Leaf chlorosis in Arabidopsis thaliana hybrids is associated with transgenerational decline and imbalanced ribosome number

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Summary

• The interaction of two parental genomes can result in negative outcomes in offspring, also known as hybrid incompatibility. We have previously reported a case in which two recessively interacting alleles result in hybrid chlorosis in Arabidopsis thaliana. A DEAD-box RNA helicase 18 (AtRH18) was identified to be necessary for chlorosis.

• In this study, we use a sophisticated genetic approach to investigate genes underlying hybrid chlorosis. Sequence comparisons, DNA methylation inhibitor drug treatment and segregation analysis were used to investigate the epigenetic regulation of hybrid chlorosis. Relative rRNA numbers were quantified using real-time quantitative PCR.

• We confirmed the causality of AtRH18 and provided evidence for the involvement of the promoter region of AtRH18 in the hybrid chlorosis. Furthermore, AtMOM1 from the second parent was identified as the likely candidate gene on chromosome 1. Chlorotic hybrids displayed transgenerational decline in chlorosis, and DNA demethylation experiment restored chlorophyll levels in chlorotic hybrids. Quantification of rRNA indicated that hybrid chlorosis was associated with an imbalance in the ratio of cytosolic and plastid ribosomes.

• Our findings highlight that the epigenetic regulation of AtRH18 causes hybrid breakdown and provide novel information about the role of AtRH18 in plant development.

Introduction

Offspring commonly resemble their parents but sometimes hybridisation can result in phenotypes that differ from the parents. These hybrid-specific phenotypes can be beneficial, neutral or deleterious in comparison to only one or both parents, and often depend on the environmental conditions (Chen et al., 2016; Vaid & Laitinen, 2019). A phenotype that is beneficial or neutral in one condition can be deleterious in another. While beneficial hybrid phenotypes often increase fitness and can be under positive selection, deleterious hybrid phenotypes can prevent hybridisation resulting in reproductive isolation and ultimately speciation (Coyne & Orr, 2004; Abbott et al., 2013). Therefore, knowledge of the genetic and molecular mechanisms underlying hybrid-specific phenotypes will help to understand the impact of genome interactions in population diversification.

Reduced fitness in hybrids, known as hybrid incompatibility, has been reported in many plant species (Bombliès & Weigel, 2007; Chen et al., 2016). The majority of genes causing hybrid incompatibilities reported so far are dominant, but incompatibilities can also be due to recessive genes and only become visible in the F2 or later generations. This phenomenon, known as hybrid breakdown, is common in many plant species including Arabidopsis, rice, wheat and rye (Mizuta et al., 2010; Matsubara et al., 2014; Okada et al., 2017; Tikhenko et al., 2017; Alhajturki et al., 2018). According to the Bateson–Dobzhansky–Müller (BDM) model, hybrid incompatibility could occur between two interacting genes that evolve independently in the parental populations. The novel interaction of a combination of different parental alleles in hybrids can result in a conflict (Bateson, 1909; Dobzhansky, 1937; Muller, 1942).

In Arabidopsis thaliana, it has been shown that due to the constant selection pressure on disease resistance loci they are likely to gather mutations, which can cause activation of an immune system response when interacting in hybrids (Bombliès & Weigel, 2007; Fishman & Sweigart, 2018) and result in hybrid necrosis (Bombliès et al., 2007; Alcázar et al., 2009, 2010, 2014; Chae et al., 2014, 2016; Todesco et al., 2014). Hybrid necrosis is the best-characterised type of post-zygotic hybrid incompatibility in A. thaliana but other mechanisms also exist (Vaid & Laitinen, 2019). We have previously reported a case of hybrid chlorosis in the F2 generation of crosses between Arabidopsis thaliana accessions Shah-dara (Sha) and Lovvik-5 (Lov-5) (Plötner et al., 2017). The phenotype is associated with severe leaf chlorosis, accompanied by a reduction in total Chl content, photosynthetic efficiency and seed yield. Similar phenotypes are typical for several A. thaliana mutants that have impaired photosynthetic or chloroplast metabolic machinery (Jeong et al., 2008; Lohscheider et al., 2010).
Hybrid chlorosis has been reported in several plant species such as wheat, rice and mimulus (Mori & Tsunewaki, 1992; Ichitani et al., 2012; Nakano et al., 2015; Zueilig & Sweigart, 2018). The causal genes underlying wheat and rice hybrid chlorosis are not known, but the white seedling phenotype in mimulus hybrids was found to be associated with an essential photosynthesis gene, *Plastid Transcriptionally Active Chromosome 14* (Zueilig & Sweigart, 2018). In the *A. thaliana* hybrids Sha and Lov-5, chlorosis was linked to two loci, one on chromosome (chr) 1 and the other on chr 5 (Plötner et al., 2017). From these, on chr 5 the *At5g05450* gene encoding a DEAD-box RNA HELICASE 18 (*AtRH18*) protein was demonstrated to be necessary for hybrid chlorosis (Plötner et al., 2017). However, we have not yet confirmed the causal role of *AtRH18*. *AtRH18* has not been functionally characterised in plants, but its yeast homologue *Spb4p* is involved in maturation of cytosolic 25S rRNA and hence is a crucial member of the ribosome biogenesis pathway (de la Cruz et al., 1998). The homozygous gene insertion mutants of *AtRH18* are embryo lethal, implying its essential role in *A. thaliana* as well (Plötner et al., 2017).

