DOCTORAL THESIS

ABCE Proteins' Role in RNA Silencing Suppression and their Evolution

Jelena Mõttus

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

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ABCE valkude roll RNA vaigistamise supressioonis ja nende evolutsioon

JELENA MÕTTUS

This work is dedicated to my parents
Anna and Jevgeni Gerassimenko.
Thank you for believing in me.

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Author's contribution to the publications

The contributions to the articles included in this thesis are:

I Conceived and designed the experiments, performed the experiments, analyzed the data.

II Conceptualization, methodology, formal analysis, investigation, data curation, writing of original draft preparation, visualization.

III Data curation, investigation, methodology, software, validation, writing original draft.

Introduction

ATP-binding cassette subfamily E (ABCE) members are one of the most evolutionary conserved proteins present in all archaea and eukaryotes. In yeast and most animals, the ABCE subfamily is represented by a single multifunctional ABCE1 protein, which is associated with different stages of protein synthesis, including the initiation and termination of translation and ribosome biogenesis. Today, the central role of ABCE1 in archaea and eukaryotes is recognized in mediating ribosome recycling, the process linking translation termination with initiation, and which is also associated with RNA degradation pathways commonly referred to as the RNA Quality Control (RQC) system. This evolutionarily conserved function seems to explain the critical role of ABCE proteins in the viability of organisms. In addition, *Arabidopsis thaliana* ABCE2 (AtABCE2) protein has been shown to act as a suppressor of RNA silencing, a complex RNA degradation mechanism. Through multiple interconnected pathways, RNA silencing mediates developmental regulation and stress responses in eukaryotes. Although there was growing data on the involvement of ABCE proteins in translation and RQC system, their role in RNA silencing suppression remained unexplored.

The first goal of this thesis was to investigate whether the suppression of RNA silencing is a plant-specific function of ABCE proteins or if it is conserved across eukaryotes. Human ABCE1 was tested for its ability to suppress RNA silencing in plant, mammalian, and heterologous animal systems. The next aim was to explore structural requirements of AtABCE2 for its suppressor function in order to shed light on the mode of its action. For this purpose, mutational analysis of AtABCE2 was performed in the well-established GFP transgene-expressing *Nicotiana benthamiana* system. Considering that multiple plants, including Arabidopsis, encode more than one ABCE protein, and with the rapid accumulation of whole-genome sequencing data of plant species, the final goal of this thesis was to provide insight into the diversity and phylogeny of the plant *ABCE* gene subfamily using bioinformatics approaches.

Altogether, the results of this thesis provide insight into yet-unexplored role of essential ribosome recycling factors ABCEs in suppressing RNA silencing and contributes to a better understanding of the relationship between distinct RNA degradation pathways and translation. Uncovering the variation in size and the phylogeny of the plant *ABCE* gene subfamily lays the foundation for further research into the functional diversification of ABCE proteins.

Abbreviations

ABCE	ATP-binding cassette subfamily E
AGO	Argonaute protein
AtABCE	*Arabidopsis thaliana* ABCE
aABCE	archaeal ABCE
DCL	Dicer-like
dpi	days post infiltration
dsRNA	double-stranded RNA
FeS cluster	iron-sulfur cluster
GFP	green fluorescent protein
HLH	helix-loop-helix
miRNA	microRNA
NBD	nucleotide binding domain
NGD	No-go decay
NMD	Nonsense-mediated decay
NSD	Non-stop decay
post-SC	post-splitting complex
PTGS	post transcriptional gene silencing
RdDM	RNA directed DNA methylation
RQC	RNA Quality Control
RDR	RNA dependent RNA polymerase (eukaryotes)
siRNA	short interfering RNA
sRNA	small non-coding RNA
ssRNA	single-stranded RNA
TGS	transcriptional gene silencing
UV	ultraviolet
WGD	whole genome duplication

1 Review of the literature

1.1 RNA silencing

RNA silencing (also called RNA interference, RNAi) is a widely conserved mechanism of sequence-specific down-regulation of gene expression in eukaryotes. Through a range of interconnected pathways, sharing a set of similar components, RNA silencing is involved in many cellular processes, including developmental gene regulation, protection of genome integrity and modulation of stress responses (Baulcombe, 2005; Li *et al*, 2017). The unifying feature for these pathways is that regulation is directed by small non-coding RNA (sRNA) molecules, which provide the effector complexes with instructions on the mode of action and the sequence specificity of the target RNA molecules (Baulcombe, 2022; Brodersen & Voinnet, 2006).

