ORRM5, an RNA recognition motif-containing protein, has a unique effect on mitochondrial RNA editing

Xiaowen Shi1,*, Benoit Castandet2, Arnaud Germain1, Maureen R. Hanson1,† and Stéphane Bentolila1,†,‡

1 Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14850, USA
2 Boyce Thompson Institute, Cornell University, Ithaca, NY 14850, USA

* Present address: Division of Biological Sciences, University of Missouri, Columbia, MO 65211, USA.
† Co-senior author
‡ Correspondence: sb46@cornell.edu

Received 4 January 2017; Editorial decision 30 March 2017; Accepted 30 March 2017

Editor: Robert Sharwood, Australian National University

Abstract

Plants have an RNA editing mechanism that prevents deleterious organelle mutations from resulting in impaired proteins. A typical flowering plant modifies about 40 cytidines in chloroplast transcripts and many hundreds of cytidines in mitochondrial transcripts. The plant editosome, the molecular machinery responsible for this process, contains members of several protein families, including the organelle RNA recognition motif (ORRM)-containing family. ORRM1 and ORRM6 are chloroplast editing factors, while ORRM2, ORRM3, and ORRM4 are mitochondrial editing factors. Here we report the identification of organelle RRM protein 5 (ORRM5) as a mitochondrial editing factor with a unique mode of action. Unlike other ORRM editing factors, the absence of ORRM5 in orrm5 mutant plants results in an increase of the editing extent in 14% of the mitochondrial sites surveyed. The orrm5 mutant also exhibits a reduced splicing efficiency of the first nad5 intron and slower growth and delayed flowering time. ORRM5 contains an RNA recognition motif (RRM) and a glycine-rich domain at the C terminus. The RRM provides the editing activity of ORRM5 and is able to complement the splicing but not the morphological defects.

Key words: Glycine-rich, mitochondria, plant development, plant editosome, plant stress response, RNA editing.

Introduction

The RNA recognition motif (RRM) is a conserved ~80 amino acid motif that binds to RNA molecules with a wide range of specificities and affinities (Kenan et al., 1991). As one of the most abundant protein motifs in eukaryotes, the RRM is involved in various processes of RNA metabolism, and also participates in plant stress responses and developmental processes (Maris et al., 2005; Lorković, 2009). The numerous biological functions of RRM-containing proteins are likely due to the structural versatility of the RRM interactions, as well as the presence of variable auxiliary motifs.

Plant organelle-targeted RRM proteins have been shown to function in a variety of RNA processes, such as RNA splicing, RNA editing and RNA stability (Schmitz-Linneweber et al., 2006; Ruwe et al., 2011; Shi et al., 2016a; Sun et al., 2016). Studies from several groups have demonstrated that plant organelle-localized RRM proteins participate also in plant development and/or stress responses. For instance, orrm4 mutants exhibited delayed growth and late flowering (Shi et al., 2016b). CP29A and CP31A, members of the chloroplast ribonucleoprotein (cpRNP) family, influence multiple chloroplast RNA processes, including RNA stability, mRNA
and rRNA processing under cold stress conditions (Tillich et al., 2009; Kupsch et al., 2012). Another member of the cpRNP family, CP33A, contributes to RNA stability, and is required for chloroplast biogenesis and plant development. cp33a null mutants survived only when provided with an external carbon source and exhibited aberrant leaf development (Teubner et al., 2016). A plastid protein named RNA-binding domain-containing protein 1 (RBD1) is involved in chilling tolerance in Arabidopsis, presumably by regulating 23S rRNA processing (Wang et al., 2016). However, the molecular mechanism underlying the participation of RRM proteins in both RNA-related processes and plant development and/or stress responses is still elusive.

Organelle RNA recognition motif-containing protein 1 (ORRM1) is essential for the post-transcriptional cytidine (C)-to-uridine (U) RNA editing in the Arabidopsis chloroplast (Sun et al., 2013). By analysing ORRM family members, we subsequently identified ORRM2, ORRM3, and ORRM4 as mitochondrial RNA editing factors whereas ORRM6 is a chloroplast RNA editing factor (Shi et al., 2015, 2016b, Hackett et al., 2017). Unlike ORRM1, which carries a truncated RNA editing factor interacting protein (RIP)-RIP motif at its N terminus and an RRM at its C terminus, ORRM2 and ORRM6 carry only an RRM, whereas ORRM3 and ORRM4 each contain an N-terminal RRM and a C-terminal glycine-rich (GR) motif (Sun et al., 2013; Shi et al., 2015, 2016b; Hackett et al., 2017). The RRM in ORRM1, ORRM3, or ORRM4 is sufficient for the protein’s function in editing, whereas the auxiliary RIP or GR motif is responsible for mediating its interaction with other trans-acting factors in the RNA editing complex (Sun et al., 2013, 2016; Shi et al., 2015, 2016b).

While investigating the function of the ORRM family members through analysis of T-DNA insertional mutants, we found that the altered expression of a gene in the ORRM family, encoded by At4g13850, causes a delayed growth and late flowering phenotype. We named this protein organelle RNA recognition motif-containing protein 5 (ORRM5). In order to investigate the cause of the morphological defects in the orrm5 mutants, we examined the effect of the ORRM5 mutations on RNA splicing, the abundance of certain transcripts, and RNA editing. ORRM5 mutations cause reduction of cis-splicing efficiency of the first intron of the mitochondrial nad5 transcript. Mutations in ORRM5 result in decreased editing efficiency at 18 mitochondrial C targets, while editing extents increased at 79 mitochondrial sites compared with the wild-type editing level. Therefore, the absence of ORRM5 results in an increase of editing extent in 14% of the mitochondrial sites surveyed. ORRM5 is the first editing factor reported to have such an inhibitory impact on plant organelle editing. Interaction data presented in this report suggest the hypothesis that the effect of ORRM5 on editing might be mediated through the sequestration of other ORRM mitochondrial editing factors.

**Materials and methods**

**Plant material and morphological analysis**

The Arabidopsis T-DNA insertion lines SALK_059714C (orrm5-2) and SALK_135802C (orrm5-3) in the ORRM5 gene were ordered from the Arabidopsis Biological Resource Center (ABRC; https://abrc.osu.edu). After 3 days of stratification, seeds from the mutant line were planted in soil and grown in a growth room (14 h of light/10 h of dark) at 26 °C. Plants were genotyped by PCR with BioMix Red (Bioline) using primers listed in Supplementary Table S1 at JXB online. The PCR products were sequenced at Cornell University Life Sciences Core Laboratories Center. Leaves were collected from 5-week-old plants for strand- and transcript-specific RNA-seq (STS-PCRseq). The ORRM5 expression level was measured by quantitative RT-PCR. All the primers used are listed in Supplementary Table S1.

The fresh weight of plants grown at 14 h of light per day was measured 26, 34, 36 and 38 days after planting. We recorded the number of days it took for visible flower buds to show in the center of the rosette, for the inflorescence stem of the plant to reach 1 cm in height, and for its first flower to open. Information regarding the fresh weight and the number of total leaves of the mutant plants, transgenic lines versus the controls, was recorded when the first flower bloomed.

