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To cite this version:

Céline Duc, Matthias Benoit, Samuel Le Goff, Lauriane Simon, Axel Poulet, et al.. The histone chaperone complex HIR maintains nucleosome occupancy and counterbalances impaired histone deposition in CAF-1 complex mutants. The Plant Journal, 2015, 81 (5), pp.707-722. 10.1111/tpj.12758 . hal-04170900

HAL Id: hal-04170900
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the plant journal

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The Plant Journal (2015) 81, 707–722 doi: 10.1111/tpj.12758

The histone chaperone complex HIR maintains nucleosome occupancy and counterbalances impaired histone deposition in CAF-1 complex mutants

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Received 3 September 2014; revised 21 November 2014; accepted 23 December 2014; published online 19 January 2015.
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SUMMARY

Chromatin organization is essential for coordinated gene expression, genome stability, and inheritance of epigenetic information. The main components involved in chromatin assembly are specific complexes such as Chromatin Assembly Factor 1 (CAF-1) and Histone Regulator (HIR), which deposit histones in a DNA synthesis-dependent or -independent manner, respectively. Here, we characterize the role of the plant orthologs Histone Regulator A (HIRA), Ubinuclein (UBN) and Calcineurin Binding protein 1 (CABIN1), which constitute the HIR complex. Arabidopsis loss-of-function mutants for the various subunits of the complex are viable, but *hira* mutants show reduced fertility. We show that loss of HIRA reduces extractable histone H3 protein levels and decreases nucleosome occupancy at both actively transcribed genes and heterochromatic regions. Concomitantly, HIRA contributes to maintenance of silencing of pericentromeric repeats and certain transposons. A genetic analysis based on crosses between mutants deficient in subunits of the CAF-1 and HIR complexes showed that simultaneous loss of both the CAF-1 and HIR histone H3 chaperone complexes severely affects plant survival, growth and reproductive development. Our results suggest that HIRA partially rescues impaired histone deposition in *fas* mutants to preserve nucleosome occupancy, implying plasticity in histone variant interaction and deposition.

Keywords: Arabidopsis, chaperone, histone, nucleosome, transcriptional silencing.

INTRODUCTION

Eukaryotic DNA is organized into chromatin. Its basic subunit, the nucleosome, consists of 146 bp of DNA wrapped around a histone octamer comprising a $(H3–H4)_2$ tetramer and two histone H2A–H2B dimers. Chromatin organization profoundly affects the accessibility of DNA to the cellular machinery, and therefore affects all cellular processes operating on DNA. To coordinate these different functions, remodeling of chromatin is required to allow access or exclusion of various factors. Remodeling can encompass movement of nucleosomes along the DNA, but also their disassembly and reassembly, as occurs during passage of the transcriptional machinery (Petesch and Lis, 2012). These processes are facilitated by factors modulating the stability of the nucleosomes or the association of histones with the DNA, such as covalent modifications and incorporation of histone variants (Jin and Felsenfeld, 2007). Except for histone H4, all histone proteins occur in non-canonical variants that differ in their primary amino acid sequence from the canonical paralogs (Talbert and Henikoff, 2010). These differences range from a few amino acids to large protein domains (Talbert *et al.*, 2012). For example, the canonical histone H3.1 and its variant H3.3 diverge by only four amino acids, but are incorporated differently during the cell cycle and show specific distribution patterns in mammals and plants (Tagami *et al.*, 2004; Goldberg *et al.*, 2010; Stroud *et al.*, 2012; Wollmann *et al.*, 2012; Filipescu *et al.*, 2013; Shu *et al.*, 2014). While nucleosomes containing H3.1 are thought to package DNA globally in a DNA synthesis-linked process, the replacement variant H3.3 is preferentially incorporated at enhancers, promoters and gene bodies of actively transcribed genes throughout the cell cycle (Ahmad and Henikoff, 2002; Jin *et al.*, 2009; Goldberg *et al.*, 2010).

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The highly basic histone proteins are accompanied from synthesis to chromatin assembly by a network of histone chaperones (De Koning *et al.*, 2007), thereby preventing uncontrolled interaction with nucleic acids or negatively charged proteins. Consequently, histone chaperones are involved in all aspects of histone dynamics, such as transport and storage, chromatin assembly and disassembly, and parental histone transfer during DNA replication (Filipescu *et al.*, 2013; Groth *et al.*, 2007; De Koning *et al.*, 2007). Histone chaperones may be classified on the basis of their preferential binding to either H3–H4 or H2A–H2B subunits. In addition, some chaperones show specificity for particular histone variants and play a crucial role in their chromatin distribution (Tagami *et al.*, 2004; Drané *et al.*, 2010; Goldberg *et al.*, 2010).

While H3 histone variants are assumed to have evolved independently in animals and plants (Ingouff and Berger, 2010), histone H3 chaperones are highly conserved through evolution. The chaperone Anti-silencing function 1 (Asf1) binds H3–H4 dimers (English *et al.*, 2006; Natsume *et al.*, 2007) in the cytoplasm, and is involved in histone import into the nucleus (Campos *et al.*, 2010). Asf1 then transfers histones to chaperone complexes involved in nucleosome assembly. In mammals, two distinct pathways control deposition of either the canonical histone H3.1 or the variant H3.3. Chromatin Assembly Factor 1 (CAF-1), consisting of the three subunits p150, p60 and p48, ensures histone deposition in a DNA synthesis-dependent manner during replication and repair (Stillman, 1989; Gaillard *et al.*, 1996). CAF-1 specifically deposits H3.1 (Tagami *et al.*, 2004; Drané *et al.*, 2010) and interacts with Asf1 (Tyler and Collins, 2001). Independently of DNA synthesis and throughout the whole cell cycle, histone deposition is promoted by Histone Regulator A (HIRA), which shows high specificity for the variant H3.3 (Ray-Gallet *et al.*, 2002; Tagami *et al.*, 2004). HIRA depletion results in reduced genome-wide loading of H3.3 (Goldberg *et al.*, 2010; Pchelintsev *et al.*, 2013). In addition, H3.3 is deposited in mammals by death-associated protein (DAXX), α-thalassemia/mental retardation X-linked syndrome protein (ATRX) (Drané *et al.*, 2010; Goldberg *et al.*, 2010) and the chaperone DEK (Sawatsubashi *et al.*, 2010). HIRA is part of a multimeric complex termed the HIR complex, which was first identified in *Saccharomyces cerevisiae* in which it consists of four subunits (Hir1, Hir2, Hir3 and Hpc2) and functions as repressor of histone genes outside S phase (Osley and Lycan, 1987). Orthologs of Hir1 and Hir2 have been identified as HIRA in *Drosophila*, mammals and Arabidopsis (Phelps-Durr *et al.*, 2005; Nie *et al.*, 2014). In humans, the complex further comprises Ubinucleins 1 and 2 (UBN1 and UBN2), orthologs of yeast Hpc2, as well as the Calcineurin Binding protein (CABIN1), an ortholog of Hir3. HIRA mediates binding to UBN1 (Balaji *et al.*, 2009) and CABIN1 (Yang *et al.*, 2011) in mammals, and binds UBN2 in plants (Nie *et al.*, 2014). HIRA further interacts with Asf1 (Tang *et al.*, 2006; Nie *et al.*, 2014).

The study of histone chaperone complexes as well as their role in histone dynamics during development is hampered in higher organisms, as these factors are essential for survival. Mice embryos deficient in p150, the large subunit of the CAF-1 complex, fail to develop beyond early embryonic stages (Houlard *et al.*, 2006). Similarly, mutants of the *Drosophila* ortholog p180 die during larval development (Klapholz *et al.*, 2009). In Arabidopsis, the CAF-1 complex consists of the subunits FASCIATA1 (FAS1), FASCIATA2 (FAS2) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) (Kaya *et al.*, 2001). Arabidopsis mutants deficient in FAS1 or FAS2 are viable, but show pleiotropic morphological abnormalities, such as fasciated stems, serrated leaves and meristem alterations (Kaya *et al.*, 2001; Exner *et al.*, 2006; Kirik *et al.*, 2006). Consistent with its role in chromatin assembly during DNA replication, CAF-1 mutants fail to maintain repressive chromatin states, as illustrated by weak transcriptional reactivation of silent endogenous repetitive sequences and the stochastic reactivation of certain transposable elements (Takeda *et al.*, 2004; Ono *et al.*, 2006; Schönrock *et al.*, 2006).

Depletion of mammalian HIRA is also lethal, and HIRA knockout mice die during embryonic development (Roberts *et al.*, 2002). Furthermore, down-regulation of HIRA in *Xenopus* embryos causes gastrulation defects (Szenker *et al.*, 2012), phenotypes that may be explained by a role for HIRA in transcription (Formosa *et al.*, 2002; Schwartz and Ahmad, 2005; Ray-Gallet *et al.*, 2011). In contrast, *Drosophila* HIRA is only required for H3.3 deposition in the male pronucleus after fertilization (Loppin *et al.*, 2005), but not for viability (Bonnefoy *et al.*, 2007). How defective histone assembly outside S phase mediated by HIRA affects plants at the molecular level has not yet been addressed.