In addition to genetic mechanisms, epigenetic mechanisms in interspecific plant hybrids can cause conflicts in expression of maternal or paternal genes in seed endosperm and result in incompatibilities (Josefsson et al., 2006; Jullien & Berger, 2009, 2010; Michalak, 2009; Lafon-Placette & Köhler, 2015). Although less numerous, intraspecific *A. thaliana* hybrids have also provided some examples of epigenetically regulated incompatibility phenotypes. For example, transcriptional silencing of the t-RNA deaminase (*TAD-3*) gene in hybrids of *A. thaliana* accessions Columbia-0 (Col-0) and Noordwijker-1 (Nok-1) causes embryo lethality (Agorio et al., 2017). Similarly, a folate transporter (*FOLT*) epiallele in Sha is associated with diminished viability of Sha × Col-0 hybrids. In yet another example, a duplicated copy of a histidine biosynthesis gene in Col-0 is epigenetically silenced, while only the corresponding copy is active in Cape Verde Island-0 (Cvi-0) (Bikard et al., 2009; Blevins et al., 2017). The Col-0 × Cvi-0 F₂ hybrids that inherit silenced copies of the histidine biosynthesis gene from both the parents show lethality (Bikard et al., 2009).

Here, we continued to investigate the mechanisms underlying F₂ hybrid chlorosis in the Sha × Lov-5 cross. We confirmed the causality of the recessive allele of Lov-5 *AtRH18* in hybrid chlorosis. Furthermore, we demonstrated that the chlorosis phenotype is associated with a reduced number of plastid ribosomes relative to cytosolic ribosomes, suggesting a role for *AtRH18* in plastid ribosome biogenesis. Additionally, the generational decline of the phenotype implied that an epigenetic mechanism underlies the hybrid chlorosis. Our findings highlight that the epigenetic regulation of two recessively interacting alleles causes hybrid breakdown and provide novel information about the role of *RH18* in plant development.

Materials and Methods

Plant material, growth conditions and phenotyping

The Arabidopsis accessions used in this study are listed in Supporting Information Table S1. All crosses done in this study and their phenotypes are listed in Table S2. In all experiments, plants were first stratified for 3 d in 0.1% agar (w/v in water) at 4°C in the dark, sown on soil and transferred to a growth chamber with long-day conditions (16 h : 8 h, 21°C: 17°C, day : night) at 180 (µmol m⁻² s⁻¹) light intensity, or in a glasshouse at 21°C : 17°C (day : night) with variable light intensities. Similar to Plötner et al. (2017), the yellowing of young leaves was adjudged as chlorosis. For determination of the stability of the chlorosis phenotype, 5-d-old seedlings of F₂ and F₄ generations were transplanted and grown in a glasshouse. The plants were observed for the chlorosis phenotype from the three-leaf stage to 15-leaf stage. Northern Swedish and southern Swedish individuals were ver- nalised at 4°C for 8 and 4 wk, respectively, and then moved to long-day conditions for flowering. For the crosses of northern and southern Swedish accessions, Sha was used as the pollen donor.

Generation and selection of transgenic plants

For identification of causal genes for the chlorosis phenotype, we amplified the genomic region of each candidate gene comprising promoter, coding and noncoding regions of the gene from the genomic DNA of the respective accession. DNA was isolated using CTAB reagent as described by Doyle & Doyle (1987). The amplified fragments were cloned using blunt-ended restriction enzyme Eco53Kl in pCambia1305 binary vector. Primers used for amplification are listed in Table S3. The strategy used for demonstrating the causality of the candidate genes is presented in Fig. S1. To study the *RH18* promoters from different accessions, the 1.5 kb region upstream of the start codon was amplified from genomic DNA of respective accessions and cloned in gateway donor. For plant transformation, *Agrobacterium tumefaciens* GV3101 containing the binary vectors were transfected in *Agrobacterium* donor.

