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H3K27me3 and H3K4me3 Chromatin Environment at Super-Induced Dehydration Stress Memory Genes of *Arabidopsis thaliana*

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Abstract

Pre-exposure to a stress may alter the plant's cellular, biochemical, and/or transcriptional responses during future encounters as a "memory" from the previous stress. Genes increasing transcription in response to a first dehydration stress, but producing much higher transcript levels in a subsequent stress, represent the super-induced "transcription memory" genes in *Arabidopsis thaliana*. The chromatin environment (histone H3 tri-methylations of Lys 4 and Lys 27, H3K4me3, and H3K27me3) studied at five dehydration stress memory genes revealed existence of distinct memory-response subclasses that responded differently to CLF deficiency and displayed different transcriptional activities during the watered recovery periods. Among the most important findings is the novel aspect of the H3K27me3 function observed at specific dehydration stress memory genes. In contrast to its well-known role as a chromatin repressive mechanism at developmentally regulated genes, H3K27me3 did not prevent transcription from the dehydration stress-responding genes. The high H3K27me3 levels present during transcriptionally inactive states did not interfere with the transition to active transcription and with H3K4me3 accumulation. H3K4me3 and H3K27me3 marks function independently and are not mutually exclusive at the dehydration stress-responding memory genes.

Keywords: dehydration stress-response genes, transcription memory genes, H3K4me3 and H3K27me3 at memory genes, CLF, *Arabidopsis thaliana*

Introduction

Plants that have been pre-exposed to a stress may produce altered cellular, biochemical, and/or transcriptional responses to a subsequent stress of a similar nature. Such a behavior could benefit the plant by making it more resistant to future stresses, leading to the idea that plants exercise a form of "memory" from the previous stress (Baldwin and Schmelz, 1996; Goh et al., 2003; Bruce et al., 2007; Ton et al., 2007; Conrath, 2011; Ding et al., 2012a). By altering the transcript levels from specific dehydration stress-responding genes, the plant finely tunes the levels of encoded proteins to meet the challenges during periods of recurring drought.

Earlier, we reported that *Arabidopsis* plants that have been previously subjected to one or more consecutive dehydration stresses of similar magnitudes and duration behave differently in a subsequent stress than plants experiencing the stress for the first time. Under our experimental design, plants lost ~35% of their initial relative water content (RWC) during the first stress treatment (S1), fully restored their RWC in recovery (R1), and, upon a subsequent exposure of similar strength and duration (S2), retained more of their internal water, losing only ~15% (Ding et al., 2012a). In addition, a subset of the dehydration stress-responding genes produced transcripts at significantly different levels when responding to a second stress (S2) relative to the levels produced in response to the first stress (S1), illustrating "transcriptional memory" responses. We refer to these genes as dehydration stress "memory" genes. Genes producing transcripts at a similar level in response to each stress represent the "non-memory" genes (Ding et al., 2012a). The operational criterion is that, in a second stress (S2), memory genes produce transcripts at levels that are different from the levels produced in S1. When the levels in S2 are significantly higher than in S1, the genes belong to the "super-induced" memory category, designated as [+/+] memory genes; non-memory genes producing similar transcript levels in both S1 and S2 are designated as [+/=]. These transcriptional patterns suggest that a plant's responses to repeated stresses were more complex than repetitive activation of the same response mechanism. The biggest challenge, then, is to identify molecular mechanisms that regulate memory-type transcription. Accumulation of signaling molecules, of proteins involved in their synthesis, and/or of transcription factors (Conrath et al., 2006; Umezawa et al., 2006; Zacharioudakis et al., 2007; Kundu and Peterson, 2010), in addition to chromatin-based (epigenetic) mechanisms (Tsuji et al., 2006; Kim et al., 2008; Jaskiewicz et al., 2011; Ding et al., 2012a; Light et al., 2013) have been proposed.

The ability of chromatin to alter gene expression rapidly and reversibly, yet to keep a gene's transcriptional potential for longer periods of time, provides a mechanism for transcription "memory" responses. We emphasize that we make a distinction between a *chromatin mark* and a *memory* (*epigenetic*) *mark* (as defined in our earlier study, Ding et al., 2012a): histone modifications that are dynamically associated with transcription, but are removed at its conclusion, are considered chromatin marks; by contrast, memory marks persist longer, after the process is no longer active. Importantly, memory marks should

affect the genes' transcriptional performances in subsequent stresses, as demonstrated for H3K4me3 and for the stalled Pol II accumulated in R1 at the [+/+] memory *RD29B* and *RAB18* genes (Ding et al., 2012a). H3K4me3 marks maintained at higher levels on drought stress–inducible genes for 5 d after rehydration (Ding et al., 2012a) and H3K4me3 accumulated at defense-response genes after chemical priming before their induction by a pathogen (Conrath et al., 2006; Conrath, 2011; Jaskiewicz et al., 2011) are examples of memory marks.

On the other hand, the H3K27me3 is considered a silencing mark counterbalancing the activating functions of H3K4me3 in both animal and plants (Kohler and Villar, 2008; Schwartz and Pirrotta, 2008; Hennig and Derkacheva, 2009). Accumulating at transcriptionally inert loci, H3K27me3 is found exclusively within euchromatin in association with the Polycomb group (PcG) complex and with epigenetic silencing effects (Kouzarides, 2007; Turck et al., 2007; Zhang et al., 2007). Substantial amounts of data are available on the role of H3K27me3 in the silencing of developmentally regulated plant genes (Molitor and Shen, 2013). Less is known about the role of PcG and its H3K27me3/CLF components at stress-responding genes when transcription dynamically changes in response to environmental stresses (Kwon et al., 2009; Sani et al., 2013).

Here, five [+/+] dehydration stress memory genes are used as a model to analyze a possible role of H3K27me3 as a memory mark. In an earlier study of 14 known dehydration stress-responding genes, we identified *LTP3*, *LTP4*, *HIPP2.2*, *RD29B*, and *RAB18* as memory genes producing significantly higher levels of transcripts during a second stress compared to the levels in the first (Ding et al., 2012a) and have designated them as [+/+] memory genes. Despite belonging in the same [+/+] memory category, the genes showed different responses to the presence/absence of CLF and different transcriptional activity during the watered (recovery) periods, suggesting internal heterogeneity and different regulatory mechanisms. The transcriptional behavior of the *AGAMOUS* (*AG*) gene during multiple exposures to dehydration stress in wild-type and *clf* mutants was also examined to compare the roles of CLF/H3K27me3 at a developmentally regulated gene and at genes that dynamically change transcription in response to repeated stresses.

Results

H3K27me3 Levels at the [+/+] Memory Genes during Transcribed and Non-Transcribed Phases of the Treatment Cycle

Three-week-old *Arabidopsis* plants grown under well-watered soil conditions were subjected to one or two dehydration stresses, as described earlier (Ding et al., 2012a). The transcript levels produced in response to a first stress (S1) and to a second stress (S2) illustrate the behaviors of [+/+] memory genes and of a non-memory [+/=] response gene RD29A (Fig. 1A).