**Generation of transgenic plants**

The coding sequence of ORRM5 was reverse-transcribed with SuperScript® III Reverse Transcriptase (Life Technologies) from RNA extracted from wild-type Arabidopsis Columbia using PureLink® RNA Mini Kit (Life Technologies), and then cloned into a PCR8/GW/TOPO vector. The N-terminal ORRM5 was amplified from the reverse-transcribed ORRM5 coding sequence with primer pair ORRM5-F and ORRM5-345RTAG. Primers used are listed in Supplementary Table S1. The fragments were subsequently shuffled into a modified pBlII121 vector using LR Clonase II. 35S-ORRM5 and 35S-nORRM5 in the pBlII121 vector were transformed into Agrobacterium tumefaciens GV3101. Floral dip transformation of homozygous orrm5 mutant plants was performed as described in Zhang et al. (2006). Plants were sprayed with Basta twice on soil for selection. The presence of the transgene and the homozygosity of the orrm5 mutant allele were verified by PCR reactions using primers listed in Supplementary Table S1. Leaves from 4- to 6-week-old transgenic plants were collected for further analysis. 35S-nORRM4 and 35S-CORRM4 transgenic lines were from a previous study (Shi et al., 2016b).

**Use of STS-PCRseq method to assay editing extent**

The STS-PCRseq technique was discussed in detail in a previous study (Bentolila et al., 2013). We amplified all transcripts encoding either plastid or mitochondrial genes with organellar transcriptspecific primers from the mutant tissue and the controls. Primers used are listed as in Bentolila et al. (2013). The RT-PCR products were mixed in equimolar ratio, shared by sonication, and then used as templates to produce an Illumina TruSeq DNA Library. All the samples in this study were pooled in one sequencing lane of an Illumina HiSeq 2500 instrument. The data we obtained were processed according to the guideline provided in a previous study (Bentolila et al., 2013). All the read numbers for each editing site determined in this study are in the Supplementary Dataset S1. Subsequent statistical analysis was mostly similar to the one performed in three previous studies (Bentolila et al., 2013; Shi et al., 2015, 2016b). The test for a significant difference in editing extent between the mutant plants and the wild-type plants varies slightly in the present study. In previous reports the data were pooled between biological replicates, either wild-type plants or mutant plants; the difference in editing extent between wild-type and mutant was then tested by a chi-square test with one degree of freedom, one test for each editing site. In this study we did not pool the reads between biological replicates. To declare a significant difference in editing extent between a wild-type and a mutant, each biological replicate had to satisfy the chi-square test, so four chi-square tests had to be positive, instead of one. Because of repetitive testing, we chose a nominal error rate of $P<1.6 \times 10^{-4}$ to achieve the desired family error rate
of $P<1 \times 10^{-3}$ when analysing 612 sites (36 plastid sites + 576 mito-
chondrial sites). In addition to this chi-square test requirement, a site
was declared significantly reduced (increased) in its editing extent in
the orrm5 mutant if the reduction (increase) compared with the
wild-type plant was $\geq 0.1$. This new methodology is more conserva-
tive and results in less difference in editing extent being called. For
the transgenic plants, a site was declared significantly affected when
the chi-square test between the transgenic plant and the correspond-
ing mutant transformed to obtain the transgenic (T5, T6, T9, T10 vs
orrm5-2-1 and orrm5-2-2; T7, T8, T11, T12 vs orrm5-3-1 and orrm5-3-
2) satisfied the threshold requirement ($P<1.6 \times 10^{-6}$). In addition,
the absolute value of the variation in editing extent had to be $\geq 0.1$
($\leq 0.1$ for an increase and $\leq 0.1$ for a decrease).

Real-time quantitative RT-PCR conditions and analysis

The real-time qRT-PCR was performed as described in a previous
study (Shi et al., 2015). Primers used in the reaction are listed in
Supplementary Table S1. The splicing efficiency was estimated by
the level of expression of two amplicons, one specific to the spliced
transcript and amplified with the primers nad5-ex1F and nad5-
ex2R, one specific to the unspliced transcript and amplified with
the primers nad5-ex1F and nad5-int1R (see Supplementary Table
S1). Splicing efficiency was determined as: (nad5-ex1F, nad5-ex2R)/
[nad5-ex1F, nad5-ex2R]+(nad5-ex1F, nad5-int1R)]

Chloroseq use and application to STS-PCRseq data

The STS-PCRseq sequencing reads were aligned to the Arabidopsis chlo-
roplast and mitochondrial genomes (TAIR10 version) using Tophat2
(Trapnell et al., 2010). The aligned reads were then used as input for
Chloroseq splicing analysis (option a-2) (Castandet et al., 2016) using
custom annotations files containing the introns and splice coordinates.
The annotation files used can be accessed online at https://github.com/
BenoitCastandet/chloroseq/tree/master/TAIR10_ChMr_files.

Yeast-two hybrid assay

The ORRM5 coding sequence with its predicted transit peptide
removed (amino acids 1–32) was amplified from the reverse-trans-
cribed ORRM5 cDNA clone with primer pairs ORRM5-97F and
ORRM5-R listed in Supplementary Table S1. The PCR product
was integrated into PCR8/GW/TOPO vectors and then shuffled
to pGADT7GW or pGBKTK7GW vectors (Horák et al., 2008). All
other constructs used in the Y2H assay were from previous studies
(Bentolila et al., 2012; Sun et al., 2013, 2015; Shi et al., 2015, 2016b).

Two mating types of yeast strain P69-4, a and $\alpha$, were trans-
formed with the constructs above as described in Gietz et al. (1995).
Double transformants were produced by mating single transfor-
mants of mating type a to mating type $\alpha$ cultured in leucine- and
tryptophan-dropout media (Clontech), and subsequently diluted
with sterile water to $1 \times 10^{9}$ and $1 \times 10^{7}$ cells ml$^{-1}$; 10 ml of each dilu-
tion was spotted on leucine-, tryptophan-, adenine- and histidine-
dropout media plates. Yeasts transformed with empty vectors were
used as negative controls to detect auto-activation. Data were col-
clected from 2 to 5 d after spotting.

Bimolecular fluorescence complementation assay

The coding sequence of ORRM5, without the stop codon, was
amplified from the full-length cDNAs as described above, and
cloned into PCR8/GW/TOPO vectors (Invitrogen). The fragment
was then shuffled into XNGW and XCGW vectors (Ohashi-Ito
and Bergmann, 2006) by LR reactions. ORRM3, ORRM4, and
RIP1 constructs were from previous studies. All the primers used
are listed in Supplementary Table S1. Final vectors were validated
by sequencing and transformed into Agrobacterium tumefaciens
GV3101. Agrobacterium infiltration and confocal imaging were as
described in (Shi et al., 2016b).

RNA blots

RNA gel blot analysis was performed as described in Germain et al.
(2011). Primers used to make the probes are listed in Supplementary
Table S1.

