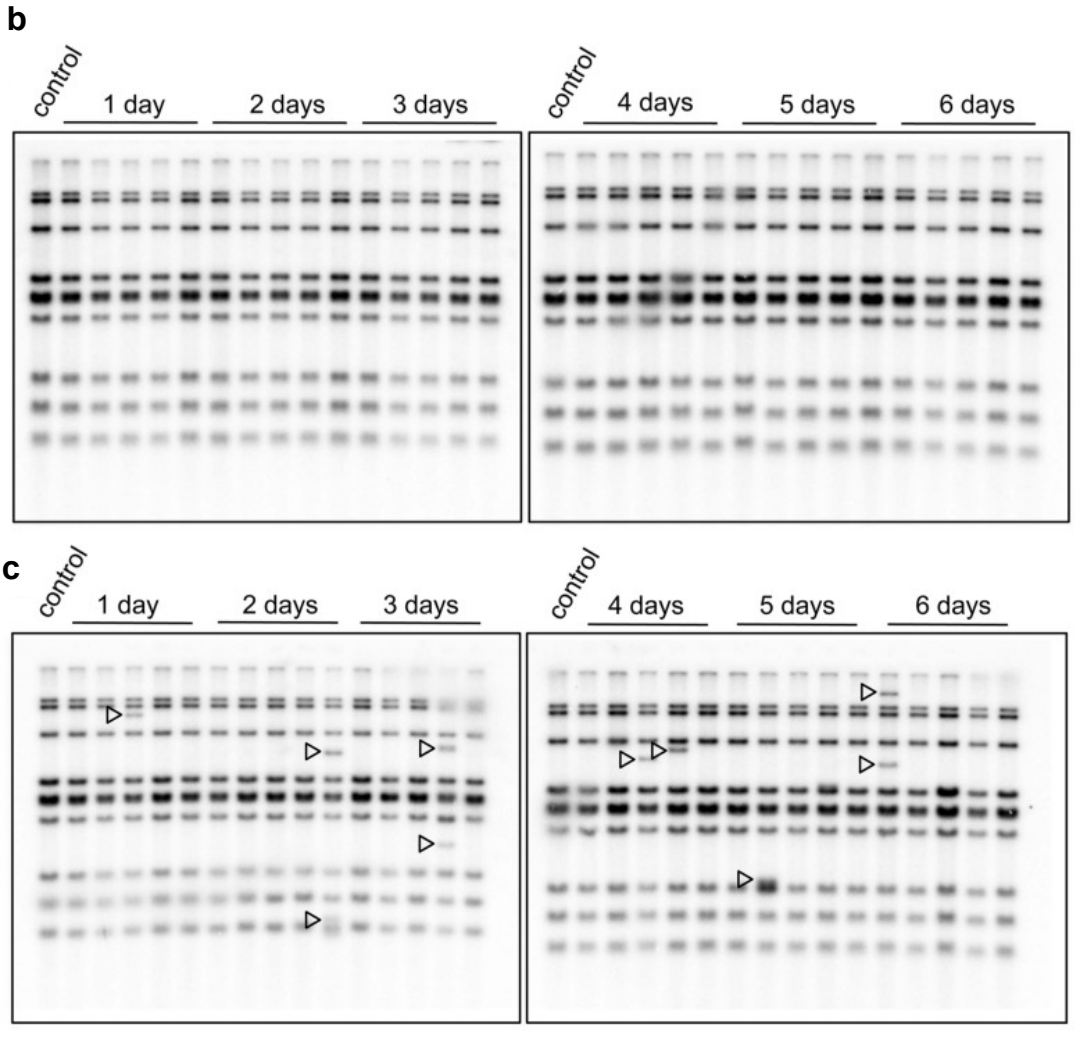

b
control
1 day
2 days
3 days
control
4 days
5 days
6 days
c
control
1 day
2 days
3 days
control
4 days
5 days
6 days