GUS assay

To investigate the activity of *AtRH18*, promoters of Sha and Lov-5 accessions were fused with β-glucuronidase (GUS) reporter gene and transformed in Sha, Lov-5 and chlorotic F₂ hybrids. For each transformation, GUS activity assay was performed on five seedlings from four independent T₃ lines. Briefly, 10-d-old seedlings were first stratified for 3 d in 0.1% agar (w/v in water) at 4°C in the dark, sown on soil and transferred to a growth chamber with long-day conditions (16 h : 8 h, 21°C: 17°C, day : night) at 180 (µmol m⁻² s⁻¹) light intensity, or in a glasshouse at 21°C : 17°C (day : night) with variable light intensities. Similar to Plötner et al. (2017), the yellowing of young leaves was adjudged as chlorosis. For determination of the stability of the chlorosis phenotype, 5-d-old seedlings of F₂ and F₄ generations were transplanted and grown in a glasshouse. The plants were observed for the chlorosis phenotype from the three-leaf stage to 15-leaf stage. Northern Swedish and southern Swedish individuals were ver- nalised at 4°C for 8 and 4 wk, respectively, and then moved to long-day conditions for flowering. For the crosses of northern and southern Swedish accessions, Sha was used as the pollen donor.
seedlings grown in half-strength MS medium were harvested and fixed in 90% acetonitrile for 1 h, and then submerged in 1 ml GUS buffer (5 mg ml⁻¹ X-Gluc in DMSO, 20 mM sodium phosphate buffer (pH 7), 0.1% Triton X-100) for 16 h, followed by destaining in 95% ethanol for 15 min at 70°C.

Ribosomal RNA analysis

To quantify the cytosolic, plastid and mitochondrial ribosomal RNA (rRNA) numbers, parents (Sha and Lov-5), the F1 hybrid and a minimum of three independent chlorotic and nonchlorotic F2 hybrids were grown on soil under long-day conditions (16 h : 8 h, 21°C : 17°C, day : night at 180 µmol m⁻² s⁻¹ light intensity). For each genotype, at the six- to eight-leaf stage, five pools, each containing the three youngest leaves from 10 plants, were sampled, flash frozen and ground in liquid nitrogen. Total RNA was extracted from 200 µg of powdered samples using a Qiagen RNeasy Plant mini kit (Qiagen) as described previously (Ishihara et al., 2017). Spike RNA with eight artificial RNA fragments (ArrayControl RNA Spikes; Thermo Fisher Scientific, Waltham, MA, USA) was added to the RNA at the beginning of the extraction. The total RNA from the spiked samples was reverse-transcribed with a mixture of oligo(dT) and random hexamers using an Invitrogen Superscript III kit as described (Ishihara et al., 2017). Cytosolic, plastid and mitochondrial rRNAs were determined by reverse transcriptase quantitative PCR (RT-qPCR) (Ishihara et al., 2017).

Zebularine drug treatment and analysis

The sterilised seeds of chlorotic and nonchlorotic F3 hybrids were grown on MS media containing either control solution (final concentration 0.025% DMSO) or 20 mM zebularine (with 0.025% DMSO carrier) for 15 d. For both chlorotic and nonchlorotic F3 hybrids, a minimum of three independent lines, each with 30 seeds, were used for experiments. The treated seedlings were then transferred to soil and observed for 7 d for the appearance of the chlorosis phenotype. On the seventh day, the three youngest leaves from each transferred plant were sampled for chlorophyll (Chl) measurements and RT-qPCR analysis. For Chl estimation, the ground samples were weighed and then extracted in 96% methanol for 14 h. The extracts were analysed spectrophotometrically at 645 and 663 nm and total Chl content was normalised against the fresh weight as described by Cross et al. (2006). For RT-qPCR analysis of AtRH18 expression, RNA was isolated using a Qiagen RNeasy Plant mini kit (Qiagen) as per the manufacturer’s instructions. RNA (1 µg) was reverse transcribed using oligo(dT) and the Invitrogen Superscript III kit as per the manufacturer’s instructions. RT-qPCR was performed using SYBR Green chemistry (Thermo Fisher Scientific) and the primers listed in Table S3.

Databases and statistical analysis

Gene polymorphisms and the methylation patterns were analysed using the online platforms Salk Arabidopsis 1001 genomes Genome Browser (http://signal.salk.edu/atg1001/3.0/gebrowser. php) and Anno-J Networked Genome Browser (http://neo morph.salk.edu/1001.aj.php). The coexpression network for the RH18 gene was built in ATTED-II (http://atted.jp/). RNA-seq profiles of transcripts were retrieved from the Transcriptome Variation Analysis database (http://travadb.org/; Klepikova et al., 2016). Statistical analysis of the data was performed using R v.3.4.1. The geographical location of Swedish individuals was mapped using ‘MAPVIEW’ and ‘LEAFLET’ libraries in R.