Results

ORRM5 mutations lead to delayed growth and late flowering

In order to characterize the function of ORRM5, we obtained two T-DNA inser-
tional mutant lines from the ABRC. The orrm5-2 mutant (SALK_059714C) contains a
T-DNA insertion in its second exon, whereas the orrm5-3 mutant (SALK_135802C) carries an insertion in its fourth intron (Fig. 1A). Both mutant lines, in the Columbia background,
are knockout mutants since the ORRM5 expression level is decreased to an undetectable level as measured by qRT-PCR (Fig. 1B). The level of ORRM5 expression in
orrm5-2 mutants is not significantly different from that in
orrm5-3 mutants (Student's t-test; $P>0.05$) (Fig. 1B). We were able to retrieve wild-type siblings (ORRM5+/-) and
orrm5-3 mutant plants (orrm5-3/-) from the orrm5-3 mutant
population while the orrm5-2 mutant population did not segregate and exhibited only homozygous mutants (orrm5-2
/-). ORRM5 mutations result in a delayed growth and late
flowering phenotype compared with wild-type Arabidopsis
plants (Col-0) (Fig. 1C–E). As shown in Fig. 1C, D, orrm5-
2 and orrm5-3 homozygous mutants grew slower and had
lower fresh weight than the wild-type plants under the long-
day conditions (14 h light day$^{-1}$). In order to examine the
flowering phenotype of the orrm5 mutants, we assessed three
flowering time-related traits. The results indicate that
orrm5-2 mutants required ~7 days more on average for their first
flower bud to become visible in the center of the rosette, for
their inflorescence stems to reach 1 cm in height, and for
their first flower to open compared with wild-type plants
(Fig. 1E). For orrm5-3 mutants, ~2 days more were required
on average for the plants to reach these stages compared with
wild-type plants (Fig. 1E).

ORRM5 mutations cause changes in mitochondrial RNA editing extents

ORRM5 is located in the mitochondrion, according to pro-
toomic and genetic analysis (Kruft et al., 2001; Vermel et al.,
2002; Heazlewood et al., 2004). In order to characterize the
role of ORRM5 in mitochondrial RNA editing, we exam-
ined the effect of ORRM5 mutations on editing extents using
an approach named strand- and transcript-specific RNA-seq
(STS-PCRseq) (Bentolila et al., 2013). Two biological repli-
cates were assayed for each sample, the two mutant plants
orrm5-2 and orrm5-3, and the wild-type siblings of ORRM5-
3. Mutations in ORRM5 cause decreased editing efficiency at
18 (30) mitochondrial sites in orrm5-2 (orrm5-3), but lead to
increased editing extents at 100 (86) mitochondrial C targets
($P<1.6 \times 10^{-6}$, $\Delta$orm5-3$\geq 10\%$, $\Delta$orm5-2$\geq 10\%$). When taking
into account the common sites affected in both orrm5-2 and
orrm5-3, ORRM5 mutations result in RNA editing increases at 79 mitochondrial sites, or 14% of the total mitochondrial sites assayed in this study. Eighteen mitochondrial sites or 3% of the total mitochondrial sites display a decrease of editing extent in both mutants. Figure 2 illustrates ten editing sites that experience significantly decreased editing extents as well as ten sites that show significantly increased editing efficiency in ORRM5 mutants.

In order to examine the distribution of the affected C targets on different transcripts, we calculated the percentage of affected edited sites per transcript in the orrm5 mutants based on where they are located and on the complex encoded by the affected transcripts (see Supplementary Fig. S1). The effect of ORRM5 mutations on RNA editing was distributed among 24 mitochondrial transcripts (Supplementary Fig. S1A).

For the majority of transcripts, the percentage of affected sites per transcript is between 10% and 40%. We categorized the sites that show editing defects upon ORRM5 mutations into two subgroups: the group that experiences reduced editing and the group that exhibits increased editing in the orrm5 mutants (see Supplementary Fig. S1B, C). Sites that experience a reduction of editing extents are distributed on five transcripts, while sites with increased editing extents were more evenly distributed on 23 transcripts. Transcripts encoding complex V, complex III, complex IV, and complex I subunits exhibit sites that experience only increased editing rather than reduced editing extent in orrm5 mutants (see Supplementary Fig. S1). Transcripts encoding the cytochrome c biogenesis complex are rather unique in their response to the orrm5 mutation since 11% of their
ORRM5, mitochondrial editing and plant development

To test whether the editing defects in the orrm5 mutants were truly caused by ORRM5 mutations, we transformed orrm5-2 or orrm5-3 mutant plants with a construct expressing the coding sequence of ORRM5 under the control of a 35S promoter, as shown in Fig. 3A. We performed genotyping of plants that survived in the Basta selection to verify the homozygosity of the two independent transgenic plants from the T0 generation, and assayed their editing extents by STS-PCRseq. We analysed the sites that were affected in orrm5-2 and orrm5-3 and determined whether their editing extents were significantly changed in the transgenic plants when compared with the respective mutants, T5 and T6 vs orrm5-2 and T7 and T8 vs orrm5-3 (Fig. 3A). Sites showing an increase in editing extent in the mutants relative to the wild-type are expected to exhibit a decrease in editing extent in the transgenic plants, while sites showing a decrease in editing extent in the mutants should show an increase in the transgenic plants. The vast majority of sites with an increase in the mutants display a significant decrease ($P<1.6 \times 10^{-6}$, $|\Delta T|>10\%$) in the transgenic plants (upper panel, Fig. 3B). Among the 86 sites that show an increase in editing extent in orrm5-3, 83 sites or 97% show the expected decrease in both transgenic plants. This percentage reaches 99% when sites showing a decrease in only one transgenic plant are included. The same observation holds true for the sites showing an increase in orrm5-2, where 93% of these display a decrease in at least one transgenic plant (upper panel, Fig. 3B). We also analysed the response observed in the transgenic lines by defining a metric we call the complementation effect. This complementation effect normalizes the difference of editing extent between the transgenic and the mutant plants to the difference observed between the wild-type and the mutant plants (see Supplementary Fig. S3). Because ORRM5 is under the control of the strong 35S promoter in the transgenic lines, the majority of the sites exhibit a transgressive response (complementation effect >1) in the transgenic lines, particularly T7 and T8, obtained by transforming orrm5-3 mutant (Supplementary Fig. S3A).

While all the sites decreased in orrm5-2 show an increase in at least one of the transgenic plants, this fraction drops to 63% for orrm5-3, presumably because of an inadequate level of expression of the transgene in T7 and T8 (Fig. 3B, lower panel). This result is also apparent when analysing the complementation effect of the sites; in T7 and T8 the majority of the sites fell below a complementation effect of 1, while in T5 and T6 the majority of the sites show a complementation effect >1 (see Supplementary Fig. S3B). When combining all the 234 sites editing sites show a reduction when compared with the wild-type (Supplementary Fig. S1). rps3 and rps4 are the only other transcripts experiencing a decrease in editing extent in the orrm5 mutants.