We show here that HIRA is an important player with respect to histone dynamics in Arabidopsis. Plants lacking HIRA are viable but show developmental defects and are impaired in the maintenance of transcriptional silencing. Loss of HIRA results in reduced H3 protein levels and affects nucleosome occupancy, not only at euchromatic but also at heterochromatic targets. Simultaneous loss of CAF-1 and HIR complexes severely affects Arabidopsis development. Surviving plants show significant defects in plant growth and reproduction, as well as an important reduction in nucleosome occupancy, without being further affected in maintenance of silencing and heterochromatin organization. Our data suggest that, in plants, the two evolutionarily conserved chromatin assembly complexes CAF-1 and HIR are involved in independent pathways of nucleosomal assembly, but show partial functional redundancy in maintenance of nucleosome occupancy.

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RESULTS

Characterization of the Arabidopsis orthologs of HIR subunits

Arabidopsis orthologs of the HIR subunits are encoded by the following genes: *HIRA* (At3g44530), *UBN1* (At1g21610), *UBN2* (At1g77310) and *CABIN1* (At4g32820) (Nie *et al*., 2014). We analyzed their protein sequences and observed conservation of the overall protein structure in Arabidopsis HIRA, which contains N-terminal WD40 repeats involved in protein–protein interaction, the C-terminal Hira domain and the B-domain required in mammals for binding to ASF1 (Figure 1a and Figure S1a,b) (Tang *et al*., 2006). As in mammals, several plant species contain two closely related UBN paralogs (Figure 1a and Figure S1e). The Arabidopsis UBN proteins are of similar length, show 59.7% sequence identity, and conservation of the Hpc2-related domain (HRD) (Banumathy *et al*., 2009) and the NHRD (N-terminal to the HRD region domain) (Figure S1c,d) (Tang *et al*., 2012). The Arabidopsis HIR complex also comprises a third subunit, CABIN1, characterized by a range of tetratricopeptide (TPR)-like bi-helical repeats, which may form a scaffold for protein–protein interaction (Balaji *et al*., 2009) (Figure 1a). Phylogenetic analysis confirmed that CABIN1 is found throughout the plant kingdom (Figure S1f). The conservation of specific domains and interaction of HIRA with Arabidopsis UBN2 and ASF1 (Nie *et al*., 2014) suggests that, as in animals, HIRA is the scaffold protein for CABIN1 and UBN recruitment and binds ASF1.

To obtain insight into the biological function of the Arabidopsis HIR complex, we confirmed expression of all four genes in several tissues (Figure 1b and Figure S1g). We obtained T-DNA insertion mutants for each gene encoding a HIR complex subunit (Figure 1c–f). As conflicting results were reported concerning the viability of *hira* mutants (Phelps-Durr *et al*., 2005; Ingouff *et al*., 2010; Nie *et al*., 2014), we obtained four T-DNA insertion lines and determined the exact insertion sites in the *HIRA* locus (Figure 1c and Figure S2a). We identified plants homozygous for all mutant alleles except SALK_143806, for which we did not identify any T-DNA insertion in various seed batches, but only the *hira-1* mutant (Ingouff *et al*., 2010; Nie *et al*., 2014) showed an effect on *HIRA* expression (Figure 1c and Figure S2b). We also generated a transgenic line expressing an artificial microRNA targeting *HIRA* transcripts ($hira^{amiRNA}$), and confirmed reduction of *HIRA* transcript levels to approximately 25% (Figure S2c). In addition to *hira* mutants, we identified T-DNA insertion alleles for *UBN1*, *UBN2* and *CABIN1*, and confirmed the absence of the corresponding full-length transcripts (Figure 1d–f). Single mutants of the HIR complex subunits display no obvious vegetative developmental phenotypes compared to wild-type (WT) plants (Figure 1g) except few cases of *hira-1* mutants with serrated leaves (Nie *et al*., 2014). In comparison, *fas1-4* and *fas2-5* mutants, which possess mutations in one of the two larger subunits of the CAF-1 complex, show pleiotropic phenotypes (Figure S2d,e). Deficiency in the CAF-1 histone chaperone affects endoreplication levels, leading to a premature switch to the endocycle and increased endopolyploidy levels (Ramirez-Parra and Gutierrez, 2007). To evaluate endopolyploidy levels in HIR complex mutants, we performed flow-cytometry analysis. Whereas *fas2-5* mutant plants show a significantly increased proportion of 8C and 16C nuclei (one and two rounds of endoreplication), endopolyploidy profiles of HIR mutants are not significantly different from those of WT plants (Figure 1h). This suggests that absence of a functional HIR complex does not affect endoreplication.

As mutants impaired in histone deposition mediated by the CAF-1 complex show reduced fertility (Figure S2e,f) (Ramirez-Parra and Gutierrez, 2007), we looked more closely at the siliques of HIR complex mutants. Seed set in self-pollinated *hira-1* mutant flowers, but not in the other HIR complex mutants, was significantly reduced, and we observed more unfertilized ovules and aborted seeds than in WT (Figure 2a,b). A similar phenotype was noted in the $hira^{amiRNA}$ line (Figure 2c). We observed standard Mendelian genetic transmission of the T-DNA mutant allele in the progeny of self-fertilized *hira-1*/*HIRA* plants (51 heterozygous and homozygous *hira-1* plants, $n = 72$, $P < 0.05$), similar to previous reports (Ingouff *et al*., 2010; Nie *et al*., 2014). Nevertheless, while anthers of HIR complex mutants develop normally (Figure 2d), in contrast to the heart-shaped *fas1-4* and *fas2-5* anthers (Figure S2g), a proportion of pollen grains are non-viable in *hira-1*, as revealed by Alexander staining (Figure 2d), but to a lesser extent than in *fas* mutants (Figure S2g). Furthermore, an increased number of unfertilized ovules were observed in heterozygous *hira-1*/*HIRA* plants compared to their WT sister plants (Figure 2e). Taken together, these results suggest that, while the HIR complex is dispensable for vegetative growth, loss of the HIRA subunit causes sporophytic and gametophytic defects, resulting in reduced fertility.

The *hira-1* mutant shows a reduced histone H3 protein pool and altered nucleosome occupancy

In analogy to yeast and animal models, Arabidopsis CAF-1 and HIR complexes are assumed to bind non-nucleosomal histones and coordinate their assembly into nucleosomes. We therefore investigated whether mutations in these two complexes affect the pool of H3 histones. We extracted proteins as described by Durut *et al*. (2014), and determined the amount of histone H3 in *fas1-4* and *hira-1* mutants relative to WT plants by Western blotting (Figure 3a). We observed reduced H3 levels in both mutants, suggesting that histone flow and the amount available for

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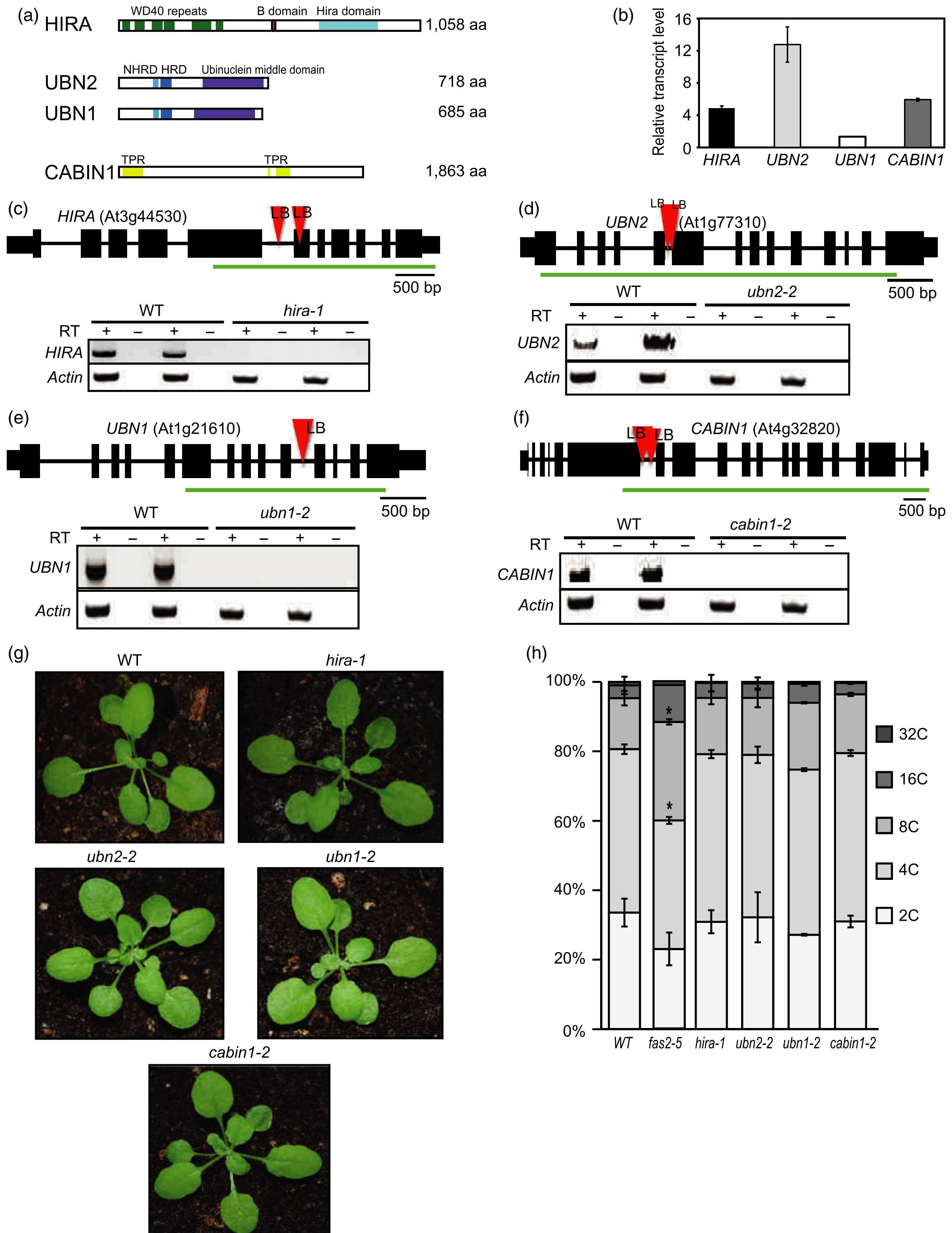