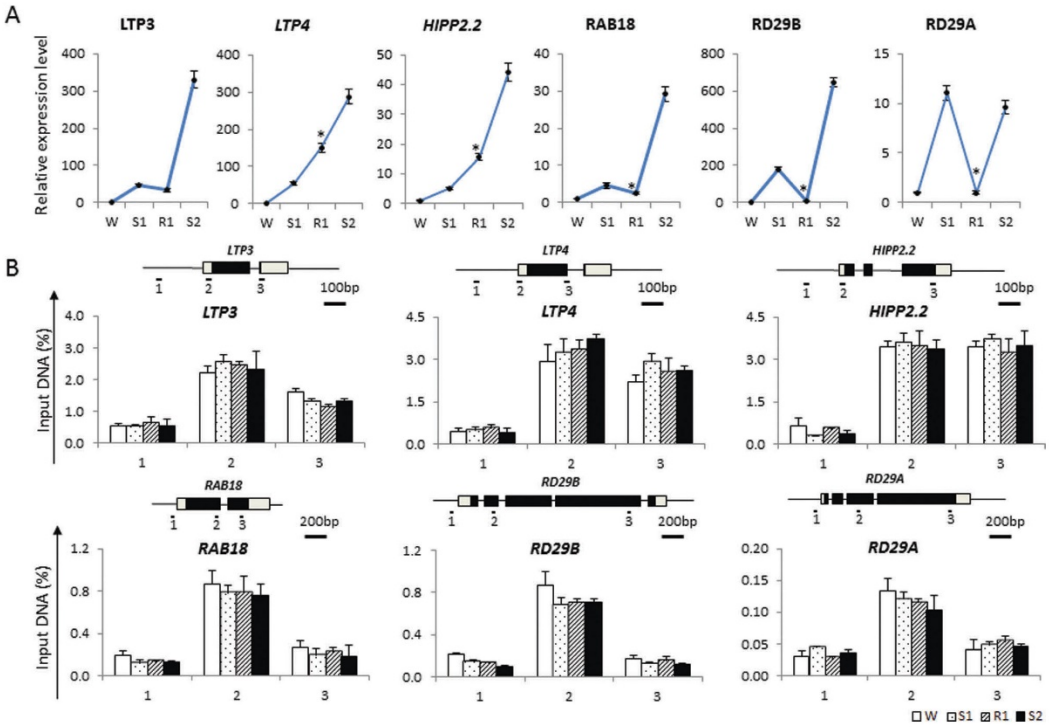

Figure 1. Transcription Patterns and H3K27me3 Distribution Levels at [+/+] Memory Genes and at a Non-Memory Gene. (A) Transcript levels from the [+/+] genes *LTP3, LTP4, HIPP2.2, RAB18*, and *RD29B* genes induced in S1 and super-induced in S2. Transcript levels from the [+/=] non-memory gene *RD29A* remain similar in S1 and in S2. The transcript levels from *RAB18*, *RD29B*, and *RD29A* are lower in R1 compared to S1, higher from *LTP4* and *HIPP2.2*, similar for *LTP3*. (B) Gene models of the genes tested by ChIP assays are shown on top. Dark boxes are exons, white boxes are non-coding regions, and connecting lines are introns. Numbered bars illustrate positions that are probed in the ChIP assays. Levels and distribution patterns of H3K27me3 at the [+/+] memory genes and the non-memory *RD29A* gene in wild-type in graphs below gene sketches. All data represent results from three independent biological replicates; error bars represents the ± SE ($n = 3$). Statistically significant differences in transcript levels between R1 and S1 based on Student's *t*-test, $p < 0.05$ are indicated by asterisks. Differences in H3K27me3 levels are not statistically significant ($p > 0.05$) at any transcriptional phase.

A possible involvement of the histone modification H3K27me3 in the [+/+] transcription memory behavior was examined by ChIP assays with anti-H3K27me3-specific antibodies and primers designed to probe multiple regions along the gene sequences (Fig. 1B). The H3K27me3 profiles at the [+/+] memory (*LTP3*, *LTP4*, *HIPP2.2*, and *RD29B*), as well as non-memory (*RD29A*) genes during the initial pre-stressed (W) conditions, when expression from all tested genes is at their basal (low) level, was taken as the baseline levels for a "high" H3K27me3 presence at each position.

Surprisingly, the H3K27me3 levels did not show substantial changes during any phase of the treatment cycle, irrespective of whether transcription was induced in S1, super-induced in S2, or showed gene-specific variable levels of activity in R1. The H3K27me3 levels in S2, remaining practically unchanged from the pre-stressed states despite super-activated transcription in S2, suggest that H3K27me3 does not prevent transcription, or play a memory-mark role, at these stress-response genes. Particularly striking was the observation that high H3K27me3 levels (at the accumulation peak for each gene) did not prevent occurrences of either the induced or super-induced transcription, implying RNA Polymerase II was able to initiate and transcribe efficiently through H3K27me3-modified nucleosomes.

The H3K27 Methyltransferase CLF in the Dehydration Stress Responses

To further explore the lack of effects of H3K27me3 on the transcription of the dehydration stress-responding genes, we analyzed their transcriptional responses during the four treatment phases in a *clf* background. The experiments performed with two *CLF* mutant lines (*clf-24*, SALK_006658) and (*clf-13*, SALK _139371, Supplemental Fig. 1; see also Fig. 6) yielded similar results.

Although the [+/+] memory genes provided similar transcriptional responses in wild-type, surprisingly, they displayed different responses in the *clf* background (Fig. 2). The *LTP3*, *LTP4*, and *HIPP2.2* transcripts in *clf* were strongly increased (in agreement with the known repressive role of CLF), while the transcript levels of *RD29B* and *RAB18* RNAs were repressed (slightly in S1 and more strongly in S2) (Fig. 2). Thereby, despite belonging in the same [+/+] memory category and despite displaying similarly unchanging H3K27me3 chromatin patterns irrespective of transcriptional states, the *LTP3*, *LTP4*, *HIPP2.2*, *RD29B*, and *RAB18* genes outline two distinct subsets that are affected differentially by the CLF deficiency. The results suggest that different mechanisms regulate the memory transcription patterns of genes from the same memory category. Of note, the levels of the non-memory *RD29A* remained practically unaffected (Fig. 2).