The effect of ORRM5 mutations on different transcripts exhibits a variety of patterns. For example, editing extents of all the C targets on the nad3 transcript are increased in the orrm5 mutants (see Supplementary Figs S1C and S2A). However, the alteration of editing extents on the nad3 transcript shows some site specificity, as the effect varies from ~15% to ~70% (Supplementary Fig. S2A). On some transcripts, the effect of ORRM5 mutation can be either inhibitory or stimulatory, depending on the site. For instance, on the rps4 transcript, the absence of ORRM5 expression causes a reduction of editing at three C targets, an increase of editing at two C targets, and leaves the remaining 15 sites unaffected (see Supplementary Fig. S2B). Like rps4, ccmB, ccmC, and rps3 transcripts carry sites that show a reduction of editing extent in the orrm5 mutant while other sites experience an increase of editing extent (Supplementary Fig. S1B, C).

Stable expression of ORRM5 complements the editing defects in the orrm5 mutants

To test whether the editing defects in the orrm5 mutants are truly caused by ORRM5 mutations, we transformed orrm5-2 or orrm5-3 mutant plants with a construct expressing the coding sequence of ORRM5 under the control of a 35S promoter, as shown in Fig. 3A. We performed genotyping of plants that survived in the Basta selection to verify the homozygosity of the T-DNA insertion allele and the presence of the ORRM5 transgene. Afterwards, we collected tissue from two independent transgenic plants from the T0 generation, and assayed their editing extents by STS-PCRseq. We analysed the sites that were affected in orrm5-2 and orrm5-3 and determined whether their editing extents were significantly changed in the orrm5 mutants. Ten sites that showed a significant decrease of editing extent ($|\Delta T|>10\%$) upon ORRM5 mutations (left), and ten sites that showed a significant increase of editing extent ($|\Delta T|>10\%$) in the orrm5 mutants (right). ORRM5-3+/+, wild-type siblings of orrm5-3 mutants; orrm5-3–/–, orrm5-3 homozygous mutants; orrm5-2–/–, orrm5-2 homozygous mutants (n=2). Editing sites are displayed according to the difference between the wild-type and the mutants, from highest to lowest. Values represent mean±SD.
affected, either increased or decreased, in both *orm5-2* and *orm5-3* mutants. 215 sites among those, or 92%, display the expected alteration of editing extent in the transgenic plants.

Numerous invariant mitochondrial sites that did not show significant change in their editing extent in the mutants when compared with the wild-type were significantly affected in the transgenic lines transformed with the full length *ORRM5*; 125 mitochondrial sites exhibited a significant alteration of their editing extent in T7 and T8 (compared with *orm5-3*), and 69 mitochondrial sites underwent significant change of editing extent in T5 and T6 (compared with *orm5-2*). Among these, the majority showed a decrease of editing extent when compared with the respective mutant plants, thereby supporting the inhibitory effect of *ORRM5* on mitochondrial editing (see Supplemental Fig. S4).

**The N-terminal RRM of ORRM5 can rescue most of the editing defects in the orrm5 mutants**

*ORRM5* carries an RNA recognition motif (RRM) at its N terminus and a glycine-rich (GR) motif at its C terminus. In order to characterize the role of the RRM in RNA editing, we transformed *orm5* mutants with a construct expressing the N-terminal RRM (amino acid 1–115) of *ORRM5* under the control of a 35S promoter but lacking the GR motif (Fig. 4A). Two independent transgenic plants surviving in the Basta selection from the T0 generation were selected, verified by genotyping, and analysed by STS-PCRseq. We first analysed the sites that showed a decrease in editing extent in the transgenic plants transformed with the full length *ORRM5* and determined how these sites behaved in the transgenic...
plants transformed with the RRM. The RRM was able to induce a decrease in editing extent in ~70% of the sites in the orrm5-3 mutant and 85% of the sites in the orrm5-2 mutant (Fig. 4B). On average, the RRM was able to cause a decrease in 77% of the sites that were decreased in editing extent in the transgenic plants transformed with the full length ORRM5. In contrast, fewer of the sites that exhibited an increase in editing extent in transgenic plants transformed with the full length ORRM5 also exhibited increases in plants expressing the RRM only (Fig. 4C). Only 49% of these sites display an increase of editing extent in the orrm5-3 mutant complemented with only the RRM, while even fewer sites experience an increase in the RRM-expressing orrm5-2 mutant. The RRM was able to cause an increase of editing extent in only 43% of the set of sites that were increased in all four transgenic plants transformed with the full-length ORRM5. The effect of the RRM construct was even opposite to the one caused by the full length ORRM5 in 14% of the sites in orrm5-3 transgenic plants (Fig. 4C). Interestingly, all these sites are located on the rps4 transcript (see below).

As an illustration of the different classes of mitochondrial editing sites we encountered during our analysis, we show the results of six mitochondrial C targets, ccmB C128, rps3 C887, and rps4 C175 with reduced editing extents, and nad7 C789, nad4 C1131, and ccmB C576 with increased editing extents in the orrm5 mutants (Fig. 5A). The editing level at sites ccmB C128, rps3 C887, and rps4 C175 is complemented to the wild-type level or even higher in the transgenic lines expressing the full length ORRM5, while the RRM is able to increase the editing extents to the wild-type level or slightly lower for the ccmB C128 and rps3 C887 sites (Fig. 5A, top). The RRM has no effect on the editing extent at the rps4 C175 site in both orrm5-3 (T11, T12) and orrm5-2 (T9, T10) transgenic plants (Fig. 5A, top). The same trend is observed for sites exhibiting an increase of editing extent in the orrm5 mutants with a more pronounced effect of the full length ORRM5 than the RRM on the editing extent in the transgenic plants (Fig. 5A, bottom). At sites nad4 C1131 and ccmB C576, both the full length ORRM5 and the RRM reduce the editing extent in transgenic plants to the wild-type level or even lower (Fig. 5A, bottom). At the nad7 C789 site, the full length ORRM5 restores the editing extent to wild-type level or slightly higher, while the RRM significantly decreases the editing extent compared with the orrm5 mutants, but does not decrease it to the wild-type level (Fig. 5A, bottom).

The last class of mitochondrial sites affected by the orrm5 mutations with a unique response in transgenic plants is present on two clustered groups of editing sites on the rps4 transcript. In these two groups, the effects of the full length ORRM5 and the RRM are in opposite directions; while ORRM5 increases the editing extent, the RRM reduces the editing extent in the transgenic plants compared with the orrm5-3 mutant (Fig. 5B).

Mutations in ORRM5 do not affect the steady-state level of RNA transcripts

A possible reason for clustering of sites on the nad3 transcript affected in the orrm5 mutants could be an alteration in the total abundance of this transcript. A change of transcript abundance is also a possible cause of the unexpected increase of editing extents observed in the mutants. We therefore examined the transcript abundance of three transcripts that exhibit diverse editing patterns upon ORRM5 mutations. All the editing sites on the nad3 transcript experience an increase of editing (see Supplementary Figs S1A and S2A). None of the sites on the rps14 transcript are affected in the orrm5 mutants (Supplementary Fig. S1). One out of 12 sites on the ccmFn-2 transcript exhibit decreased editing extent in the orrm5 mutants (Supplementary Figs S1 and S5). In all three
circumstances, we did not observe any change of transcript abundance in the mutants vs the wild-type plants as shown in Supplementary Fig S6. We also assayed the steady-state level of nad3, rps14, and ccmFl-2 transcripts in the transgenic lines expressing ORRM5 or nORRM5 in either orrm5-2 or orrm5-3 mutant background by RNA blots. Again we did not observe any alteration of transcript abundance (Supplementary Fig S6).