Results

Lov-5 allele of RH18 is causal for hybrid chlorosis

We have previously reported that the chlorosis phenotype segregating in the F2 generation of the Sha and Lov-5 cross is linked to two genomic loci, one on chr 1 and the other on chr 5 (Plönter et al., 2017). On chr 5, At5g05450 from the Lov-5 parent, encoding a DEAD-box RNA HELICASE 18 (AtRH18) protein, was found to be necessary for the hybrid chlorosis phenotype (Plönter et al., 2017). Here we further investigated the causality of the Lov-5 AtRH18 gene (RH18Lov-5) in chlorosis. Because the causal allele acts recessively, and its homozygous mutants are embryo lethal, we used the following strategy. We transformed both RH18Lov-5 and RH18Sha alleles into the F1 hybrid and screened the transgenic F2 generation for the chlorosis phenotype (Fig. S1). We reasoned that if RH18Lov-5 is causal for the phenotype, then the introduction of an additional copy of the nonchlorosis-causing RH18Sha to the F1 hybrid should reduce the proportion of chlorotic hybrids segregating in the F2 generation (Fig. S1). By contrast, introduction of the chlorosis-causing RH18Lov-5 allele should not affect the number of chlorotic hybrids in the F2 generation (Fig. S1). For the RH18Sha allele, we obtained 111 transgenic F2 plants which we further genotyped for the chr 1 and chr 5 loci (Table S4). We found that four of the transgenic plants had the homozygous genotypes of Sha on the chr 1 locus and Lov-5 on the chr 5 locus, known to be linked to chlorosis in nontransgenic F2 hybrids (Table S4). Interestingly, none of these four plants showed the chlorosis phenotype (Table S4; Fig. 1a), confirming that the additional RH18Sha copy abolished the chlorosis phenotype in these hybrids. In addition, from the 106 independent F2 plants carrying the additional RH18Lov-5 allele, five transgenic plants had the genotype that was linked to chlorosis and all were chlorotic (Table S4; Fig. 1a), confirming that only the RH18Sha allele is able to abolish the chlorosis phenotype, indicating a causal role of RH18Lov-5 in hybrid chlorosis.

Next, we investigated if the RH18 expression pattern could underlie the chlorosis phenotype. For this, we transformed two different promoters of RH18 (pRH18, pRH18Lov-5 and pRH18Sha) driving the expression of the beta-glucuronidase (GUS) reporter gene in Sha, Lov-5 and three chlorotic F3 lines. For all genotypes, we harvested five seedlings from four independent transgenic lines for GUS staining. GUS staining revealed that while pRH18Lov-5 drove high expression in Lov-5, its activity was highly diminished in both Sha and the chlorotic F3 hybrids.
By contrast, pRH18Sha displayed less GUS activity in Sha and F3 hybrids in comparison to the Lov-5 parental background (Fig. 1b). These results indicate that there is interference in RH18Lov-5 expression by the Sha background and that the region of interference, at least partially, resides in the promoter region of RH18Lov-5.

Chlorotic hybrids showed decreased accumulation of chloroplast ribosomes in relation to cytosolic ribosomes

A coexpression network of AtRH18 identified several genes involved in ribosome biogenesis pathways (Fig. S2). Yet, in our previous study, we did not find evidence for the involvement of AtRH18 in cytosolic ribosome biogenesis (Plötner et al., 2017). To further examine the involvement of AtRH18 in ribosome biogenesis, we quantified the small (16S and 18S) and large subunits (23/25/26S) of plastid (rRNAPla), cytosolic (rRNACy) and mitochondrial (rRNAMit) tRNA numbers in the Sha and Lov-5 parents, in the F1 hybrid, and in the chlorotic and nonchlorotic F3 hybrids using RT-qPCR (Figs 2, S3). The tRNA number can be used as a proxy for ribosome number. Quantification of the tRNA number was achieved by spiking eight different concentrations of artificial RNA molecules into the sample at the beginning of the RNA isolation (Ishihara et al., 2017). To make the differences between the genotypes more evident, we normalised the tRNA numbers for each genotype relative to the tRNA number...
in Sha (Fig. 2a). The un-normalised data with statistical significance are presented in Fig. S3. Our analysis revealed that the chlorotic hybrids had significantly less 16S plastid rRNA than the Lov-5 parent while the cytosolic and mitochondrial rRNAs did not show significant differences between the genotypes (Figs 2a, S3a,b). Lov-5 also accumulated higher but nonsignificant amounts of rRNACyt, rRNAPla and rRNAMit compared to Sha (Figs 2a, S3a,b). rRNA abundances in the F1 hybrid were similar to Sha, indicating a dominant inheritance of the number of ribosomes from Sha. rRNA abundances in the nonchlorotic F3 hybrid were intermediate to Sha and Lov-5 (Figs 2a, S3a). In the chlorotic F3 hybrids, while the amount of rRNAPla and rRNAMit were higher than in Sha and the F1 and similar to the Lov-5 parent, rRNAPla had similar abundances to Sha (Figs 2a, S3a). This implies that while the chlorotic F3 hybrids seem to have the recessively inherited number of cytosolic and mitochondrial ribosomes from the Lov-5 parent, a factor from the Sha parent specifically influenced the abundance of rRNAPla. Next, we investigated if the reduced 16S plastid rRNA number in chlorotic hybrids resulted in an imbalance in the ratio between cytosolic, mitochondrial and plastid rRNA numbers. Interestingly, the chlorotic F3 hybrids had significantly higher ratios only between cytosolic 18S or 25S and 16S plastid rRNA numbers in comparison to all other nonchlorotic genotypes (Figs 2b, S3c). The reduced rRNAPla number further had a significant positive correlation with the reduced Chl content (Fig. 2c). These results indicate that chlorotic hybrids are not able to accumulate as much rRNAPla as Lov-5, resulting in an imbalance in cytosolic and plastid rRNA numbers. However, we cannot conclude from these experiments whether these rRNA perturbations are the cause or consequence of the chlorosis phenotype.