Fig.16

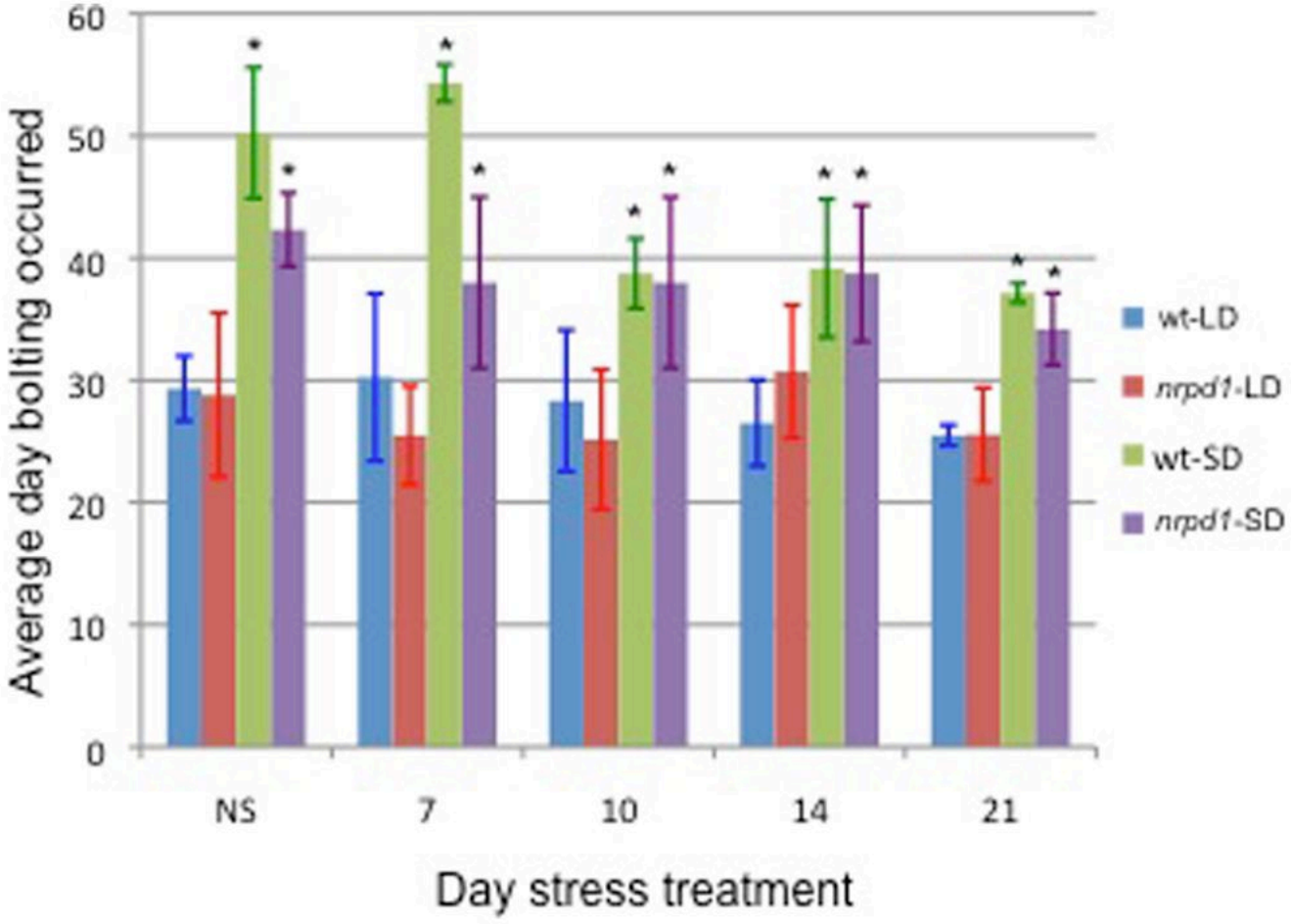