Stable expression of ORRM5 complements the morphological defects caused by ORRM5 mutations

In order to assay whether the morphological defects in the orrm5 mutants are caused by the ORRM5 mutation, we compared the morphology of the orrm5 homozygous mutants, their wild-type siblings, and the transgenic lines expressing the ORRM5 or nORRM5 transgene under the control of a 35S promoter. We planted the seeds collected from the transgenic lines used for the editing assay, and recorded the growth phenotype of the segregating T1 plants. The presence of the transgene was confirmed by genotyping. As shown in Fig. 6A, stable expression of ORRM5 makes the plants grow faster compared with the non-transgenic orrm5 mutants, whereas the expression of nORRM5 does not change the morphology of the orrm5 mutants. We also observed the complementation of the delayed growth phenotype in the transgenic lines expressing ORRM5, as the fresh weight of the 35S-ORRM5 transgenic lines is significantly increased compared with the non-transgenic orrm5-3 mutants (Fig. 6B). Stable expression of ORRM5 results in an increase of fresh weight in plants grown in long-day conditions. The fresh weight rises from 0.47 to 0.86 g on day 34 and from 1.07 to 1.50 g on day 38 (Fig. 6B). However, the expression of 35S-nORRM5 did not affect the fresh weight of the plants (Fig. 6B). We used three additional flowering time-related traits to characterize the contribution of ORRM5 to late flowering. Again, the results demonstrate that the expression of the ORRM5 transgene complements the late flowering caused by ORRM5 mutations, whereas nORRM5 expression does not (Fig. 6C).
To determine whether the delayed growth phenotype is the cause of late-flowering phenotype, we recorded the fresh weight and the number of total leaves of the orrm5 mutants, their wild-type siblings as well as the transgenic lines expressing ORRM5 or nORRM5. As shown in Fig. 6B, C, orrm5-3 mutants have higher fresh weight and total number of leaves than their wild-type siblings when their first flower opened. Given that the mutants have a greater mass than the wild-type when the first flower opens, late flowering is not the sole consequence of delayed growth in the mutants. However, the fresh weight and total number of leaves of the transgenic lines are not significantly different from the orrm5-3 mutants (Fig. 6C) even though the complemented lines flowered at the same time as wild-type.

**ORRM5 mutations cause reduction of cis-splicing efficiency of nad5 transcripts**

Pleiotropic effects are frequently observed in mutants affected in organelle gene expression. We therefore considered whether ORRM5 might play additional roles in RNA metabolism. Chloroseq, an optimized chloroplast RNA-seq bioinformatic pipeline, has been recently developed to analyse features of chloroplast RNA metabolism including processing, editing, splicing, and relative transcript abundance (Castandet et al., 2016). While this tool was built to process chloroplast RNA-seq data, we were able to adapt it to analyse our STS-PCRseq data.

We analysed the known cis-splicing events and observed a very significant reduction of cis-splicing efficiency of the first intron of the nad5 transcript in both orrm5 mutants, as shown in Fig. 7A. Expression of ORRM5 or nORRM5 in either orrm5-2 or orrm5-3 mutant background rescues the defective cis-splicing of the first nad5 intron, with the splicing efficiency increasing from ~50% to >95% (Fig. 7A). The nad5 transcript is composed of five exons; the maturation of the transcript requires two cis-splicing events to join exons 1 and 2 on the one hand and exons 4 and 5 on the other hand, and two trans-splicing event to join the 22-nucleotide-long exon 3.
to the other two parts (see Supplementary Fig. S7) (Knoop et al., 1991). Since Chloroseq was not originally developed to analyse STS-PCRseq data by using ChloroSeq, a bioinformatic pipeline developed for chloroplast RNA-Seq, we validated the defect in splicing of the first intron in nad5 transcript by measuring splicing efficiency with a qRT-PCR assay. The same RNAs from the ORRM5-3 wild-type and orrm5-3 mutant plants used for the STS-PCRseq and Chloroseq analysis were assayed by qRT-PCR and showed a similar defect in splicing of nad5 first intron (Fig. 7B). In addition, a new batch of wild-type and mutant plants were grown; the qRT-PCR assay confirmed the defect in nad5 splicing of the first intron (Fig. 7B).

**ORRM5 interacts with ORRM2, ORRM3, and ORRM4**

RNA editing is carried out by a protein complex 200–440 kDa in size (Bentolila et al., 2012). ORRM5’s involvement in mitochondrial RNA editing indicates that it may interact with other components of the mitochondrial editosome. Therefore, we performed yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays to examine the physical interactions between ORRM5 and other editing factors. BiFC assays were performed in Nicotiana benthamiana by transiently coexpressing a protein fused to the N-terminal half of green fluorescent protein (GFP) (GFPN) with another protein fused to the C-terminal half of GFP (GFPs). Positive protein–protein interaction signals were observed when ORRM5-GFPs was co-inoculated with ORRM2-GFPs, ORRM3-GFPs, or ORRM4-GFPs (Fig. 8A). We also tested RIP1 (RNA-editing factor Interacting Protein 1), a major mitochondrial editing factor which affects editing at 474 mitochondrial C targets (Bentolila et al., 2012; Bentolila et al., 2013). However, no signal was observed when ORRM5-GFPs was coexpressed with RIP1-GFPN or ORRM5-GFPs (Fig. 8B). In our Y2H assays, the predicted mature coding sequence (with the predicted transit peptide removed) was fused to the AD or BD domain. ORRM5 interacts with ORRM3 and ORRM4, but ORRM5 does not interact with RIP1 or itself in Y2H assays (Fig. 8C, D). Interaction between ORRM2 and ORRM5 cannot be determined in yeast due to auto-activation when fused to the BD domain.

**Discussion**

**ORRM5 affects both RNA editing and RNA splicing**

In this study, we demonstrate that ORRM5 is a mitochondrial editing factor that affects about 17% of mitochondrial sites in Arabidopsis. ORRM5 is unique in its effect on mitochondrial editing, as its absence results primarily in an increase of editing extent at the targeted C that it affects. The mutants of all the other mitochondrial factors characterized in our lab by the STS-PCRseq method show a much higher percentage of sites with decreased than increased editing extent (Table 1). Some editing sites were impaired in the mutants, but considerable editing remained. The weak effect on editing may be due to the presence of a different RRM protein that can act as an editing factor in the absence of ORRM5.