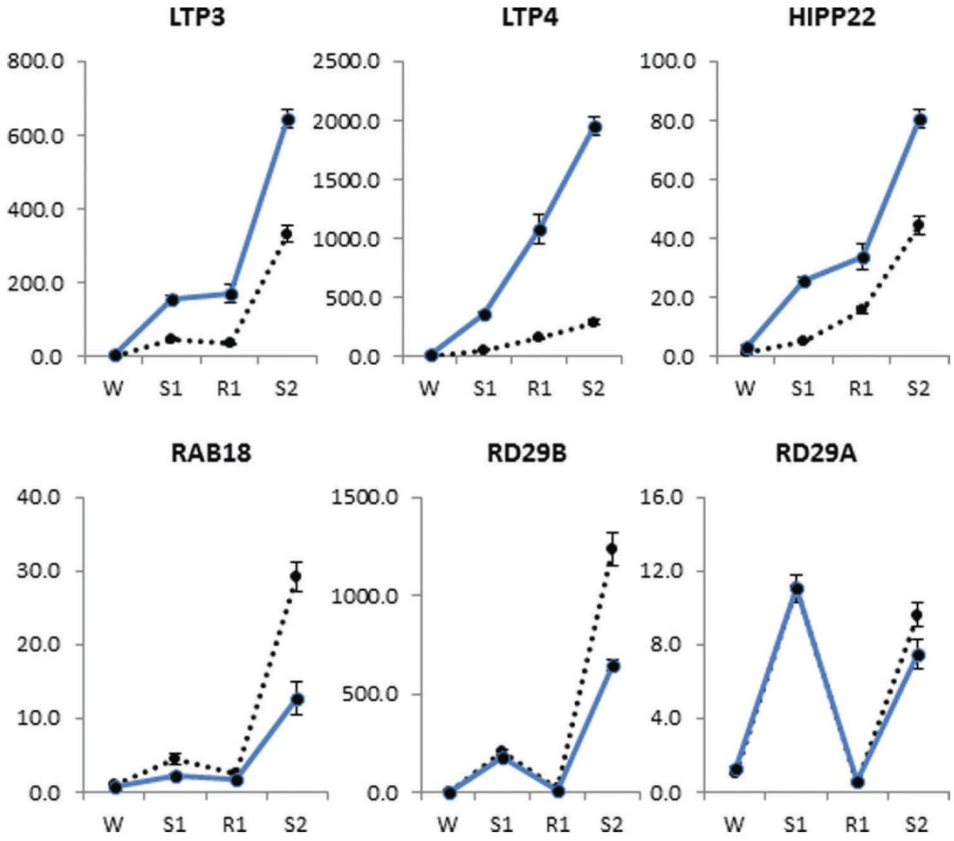

Figure 2. Transcript Levels during the Four Phases of the Treatment Cycle in *clf* Background. Solid lines illustrate transcripts in *clf-24* background; dotted lines show the levels in wild-type. Results are the average of three independent experiments, each with two replicates. Error bars indicate the standard error of the mean.

H3k27me3 and Dehydration Stress Responses in the clf *Background*

Collectively, the results presented an apparent paradox: H3K27me3 levels did not correlate with the transcriptional status of any of the tested genes and, yet, their transcriptional responses in the absence of CLF were affected in a gene-, or subset-, specific manner. To understand whether the effect of CLF on the transcriptional performance of the dehydration stress-responding genes was mediated by altered levels of H3K27me3, we measured H3K27me3 in the different stress/recovery phases in the *clf* background (Fig. 3). The non-memory gene *RD29A* is most likely not regulated by CLF, as suggested by the lack of significant changes in H3K27me3 levels during W and R1 (Fig. 3) and by its transcription patterns in *clf* mutants (Fig. 2). In contrast, the H3K27me3 presence at the *LTP3*, *LTP4*, and *HIPP2.2* genes was substantially reduced in *clf* mutants (Fig. 3). Together with their concomitantly de-repressed transcription in the absence of CLF (see Fig. 2), the results suggest *LTP3*, *LTP4*, and *HIPP2.2* are regulated by CLF. Interestingly, the H3K27me3 marks at *RD29B* and *RAB18* in *clf* mutants did not change significantly from wild-type levels (Fig. 3), although their transcription was reduced (Fig. 2). Therefore, based on their different

responses to CLF depletion, the five [+/+] memory genes outline two distinct subsets. Furthermore, the results suggested CLF was not involved in modifying the *RD29B* and *RAB18* nucleosomes but decreased transcription in *clf* mutants implied the effect was indirect. Possible factor(s) contributing to the lower transcript levels of *RD29B* and *RAB18* in the *clf* background were examined next.

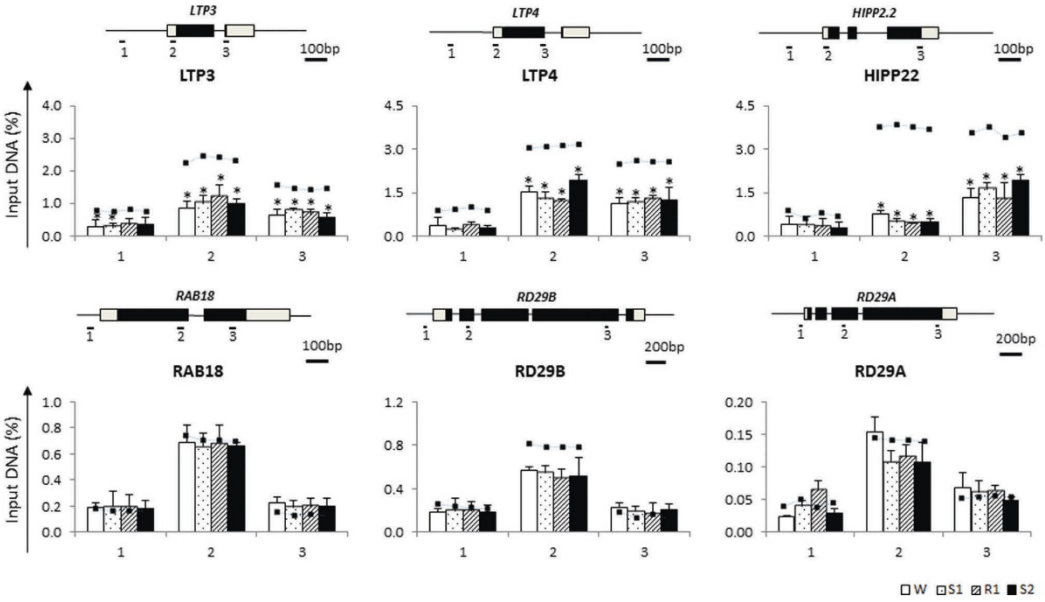

Figure 3. Levels and Distribution Patterns of H3K27me3 at the [+/+] Memory Genes and the Non-Memory *RD29A* Gene in the *clf* Background. H3K27me3 levels in *clf* measured at the same regions as those tested in wild-type, indicated by numbered bars under the gene models. Included for comparison are wild-type H3K27me3 levels at each position, marked by black squares (see Fig. 1B). Results are the average of three independent experiments, each with two replicates. Error bars indicate the standard error of the mean. Asterisks indicate statistical significance between *clf* mutant and wild-type counterpart based on Student's *t*-test ($p < 0.01$). Gene models shown on top are as described in Figure 1.