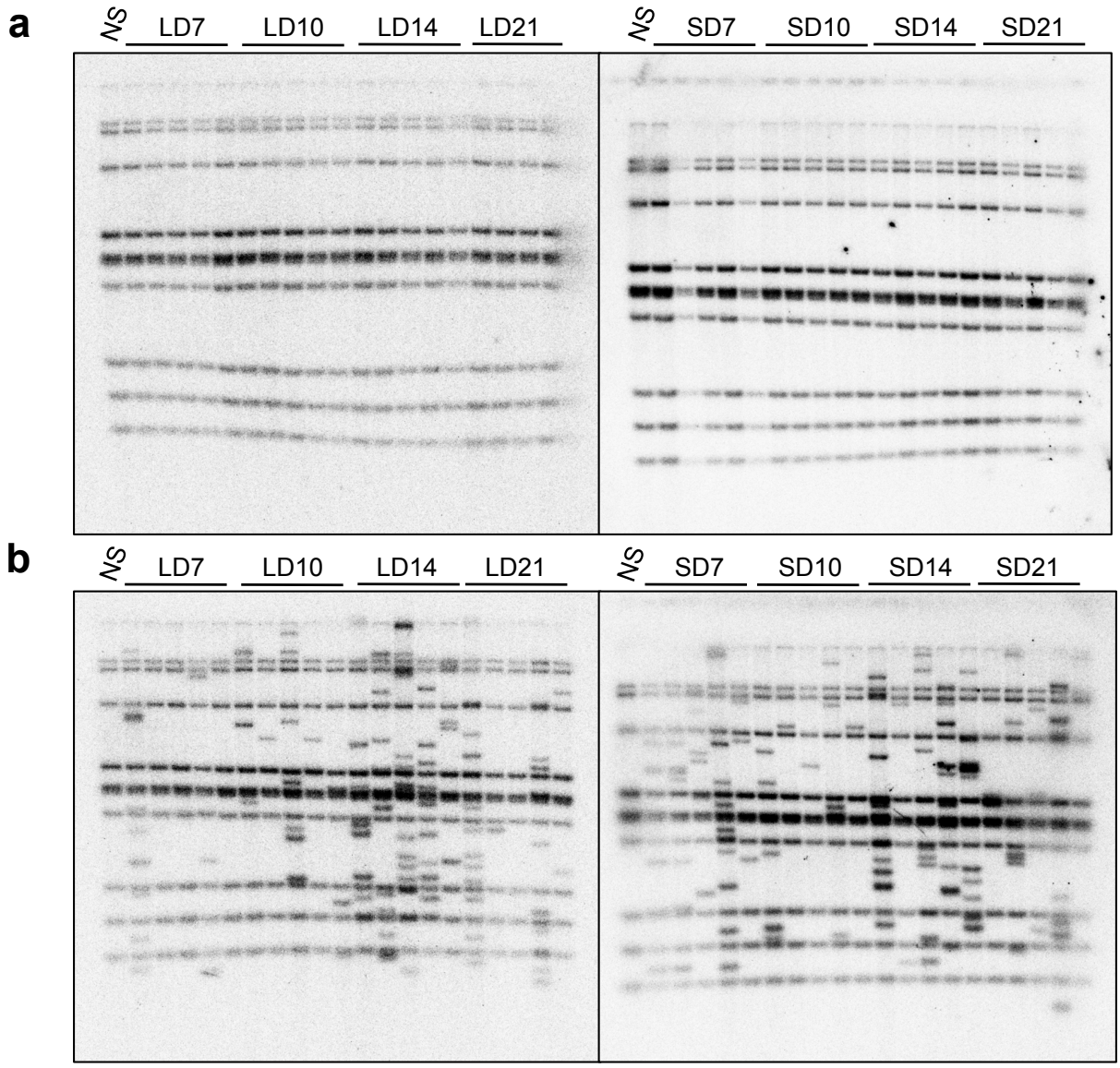