Chlorosis-causing allele of RH18 lacks unique polymorphisms

Next, to investigate the role of AtRH18 in causing hybrid chlorosis, we compared the sequences of AtRH18 alleles from Sha and Lov-5. While the promoter region comparison of Sha and Lov-5
sequences revealed several polymorphisms, only one polymorphism was found in the coding region (Plötner et al., 2017). A more detailed analysis of the promoter region revealed that it contains a transposable element (TE; At5TE05820) and that several parts of the promoter were deleted in the Lov-5 accession in comparison to Sha (Fig. S4). Plötner et al. (2017) showed that several A. thaliana accessions carried the Sha-like allele on chr 1, but theLov-5 chr 5 allele was not found in global A. thaliana accessions. Nevertheless, the chr 5 chlorosis-causing allele was very common among accessions collected in northern Sweden from where Lov-5 originates (Plötner et al., 2017; Fig. S5; Table S5). To determine if the chlorosis was linked to the promoter region polymorphisms, we sequenced and compared the AtRH18 promoters of eight northern Swedish individuals. The promoters of both chlorosis-causing and nonchlorosis-causing individuals had high sequence similarity with each other for the AtRH18 locus (Fig. S6) and the few polymorphisms observed within the individuals did not explain the chlorosis phenotype. The polymorphism in the coding region of AtRH18 was in the Q-motif region of the helicase, which is involved in substrate binding and ATP hydrolysis (Cordin et al., 2006). However, this polymorphism was also found in several northern Swedish individuals that did not display the hybrid chlorosis phenotype with Sha and therefore it does not seem to be linked to chlorosis. These analyses suggest that the chlorosis phenotype is not directly caused by differences in the DNA sequences, raising the possibility that the variation is present at the epigenetic level.

Finally, we investigated if the chlorosis-causing RH18 allele is exclusive only to the Lov-5 region of origin. To identify this, we crossed 140 southern Swedish accessions with Sha and screened the F2 hybrids for chlorosis (Table S5). From these, on average 28.5% F2 hybrids showed chlorosis, indicating that the chlorosis-causing allele is frequently present in A. thaliana in Sweden, and it is not restricted to northern Sweden.