Lower Endogenous ABA Levels in clf *Background under Stress*

ABA is a signaling molecule that is synthesized under dehydration stress and is critically required to induce the transcription from a large number of dehydration stress-responding genes (Cutler et al., 2010). To establish whether ABA levels were affected by the CLF loss-of-function, we measured the endogenous ABA in the *clf* background and found ABA levels were 40%–60% of the levels in wild-type (Fig. 4A). Diminished presence of ABA suggests a plausible explanation for the reduced *RD29B* and *RAB18* transcript levels upon induction. Consistent with the lower endogenous ABA production in *clf*, mutant plants displayed higher sensitivity to dehydration stress (Fig. 4B) and lost water upon exposure to dry air at higher rates than wild-type plants (Supplemental Fig. 2).

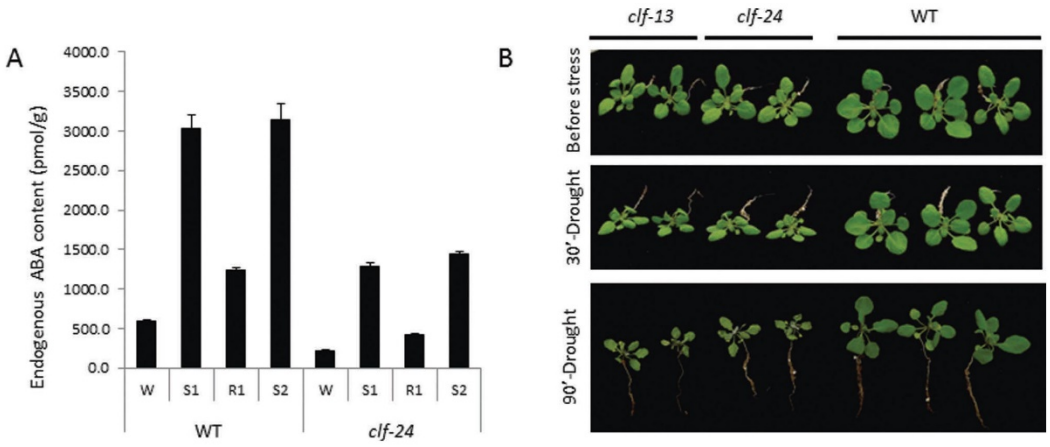

Figure 4. Endogenous ABA Levels in the Different Backgrounds and Plants' Responses to Drought Stress. (A) Endogenous ABA levels in wild-type and in *clf* backgrounds under stress and in watered recovery. (B) Wild-type and *clf* mutant seedlings before and after drought stress treatment.

We conclude that reduced transcription of *RD29B* and *RAB18* in *clf* mutants results, at least partly, from decreased ABA levels, indicating CLF is required for wild-type-level synthesis of this hormone during dehydration stress. It is important to note, however, that *LTP3*, *LTP4*, and *HIPP2.2* are also induced by ABA (Arondel et al., 2000; Huang et al., 2008) but that, in contrast to *RD29B* and *RAB18*, their strong upregulation in the *clf* background indicates de-repression from the loss of CLF overrides the effects from diminished ABA. The results clearly outline at least two distinct subsets among the five [+/+] memory genes that are regulated by different molecular mechanisms when responding to repeated stresses. This internal heterogeneity among the [+/+] genes was explored next.

H3K4me3 Marks and Transcription during the Four Phases of the Stress Treatment

Generally, the H3K4me3 marks accumulate as a distinct peak at the 5′-end of plants genes, immediately downstream of the transcription start site, and their levels correlate with the transcriptional activity of the genes (van Dijk et al., 2010; Ding et al., 2012a, 2012b). Earlier, we established that higher H3K4me3 levels during S1-induced transcription of *RD29B* and *RAB18* genes were retained during the transcriptionally less active recovery periods (R1–R3) (Ding et al., 2012a). Consistent with our definition, the H3K4me3 behaved as memory (epigenetic) marks at the *RD29B* and *RAB18* genes. Here, we measured the H3K4me3 levels at *LTP3*, *LTP4*, and *HIPP2.2* genes during the four phases of the treatment cycle to determine whether H3K4me3 functions as a memory mark in their transcriptional responses. ChIP assays with anti-H3K4me3 antibodies and primers overlapping the 5′-ends of the genes (regions 1 and 2 in Fig. 5A) were performed.

Upon a first stress treatment (S1), H3K4me3 levels increased at the 5′-ends of coding sequences for all genes in correlation with increased transcription (Fig. 5B); during watered recovery (R1), the H3K4me3 levels at *RD29B* and *RAB18* remained similar to the levels in

S1, despite lower transcription in R1 (see also Ding et al., 2012a). In contrast, H3K4me3 levels at *LTP3*, *LTP4*, and *HIPP2.2* were significantly higher compared to S1, correlating with the genes' increased transcript levels in R1 (see also Fig. 1A). Importantly, these transcript levels in R1 resulted from continuing transcription of *LTP3*, *LTP4*, and *HIPP2.2*, as evidenced by the presence of the actively elongating RNA Polymerase II (Pol II) (Fig. 5C).

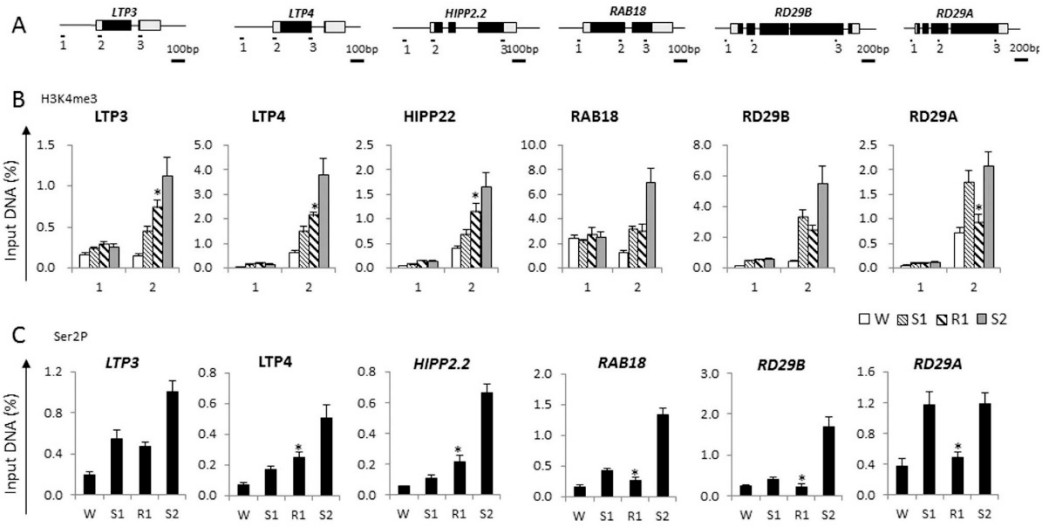

Figure 5. H3K4me3 and Ser2P Pol II Distribution Levels at the [+/+] Memory Genes. (A) Gene models of the genes tested by ChIP assays shown on top are as described in Figure 1. (B) H3K4me3 levels measured at regions indicated 1 and 2 on the models (on the *x*-axis) during the four phases of the stress cycle. (C) Ser2P Pol II levels are measured at the regions indicated by the number 3 for each gene during the four phases of the stress cycle. Results are the average of three independent experiments, each with three replicates. Error bars indicate the standard error of the mean. Asterisks indicate statistical significant differences in values for both the H3K4me3 and the Ser2P Pol II levels measured in R1 and S1, based on Student's *t*-test ($p < 0.05$).