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Figure 1. Characterization of the HIR complex subunits in plants.
(a) Functional domains of the HIR complex proteins. HRD, Hpc2-related domain; NHRD, N-terminal to the HRD region domain; TPR, tetratricopeptide-like bi-helical repeats.
(b) Quantitative RT-PCR analysis of *HIRA*, *UBN2*, *UBN1* and *CABIN1* transcript levels in 4-week-old plants.
(c–f) Gene structures and characterization of the Arabidopsis HIR complex mutants. Exons, boxes; UTRs, narrower boxes; introns, lines; T-DNA insertion, red triangle; LB, left border. Absence of full-length transcripts is revealed by RT-PCR on two biological replicates: the amplified region is indicated by a green line.
(g) Representative 25-day-old plantlets of HIR complex mutants grown on soil.
(h) Ploidy level distribution of CAF-1 and HIR complex mutant nuclei from 10-day-old plants.

de novo deposition are altered upon loss of CAF-1 or HIR chromatin assembly complexes.

To analyze nucleosome occupancy at specific genomic sites in WT, *fas1-4* and *hira-1* mutants in a quantitative manner, we used H3-ChIP combined with quantitative PCR. Based on one of the available whole-genome ChIP-Seq datasets (Stroud *et al.*, 2012), we selected euchromatic, intergenic and heterochromatic sites differentially enriched in H3.3 and H3.1 (Figure S3b–h). We first analyzed nucleosome occupancy at three constitutively active genes (*UBC28*, *UEV1C* and *HXK1*) with different expression levels (Figure S3a) that are enriched in H3.3 in their middle and 3′ regions (Figure S3b–d) (Stroud *et al.*, 2012), suggesting that they are preferential targets for H3 deposition mediated by HIRA, which co-immunoprecipitates with H3.3 (Nie *et al.*, 2014). In 3-week-old *fas1-4* mutant plants grown *in vitro*, nucleosome occupancy was unaffected, but in *hira-1* mutants, *UEV1C* and *HXK1* show mildly reduced H3 levels in the middle and 3′ regions (Figure 3b–d), but not in the 5′ region at which neither H3.1 nor H3.3 are enriched (Figure S3b–d). This difference is not due to altered nucleosome displacement associated with increased expression (Figure S3i), revealing a specific role for HIRA in H3 deposition at these genic regions.

As *fas1* mutants were previously shown to have moderately reduced nucleosome occupancy at selected pericentromeric sequences (Pecinka *et al.*, 2010), we also included heterochromatic repetitive elements (180 bp repeat, 106B centromeric satellites and an endogenous family of transcriptionally repressed repeats called transcriptionally silent information (TSI) (Steimer *et al.*, 2000), as well as an intergenic region (Pecinka *et al.*, 2010) in our analysis. These heterochromatic regions are enriched in H3.1 (Figure S3e–g), but neither H3.1 nor H3.3 are particularly enriched at the intergenic region (Figure S3h). We observed reduced nucleosomal occupancy at 106B and TSI in *fas1-4* mutants (Figure 3e). Unexpectedly, we also found that nucleosome occupancy is reduced in *hira-1* mutants in these two heterochromatic regions and in the intergenic region (Figure 3e).

We conclude that loss of function of either of the two chaperone complexes CAF-1 and HIR affects the extractable H3 histone pool. Furthermore, loss of HIRA affects nucleosome occupancy at both euchromatic and heterochromatic regions, while loss of CAF-1 mainly affects heterochromatic sequences.

Loss of HIRA interferes with maintenance of transcriptional silencing but not with gene induction upon salt stress

The observed differences in nucleosome occupancy prompted us to investigate the functional consequences of altered histone dynamics in HIR complex mutants. We first analyzed the impact on maintenance of transcriptional silencing at the heterochromatic regions by ChIP combined with quantitative PCR. Quantification of transcript levels by quantitative RT-PCR revealed partial silencing release of TSI in *fas* mutants, as expected. In agreement with the changes in nucleosome occupancy, the *hira-1* mutants also show alleviation of TSI silencing (Figure 4a), which is not seen in *ubn2-2*, *ubn1-2* and *cabin1-2* mutants. None of the mutants of HIR complex subunits reactivates silencing at 106B, 180 bp or a multicopy transgenic locus (Morel *et al.*, 2000) (Figure 4a and Figure S4a). To examine whether the silencing release in *hira-1* is restricted to TSI sequences or is more general, we tested additional targets and observed alleviation of silencing of the Ta3 retrotransposon and a Mutator-like DNA transposon (*Mule*, At2g15810) (Figure 4b) in *hira-1* compared to WT plants, concomitant with reduced nucleosome occupancy in these two regions (Figure 4c).

Given the suggested function of HIRA in transcription in other species, we wished to determine whether HIRA is required to rapidly activate gene expression in response to an environmental stimulus. We exposed plants to salt stress and analyzed the expression of four genes induced under this stress condition (Zeller *et al.*, 2009). Under normal growth conditions, *Protein Phosphatase 2C* (*PP2C*, At3g16800) and *Ethylene Responsive Factor/APETALA 2* (*ERF/AP2*, At1g74930) are moderately expressed, while *MYB domain protein 41* (*MYB41*, At4g28110) and *C-Repeat/DRE binding factor 1* (*CBF1*, At4g25490) are not expressed (Zeller *et al.*, 2009; Duc *et al.*, 2013). In WT plants, all four genes are induced after 1 h of exposure to high-salt medium, undergoing changes in transcript levels from twofold to several hundred times, depending on the gene (Figure 4d). However, the *hira-1* mutant plantlets are not impaired in the rapid transcriptional response, and up-regulate salt-responsive genes similarly to WT (Figure 4d). We next analyzed three genes that are repressed by salt stress (Zeller *et al.*, 2009): At4g12510 and At4g12520, which are expressed at a low level under normal growth