The chr 1 locus was narrowed down to two candidate genes, Sha MOM1 and Sha CHX6A

Sequence comparison of the chr 1 locus (2.49–2.58 Mb) from the Sha accession with the 1135 fully sequenced accessions...
(1001genomes.org) revealed 18 accessions with high sequence similarity (Fig. 3a,b; Table S2). These accessions were crossed with Lov-5 and 96 F2 plants from each cross were phenotyped for chlorosis. In addition to three previously identified accessions, Sijak-1 (Sij-1), Sijak-2 (Sij-2) and Shiguljovsk-2 (Shigu-2) (Plötner et al., 2017), three additional accessions, Westkar-4, Neo-6 and Novojegorjevskoje-2 (Noveg-2), displayed F2 hybrid chlorosis when crossed to Lov-5 (Fig. 3a,b; Table S2). Based on sequence comparisons of these accessions, two of the seven candidate genes (Plötner et al., 2017), At1g08060 encoding for MORPHEUS MOLECULE 1 (MOM1) and At1g08140 encoding for a putative Na+/H+ antiporter (CHX6A), shared single nucleotide polymorphisms (SNPs) with Sha and the six other chlorosis-causing accessions, but not with Lov-5 (Fig. 3b). The shared SNP in the MOM1 gene of chlorosis-causing accessions is chr1:2506786 G→C, resulting in a T900R mutation in the protein product. T900 is not located in a known functional region (Nishimura et al., 2012). The SNP in CHX6A common to chlorosis-causing accessions is chr1:2552246 T→C, resulting in a Y806C mutation in the CHX6A protein. This mutation is the hydrophilic carboxyl-terminal domain, which is proposed to have regulatory functions in CHX family proteins (Sze et al., 2004). CHX6A has only been shown to be expressed in pollen (Sze et al., 2004) while MOM1 is expressed throughout development including embryos, cotyledons and leaves (Schmid et al., 2005), leaves also being the tissue showing phenotypes in the chlorotic hybrids. This was confirmed by publicly available RNA-sequencing expression profile data for MOM1 and CHX6A (Table S6). We further analysed expression of MOM1 and CHX6A in leaf tissue at different developmental stages and only MOM1 was expressed in parents and hybrids (Fig. S7). Therefore, we investigated MOM1 further as the most likely candidate gene for chr 1.

To confirm causality, we introduced the genomic constructs of MOM1<sub>Lov-5</sub> and MOM1<sub>Sha</sub> into the F1 hybrid. As for RH18 on chr5 (Fig. S1), introduction of the nonchlorosis-causing MOM1<sub>Lov-5</sub> allele to the F1 hybrid should reduce the number of chlorotic plants segregating in the F2 generation. After transformation, we genotyped the F2 plants for the transgene and for the chlorosis-causing genomic combination. In the case of MOM1<sub>Lov-5</sub>, five out of the 160 independent F2 plants carrying the transgene had the chlorosis-causing genetic combination, and from these, three had reverted the chlorosis phenotype (Fig. 3c). Partial reversion of the phenotype did not support the causal role of the MOM1 gene. To understand if the partial reversion of the chlorosis phenotype in F2 transformants was a spontaneous event, we grew at least 20 F3 plants from each of the five MOM1<sub>Lov-5</sub> transgenic F2 lines without antibiotic selection. From the three independent nonchlorotic F2 MOM1<sub>Lov-5</sub> transgenic lines, nearly all F3 plants carrying the transgene remained nonchlorotic in the F3 generation (Fig. 3d, right panel; Table S2), while the segregating nontransgenic plants from these lines showed the chlorosis phenotype in <i>c.</i> 50% of individuals (Fig. 3d, right panel; Table S2). This indicates that an additional copy of the MOM1<sub>Lov-5</sub> transgene further reduced the number of plants with the chlorosis phenotype. We further reasoned that if reversion of the chlorosis phenotype was uniquely associated with the MOM1<sub>Lov-5</sub> allele, introduction of the MOM1<sub>Sha</sub> allele in the F1 hybrids should not exhibit a reversion phenotype. Interestingly, introduction of the MOM1<sub>Sha</sub> allele induced the chlorosis phenotype in 75% of 80 independent F2 transgenic lines (Table S2). These results point strongly towards the role of MOM1<sub>Sha</sub> as the causal gene for chlorosis.

An epigenetic mechanism underlies F2 hybrid chlorosis in Sha × Lov-5 hybrids

In our experiments, we had observed a decline in the chlorosis phenotype after the third generation of hybrids. Transgenerational instability is often associated with phenotypes related to epigenetic mechanisms (Cubas et al., 1999; Agorio et al., 2017). To quantify the transgenerational instability of chlorosis, we recorded the frequency of chlorosis in the F3 and F4 generations of five randomly selected chlorotic F2 individuals from a cross between Sha and Lov-5 (Figs 4a, S8). In the F3 generations of the five chlorotic F2 hybrids, 98–100% of individuals were chlorotic (Fig. S8). We took at least four chlorotic F3 plants from the five chlorotic F2 hybrids and phenotyped 35 F4 individuals from each of the 23 F3 lines. The proportion of chlorosis in the F4 lines varied from 0% to 100%, with an average of 47% of nonchlorotic individuals (n = 1058) (Fig. 4a). Most of the F4 lines (19 lines out of 23) showed some degree of transgenerational decline of chlorosis with <90% chlorotic individuals. The observed transgenerational decline indicates epigenetic regulation of the chlorosis phenotype in hybrids.