The levels of Pol II phosphorylated at serine 2 of its C-terminal domain (Ser2P Pol II), accumulating as a peak at the 3′-ends of genes, is a diagnostic marker of ongoing transcription (Nechaev and Adelman, 2011; Ding et al., 2012a, 2012b).

ChIP assays with anti-Ser2P Pol II-specific antibodies and primers from the genes' 3′-end regions indicated Ser2P Pol II levels in S1 are higher than pre-stressed levels (in W) and are highest in S2 (Fig. 5C). These Ser2P Pol II distribution patterns reflect the induced and super-induced transcription during the first and the second stresses, respectively, which are the signature feature for the [+/+] memory behavior (Ding et al., 2012). In contrast, the levels of Ser2P Pol II at the non-memory *RD29A* gene are similar in S1 and in S2, consistent with the constant transcript levels produced by the gene (Fig. 5C).

As transcription from *LTP3*, *LTP4*, and *HIPP2.2* continues during watered recovery (R1) in the absence of stress, the high H3K4me3 levels at *LTP3*, *LTP4*, and *HIPP2.2* in R1 reflect

their transcriptional activity and, thus, represent a dynamically changing chromatin modification, not a memory mark, for these genes. Combined analysis of the five [+/+] memory-response genes revealed two distinct subsets within this memory category that are defined most clearly by their responses to CLF. However, they also display different transcriptional activity in R1, as supported by their transcript levels and the accumulation profiles of Ser2P Pol II and H3K4me3.

CLF/H3K27me3 in the Transcriptional Responses of AG during Multiple Exposures to Dehydration Stresses

The flower-specific gene *AGAMOUS* (*AG*) is a signature CLF/ H3K27me3-regulated *Arabidopsis* gene that is kept silent in vegetative tissues of the plant by the repressive function of CLF/H3K27me3. *AG* is de-repressed and ectopically expressed in rosette leaves of *clf* mutant plants (Goodrich et al., 1997; Schubert et al., 2006).

AG was not expressed in the initial pre-stressed (W) phase or during any of the dehydration stress/recovery treatments in wild-type rosette leaves (Fig. 6A). Together with the high H3K27me3 levels present during the four stress/recovery phases in wild-type rosette leaves (Fig. 6B and 6C), the results are in full agreement with the known silencing functions of CLF/H3K27me3 at *AG*. However, in addition to being strongly de-repressed in the watered state in the *clf* background, as expected, *AG* behaved as a negatively regulated dehydration-stress-responding gene under repeated stress: *AG* transcripts decreased in S1, returned to pre-stressed levels in R1, and decreased again in S2 (Fig. 6A). Low H3K27me3 levels in *clf* mutants (Fig. 6C), correlating with up-regulated *AG* transcription in the W and R1 states (Fig. 6A), are consistent with the known CLF/H3K27me3-imposed repression of *AG*. However, *AG*'s dynamically attenuated transcription in response to stress in the *clf* background was not mediated by H3K27me3 levels, as these were low throughout. Therefore, CLF and H3K27me3 are critical for suppressing *AG*'s ectopic expression in wild-type rosette leaves. However, the transcription factors (TFs) activating *AG* during the watered W and R1 states in a *clf* background are less active or are counterbalanced by dehydration-responsive repressors during dehydration stress.

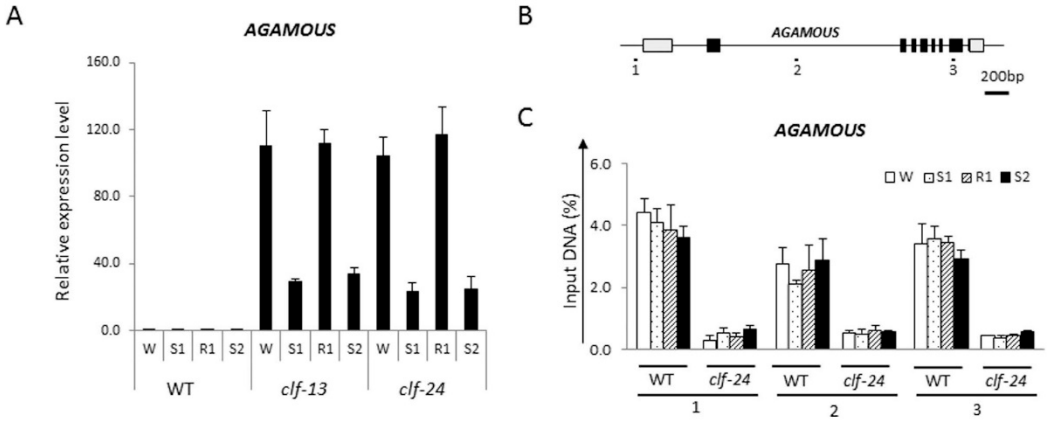

Figure 6. *AG* Transcript and H3K27me3 Levels in Responses to Water-Withdrawal Stress Measured in Wild-Type and *clf* Backgrounds. (A) *AG* transcript levels in wild-type and in *clf* backgrounds under stress and in watered recovery. (B) Gene model of the *AGAMOUS* gene. Dark boxes are exons, white boxes non-coding regions, connecting lines are introns, numbered bars illustrate positions probed for H3K27me3 levels by the ChIP assay. (C) H3K27me3 levels in wild-type and *clf* mutant plants during the four phases of the treatment cycle. Results are the average of three independent experiments, and each with two replicates. Error bars indicate the standard error of the mean.

Discussion

[+/+] Memory Genes Are Regulated by Different Molecular Mechanisms

Five dehydration stress-response genes, *LTP3*, *LTP4*, *HIPP2.2*, *RD29B*, and *RAB18*, increase their transcription when experiencing a 2-h exposure to dry air (S1). When encountering a similar second stress (S2) (after a 22-h watered recovery, R1), the transcripts from all five genes dramatically increased. This behavior defined them as [+/+] memory-response genes. A more detailed analysis revealed that in R1 (under low ABA and fully restored RWC conditions, Ding et al., 2012a), three genes, *LTP3*, *LTP4*, and *HIPP2.2*, continued transcription (Figs. 1A and 5C), while transcription from *RD29B* and *RAB18* in R1 was decreased (Figs. 1A and 5C; Ding et al., 2012a). These results suggest that different mechanisms regulate their transcription patterns, despite belonging in the same [+/+] memory category. Strong supporting evidence was provided by the responses of the genes to a CLF deficiency. Thus, in accordance with the known repressive role of CLF, transcription from *LTP3*, *LTP4*, and *HIPP2.2* was strongly increased in the *clf* background; in contrast, the transcript levels from *RD29B* and *RAB18* were decreased (slightly in S1 and more strongly in S2) (Fig. 2). Furthermore, the H3K27me3 levels at the *LTP3*, *LTP4*, and *HIPP2.2* genes were strongly decreased in *clf* mutants, suggesting CLF is involved in modifying their nucleosomes. The decreased transcription of *RD29B* and *RAB18* in *clf* and retention of H3K27me3 at wild-type levels, however (Figs. 1–3), suggested the CLF's role at these genes was indirect. Decreased levels of endogenous ABA in *clf* mutants (Fig. 4) provide a plausible explanation for the reduced transcription of *RD29B* and *RAB18*. For *LTP3*, *LTP4*, and

HIPP2.2, the de-repressive effects from the CLF loss override the effects from diminished ABA.