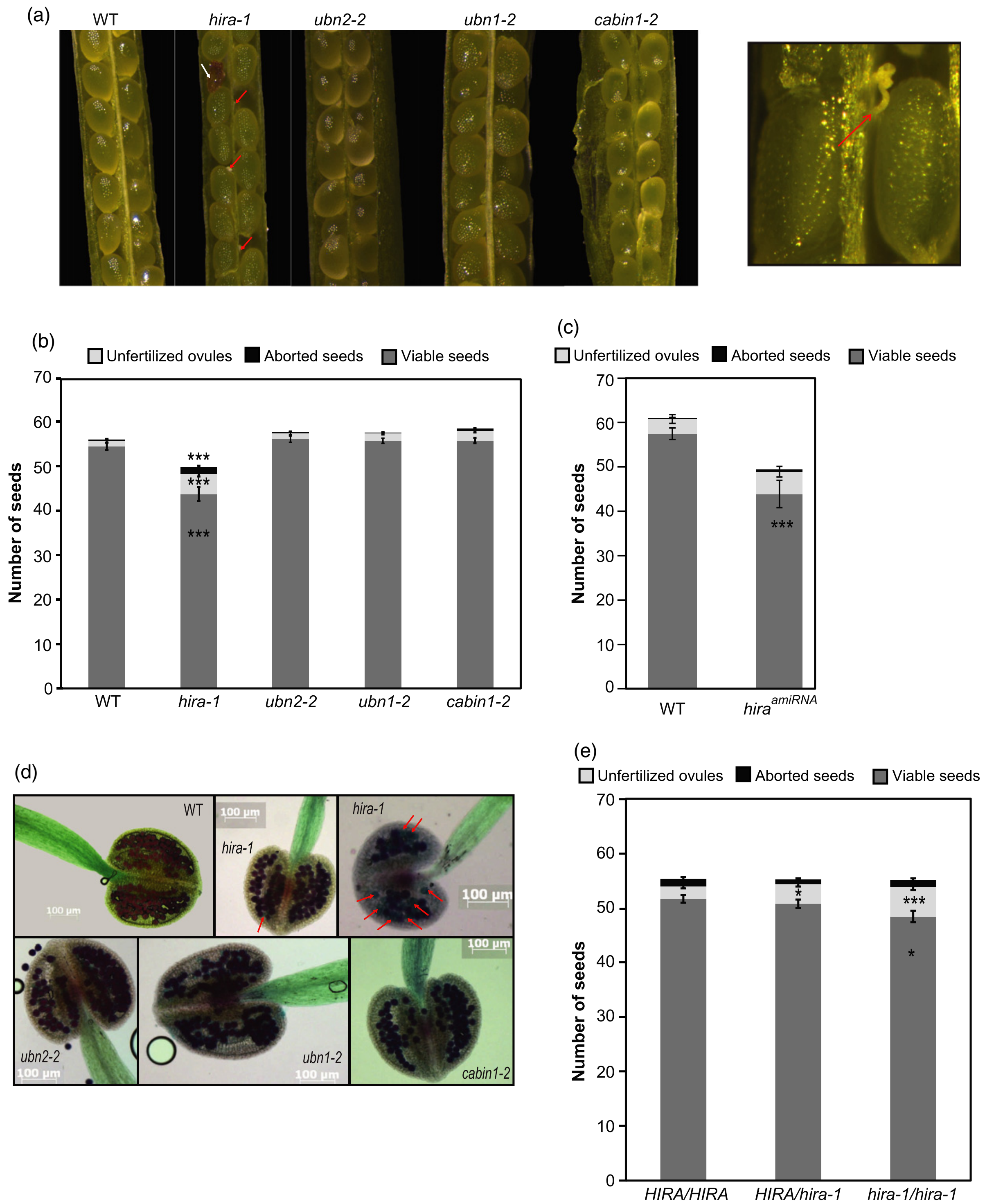

Figure 2. Analysis of Arabidopsis mutants in the HIR complex.
(a) Representative dissected siliques from HIR complex mutants. Red arrows indicate unfertilized ovules, and the white arrow indicates an aborted seed. Right, close-up of an unfertilized ovule.
(b) Quantification of seed content in HIR complex mutant siliques. Quantifications were obtained from 30 pooled siliques from at least four plants.
(c) Quantification of seed content in the *hira*^{amiRNA} line. Quantifications were obtained from 16 WT and 19 *hira*^{amiRNA} pooled siliques from four plants.
(d) Pollen viability assessed by Alexander staining. Only *hira-1* mutant anthers contain non-viable pollen (green color), as indicated by red arrows.
(e) Quantification of seed content in WT (*HIRA/HIRA*), heterozygous (*HIRA/hira-1*) and homozygous (*hira-1/hira-1*) siliques. Quantifications were obtained from 30 pooled siliques from at least six plants.

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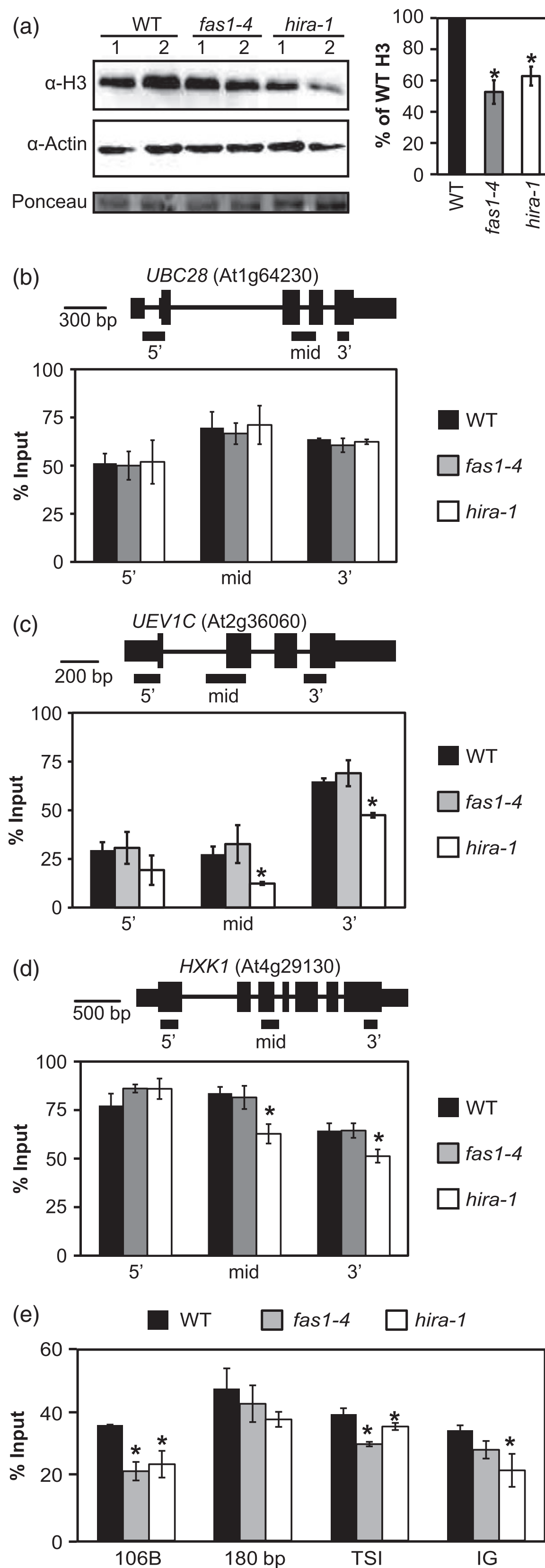

Figure 3. HIRA loss affects the extractable histone pool and nucleosome occupancy.
(a) Left: Histone H3 protein levels quantified by Western blotting. Twenty micrograms of proteins extracted from two independent biological replicates of 3-week-old *in vitro* grown plants were loaded per lane. Right: Quantification of H3 band intensities relatively to actin from three independent experiments.
(b–e) Histone H3 occupancy at various positions along three active genes [*UBC28* (b), *UEV1C* (c) and *HXK1* (d)] and at heterochromatic repeats and an intergenic region (e) assessed by H3-ChIP combined with quantitative PCR in 3-week-old *in vitro* grown WT, *fas1-4* and *hira-1* mutant plants.

conditions, and *Small Auxin Upregulated RNA 14* (*SAUR14, At4g38840*), which is moderately expressed under normal growth conditions (Zeller *et al*., 2009; Duc *et al*., 2013). Similarly to salt-induced genes, *hira-1* mutant plantlets down-regulate the expression of these genes, as in WT (Figure 4e).

Taken together, HIRA is implicated in the maintenance of transcriptional gene silencing at a selection of endogenous repeat elements and transposons, but is dispensable for rapid gene expression changes of the examined genes upon salt exposure.

Genetic analyses identify HIRA as the central subunit of the HIR complex

To obtain further insight into the relative importance of the various HIR complex members and to examine the epistatic relationship between CAF-1 and HIR complexes, we first crossed *fas1* and *fas2* mutants with each mutant for the various HIR complex subunits (Figure S5a). We performed segregation analyses on F_2 progeny from several independent F_1 plants obtained for each cross by selecting homozygous *fas* plants which display serrated leaf margins and genotyping for the corresponding HIR complex mutation (Table 1). Double mutants of *cabin1-2*, *ubn2-2* or *ubn1-2* with *fas1-4* show the expected segregation ratio (Figure S5a and Table 1) worsening the *fas1-4* growth phenotypes and fertility defects. (Figure 5a,b and Figure S5b). In contrast, double mutants of *cabin1-2*, *ubn2-2* and *ubn1-2* with *fas2-5* were recovered with reduced frequency (Table 1) but were indistinguishable from single *fas2-5* mutants (Figure 5c and Figure S5c). Strong reduction in seed set was observed for *fas2-5 ubn2-2* and *fas2-5 cabin1-2* plants (Figure 5d and Figure S5d), and very little viable pollen was detected in anthers, which were frequently aberrantly shaped in *fas2-5 ubn2-2* plants (Figure S5e,f). In comparison with the other HIR complex subunits, the double mutants generated between *hira-1* and CAF-1 mutants revealed more severe phenotypes. Only a few *fas2-5 hira-1* plants were obtained with and without prior selection of the *fas2* phenotype (Table 1 and Table S1a), and their development was arrested before formation of the first leaves (Figure 5c). Furthermore, *fas2-5 hira-1/HIRA* plants