As a rule, RNA silencing is triggered by a double-stranded RNA (dsRNA), which may be either bimolecular or single-stranded "hairpin" RNA of exogenous origin, like viruses or derived from endogenous loci. In some cases, however, the trigger is an "aberrant" RNA, lacking itself dsRNA structure, though because of specific characteristics may be targeted by RNA dependent RNA polymerase (RdRp, called RDR in eukaryotes) and converted into dsRNA (Wassenegger & Krczal, 2006). In the first step, dsRNA is processed by Dicer endonuclease into sRNA duplexes ranging from 21 to 26 nucleotide (nt) in length (Brodersen & Voinnet, 2006; Finnegan & Matzke, 2003). Next, sRNA duplex is incorporated into Argonaute (AGO) protein to form RNA-induced silencing complex (RISC). After unwinding and separation of the sRNA duplex, one strand is removed, while remaining strand acts as a sequence-specificity determinant and guides RISC to either the cleavage or translational repression of the complementary mRNAs and thus it is called post transcriptional gene silencing (PTGS) (Iwakawa & Tomari, 2022; Meister, 2013). There is also a class of sRNAs that binds to another silencing complex and guides the methylation of the cognate DNA sequence, resulting in transcriptional gene silencing (TGS) at a specific locus (Erdmann & Picard, 2020; Sigman *et al*, 2021).

There are pathways of RNA silencing that are present in the most of eukaryotes, while others are lineage specific. In plants and many animal RNA silencing constitutes the primary immune system against viruses, which is widely accepted to be its most ancient function (Jin *et al*, 2022). However, chromatin-associated regulatory functions are arguably the most conserved among species, suggesting that RNA silencing could evolve from prokaryotic antisense RNA regulatory machinery to maintain self-genome and later specialized as a defense mechanism (Borges & Martienssen, 2015; Torri *et al*, 2022). Some unicellular eukaryotes, including *Saccharomyces cerevisiae* (*S. cerevisiae*) have lost RNA silencing during evolution, while organisms of higher organizational level, especially plants, have adapted it to the most if not all aspects of life (Olina *et al*, 2018; Torri *et al*, 2022).

In plants RNA silencing was discovered in 1990 as a co-suppression of chalcone synthase, responsible for an anthocyanin production in flowers, when extra copy of the gene was introduced in petunia. Instead of a stronger color, the disappearance of pigmentation was observed in transgenic plants (Napoli *et al*, 1990; van der Krol *et al*, 1990). Later, the expression of a viral genome fragment as a transgene in plants was shown to mediate the restriction of a particular virus infection by the same mechanism. Furthermore, this virus specific resistance could spread systemically to the distant organs leading to the recovery of the plant (Lindbo *et al*, 1993; Mueller *et al*, 1995; Palauqui,

1997). In 1999 sRNAs were described for the first time and it was confirmed that they are responsible for transgene as well as antiviral PTGS (A. J. Hamilton & Baulcombe, 1999). To date RNA silencing is recognized as an essential part of plant immunity against viruses and other pathogens, including bacteria and fungi (Qiao *et al*, 2021; J. Zhao & Guo, 2022). Moreover, through the action of different classes of endogenous sRNAs, sometimes in a cascade manner, RNA silencing is involved in vital processes, such as development, growth and adaptation to environmental changes (Bologna & Voinnet, 2014; de Felippes, 2019).

Along with RNA silencing there are other conserved RNA decay mechanisms, including RNA Quality Control system, which together maintain RNA homeostasis in a cell. In some cases, these pathways may compete for the substrate with RNA silencing machinery and limit its activity, thereby protecting a cell from potentially harmful consequences (Hung & Slotkin, 2021; Moreno *et al*, 2013).