The transcriptional behavior in R1 and the different responses to the lack of CLF outlined two distinct memory subsets were within the five [+/+] memory genes. Therefore, the mechanisms that regulate the memory behavior of dehydration stress-responding genes, even from the same memory category, will, most likely, function in a gene- (or subset-) specific manner.

Chromatin, Epigenetics, and Responses to the Environment

Chromatin-based mechanisms, involving changes in histone modifications and nucleosome occupancy in response to cold, high salinity, hypoxia, and drought-induced transcription, have been proposed as epigenetic regulators of stress-responding genes in a variety of plant species and tissue cultures (Tsuji et al., 2006; Sokol et al., 2007; Chinnusamy et al., 2008; Kim et al., 2008, 2012; Light et al., 2010; Jaskiewicz et al., 2011; Light et al., 2013). According to our definition, however, a memory mark should persist longer than the initial stimulus and should affect a gene's performance upon a subsequent stress (Ding et al., 2012a). In this context, H3K4me3 is a memory mark for the *RD29B* and *RAB18* genes (it remains high in R1 when transcription is low) but not for *LTP3*, *LTP4*, and *HIPP2.2*, where it dynamically changes in correlation with transcription (Fig. 5C, Ding et al., 2012a). The H3K27me3 modification also does not serve as a memory (epigenetic) mark, as its levels remain unchanged during changing transcriptional activities of the response genes.

Of note, small (but significant) changes in overall H3K27me3 levels were measured at about 100 genes after pre-treatment of *Arabidopsis* seedlings with high-salt solution (referred to as "Priming-induced 'etching' of H3K27me3 islands," Sani et al., 2013); at the promoter of the cold-response gene *COR15A*, the H3K27me3 levels decreased slightly upon induction (Kwon et al., 2009). Importantly, however, decreased H3K27me3 levels did not affect the genes' transcriptional induction upon a subsequent exposure to the stress (Kwon et al., 2009; Sani et al., 2013) and, according to our definition, do not meet the criterion for a memory mark at the tested genes.

Histone H3K4me3 and H3K27me3 Marks Function Independently and Are Not Mutually Excluded during Active Transcription of Dehydration Stress-Responding Memory Genes

Although the H3K27me3 and H3K4me3 modifications are considered counterbalancing and mutually exclusive (Schwartz and Pirrotta, 2007; Bouyer et al., 2011; Roudier et al., 2011), our results indicated that, for the response genes analyzed here, they were neither. *First*, the levels of H3K27me3 and of H3K4me3 were independent of each other's presence. The H3K27me3 levels remained unchanged, regardless of whether the [+/+] memory genes were transcribed or not, while the H3K4me3 levels dynamically correlated with the transcription, indicating the two marks were not counterbalancing. S*econd*, high-level presence of H3K27me3 at the stress-response genes did not preclude accumulation of H3K4me3 when genes were actively transcribed (Figs. 1 and 5).

These results and conclusions seem to contradict the "gain" in H3K4me3 upon a H3K27me3 loss in *fie* mutants reported for about 5.5% of the *Arabidopsis* genes marked by H3K27me3 (Bouyer et al., 2011) or the "switch" from H3K27me3 to H3K4me3 observed at

the *AG* locus (Carles and Fletcher, 2009). This apparent contradiction may be resolved by results from our earlier studies establishing that the H3K4me3 levels accumulated at the genes' 5′-ends are determined by the degree of initiated transcription: the higher transcription rates, the higher amounts of H3K4me3 are deposited at the gene's transcription start site (TSS) (Ding et al., 2011, 2012b). Thus, the substantial H3K4me3 increase during S2, compared to its levels in S1, is a consequence of the super active transcription in S2. The "gained" increase in H3K4me3observed upon decreased H3K27me3, then, reflects the increased transcription resulting from de-repression in a PcG mutant background (Schwartz et al., 2010).

CLF and Its Product H3K27me3 at Dehydration Stress-Responding Genes

Among the most unexpected findings of this study was that the H3K27me3 levels present during the genes' transcriptionally inactive states remained at similarly high levels after the genes were activated in S1, or super-activated in S2 (Fig. 1A and 1B). Apparently, high-level H3K27me3 presence at transcribed sequences did not block the passage of the elongating Ser2Pol II (Fig. 5C; see also Buzas et al., 2012), nor did H3K27me3 serve as a repressive mark for the memory response in S2 or the high-level accumulation of H3K4me3. On this background, the strong transcriptional activation (de-repression) of the *LTP3*, *LTP4*, and *HIPP2.2* genes (Fig. 2), together with the decreased H3K27me3 at their nucleosomes in *clf* mutants (Fig. 3), were particularly important, as they suggested CLF/H3K27me3 acted as a mechanism that limits, rather than prevents, transcription of these genes when responding to dehydration stress. Therefore, CLF and H3K27me3 limit the absolute level of the *LTP3*, *LTP4*, and *HIPP2.2* transcription, while allowing a range of dynamic responses at stress-responding genes. The H3K27me3 levels do not change between these conditions but their presence restricts the levels of transcripts produced. This is a major difference from the "on/off" repressive role of PcG at developmentally regulated genes and reveals a novel aspect of the PcG (CLF/H3K27me3) function: to define the dynamic range of expression of specific memory genes.

To pursue further the different silencing roles of CLF/H3K27me3 in the transcription of developmental and of dehydration stress memory genes, the behavior of *AG* under repeated dehydration stresses was analyzed. *AG* is the paradigm for the CLF and H3K27me3 roles in keeping a flower-specific gene silent in vegetative tissues of the plant (Goodrich et al., 1997; Schubert et al., 2006). As expected, *AG* was not expressed in wild-type rosette leaves during the initial pre-stressed (W) phase, nor was it expressed during any of the dehydration stress/recovery treatments (Fig. 6A). In *clf* mutants, however, in addition to being strongly de-repressed in the watered state (as expected), unexpectedly, *AG* responded to the dehydration stress behaving like a non-memory [–/=] gene (Fig. 6A). These changes in transcription were taking place in the absence of H3K27me3 marks (Fig. 6B), suggesting the dynamic transcriptional responses were regulated by specific activating or repressing TFs. The important implication of this result is that CLF/H3K27me3 are sufficient to suppress *AG*'s ectopic expression in wild-type rosette leaves and to overcome the effects from TFs that are, apparently, present under normal watered conditions. At *LTP3*, *LTP4*, and *HIPP2.2*, however, CLF/H3K27me3 cannot fully suppress the activating effects from dehydration stress-regulated TFs: transcription could be induced but at levels below

the maximum capacity of these genes to be transcribed. Importantly, the basal transcription under noninduced (W) conditions remained low in the *clf* mutants (Fig. 2), indicating that *LTP3*/*LTP4*/*HIPP2.2*-specific dehydration stress/ABA-dependent transcription factors must be activated and that they play the primary role in inducing transcriptional responses from the involved genes. On the contrary, *AG* is de-repressed in *clf* mutants under watered conditions but is apparently repressed by transcriptional repressors activated by the stress (Fig. 6A). The effects from CLF are gene-specific, as CLF is not involved in modifying the nucleosomes of *RD29B* and *RAB18*. It will be interesting to identify the methyltransferase responsible for their modification in order to establish whether a decrease in H3K27me3 would result in a similar de-repression of their basal and induced transcription when responding to stress.