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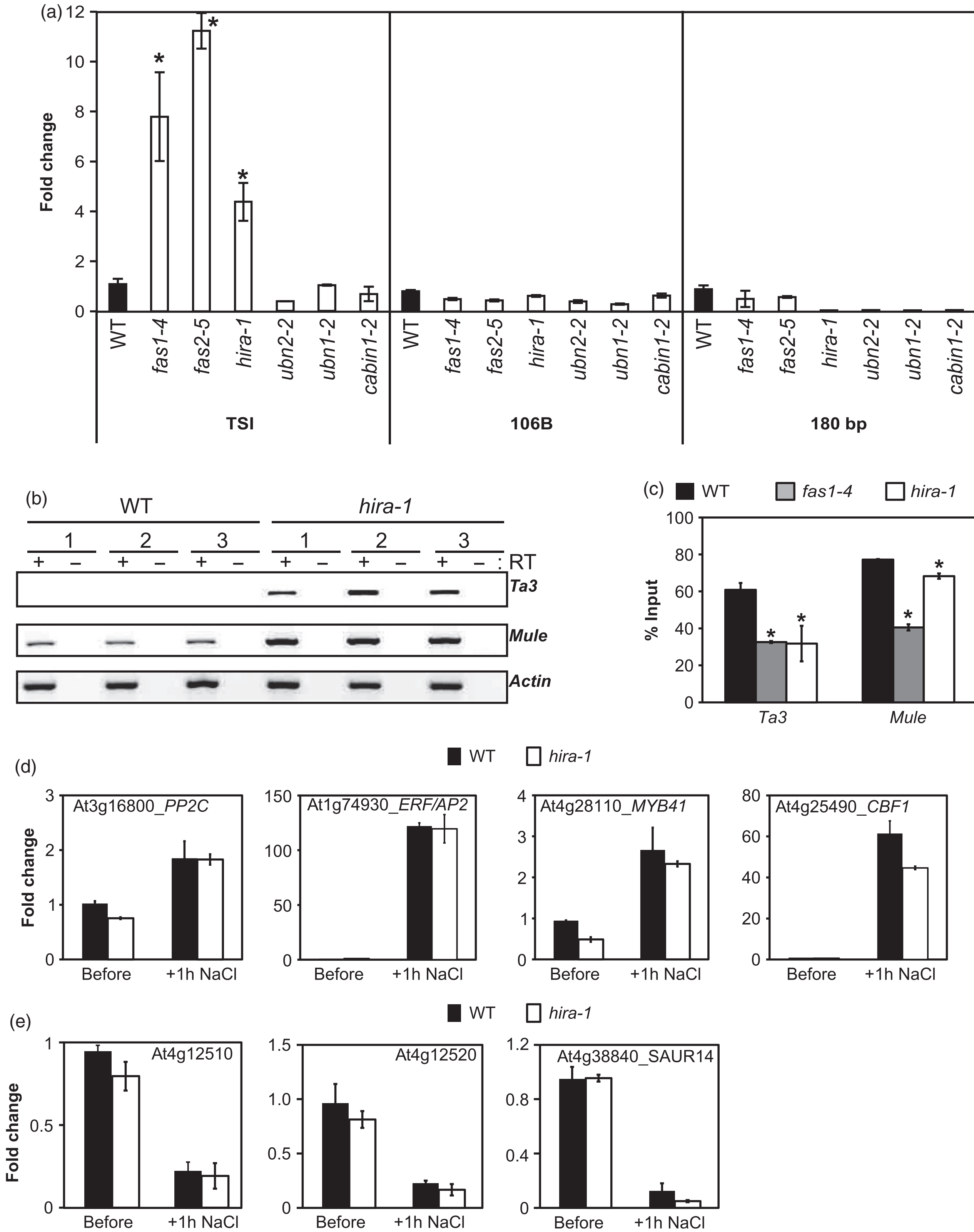

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Figure 4. Release of transcriptional gene silencing and induction of gene expression in *hira-1* mutants.
(a) Quantitative RT-PCR analysis of TSI, 106B and 180 bp transcripts in 18-day-old CAF-1 and HIR complex mutant plants grown on soil.
(b) RT-PCR analysis of *Ta3* and *Mule* (At2g15810) transcripts of three independent biological replicates from WT and *hira-1* plants. *Actin* was used for normalization.
(c) Histone H3 occupancy at a heterochromatic pericentromeric region (*Ta3*) and an intergenic transposon (*Mule*, At2g15810) assessed by H3-ChIP combined with quantitative PCR in 3-week-old *in vitro*-grown WT, *fas1-4* and *hira-1* mutant plants.
(d,e) Quantitative RT-PCR analysis of transcript levels of salt-induced genes (*PP2C*, *ERF/AP2*, *MYB41* and CBF1) (d) and salt-repressed genes (At4g12510, At4g12520 and *SAUR14*) (e) before and after exposure of 10-day-old WT and *hira-1* mutant plantlets to 200 mM NaCl for 1 h (+1 h NaCl). The value for WT before salt treatment was set to 1.

were almost sterile (Figure S5g) and showed little viable pollen (Figure S5h). Similarly, significantly fewer *fas1-4 hira-1* double homozygous mutants were obtained (Table 1 and Table S1b). They develop beyond the state of *fas2-5 hira-1* mutant plants but are dwarf and dark green (Figure 5e), show flowers harboring mis-shapen carpels and short stamens with aberrant anthers (Figure 5f), and do not produce siliques. This finding is in agreement with the complete male sterility revealed by Alexander staining (Figure 5g), in comparison to their heterozygous sister plants (Figure S5i). We anticipated that absence of either FAS1 or FAS2 would render the CAF-1 complex non-functional, and therefore expected similar phenotypes for the two sets of crosses. However, all plants carrying the *fas1-4* allele show less severe defects, suggesting that *fas1-4* is not a complete loss-of-function mutant. Indeed, while RT-PCR analysis confirmed the absence of *FAS2* full-length transcripts in *fas2-5* mutants (Figure S5j), remaining full-length *FAS1* transcripts were detected in *fas1-4* mutants (Figure S5k,l) (Ramirez-Parra and Gutierrez, 2007). Therefore, *fas1-4 hira-1* plants retained residual CAF-1 activity, permitting survival and flowering.

Table 1 Epistatic relationship between CAF-1 and HIR complexes

Genotype		+/+	+/−	−/−	*n*	*N*
fas1-4	*cabin1-2*	17	27	19	63	3
	ubn2-2	28	28	3	59	3
	ubn1-2	22	27	9	58	3
	hira-1[b]	33	76	3	112	[a]
fas2-5	*cabin1-2*[b]	18	68	19	105	3
	ubn2-2	27	44	14	85	4
	ubn1-2[b]	22	73	19	114	3
	hira-1[b]	10	61	0	71	4

Number of F_2 plants with the indicated genotype. F_2 plants with the serrated leaf phenotype associated with the *fas* mutation were selected from the progeny of several independent F_1 plants, and genotyped for the corresponding HIR complex mutation (+/+, WT; +/−, heterozygous; −/−, homozygous for the studied mutation). The expected segregation ratio of WT to heterozygous to homozygous is 1:2:1 for genetically non-linked mutations. *n*, total number of plants analyzed; *N*, number of independent F_1 plants used in this study. The genes *FAS1*, *UBN2* and *UBN1* are located on the same chromosome (Figure S5a).
[a]Segregation analysis from a pool of seeds derived from several independent F_1 plants.
[b]Segregation significantly different from the expected ratio (chi-square test).

We conclude that the simultaneous mutation of CAF-1 and HIR complexes causes strong defects ranging from severe growth and developmental difficulties to lethality. While UBN1 appears to be dispensable for plant survival and reproduction, loss of HIRA causes the severest morphological aberrations, suggesting that HIRA is essential for HIR complex function. We assessed this hypothesis by generating *hira-1 ubn2-2*, *hira-1 ubn1-2* and *hira-1 cabin1-2* double mutants. We found that the double mutants show seed set similar to that of single *hira-1* mutant plants, with slightly more aborted and fewer viable seeds for *hira-1 ubn2-2* and *hira-1 cabin1-2* double mutants, respectively (Figure 5h). Furthermore, we observed comparable TSI levels in *hira-1*, *hira-1 ubn1-2* and *hira-1 cabin1-2* mutants, while *hira-1 ubn2-2* plants showed stronger reactivation of TSI repeat expression (Figure 5i). These observations, together with our data derived from crosses with mutants of the CAF-1 complex, suggest a central role of HIRA in HIR complex function, but do not exclude HIR complex-independent roles for the various subunits of the complex.

CAF-1 and HIR complexes are involved in independent but complementary pathways of chromatin assembly

The *fas1-4 hira-1* plants offer the unique opportunity to study the molecular consequences of simultaneous mutation of CAF-1 and HIR complexes. To obtain sufficient plant material, we selected double mutants grown on soil by phenotype from the segregating progeny of *fas1-4/FAS1 hira-1* plants (Figure S6a). We first analyzed H3 levels in *fas1-4 hira-1* plants. We found that the extractable pool of H3 proteins is significantly reduced compared to WT (Figure 6a). Histone H3 proteins are stored and transported by ASF1 proteins that donate histones to the chromatin complexes mediating assembly. The balance of *ASF1* expression is altered in *fas1-4 hira-1* mutants compared to WT: *ASF1A* expression is down-regulated while that of *ASF1B* is up-regulated (Figure 6b). Such a change in ASF1A and B protein levels may adjust histone flow to the remaining assembly complexes.