Like ORRM3 and ORRM4, ORRM5 carries an N-terminal RRM and a C-terminal GR motif. Previously we showed that the GR domain of ORRM4 is required for mediating its interaction with ORRM3 and itself (Shi et al., 2016b). Another group studied NtGR-RBP1, which contains an N-terminal RRM and a C-terminal GR motif like ORRM4, and demonstrated that the GR domain is responsible for its self-association by transient interactions with its RRM (Khan et al., 2014). Protein–protein interaction assays
showed that ORRM3 and ORRM4 can form homodimers, whereas ORRM2 cannot (Shi et al., 2015, 2016b). Therefore, we hypothesized the inability of ORRM2 to form homodimers to its lack of the GR motif. Surprisingly, despite the presence of a GR motif, ORRM5 does not form homodimers in our Y2H and BiFC assays. Perhaps the GR motif in ORRM5, which is much shorter than that in ORRM3 or ORRM4, has insufficient length to mediate such interactions. A short GR region could also possibly explain why ORRM5 does not interact with RIP1, whereas ORRM3 and ORRM4 do.

One possible explanation for the action of ORRM5 on mitochondrial editing extent is sequestration of other bona fide editing factors such as ORRM2, ORRM3, and ORRM4, preventing them from fulfilling their role. In this scenario, in orrm5 mutants, the absence of ORRM5 releases the other ORRM mitochondrial editing factors, resulting in an increase of editing extent of the sites under their control. This hypothesis is strongly supported by the significant dependence of the editing extent in orrm5 mutant and in orrm2 or orrm3 mutants ($P<10^{-7}$, Supplementary Fig. S8). The large value of the $\chi^2$ is mostly due to the excess of observed sites experiencing both an increase of editing extent in orrm5 mutants and a decrease of editing extent in either orrm2 or orrm3 mutants (see Supplementary Fig. S8).

In the transgenic lines overexpressing ORRM5, numerous mitochondrial sites that were invariant in the mutant lines exhibit a significant alteration of their editing extents.
Among these, a majority, 27% vs 6% in orrm5-3 transgenic plants and 15% vs 6% in orrm5-2 transgenic plants, show a decrease of editing extent vs an increase of editing extent (see Supplementary Fig. S4). This result supports the inhibitory effect of ORRM5 on the mitochondrial editing extent.

A possible explanation is that increasing chaperone activity by using 35S overexpression causes more changes in RNA structure and thus potentially in RNA editing. However, an analysis of the mitochondrial sites showing a decrease of their editing extent in the transgenic lines together with the effect of other mitochondrial editing factors, ORRM2 or ORRM3, shows a strong dependency relationship (Supplementary Fig. S9). The large value of the χ² is mostly due to the excess of observed sites experiencing both a decrease of editing extent in orrm5 transgenic lines and a decrease of editing extent in either orrm2 silenced or orrm3 mutant plants (Supplementary Fig. S9). Taken together, these results support a model in which an excess of ORRM5 in the transgenic lines might sequester more mitochondrial editing factors than in the wild-type, thus decreasing preferentially the editing extent of the sites under the control of the sequestered editing factors.

Our results indicate that the RRM of ORRM5 is sufficient for ORRM5’s function in RNA editing, at least for the majority of the sites that are increased in editing extent in the mutant. Complementation of editing defects by the RRM alone was also true in the cases of ORRM1, ORRM3, and ORRM4 (Shi et al., 2015, 2016b). We also demonstrate that the RRM of ORRM5 is essential for efficient cis-splicing of the first intron on the nad5 transcript. However, the expression of the RRM could not compensate the morphological defects caused by the orrm5 mutants. We previously found that the expression of the GR domain of ORRM4 complements the late flowering phenotype in the orrm4 mutants (Shi et al. 2016b). Taken together, these results indicate that the GR domain may participate in ORRM5’s role in plant growth and development. Given that the RRM could only rescue the molecular defects rather than the physiological changes in orrm5 mutants, it is quite possible that the physiological changes observed in orrm5 mutants are not caused by its defects in RNA processing. However, how ORRM5 influences plant growth and development is not yet identified.

ORRM5 mutations cause both RNA editing and RNA splicing defects. These processes both occur post-transcriptionally in mitochondria of flowering plants. Several studies have addressed the question of the temporal relationship of RNA editing and RNA splicing. Partially edited spliced cDNA transcripts or DNA, and its expression could be induced by cold treatment, but not by wound, drought or ABA treatment (Vermel et al., 2002). ORRM5 was also characterized as glycine-rich RNA-binding protein 2 (GRP2), glycine-rich RNA binding protein 2 (GR-RBP2) or RNA-binding glycine-rich subclass A 5 (RBGA5) (Lorković and Barta, 2002; Kim et al., 2007b; Krishnamurthy et al., 2015). It has been reported to affect seed germination of Arabidopsis plants under salt stress, accelerate seed germination and seedling growth under cold stress, and enhance the cold tolerance in Arabidopsis plants (Kim et al., 2007b). In another study, expression of the Arabidopsis ORRM5/GRP2 in rice (Oryza sativa) was able to improve rice grain yield under drought stress conditions (Yang et al., 2014). Additionally, ORRM5/
GRP2 complements the cold sensitivity of an *Escherichia coli* BX04 mutant and exhibits transcription anti-termination activity, indicating that it has a RNA chaperone activity during the cold adaptation process (Kim et al., 2007a; Kim et al., 2007b). The anti-termination activity requires melting of an RNA secondary structure in *E. coli*. If ORRM5 can similarly alter RNA secondary structure in mitochondria, orrm5 mutation may lead to changes in RNA structure that might cause indirect effects in RNA editing/splicing. Alternatively, ORRM5 may perform multiple functions depending on the environment, acting as RNA chaperone under stress conditions while functioning as an RNA editing/splicing factor under normal conditions. What role ORRM5 plays in stress response is not evident from our analysis of its functions in RNA editing and splicing.

Results from several studies indicate that stress conditions, such as exposure to heat, cold, or heavy metals, could affect the efficiency or patterns of RNA splicing and/or RNA editing (Luehrsen et al., 1994; Simpson and Filipowicz, 1996). CP31A, a chloroplast ribonucleoprotein that protects plant against cold stress, is involved in the splicing of ndhB and ycfβ mRNAs under cold stress (Kupsch et al., 2012). Additionally, a cold stress-upregulated nuclear protein, STABILIZED1 (STA1), contributes to the splicing of transcripts encoded by the *COR15A* gene in cold-treated Arabidopsis plants (Lee et al., 2006). Results from a recent study that analysed published RNA-Seq datasets derived from Arabidopsis grown under stress conditions demonstrated that heat stress results in a global reduction in splicing and editing efficiency in Arabidopsis chloroplast (Castandet et al., 2016). RNA editing and splicing of the wheat mitochondrial *cox2* transcript were also impaired under low-temperature conditions (Kurihara-Yonemoto and Kubo, 2010).