60
50
40
30
20
10
0
Average day bolting occurred
NS
7
10
14
21
Day stress treatment
wt-LD
nrpd1-LD
wt-SD
nrpd1-SD

Fig.17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LD7
LD10
LD14
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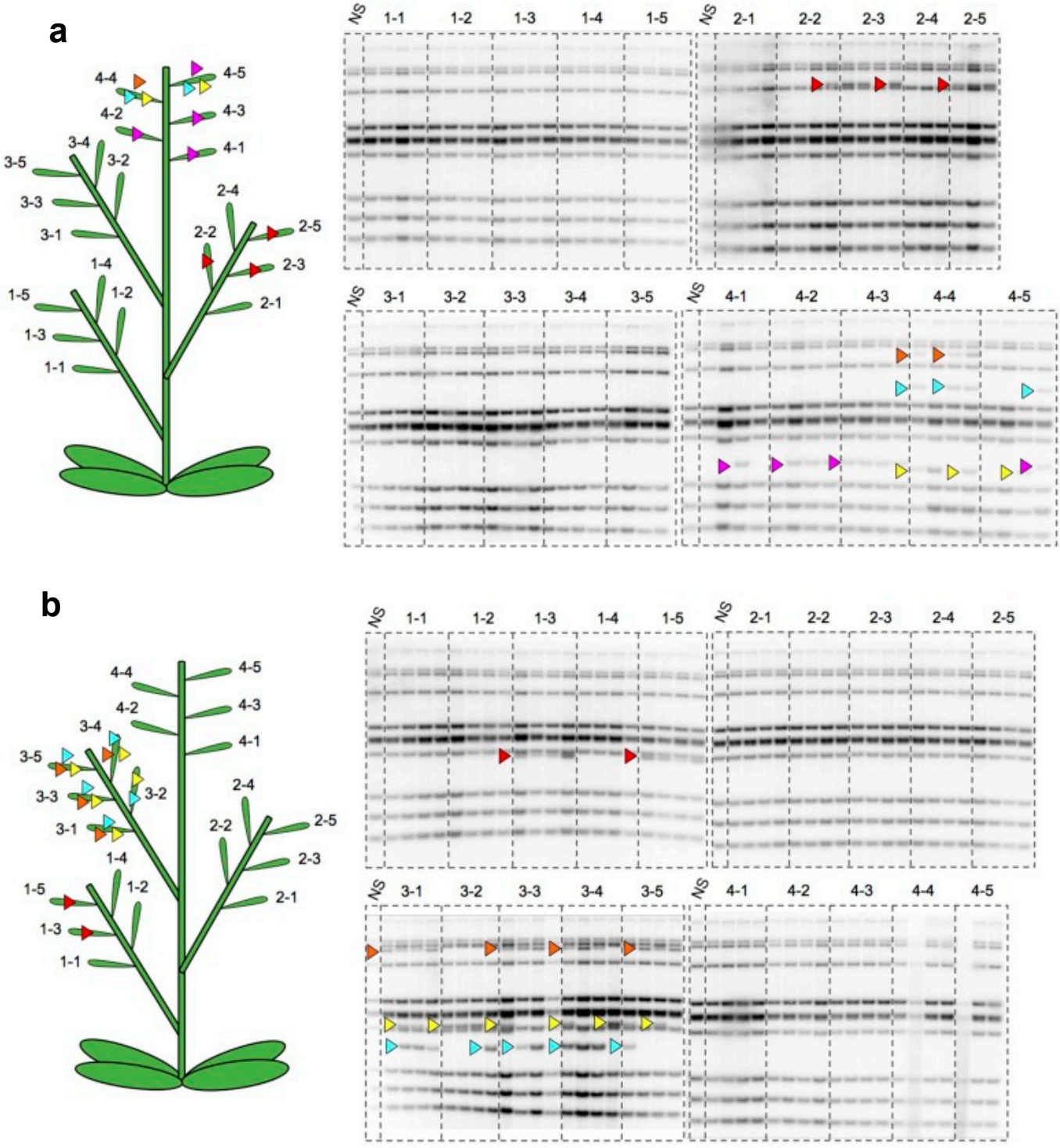
NS
SD7
SD10
SD14
SD21
b
NS
LD7
LD10
LD14
LD21
NS
SD7
SD10
SD14
SD21