Therefore, CLF and its product, H3K27me3, play different roles at developmental genes and at dehydration stress-responding genes. CLF/H3K27me3 and other PcG components are critically required for the maintenance of cell fates, cellular differentiation, and pluripotency as well as in regulating flowering time, seed development, in transition from embryonic to vegetative growth, and defining the domains of expression of developmental genes (Henderson and Dean, 2004; Dennis and Peacock, 2007; Kohler and Villar, 2008; Amasino, 2010; Schatlowski et al., 2010; He et al., 2012). In PcG mutants, developmental gene expression extends to other domains (Goodrich et al., 1997; Chanvivattana et al., 2004; Schubert et al., 2006; Calonje et al., 2008; Simon and Kingston, 2009; Adrian et al., 2010; Bratzel et al., 2012) or is reduced to a small group of cells (Farrona et al., 2011). At the dehydration stress-response *LTP3*, *LTP4*, and *HIPP2.2* genes, CLF/H3K27me3 define the scope of their expression in wildtype by limiting the maximal possible induction of their transcription without fully repressing these genes.

Collectively, among five [+/+] memory genes, the existence of two distinct subtypes regulated by different molecular mechanisms was revealed. The two subsets differ by their transcriptional activity in R1 and opposite responses to the CLF deficiency. Common for both subtypes, however, is that baseline levels of H3K27me3 in the transcriptionally inactive (W) states did not change during the induced and super-induced transcription in S1 and S2. High-level presence of H3K27me3 does not prevent transcription initiation or progression of Pol II and/or accumulation of H3K4me3, indicating the two modifications were not mutually exclusive. CLF/H3K27me3 do not prevent dehydration stress-regulated expression from specific genes but limit their capacity to be transcribed at their maximum potential under noninduced or induced conditions in wild-type plants. This result is the most important contribution of this research, as it reveals a new role for CLF/H3K27me3 in setting the range of transcription levels at specific genes. The results imply that the chromatin environment, determined by PcG (H3K27me3 and CLF, in particular), plays different and gene-specific roles in the transcriptional performance of developmentally regulated and environmentally responsive genes, namely restricting the cellular specificity of developmentally regulated genes and setting the dynamic range of environmentally responsive genes.

Methods

Plant Growth and Treatments

Wild-type *Arabidopsis thaliana* plants and *clf* mutants analyzed here were grown in potting soil in controlled environment rooms at 22°C with a 12-h light photoperiod and light intensity of 180 μmol m^{-2} s^{-1}. Dehydration stress and full watered recovery were performed as described by Ding et al. (2012a) with some modifications. Briefly, after removing the plants from soil and washing any remaining soil from their roots, plants were placed in humid chambers overnight to recover from potential root wounding during extraction from soil and to exclude possible effects on the transcriptional responses. Transcript levels measured in recovered plants before initiating stress treatments are designated as pre-stressed (W) levels. The first stress (S1) treatment is achieved by exposing plants to dry air for 90 min, followed by recovery (R1), achieved by placing plants in humid chambers for 22 h with their roots in a few drops of water. For a subsequent stress treatment, R1 plants were gently blotted onto filter paper to remove water and air-dried for 90 min (S2) followed by a recovery (R2). The same procedures were repeated for S3. The T-DNA insertion lines of *clf-24* (Salk_006658) and *clf-13* (Salk_139371) were kindly provided by Dr. Zhang X. (University of Georgia), and the alleles are in Col-0 background.

Reverse Transcription and Real-Time PCR

Total RNA isolation and reverse transcription with oligo(dT)15 primer (C1101, Promega) were performed as described previously (Ding et al., 2012a). The amounts of individual genes were measured with gene-specific primers by real-time PCR analysis with a CyclerIQ real-time PCR Instrument (Bio-Rad) and SYBR Green mixture (Bio-Rad). The relative expression or amount of specific genes was quantitated with the $2^{-\Delta\Delta C_t}$ calculation (Livak and Schmittgen, 2001), according to the manufacturer's software (Bio-Rad), where $\Delta\Delta C_t$ is the difference in the threshold cycles and the reference housekeeping gene, which was ubiquitin for expression analyses. The specific primers used are shown in Supplemental Table 1.

Chromatin Immunoprecipitation (ChIP)

The ChIP assay was performed according to the described method (Saleh et al., 2008; Ding et al., 2012a). The specific antibodies (1:150 dilution) used for Ser2P Pol II (ab5095, Abcam, Cambridge, Massachusetts, USA, Lot: 703307), trimethyl-H3K4 (ab1012, Abcam, Lot: GR561731-1), trimethyl-H3K27 (#07- 449, Millipore, Lot: JBC1924326), or H3 (ab1791, Abcam, Lot: 517990) were used. Each immunoprecipitation was performed in at least three separate experiments. Enriched DNA was quantified as the proportion of the input DNA that was recovered in the IP sample. The gene-specific primers used in quantitative real-time PCR were shown in Supplemental Table 2.

Endogenous ABA Assay

Leaves were harvested from plants that were watered or air-dried during stress-recovery trainings. The leave tissues were ground in liquid nitrogen immediately and homogenized in 90% (v/v) methanol containing 200 mg l^{-1} of diethydithiocarbamic acid sodium salt. The

extracts were then incubated overnight in a covered, silanized borosilicate tube in darkness at 4°C, followed by a low-speed centrifugation. The methanolic supernatant was recovered and evaporated, and the residue was dissolved by methanolic Tris buffer (10% methanol, 50 mM Tris, pH 8.0, 1 mM $MgCl_2$, and 150 mM NaCl). An ELISA kit was used for the determination of ABA following the manufacturer's instructions (Agdia, USA).