Next, we investigated if the hybrid chlorosis phenotype is dependent on DNA methylation. We compared chlorosis in F3 hybrids grown on control or zebularine-supplemented growth medium. Zebularine is a transient inhibitor of cytosine methylation (Baubec et al., 2009) that acts as a demethylating agent and can reactivate a gene previously silenced by methylation (Cheng et al., 2003). If a methylation-dependent genetic pathway mediated the hybrid chlorosis phenotype, demethylation would restore the amount of Chl in the chlorotic F3 hybrids. We grew the chlorotic and nonchlorotic control plants for 2 wk on plates supplemented either with zebularine or with a mock treatment. We then transferred the seedlings to soil to grow for an additional 7 d. Analysis of the total Chl content of the three youngest leaves demonstrated that the zebularine-treated chlorotic F3 hybrids had significantly higher total Chl content (P < 0.001) in comparison to the mock-treated chlorotic F3 hybrids (Fig. 4b,c). Drug treatment had no influence on the total Chl content in the nonchlorotic control hybrids (Fig. 4b,c). These results indicate a role for DNA methylation in regulating the F3 chlorosis phenotype. Next, we investigated if the drug treatment influenced RH18 expression. We found a reduced RH18 expression to the level of the nonchlorotic untreated hybrids in the treated chlorotic hybrids with reverted chlorosis phenotype (Fig. S9). However, RH18 expression was also reduced in treated nonchlorotic hybrids in comparison to nontreated nonchlorotic hybrids, indicating that the drug treatment also reduces RH18 expression independent of the phenotype (Fig. S9).
We confirmed that RH18Lov-5 is not only necessary but also causal for chlorosis in the F2 hybrid of Sha and Lov-5. The homology of AtRH18 with yeast ribosome biogenesis factor Spb4p and its coexpression with ribosome biogenesis genes in A. thaliana suggests that it has a role in ribosome biogenesis. Indeed, a similar chlorosis phenotype has been observed in several A. thaliana mutants with defects in plastid ribosome assembly (Komatsu et al., 2010; Liu et al., 2010; Tiller et al., 2012; Fristedt et al., 2014; Janowski et al., 2018). AtRH18 lacks a chloroplast transit peptide and is not found in the chloroplast proteome (http://ppdb.tc.cornell.edu; http://suba.live/; Sun et al., 2008; Hooper et al., 2016), indicating that it is not likely to be localised in the chloroplast. Although chloroplast, mitochondrial and cytosolic ribosomes are largely autonomous organelles, their translation products are known to interact and form chimeras (Chi et al., 2013; Couvillion et al., 2016; Quiros et al., 2016). For example, impairment of chloroplast biogenesis and Chl accumulation were recently reported to be caused by a defect in a cytosolic ribosome biogenesis protein (Wang et al., 2018). Our analysis demonstrated that Lov-5 parents contained a significantly higher number of chloroplast, mitochondrial and cytosolic ribosomes than the Sha parent (Fig. 2a). Moreover, our results indicated that while the chlorotic F3 hybrids seemed to inherit the number of cytosolic and mitochondrial ribosomes from the Lov-5 parent, the plastid ribosome number was reduced and specifically inherited from the Sha parent (Fig. 2). By contrast, the nonchlorotic F3 hybrids had plastid ribosome abundance intermediate to that of the parents. This would imply that the factor causing the imbalance between the nuclear and plastid ribosome abundance is inherited from the Sha parent and it specifically reduces the synthesis of plastid ribosomes in the chlorotic hybrids. Yet, additional experiments are required to determine the role of AtRH18 in ribosome biogenesis and whether it is involved in cytosolic or plastid ribosome biogenesis.