Fig.18


a
4-4
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2-2
2-3
1-4
1-5
1-2
2-1
1-3
1-1
NS
1-1
1-2
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1-4
1-5
NS
2-1
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2-3
2-4
2-5
NS
3-1
3-2
3-3
3-4
3-5
NS
4-1
4-2
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b
4-4
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4-2
4-3
3-4
4-1
3-5
3-3
3-2
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2-2
2-5
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NS
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NS
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2-5
NS
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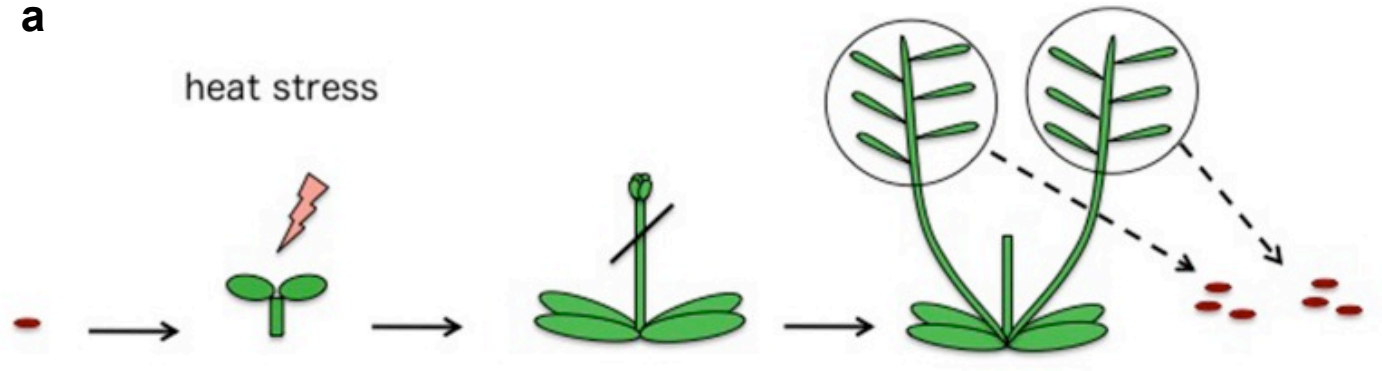
3-4
3-5
NS
4-1
4-2
4-3
4-4
4-5