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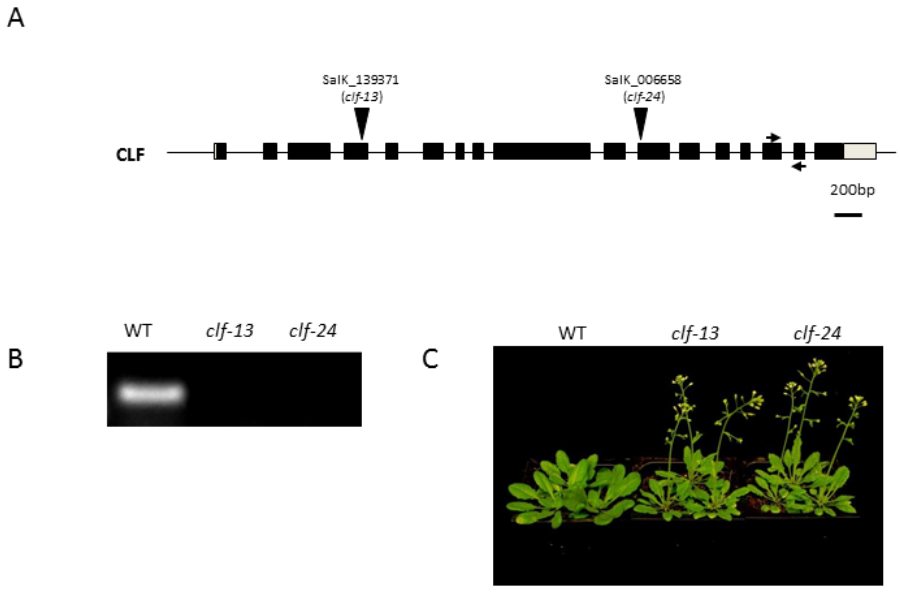
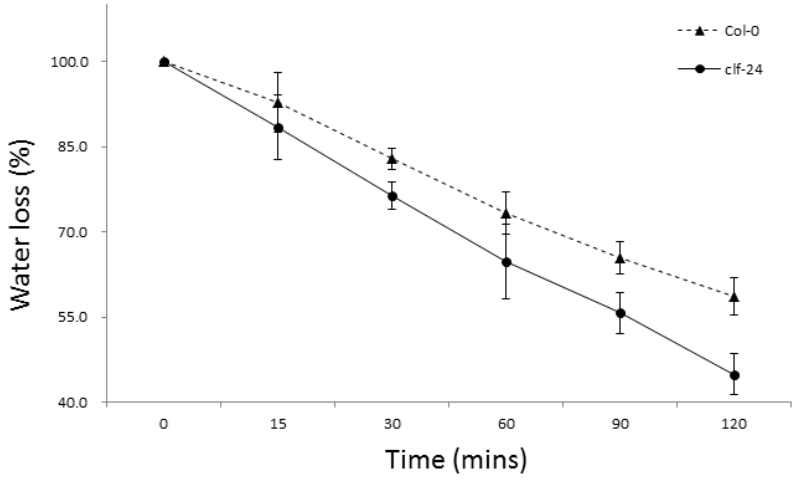


Supplementary Figure 1. Characterization of *CLF* T-DNA insertion lines.

A) Molecular structure of Curly Leaf (*CLF*) locus. Exons are depicted as black boxes, UTRs are shown as grey bars, and introns as black lines. The sites of T-DNA insertion are shown as a triangle. Horizontal arrows indicate the positions of PCR primers; **B)** RT-PCR products derived from primer pairs indicated on the right, and seedling lines as indicated above lanes. **C)** 4-week old *clf* mutants and wild type control seedling grown on soil under standard conditions.



Supplementary Figure 2. Water loss rate of *clf-24* and wild type seedling after air drying for the indicate time points. Experiments were preformed with three biologicalreplicates, each containing 10 seedlings. The results indicate the mean ± SE (n = 3).

Supplementary Table 1: List of primers used for RT-qPCR analysis

Genes	Sequence (5'-->3')
RD29A	CTGATGAGGTGAAGCCAGAA
	GAGCCAAGTGATTGTGGAGA
RD29B	ACGAGCAAGACCCAGAAGTT
	AGGAACAATCTCCTCCGATG
RAB18	TAGCCACCAGCATCATATCC
	AAGGAGGGAGGAGGAAGAAG
LTP3	AACGGTGTGCATAGTTGCAT
	TGACTCCTGCACAACATGAA
LTP4	AGCATGCAGATGCTTACAGTC
	AGTTGGTGCTCGTGGAGAT
HIPP2.2	TCCATACACAATGGTGGCTT
	GGTTCTCGTCGCTAAAGAGG
AGAMOUS	TCTCCCAAAGAGCTCAGGAA
	TTCTGCATGTAGTCGATTTCAGA

Supplementary Table 2. List of primers used for ChIP-qPCR analysis

Genes		Sequence (5'-->3')
RD29A	Region 1	ACCGACATCAGTTTGAAAGAAA
		TGGTGTGACGTCAAAGTCATT
	Region 2	TCACTAAACATGGACAAAGCAA
		TGCATCGATCACTTCAGGTT
	Region 3	CTCCATCAAGAAGCCATGAA
		GGCGAATACTCGTTTCTTCC
RD29B	Region 1	CGTAGAGAGCAACTGGCTGA
		ATTCTGACACGTACGATGCG
	Region 2	AATGGAGTCACAGTTGACACG
		GGATGGTGAATTCTGATTGGT
	Region 3	TGGAAGTGACGGTTGAGAAG
		ACCGCTCCTTTAACTTTCCC
RAB18	Region 1	TGAGCTTTCATGTCGATACCA
		AGGAGGAGCATTACGTGTCC
	Region 2	TCCTTGTGGAGTTGCTCTTG
		GGACTGAAGGCTTTGGAACT
	Region 3	TAGCCACCAGCATCATATCC
		AAGGAGGGAGGAGGAAGAAG
LTP3	Region 1	TGGCCACAGTTAATTAAAAGCA
		GACGGACACGTGTACCCAAC
	Region 2	TCAGTCGATGCTGCAATCTC
		TCCTGCACAACATGAAGGTG
	Region 3	TTGATCTAGAGACCATGAATTATTTTT
		CGACGTAAGCTTCCATTTCA
LTP4	Region 1	CATGCCGTCTGATTTAATGC
		GAAAGGTGGTCCAATGGAAA
	Region 2	CCCATCATCATCTCCCACTT
		TTGCTCTTCTCTTTTGGGTGA
	Region 3	CGACATCATTTGCCTGAAGA

		CAAAGCCATCAAGACAAACAAA
HIPP2.2	Region 1	ATGCATCCTCAGCATTGGTT
		GCGACCCACACGTATACAAA
	Region 2	CATCTCTCTGCTCGCTTGTG
		TTTACGTTTCTTCCGTTTTCG
	Region 3	TCCCTCTTTAGCGACGAGAA
		TGTTGCGTTGCTTCTCACAT
AGAMOUS	Region 1	CAAGTAATGGTAAGTAGAGTCTGCATC
		GGTGGGTAGTTCTTGTGTGGT
	Region 2	TAATTCGACACGCAATTTCC
		GTTGACAACTGTAAACGATTAAGAAA
	Region 3	ACTACGAGCAGCTTATGCCA
		TAACTGGAGAGCGGTTTGGT