Given that *fas1-4 hira-1* plants are severely affected in growth and development, we then assessed whether heterochromatin organization and silencing are impaired in the double mutants. RT-PCR analysis on 4-week-old soil-grown plants showed that *fas1-4 hira-1* mutants release *TSI*, *Ta3* and *Mule* silencing to similar levels as single

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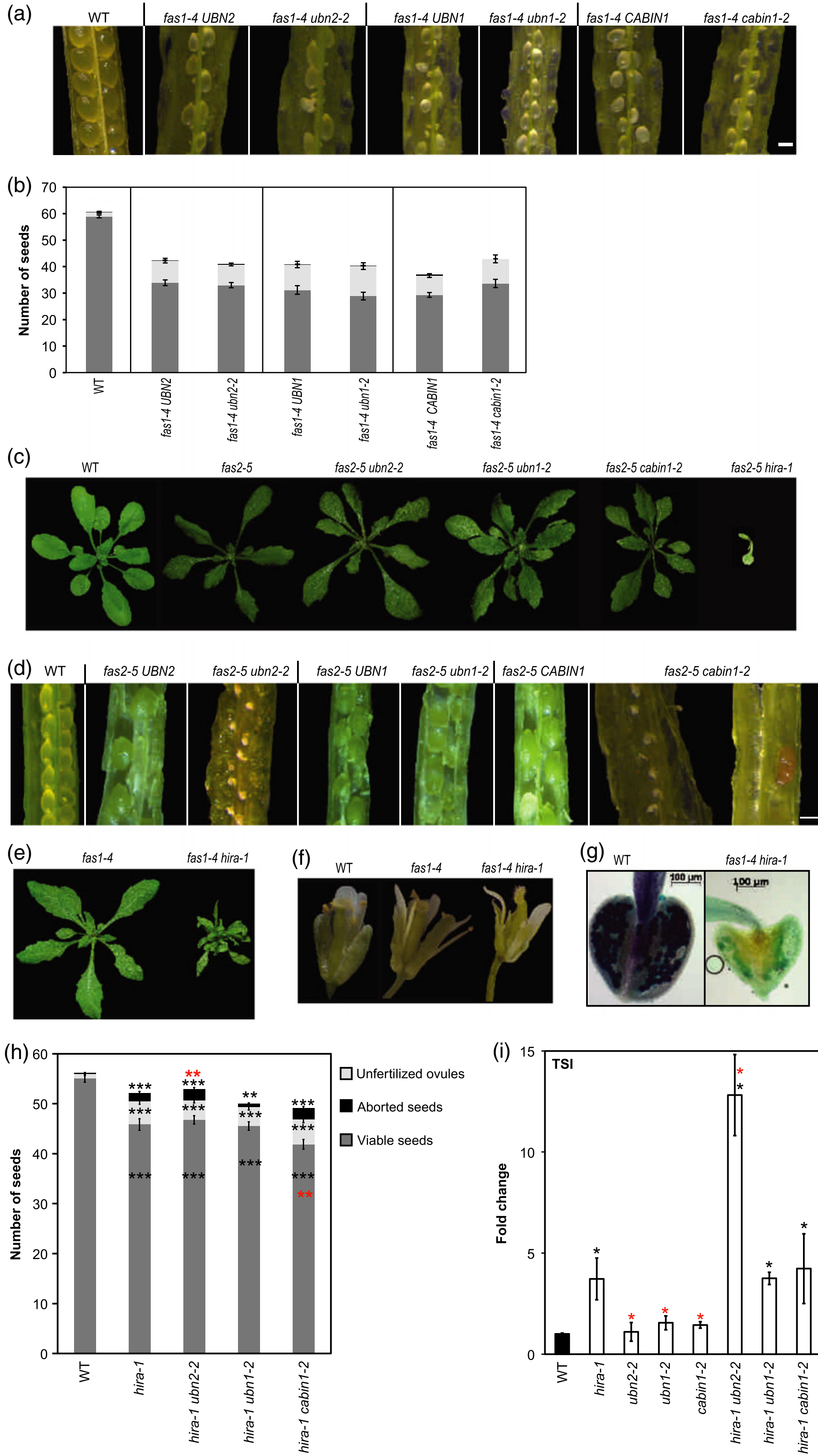

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Figure 5. Genetic interactions between members of the CAF-1 and HIR complexes.
(a,d) Dissected siliques from F_2 progeny of *fas1-4* (a) and *fas2-5* (d) crosses with *ubn2-2*, *ubn1-2*, and *cabin1-2* mutants. For each cross, a representative silique from the *fas* single mutant and double mutant sister plants is shown. Scale bars = 0.3 mm.
(b) Quantification of seed content in siliques of *fas1-4* single and double mutants compared to WT. Thirty siliques pooled from at least four F_3 plants derived from F_2 plants homozygous for *fas1-4* and heterozygous for the respective mutation of the HIR complex were analyzed. No statistically significant difference was detected in comparison with the respective *fas1-4* sister plant (Student's *t* test).
(c,e) Representative WT, *fas2-5*, *fas2-5 ubn2-2*, *fas2-5 ubn1-1*, *fas2-5 cabin1-2 and fas2-5 hira-1* (c) and *fas1-4* and *fas1-4 hira-1* (e) mutant plants grown on soil.
(f) Representative flowers from WT, *fas1-4* and *fas1-4 hira-1* mutants.
(g) Representative WT and *fas1-4 hira-1* mutant anthers after Alexander staining, revealing absence of viable pollen in the double mutant.
(h) Quantification of seed content in WT, *hira-1*, *hira-1 ubn2-2*, *hira-1 ubn1-2* and *hira-1 cabin1-2* siliques. Quantifications were obtained from 30 pooled siliques from at least four plants. Red and black asterisks indicate comparisons with *hira-1* or WT, respectively.
(i) Quantitative RT-PCR analysis of TSI expression in WT and HIR complex single and double mutants. Red and black asterisks indicate comparisons with *hira-1* or WT, respectively.

mutants. Silencing at 106B and 180 bp repeats is not affected (Figure 6c,d). Furthermore, fluorescence *in situ* hybridization revealed that the global organization of 180 bp and TSI repetitive elements into chromocenters is not altered in *fas1-4 hira-1* nuclei (Figure S6b). Therefore, our data indicate that organization and silencing of repetitive sequences is largely maintained despite altered histone flow in the presence of CAF-1 and HIR complex mutations, suggesting that alternative silencing mechanisms counterbalance impaired nucleosome assembly in plants.

To investigate whether histone deposition mediated by the HIR complex rescues deficient replication-dependent nucleosome assembly in CAF-1 mutants, we assessed nucleosome occupancy by H3-ChIP combined with quantitative PCR on 4-week-old soil-grown *fas1-4 hira-1* plants. We found that nucleosome occupancy was reduced at all targets tested, including heterochromatic repeats, Ta3 and *Mule* transposons and an intergenic region, as well as at most analyzed regions of the euchromatic loci tested (Figure 6e–g). We conclude that, when CAF-1 and HIR-mediated assembly pathways are simultaneously impaired, plants fail to maintain nucleosome occupancy in both actively transcribed and transcriptionally repressed genomic regions. This suggested that deficient CAF-1-mediated incorporation of canonical histones could be rescued by alternative pathways, such as HIR complex-mediated assembly. Given the molecular and phenotypic defects caused by loss of both chaperone complexes, we conclude that Arabidopsis CAF-1 and HIR complexes are involved in independent pathways of chromatin assembly that concomitantly contribute to maintenance of nucleosome occupancy.

DISCUSSION

Proper packaging of DNA into chromatin is essential for genome structure, and ensures stability and inheritance of epigenetic information. A particular role in these processes may be assigned to factors responsible for histone deposition. Indeed, the various chromatin assembly factors, as well as other histone chaperones involved in histone transport and storage, are highly conserved through evolution.

Distinct contribution of the various members of the HIR complex

Various studies (Phelps-Durr *et al.*, 2005; Ingouff *et al.*, 2010; Nie *et al.*, 2014) have reported developmental defects ranging from WT appearance to distinctive leaf and flower phenotypes, suggesting that different growth conditions may play a role in the penetrance of *hira* mutant phenotypes. Although vegetative development was barely affected in single mutants for the various subunits under our growth conditions, we found defects in reproductive development in *hira-1* plants. Fewer viable seeds were also observed in the $hira^{amiRNA}$ line and in out-crosses of the *hira-1* mutant, suggesting that this defect is not caused by an epimutation. The simultaneous occurrence of fewer viable seeds, and an increased number of unfertilized ovules and aborted seeds in *hira* mutants, suggests a complex phenotype that is likely to have both gametophytic and sporophytic origin, and may result from alterations in the transcriptome (Nie *et al.*, 2014). Another intriguing hypothesis is the implication of HIRA in reprogramming of the histone variant repertoire (Ingouff *et al.*, 2007, 2010), as essentially only H3.3 and H3.3-like variants are present in sperm and egg cells (Ingouff *et al.*, 2010).