Fig.19

a



b

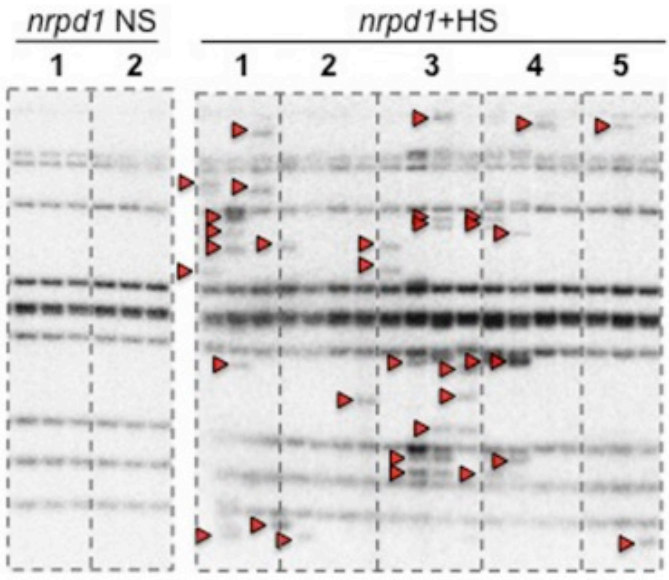


Fig.20