Several organelle RNA editing factors have been reported to participate in plant stress responses. A member of the Arabidopsis PPR protein family, MEF11/LO11, participates in mitochondrial RNA editing as well as ABA and stress responses (Sechet et al., 2015). ORRM1, the founder member of the ORRM family, is essential for chloroplast RNA editing in Arabidopsis and maize (Sun et al., 2013). A recent study reports that ORRM1 is involved in cold stress tolerance in Arabidopsis (Wang et al., 2016). The expression of mitochondrial RNA editing factor *ORRM3/GR-RBP3* could also be induced by cold treatment. ORRM5 is relevant to cold and drought stress responses as discussed earlier (Kim et al., 2007a; Kim et al., 2007b; Yang et al., 2014). However, the association between stress tolerance and RNA splicing and/or RNA editing awaits further investigation.

**Supplementary data**

Supplementary data are available at *JXB* online.

Dataset S1. Number of reads at each editing site for each plant assayed by STS-PCRSeq.

Fig. S1. Percentage of affected edited sites/transcript in the *orrm5* mutants.

Fig. S2. Editing on the *nad3* and *rps4* transcripts is affected by *ORRM5* mutation.

Fig. S3. Distribution of the complementation effect in the transgenic lines transformed with the full length *ORRM5*.

Fig. S4. Distribution and behavior of the editing sites that did not show any significant change in the mutant lines (vs the wild-type) in the transgenic lines transformed with the full length *ORRM5*.

Fig. S5. Editing defect at the *ccmFn*-2 C320 site in the *orrm5* mutants.

Fig. S6. Examples of transcript abundance in mutant, wild-type, and transgenic lines.

Fig. S7. Representation of the complex structure of the *nad5* transcript.

Fig. S8. Contingency tables of the number of mitochondrial sites experiencing editing change in *orrm* mutants.

Fig. S9. Contingency tables of the number of mitochondrial sites experiencing editing decreases in transgenic lines overexpressing *ORRM5* and in *orrm2* silenced or *orrm3* mutants.

Table S1. Primers used in this study.

**Acknowledgements**

We thank Robert Bukowski of the Computational Biology Service Unit at Cornell University for performing the primary analysis of the Illumina sequencing reads. This work was supported by a grant to MRH and SB from the National Science Foundation, Division of Molecular and Cellular Biosciences, the Genetic Mechanisms Program (NSF-MCB-1615393). BC is supported by the grant DE-FG02-10ER20015 from the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences, of the US Department of Energy.

**References**

Bentollia S, Heller WP, Sun T, Babina AM, Friso G, Wijk KJv, Hanson MR. 2012. RfP1, a member of an Arabidopsis protein family, interacts with the protein RARE1 and broadly affects RNA editing. Proceedings of the National Academy of Sciences, USA 109, E1453–E1461.

Bentollia S, Oh J, Hanson MR, Bukowski R. 2013. Comprehensive high-resolution analysis of the role of an Arabidopsis gene family in RNA editing. PLoS Genetics 9, e1003584.

Borner GV, Mori M, Wissinger B, Brennicke A, Schmelzer C. 1995. RNA editing of a group II intron in *Onothera* as a prerequisite for splicing. Molecular Genetics and Genomics 246, 739–744.

Castandet B, Choury D, Bégu D, Jordana X, Araya A. 2010. Introrn RNA editing is essential for splicing in plant mitochondria. Nucleic Acids Research 38, 7112–7121.

Castandet B, Hotto AM, Strickler SR, Stern DB. 2016. ChloroSeq, an optimized chloroplast RNA-Seq bioinformatic pipeline, reveals remodeling of the organelar transcriptome under heat stress. G3 6, 2817–2827.

Colas des Francs-Small C, Falcon de Longevialle A, Li Y, Lowe E, Tanz SK, Smith C, Bevan MW, Small I. 2014. The pentatricopeptide repeat proteins TANG2 and ORGANELLE TRANSCRIPT PROCESSING439 are involved in the splicing of the multipartite nad5 transcript encoding a subunit of mitochondrial complex I. Plant Physiology 165, 1400–1416.

Farré JC, Akinin C, Araya A, Castandet B. 2012. RNA editing in mitochondrial trans-introns is required for splicing. PLoS One 7, e52644.

Germain A, Herlich S, Larom S, Kim SH, Schuster G, Stern DB. 2011. Mutational analysis of Arabidopsis chloroplast polynucleotide phosphorylase reveals roles for both RNAse PH core domains in polyadenylation, RNA 3’-end maturation and intron degradation. The Plant Journal 67, 381–394.
Giet RD, Schiestl RH, Willems AR, Woods RA. 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11, 355–360.

Gray MJ, Ouellet PS. 1993. RNA editing in plant mitochondria and chloroplasts. The FASEB Journal 7, 64–71.

Guadaldo JM, Bonnard G, Lamattina L, Grienberger JM. 1991. Expression of the wheat mitochondrial nad3-rps12 transcription unit: correlation between editing and mRNA maturation. The Plant Cell 3, 1109–1120.

Hackett JB, Shi X, Kobylarz AT, Lucas MK, Wessendorf RL, Hines KM, Bentolila S, Hanson MR, Lu Y. 2017. An organellar RNA recognition motif protein is required for photosynthetic subunit psbF transcript editing. Plant Physiology 173, 2278–2293.

Heazlewood JL, Tonti-Filippini JS, Gout AM, Day DA, Whelan J, Millar AH. 2004. Experimental analysis of the Arabidopsis mitochondrial prelome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. The Plant Cell 16, 241–256.

Horak J, Grefen C, Berendzen KW, Hahn A, Stierhof YD, Stadelhofer B, Stahl M, Koncz C, Harter K. 2008. The Arabidopsis thaliana response regulator ARR22 is a putative AHP phospho-histidine phosphatase expressed in the chalaza of developing seeds. BMC Plant Biology 8, 77.

Kenan DJ, Query CC, Keene JD. 1991. RNA recognition: towards identifying determinants of specificity. Trends in Biochemical Sciences 16, 214–220.

Khan F, Daniëls MA, Folkers GE, Boelens R, Saqlan Naqvi SM, van Ingen H. 2014. Structural basis of nucleic acid binding by Nicotiana tabacum glycine-rich RNA-binding protein: implications for its RNA chaperone function. Nucleic Acids Research 42, 8705–8718.

Kim JS, Park SJ, Kwak KJ, Kim YO, Kim JY, Song J, Jang B, Jung CH, Kang H. 2007a. Cold shock domain proteins and glycine-rich RNA-binding proteins from Arabidopsis thaliana can promote the cold adaptation process in Escherichia coli. Nucleic Acids Research 35, 506–516.

Kim JY, Park SJ, Jang B, Jung CH, Ahn SJ, Goh CH, Cho K, Han O, Kang H. 2007b. Functional characterization of a glycine-rich RNA-binding protein 2 in Arabidopsis thaliana under abiotic stress conditions. The Plant Journal 50, 439–451.

Knoopp V, Schuster W, Wissinger B, Brennicke A. 1991. Trans splicing integrates an exon of 22 nucleotides into the nad5 mRNA in higher plant mitochondria. The EMBO Journal 10, 3483–3493.