We revealed various levels of importance for the distinct subunits of the complex, with strongest defects observed in *hira-1* mutants. Given that HIRA comprises the conserved Hira domain and WD40 repeats involved in interactions with CABIN1 and UBN1 in mammals (Rai *et al.*, 2011; Tang *et al.*, 2012), and that Arabidopsis HIRA mediates the interaction with UBN2 and ASF1 (Nie *et al.*, 2014), its absence may destabilize the complex. This is the case in yeast, where loss of Hir1 or Hir2 reduces Hir3 protein levels (Song *et al.*, 2013), and in mammals, where HIRA depletion leads to a concomitant decrease in UBN1 and CABIN1 (Ray-Gallet *et al.*, 2011). However, our data provide evidence that the Arabidopsis HIR complex is at least still partly functional in the absence of UBN2 or CABIN1, and can sustain vegetative development in the absence of CAF-1. This reflects the situation in mammals, in which CABIN1 plays only a limited role in H3.3 deposition, followed by UBN1 and finally HIRA, which is crucial for H3.3

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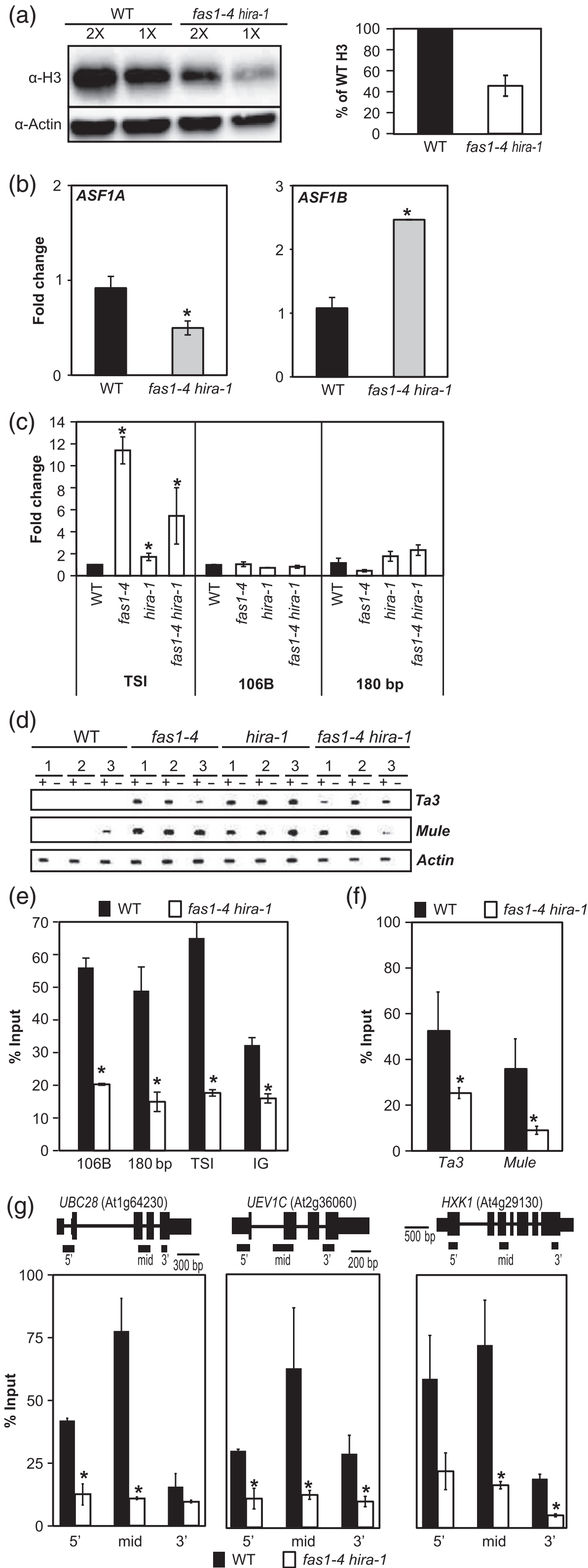

Figure 6. Molecular consequences of simultaneous mutation of CAF and HIR complexes.
(a) Left: Histone H3 protein levels quantified by Western blotting. Proteins extracted from WT and *fas1-4 hira-1* 4-week-old mutant shoots were loaded in each lane (2X, 20 μg; 1X, 10 μg). Right: Quantification of H3 band intensities relatively to actin from two independent experiments.
(b) Quantitative RT-PCR analysis of *ASF1A* and *ASF1B* expression in *fas1-4 hira-1* 4-week-old mutants.
(c) Quantitative RT-PCR analysis of TSI, 106B and 180 bp expression in WT, *fas1-4*, *hira-1* and *fas1-4 hira-1* 4-week-old plants.
(d) RT-PCR analysis of *Ta3* and *Mule* (At2g15810) transcript levels in WT, *fas1-4*, *hira-1* and *fas1-4 hira-1* 4-week-old plants. Three independent biological replicates were analyzed. *Actin* was used as a control.
(e–g) Histone H3 occupancy assessed by H3-ChIP combined with quantitative PCR relative to input in WT and *fas1-4 hira-1* 4-week-old mutant shoots at heterochromatic and intergenic regions (e,f) and at various positions along three active genes (g).

incorporation (Ray-Gallet *et al.*, 2011). Furthermore, transcriptional repression of yeast histone genes depends on Hir1 and Hir2, but only partly on Hir3 (Osley and Lycan, 1987; Spector *et al.*, 1997), and the *Drosophila* HIR complex does not comprise a CABIN1 ortholog (Amin *et al.*, 2011). An alternative but not exclusive hypothesis is that HIRA and other subunits of the complex also have HIR complex-independent functions. Indeed, genome-wide ChIP-Seq analysis in human cells localized HIRA to several chromosomal positions that are not co-occupied by UBN1 or ASF1 (Pchelintsev *et al.*, 2013), and roles as transcriptional co-repressors have been demonstrated for UBN1 and CABIN1 (Aho *et al.*, 2000; Jang *et al.*, 2007). We expect that specific functions of the various Arabidopsis HIR complex subunits may be revealed only under certain growth and environmental conditions, or in particular cell types.

Role of HIRA in nucleosome dynamics

Loss of HIRA or FAS1 subunits results in reduced levels of an extractable histone pool. In comparison to methods developed for mammalian cell cultures (Loyola *et al.*, 2006), this fraction of extractable histones probably presents a combination of the cytosolic and nucleoplasmic pool of histones, while nucleosomal histones were precipitated during the centrifugation step. Therefore, the reduced levels of this histone pool in *fas1-4* and *hira-1* mutants suggest that absence of histone deposition complexes affects histone flow, and may render prone to degradation those histones that are not readily assembled into chromatin. This alteration in the extractable histone pool is then reflected at the level of nucleosomal H3 occupancy. Indeed, by analyzing H3 enrichment in a locus-specific manner, we found that loss of HIRA affects nucleosome occupancy at certain active genes, specifically within the body and 3′ ends, regions that were previously shown to be enriched in H3.3 (Stroud *et al.*, 2012; Wollmann *et al.*, 2012). This nucleosome loss at active genes may be explained by failure to restore nucleosomes using H3.3 after passage of the transcriptional machinery. Interestingly, similar to CAF-1, HIRA also affects H3 occupancy at heterochromatic targets. Despite heterochromatin being not (or very poorly) transcribed, and generally considered to have slow histone

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exchange rates, the presence of H3.3 has been reported in centric and pericentric heterochromatin and telomeres in mouse and human (Wong *et al*., 2009; Drané *et al*., 2010; Goldberg *et al*., 2010; Lewis *et al*., 2010; Dunleavy *et al*., 2011; Morozov *et al*., 2012). It is therefore possible that HIRA is implicated in histone deposition at heterochromatic sites, a process that may be particularly important in cells that undergo neither replication nor endoreplication. Such a requirement for the HIR complex may explain the transcriptional reactivation of endogenous pericentromeric repeats and transposons observed in *hira-1* mutants, and implies that nucleosome occupancy maintenance contributes to transcriptional gene silencing. Interestingly, HIRA orthologs in fission yeast are also involved in transcriptional silencing at pericentromeric heterochromatin (Blackwell and Martin, 2004; Yamane *et al*., 2011). Further studies that take the cellular properties of various tissues and developmental variations into account should shed further light on cell-specific roles of the HIR complex.

Several lines of evidence have linked HIRA and deposition of histone replacement variants to transcription control (Ahmad and Henikoff, 2002; Formosa *et al*., 2002; Sakai *et al*., 2009; Ray-Gallet *et al*., 2011; Schneiderman *et al*., 2012). However, when we analyzed the capacity of HIRA-deficient plants to activate or repress gene transcription in response to salt stress, we found no difference compared to WT, either for genes with basal expression levels or for genes that were silent before induction. To obtain a more general view regarding the role of HIRA in the transcriptional response to environmental stimuli, additional salt-responsive genes should be analyzed and different abiotic stresses considered in the future. Nevertheless, based on the available data, we speculate that either HIR-mediated histone dynamics or H3.3 incorporation during transcription are dispensable for proper expression at these sites. The possibility that H3.3 incorporation is dispensable is in agreement with observations from both *Tetrahymena* and *Drosophila*, which survive and show correct gene expression in the absence of H3.3 (Cui *et al*., 2006; Hödl and Basler, 2009), and the lack of strong phenotypes in *hira-1*. It is likely that alternative histone chaperone complexes are also implicated in deposition of replacement variants. While no DAXX ortholog has been identified in plants (Zhu *et al*., 2013), Arabidopsis encodes several DEK proteins (Waidmann *et al*., 2014), and other as yet unidentified histone chaperones may play a role in histone variant dynamics (Otero *et al*., 2014).

Functional redundancy of CAF-1 and HIR complexes in histone deposition?