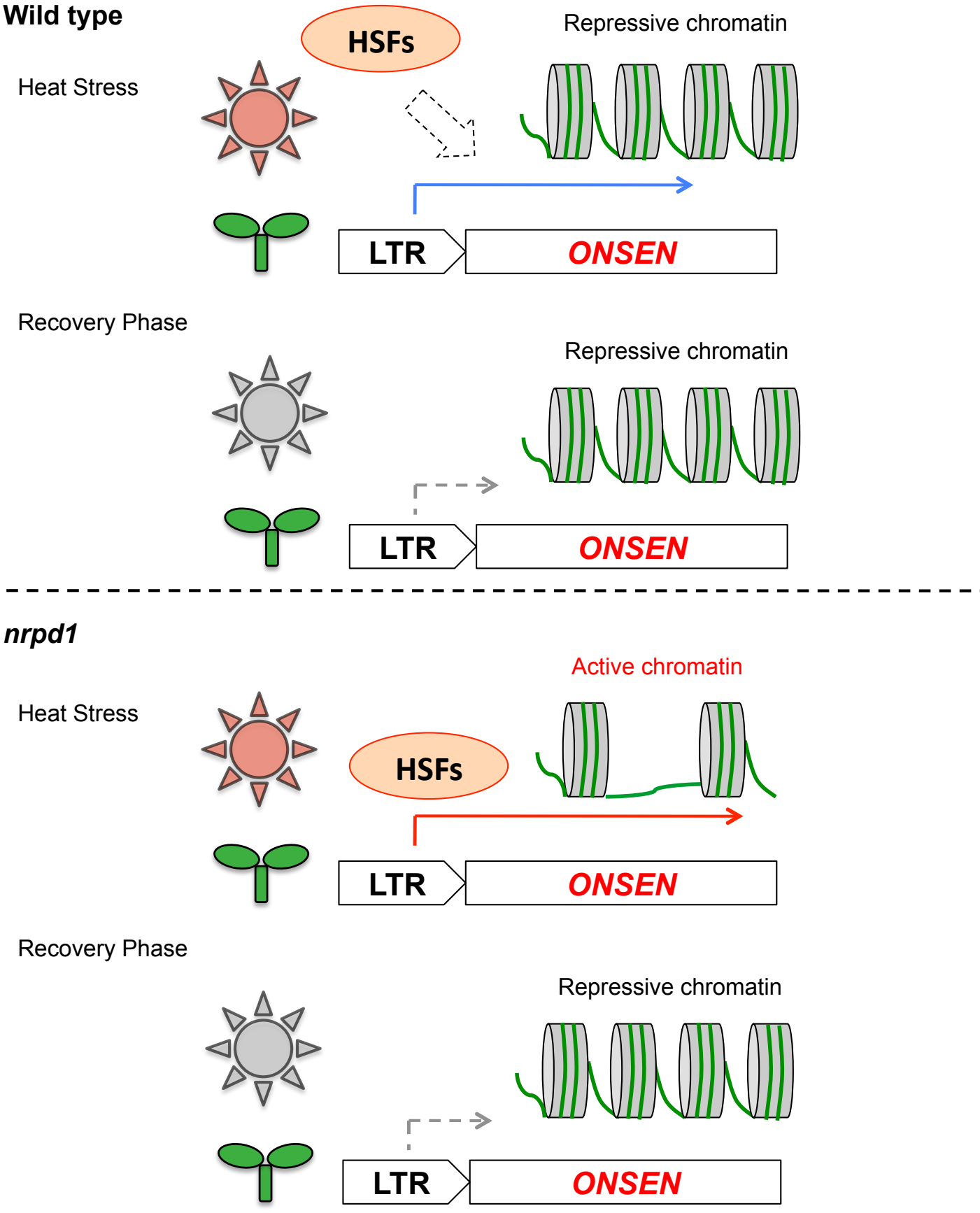
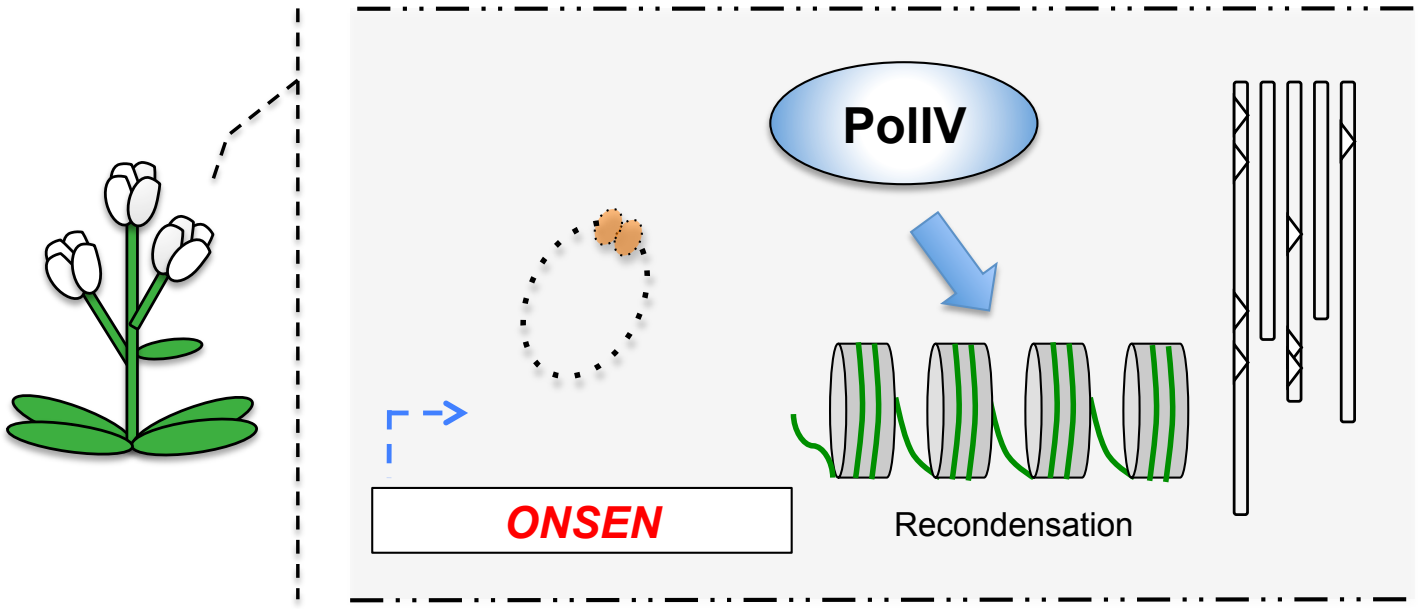