Interestingly, we observed that the number of chlorotic hybrids was reduced in later generations and the segregation did not follow Mendelian inheritance, indicating that the hybrid chlorosis is epigenetically regulated. Epigenetic regulation would also explain the lack of detectable functional polymorphisms between the parents at the AtRH18 locus. Similar spontaneous disappearance of the incompatibility phenotype was previously reported in Col-0 × Nok-1 hybrids, which was further associated with the loss of epigenetic marks on the t-RNA deaminase gene (Agorio et al., 2017). We mimicked the transient loss of DNA methylation marks by growing the chlorotic and nonchlorotic F3 hybrids on the cytidine analogue zebularine (Baubec et al., 2009). Indeed,
TSS (Numa AtRH18 in independent pathways dually regulate the MOM1 acts on a one such dually regulated locus in ribosome biogenesis pathway (Vaillant 2010; He thaliana hybrids has been reported previously (Martienssen, et al. 2010; Yokthongwattana Habu, 2010; Numa New Phytologist /C211 reported by Pl et al. methylation marks (Probst hybrids. Yet, MOM1 is known to act largely independently of and cytosolic ribosome abundances observed in the chlorotic changes have been reported previously in Arabidopsis (Michalak, 2009; Groszmann, et al., 2011; Lafon-Placette & Köhler, 2015). Our genetic analysis narrowed down the candidates on chr 1 to two genes, At1g08060 coding for MORPHEUS MOLECULE 1 (MOM1) and At1g08140 coding for a putative Na+/H+ antiporter (CHX6A). From these, MOM1 is a known epigenetic gene silencer in plants (Habu, 2010). Furthermore, the presence of an extra copy of the incompatible MOM1Shah allele in the F2 hybrids could cause chlorosis in genetic combinations that otherwise remained nonchlorotic. These results make MOM1 the most prominent candidate for the second causal gene of hybrid chlorosis. It is intriguing to speculate that MOM1Shah by epigenetic regulation of AtRH18L1ov-5 causes the imbalance between the plastid and cytosolic ribosome abundances observed in the chlorotic hybrids. Yet, MOM1 is known to act largely independently of methylation marks (Probst et al., 2003; Vaillant et al., 2006; Habu, 2010; Numa et al., 2010; Yokthongwattana et al., 2010; Cambiagno et al., 2018). Interestingly, the promoter of AtRH18 contains one TE from the rolling circle/helitron class of transposons. TEs are also among the major targets of MOM1-mediated regulation (Vaillant et al., 2006; Habu, 2010; Numa et al., 2010; Yokthongwattana et al., 2010; Cambiagno et al., 2018). Indeed, the repressive nature of TEs on gene expression in A. thaliana hybrids has been reported previously (Martienssen, 2010; He et al., 2011; Li et al., 2012). Another possibility is that MOM1 acts on a trans-locus several kilobases upstream of the AtRH18 TSS (Numa et al., 2010). However, it is also possible that methylation-dependent and MOM1-mediated methylation-independent pathways dually regulate the AtRH18. Intriguingly, one such dually regulated locus in A. thaliana is 5S rRNA of the ribosome biogenesis pathway (Vaillant et al., 2006). Our observation of the common occurrence of F2 hybrid chlorosis between the Sha and northern Swedish accessions and the lack of F2 chlorosis between Shah and Sha accessions reported by Plötner et al. (2017) led us to hypothesise that the RH18L1ov-5 alleles could have become common through either local adaptation or genetic drift for northern Swedish accessions. To investigate whether the chlorosis-causing RH18 allele was exclusively specific to the local environment in northern Sweden, we crossed 140 southern Swedish accessions to Shahdara. Interestingly, > 25% of these crosses showed hybrid chlorosis, indicating that the chlorosis-causing allele of RH18 is not specific to northern Sweden but is also commonly found in southern Sweden. Northern and southern Swedish accessions are genetically very distinct. The northern Swedish accessions show more genetic similarity to Finnish accessions, while southern Swedish Arabidopsis show similarity to the central European accessions (Nordborg et al., 2005). This indicates that local environmental conditions are more likely to underlie the maintenance of chlorosis-causing alleles in these genetically diverse accessions.

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Author contributions

NV performed most of the experiments, and collected and analysed most of the data; HI performed rRNA experiments and analysed the data; BP performed crosses with NV. KS-F and NV screened the transgenic plants. AW analysed some of the data; RAEL supervised all experiments; and RAEL and NV conceived and designed the experiments and wrote the paper with contributions from all the authors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Schematic representation of expected phenotypes upon transformation of the candidate genes in *F1* hybrids.

**Fig. S2** Coexpression network of *AtRH18*.

**Fig. S3** Analysis of rRNA abundances in different lines.

**Fig. S4** Sequence polymorphisms in the promoter of *RH18* in Sha and Lov-5 relative to Col-0.

**Fig. S5** Prevalence of the chlorosis-causing allele in northern Sweden and in southern Sweden.

**Fig. S6** Sequence similarity of the *RH18* promoter within Lovvik individuals.

**Fig. S7** Chromosome 1 candidate gene expression in hybrid parents and chlorotic F3 hybrids.

**Fig. S8** Schematic presentation of the analysis of chlorosis across generations.

**Fig. S9** Relative expression of *AtRH18* in nonchlorotic (NC) and chlorotic (Chl) hybrids treated with zebularine or mock control.

**Table S1** List of accessions used in this study.
Table S2 List of crosses and corresponding phenotypes used in this study. Parents of crosses resulting in F2 chlorosis are in bold type.

Table S3 List of primers used in this study.

Table S4 Details of transgenic lines that were screened for identification of the causal gene for the chlorosis phenotype.

Table S5 Northern and southern Sweden collection sites, individuals collected from each site and proportion of individuals that had the chlorosis phenotype in the F2 generation when crossed with Sha.

Table S6 MOM1 and CHX6A transcript read counts at different developmental stages based on RNA-seq profiling.

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