The combination of the *fas2-5* and the *hira-1* mutations causes post-germination lethality, while *fas1-4 hira-1* mutants are viable but sterile. The survival of *fas1-4 hira-1* mutants may be explained by residual CAF-1 activity, concomitant with the slow growth and potentially the premature switch to endoreplication in *fas* mutants (Ramirez-Parra and Gutierrez, 2007). In addition, the shuttling of histones primarily to the CAF-1 complex by changing the ASF1A/B balance may help to sustain sufficient nucleosome assembly to permit plant survival. Maintenance of heterochromatin in the absence of CAF-1 function in yeast requires the HIR complex (Osley and Lycan, 1987; Kaufman *et al*., 1998). However, despite a reduction in H3 occupancy in the *fas1-4 hira-1* double mutant, silencing of an endogenous repeat sequence and certain transposons as well as heterochromatin organization is not further affected compared to the single mutants. This is surprising, as appropriate nucleosome assembly is not only required for DNA packaging but is also important for propagating epigenetic information. We interpret this finding as due to the presence of alternative mechanisms required for gene silencing and heterochromatin maintenance, such as DNA methylation, which is of primary importance in plants in directing histone modifications and silencing (Rigal and Mathieu, 2011), and which is unaffected in *fas* mutants (Schönrock *et al*., 2006).

The nucleosomal loss and accentuated phenotypic defects in *fas1-4 hira-1* double mutants, together with the viability of the respective single mutants, suggest partial functional redundancy between the histone deposition complexes. Hence, we speculate that the Arabidopsis HIR complex can at least partially rescue a deficiency in replication-coupled histone assembly, potentially by nucleosomal gap-filling mechanisms. Such a mechanism has been proposed in mammals, where, in the absence of functional CAF-1, the HIR complex recognizes naked DNA stretches remaining after replication via its DNA-binding properties, and fills the gaps through H3 deposition (Ray-Gallet *et al*., 2011). Whether this mechanism occurs in plants remains to be determined. Given the moderate nucleosomal reduction observed in *hira-1* mutants, we envisage the occurrence of histone deposition by other complexes dedicated to replacement variant assembly or even H3.3 incorporation by the CAF-1 complex. Indeed, in DAXX- or HIRA-depleted mammalian cells, CAF-1 co-purifies with H3.3 (Drané *et al*., 2010; Lewis *et al*., 2010).

In conclusion, several lines of evidence support the notion that the Arabidopsis HIR complex functions as a histone chaperone: loss of HIRA reduces the pool of extractable H3 histones, affects nucleosome occupancy at both active and repressed chromosomal regions, and alleviates transcriptional silencing. In addition, synthetic lethality is observed when the *hira* mutant is combined with complete loss-of-function *fas* alleles. Taken together, our results imply an evolutionarily conserved mode of action of these two plant histone H3 assembly complexes. Indeed, the presence of Arabidopsis H3.1 and H3.3 variants at

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transcriptionally repressed or active genomic regions, respectively, is similar to *Drosophila* and mammals (Mito *et al*., 2005; Goldberg *et al*., 2010; Stroud *et al*., 2012; Wollmann *et al*., 2012; Shu *et al*., 2014). This observation was unexpected, given that the evolution of functionally divergent H3 variants is thought to have occurred independently in plant and animal kingdoms. However, the highly conserved function of plant histone chaperone complexes suggests that histone chaperones may have contributed to functional diversification of the histones they transport and deposit.

EXPERIMENTAL PROCEDURES

Plant material

Mutant Arabidopsis lines were obtained from the Nottingham Arabidopsis Stock Center (http://arabidopsis.info/) and/or were gifts from other laboratories. Homozygous mutants *fas1-4* (SAIL-662-D10) (Kirik *et al*., 2006), *fas2-5* (SALK_147693), *hira-1* (WiscDsLox362H05) (Ingouff *et al*., 2010), *ubn2-2* (GABI_018D02), *ubn1-2* (GABI_130H01), *cabin1-2* (SALK_099927), SALK_019573 (Phelps-Durr *et al*., 2005), GABI_775H03 and SALK_143806 (Phelps-Durr *et al*., 2005) were identified by PCR-based genotyping (Table S2, Data S1). All mutants are in the Columbia background. Plants were grown on soil in a growth chamber under 16 h light/8 h dark cycles at 22°C. For *in vitro* culture, seeds were sterilized and sown on germination medium containing 0.8% w/v agar, 1% w/v sucrose and Murashige & Skoog salts (M0255; Duchefa Biochemie; www.duchefa-biochemie.com). After 2 days of stratification at 4°C at dark, plants were grown under 16 h light/8 h dark cycles at 23°C.

Salt treatment

Plantlets grown for 10 days *in vitro* were transferred into liquid MS medium containing 200 mM NaCl. Samples were shock-frozen in liquid nitrogen before treatment and after 1 h of salt exposure.

RNA extraction and RT-PCR

RNA was extracted using Tri-Reagent (Euromedex; www.euromedex.com) according to the manufacturer's instructions, treated with RQ1 DNase I (Promega; www.promega.com), and purified using phenol/chloroform extraction. Reverse transcription was performed using either oligo$(dT)_{15}$ or random hexamers and M-MLV reverse transcriptase (Promega). The resulting cDNAs were used in standard PCR (Promega Flexi) or in quantitative PCR using the LightCycler® 480 SYBR Green I master kit on the Roche LightCycler® 480 (lifescience.roche.com). Transcript levels of interest were normalized to those for At2g28390 (Czechowski *et al*., 2005) using the comparative threshold cycle method. Quantitative RT-PCR histograms show means of transcript levels ± SEM obtained for two independent PCR amplifications of three biological replicates. The *y* axis shows the fold change relative to WT (WT set to 1) after normalization to expression of At2g28390.

Statistical analysis

Student's *t* test was used for mean comparison for quantitative PCR analyses and seed counting: Asterisks in the figures indicate statistically significant differences (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

Ploidy analysis

Nuclei were prepared using a modified version of the original Galbraith method (Galbraith *et al*., 1983). For ploidy analysis of each genotype, two independent preparations of pooled shoot material from 15 plants were investigated. Cytometric analysis was performed using an Attune® acoustic focusing cytometer (Thermo Fisher Scientific; www.lifetechnologies.com). For statistical analysis of differences in ploidy levels, we performed a Shapiro–Wilk test, followed by a Bartlett's test, and finally Tukey's HSD (honest significant difference) test was applied in conjunction with ANOVA to find means that were significantly different (indicated by an asterisk).

ChIP analysis

Chromatin of plantlets was formaldehyde cross-linked, and chromatin immunoprecipitation was performed as previously described (Bowler *et al*., 2004) with minor modifications. Chromatin was sheared using a Diagenode bioruptor (ten cycles of 30 sec on and 1.5 min off). Protein A-coupled magnetic beads (Diagenode; www.diagenode.com) or Protein A-coupled Dynabeads (Invitrogen) were used, and the sonicated chromatin was pre-cleared in presence of magnetic beads for 3 h, before immunoprecipitation with anti-H3 antibody (Abcam, ab1791; www.abcam.com). DNA was quantified using quantitative PCR (Roche) and normalized relative to input.

Protein extraction and western blotting

Proteins were extracted from 100 mg of plantlet material as described previously (Durut *et al*., 2014). Western blots were probed using anti-H3 antibody (Abcam, ab1791, 1/5000). Equal loading was confirmed using anti-actin antibody (1/1000; Sigma; www.sigmaaldrich.com) and Ponceau staining. Primary antibodies were revealed by incubation with anti-rabbit (1/25 000; Sigma) or anti-mouse (1/5000; Sigma) secondary antibodies. Immunoblot analysis was performed using ECL Western blotting detection reagents (GE Healthcare Bio-Sciences; www.gelifesciences.com). Densitometric analysis of immunoreactive protein bands was performed on non-saturated signals using MultiGauge software (Fujifilm; www.fujifilm.com), and H3 levels normalized to actin in WT were set to 100%.

ACKNOWLEDGEMENTS

C.D., S.L.G. and A.V.P. were supported by Agence Nationale de la Recherche grant 'Dynam'Het' ANR-11 JSV2 009 01, and S.C. and A.V.P. were supported by Agence Nationale de la Recherche grant 'SINODYN' ANR-12 ISV6 0001. M.B., A.P. and L.S. are supported by stipends from the Region Auvergne and M.B. is supported by an Association pour la Recherche sur le Cancer doctoral fellowship. This work was supported by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Université Blaise Pascal and the Université d'Auvergne. We thank M.-C. Espagnol and L. Borao for technical assistance, S. Tourmente for stimulating discussion, and O. Mittelsten Scheid and J. Bassler (Gregor Mendel Institute, Vienna) for critical comments on the manuscript.

SUPPORTING INFORMATION

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Figure S1. Evolutionary conservation and expression of the HIR complex subunits.

Figure S2. Characterization of HIR and CAF-1 complex mutants.

Figure S3. Enrichment in H3.1 and H3.3 at targets analyzed by H3-ChIP combined with quantitative PCR.

Figure S4. Analysis of transcriptional silencing release in CAF-1 and HIR complex mutants.

Figure S5. Genetic interactions between members of the CAF-1 and HIR complexes.

Figure S6. Generation and study of *fas1-4 hira-1* double mutant material.

Table S1. *fas hira-1* double mutants are under-represented in segregating F_2 populations.

Table S2. List of primers used in the study.

Data S1. Supplementary experimental procedures.

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