Wild type
HSFs
Repressive chromatin
Heat Stress
LTR
ONSEN
Recovery Phase
Repressive chromatin
LTR
ONSEN
nrpd1
Active chromatin
Heat Stress
HSFs
LTR
ONSEN
Recovery Phase
Repressive chromatin
LTR
ONSEN

Fig.21

Wild type



nrpd1

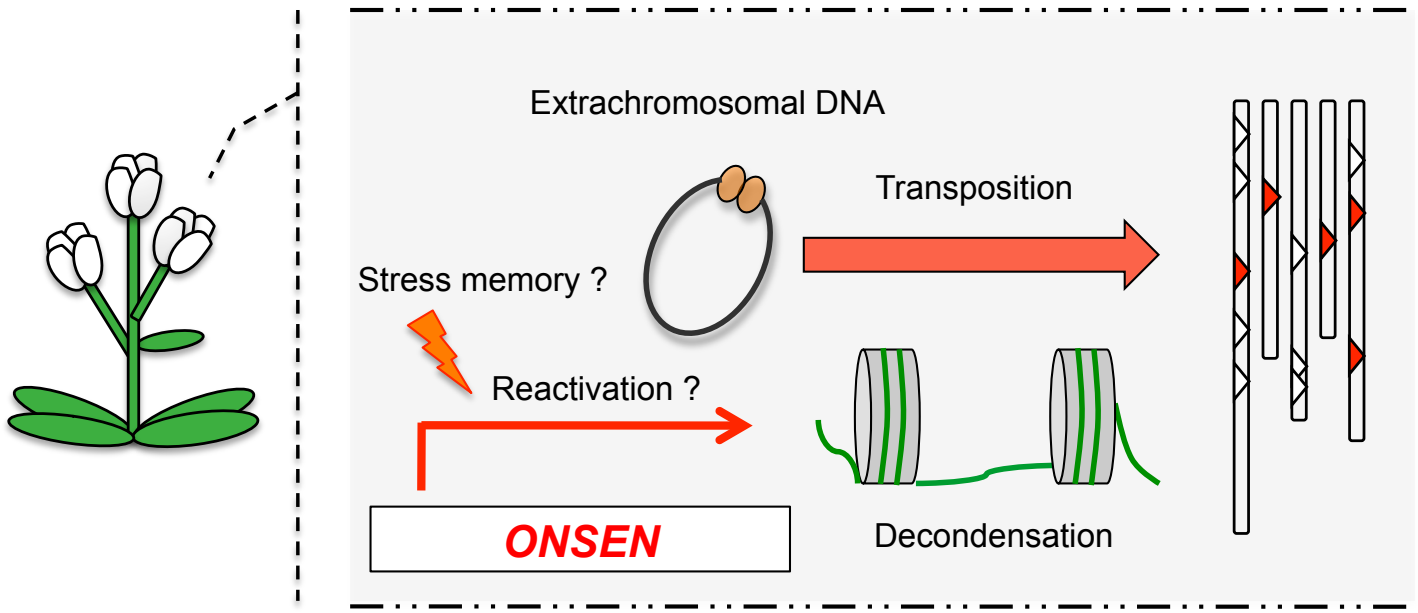


Table.1 Primers

Experiment	Name	Sequence (5'-3')
Southern probe	ONSEN-F	TAATGTTCCCTTCCAAGTCCC
	ONSEN-R	GCTTGTAATGACCCAAGAAGT
	mgfp-F	TGGTGGTGGTGGTGTTTGTAT
	mgfp-R	TTTCACTGGAGTTGTCCCAA
Real-time PCR	COPIA78_4129F_RT	CCACAAGAGGAACCAACGAA
	COPIA78_4300R_RT	TTCGATCATGGAAGACCGG
	GFP_qPCR_F	TGGCTTTGATGCCGTTCTTTTG
	GFP_qPCR_R	CGATTTCAAGGAGGACGGAAACAT
	18Sr_FW	CGTCCCTGCCCTTTGTACAC
	18Sr-RV	CGAACACTTCACCGGATCATT
RT-PCR	wus_RT_F	AGATCCATAGACATGGCTACTCA
	wus_RT_R	AGAAAGCGGCAACAACAACA
	ACT2_F	GTTGCACCACCTGAAAGGAAG
	ACT2_R	CAATGGGACTAAAACGCAAAA
Cloning	AT5G13205-GFP-F1	CACCTGAGAAGCAGCAGAAACCAA
	AT5G13205-GFP-R1	AGGGAACATTGTTACTCGCCA
TAIL-PCR	GSP1	TGAAAAAGCCTGAACTCACCGCGA
	GSP2	CGGTCAATACACTACATGGCGTGATTTC
	GSP3	TGACGGCAATTTCGATGATGCAG
	A1	NGTCGASWGANAWGAA
	A2	GTNCGASWCANAWGTT
	A3	WGTGNAGWANCANAGA
Genotyping PCR	28/3 flanking-F	AGATTGTTGTCGGTGGGAAA
	28/3 flanking-R	TGTTCAGAGGTATGTTCTTCCCT
	pMDC107 insert	ATTTCGATGATGCAGCTTGG
	SDE4_3021_F	TGTTCATTCAGTTACAAGTCGTG
	SDE4_4293_R	TTCACCTTTTTGATCCCTTGATC
	LBa1	TGGTTCACGTAGTGGGCCATCG

Table.2 TAIL-PCR protocol

1st PCR				
40-200ng gDNA	1ul		94ºC 1min → 95ºC 1min	
10x ExTaq buffer	2ul		94ºC 1min → 65ºC 1min → 68ºC 3min	x5
2.5mM dNTPs	1.6ul		94ºC 1min → 30ºC 3min → 68ºC 3min	
10pmol/ul GSP1	0.4ul		94ºC 30sec → 68ºC 1min → 68ºC 3min	
100pmol/ul A1	1ul		94ºC 30sec → 68ºC 1min → 68ºC 3min	x13
5U/ul ExTaq	0.2ul		94ºC 30sec → 44ºC 1min → 68ºC 3min	
dH_2O	13.8ul		68ºC 5min	
	20ul		4ºC	

2nd PCR				
1st PCR product (DF=50-200)	1ul		94ºC 1min → 95ºC 1min	
10x ExTaq buffer	2ul		94ºC 30sec → 68ºC 1min → 68ºC 3min	
2.5mM dNTPs	1.6ul		94ºC 30sec → 68ºC 1min → 68ºC 3min	x13
10pmol/ul GSP2	0.4ul		94ºC 30sec → 44ºC 1min → 68ºC 3min	
100pmol/ul A1	0.8ul		68ºC 5min	
5U/ul ExTaq	0.16ul		4ºC	
dH_2O	14.04ul			
	20ul			

3rd PCR				
2nd PCR product (DF=50-200)	1ul		94ºC 1min → 95ºC 1min	
10x ExTaq buffer	2ul		94ºC 30sec → 68ºC 1min → 68ºC 3min	
2.5mM dNTPs	1.6ul		94ºC 30sec → 68ºC 1min → 68ºC 3min	x13
10pmol/ul GSP3	0.6ul		94ºC 30sec → 44ºC 1min → 68ºC 3min	
100pmol/ul A1	0.6ul		68ºC 5min	
5U/ul ExTaq	0.1ul		4ºC	
dH_2O	14.1ul			
	20ul			