Krishnamurthy P, Kim JA, Jeong MJ, Kang CH, Lee SI. 2015. Defining the RNA-binding glycine-rich (RBG) gene superfamily: new insights into nomenclature, phylogeny, and evolutionary trends obtained by genome-wide comparative analysis of Arabidopsis, Chinese cabbage, rice and maize genomes. Molecular Genetics and Genomics 290, 2279–2295.

Kruft V, Eubel H, Jänsch L, Werhahn W, Braun HP. 2001. Promoteric approach to identify novel mitochondrial proteins in Arabidopsis. Plant Physiology 126, 1694–1710.

Kupsch C, Ruwe H, Gusewski S, Tillich M, Small I, Schmitz-Linneweber C. 2012. Arabidopsis chloroplast RNA binding proteins CP31A and CP29A associate with large transcript pools and confer cold stress tolerance by influencing multiple chloroplast RNA processing steps. The Plant Cell 24, 4266–4280.

Kurihara-Yonemoto S, Kubo T. 2010. Increased accumulation of intron-containing transcripts in rice mitochondria caused by low temperature: is cold-sensitive RNA editing implicated? Current Genetics 56, 529–541.

Lee BH, Kapoor A, Zhu J, Zhu JK. 2006. STABILIZED1, a stress-upregulated nuclear protein, is required for pre-mRNA splicing, mRNA turnover, and stress tolerance in Arabidopsis. The Plant Cell 18, 1736–1749.

Lorković ZJ. 2009. Role of plant RNA-binding proteins in development, stress response and genome organization. Trends in Plant Science 14, 229–236.

Lorković ZJ, Barta A. 2002. Genome analysis: RNA recognition motif (RRM) and K homology (KH) domain RNA-binding proteins from the flowering plant Arabidopsis thaliana. Nucleic Acids Research 30, 623–635.

Luehrsens KR, Taha S, Walbot V. 1994. Nuclear pre-mRNA processing in higher plants. Progress in Nucleic Acid Research and Molecular Biology 47, 149–193.

Maris C, Dominguez C, Allain FH. 2005. The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression. The FEBS Journal 272, 2118–2131.

Matsushita K, Bergmann DC. 2006. Arabidopsis FAMA controls the final proliferation/differentiation switch during stomatal development. The Plant Cell 18, 2493–2505.

Paluska SG, Ali GS, Reddy AS. 2007. Alternative splicing of pre-mRNAs of Arabidopsis serine/arginine-rich proteins: regulation by hormones and stresses. The Plant Journal 49, 1091–1107.

Ruwe H, Kupsch C, Teubner M, Schmitz-Linneweber C. 2011. The RNA-recognition motif in chloroplasts. Journal of Plant Physiology 168, 1361–1371.

Schmitz-Linneweber C, Tillich M, Herrmann RG, Maier RM. 2001. Heterologous, splicing-dependent RNA editing in chloroplasts: allotetraploidy provides trans-factors. The EMBO Journal 20, 4874–4883.

Schmitz-Linneweber C, Williams-Carrier RE, Williams-Voelker PM, Kroeger TS, Vichas A, Barkan A. 2006. A pentatricopeptide repeat protein facilitates the trans-splicing of the maize chloroplast rps12 pre-mRNA. The Plant Cell 18, 2650–2663.

Sechet J, Roux C, Plessis A, et al. 2015. The ABA-deficiency suppressor locus HAS2 encodes the PPR protein L01/MEF11 involved in mitochondrial RNA editing. Molecular Plant 8, 644–656.

Shi X, Bentolila S, Hanson MR. 2016a. Organelle RNA recognition motif-containing (ORRM) proteins are plastid and mitochondrial editing factors in Arabidopsis. Plant Signaling & Behavior 11, e1167299.

Shi X, Germain A, Hanson MR, Bentolila S. 2016b. RNA recognition motif-containing protein ORRM4 broadly affects mitochondrial RNA editing and impacts plant development and flowering. Plant Physiology 170, 294–309.

Shi X, Hanson MR, Bentolila S. 2015. Two RNA recognition motif-containing proteins are plant mitochondrial editing factors. Nucleic Acids Research 43, 3814–3825.

Simpson GG, Filipowicz W. 1996. Splicing of precursors to mRNA in higher plants: mechanism, regulation and sub-nuclear organisation of the spliceosomal machinery. Plant Molecular Biology 32, 1–41.

Sun T, Bentolila S, Hanson MR. 2016. The unexpected diversity of plant organelle RNA editosomes. Trends in Plant Science 21, 962–973.

Sun T, Germain A, Giloteaux L, Hammami K, Barkan A, Hanson MR, Bentolila S. 2013. An RNA recognition motif-containing protein is required for plastid RNA editing in Arabidopsis and maize. Proceedings of the National Academy of Sciences, USA 110, E1169–E1178.

Sun T, Shi X, Friso G, Van Wijk K, Bentolila S, Hanson MR. 2015. A zinc finger motif-containing protein is essential for chloroplast RNA editing. PLoS Genetics 11, e1005028.

Sutton CA, Conklin PL, Pruitt KD, Hanson MR. 1991. Editing of pre-mRNAs can occur before cis-splicing and trans-splicing in Petunia mitochondria. Molecular and Cellular Biology 11, 4274–4277.

Teubner M, Fuss J, Kuhn K, Krause K, Schmitz-Linneweber C. 2016. The RRM protein CP33A is a global ligand of chloroplast mRNAs and is essential for plastid biogenesis and plant development. The Plant Journal 89, 472–485.

Tillich M, Hardel SL, Kupsch C, et al. 2009. Chloroplast ribonuclease ACP31A is required for editing and stability of specific chloroplast mRNAs. Proceedings of the National Academy of Sciences, USA 106, 6002–6007.

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature Biotechnology 28, 511–516.

Vermel M, Guerrmann B, Delage L, Grienberger JM, Marezolle-Drouraud L, Guadaldo JM. 2002. A family of RRM-type RNA-binding proteins specific to plant mitochondria. Proceedings of the National Academy of Sciences, USA 99, 5866–5871.

Wang S, Bai G, Wang S, Yang L, Yang F, Wang Y, Zhu JK, Hua J. 2016. Chloroplast RNA-binding protein RB1D1 promotes chilling tolerance through 23S rRNA processing in Arabidopsis. PLoS Genetics 12, e1006027.
Yang AJ, Mulligan RM. 1991. RNA editing intermediates of cox2 transcripts in maize mitochondria. Molecular and Cellular Biology 11, 4278–4281.

Yang DH, Kwak KJ, Kim MK, Park SJ, Yang KY, Kang H. 2014. Expression of Arabidopsis glycine-rich RNA-binding protein AtGRP2 or AtGRP7 improves grain yield of rice (Oryza sativa) under drought stress conditions. Plant Science 214, 106–112.

Zahler AM, Neugebauer KM, Lane WS, Roth MB. 1993. Distinct functions of SR proteins in alternative pre-mRNA splicing. Science 260, 219–222.

Zhang X, Henriques R, Lin SS, Niu QW, Chua NH. 2006. Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nature Protocols 1, 641–646.