1.1.1 Small RNAs

Small RNAs, a hallmark of RNA silencing, regulate broad range of biological processes through repression of gene expression at transcriptional and posttranscriptional levels. Based on the differences in the origin, biosynthesis pathways and molecular functions, sRNAs can be divided into two major classes: short interfering RNAs (siRNA) and microRNAs (miRNA). Plants possess an additional layer of complexity through the secondary siRNAs, which may be produced upon both siRNA and miRNA pathways (Bologna & Voinnet, 2014; Deng *et al*, 2018; Lopez-Gomollon & Baulcombe, 2022).

- **siRNAs**

In the classical RNA silencing, siRNAs derive from perfectly matched longer dsRNA of exogenous origin with the aim to eliminate potentially dangerous invading nucleic acids. The source may be a viral replication intermediate, RNA with secondary structure containing extensive dsRNA region or a transgene's aberrant transcript, converted into dsRNA by RDR enzyme (D. Baulcombe, 2004; Deleris *et al*, 2006; Ding, 2010). However, numerous siRNAs are also generated from endogenous transcripts, aimed to target host and/or pathogen gene expression or to silence reactivated transposons (). Dicer proteins process given dsRNAs into perfectly paired siRNA duplexes of specific size with two nucleotide overhangs at 3'-ends and monophosphate at 5' ends (Cullen, 2004; Schwarz *et al*, 2003). In plants, siRNAs with lengths of 21, 22 and 24 nt are produced by distinct Dicer-like (DCL) proteins and further loaded onto specific AGO proteins to exert their function. As a rule, siRNAs recognize perfectly complementary part of the target RNA or DNA and guide its silencing. While 21 and 22 nt siRNAs mainly contribute to PTGS through target mRNA degradation, 24 nt siRNAs are involved in RNA directed DNA methylation (RdDM) and subsequent TGS (Bologna & Voinnet, 2014).

During viral infection all three classes of siRNAs are produced from viral dsRNA with most abundant - 21 nt long siRNAs, responsible for the degradation of viral RNAs. The 22 nt viral siRNA are also able to guide the target RNA cleavage, however, their role in antiviral immunity is rather to strengthen the silencing response through the triggering of siRNA amplification (H.-M. Chen *et al*, 2010; Deleris *et al*, 2006).

There is a large diversity in plant endogenous siRNAs with the vast majority, especially in the case of large genomes, of 24 nt long heterochromatic siRNAs (hc-siRNAs), derived from transposons and repetitive sequences (Chen, 2009). Many other siRNAs of different biogenesis and function are generated in response to the invasion by a pathogen, such

as fungi or bacteria, or as a part of developmental program. These include 22 nt long natural antisense transcript siRNAs (nat-siRNAs), which result from processing of dsRNA formed through annealing of two complementary transcripts. NAT-transcript pairs derived from the same locus (cis-NAT transcripts), are widespread in eukaryotic genomes and are thought to regulate stress responses (Bologna & Voinnet, 2014). Another large class of plant endogenous siRNAs is represented by phased secondary siRNAs (phasiRNAs), generated from *PHAS* precursor transcripts, converted into dsRNA and processed in a certain phased manner. The most studied of them are 21 nt long trans-acting siRNAs (tasiRNA), which derive from long noncoding *TAS* transcripts and through targeting complementary mRNAs mediate numerous developmental processes (Borges & Martienssen, 2015; Bouché *et al*, 2006; Y. Liu *et al*, 2020). For instance, tasiRNAs from *TAS3* transcript, which target AUXIN-RESPONSE FACTOR 3 (ARF3) and ARF4, regulate the transition of plant from juvenile-to-adult phase (Bologna & Voinnet, 2014).

- **miRNAs**

The second major class of sRNAs is represented by miRNAs, which are found in all eukaryotes and are recognized as essential regulators of growth and development as well as environmental adaptation (Bartel, 2004; Brodersen & Voinnet, 2006; de Felippes, 2019). In contrast to siRNAs, miRNAs originate from their own genes, transcribed in the nucleus by RNA pol II into stem-loop containing precursors – primary miRNAs (pri-miRNA). Pri-miRNA undergoes two sequential processing by Drosha and Dicer proteins in animals and DCL proteins in plants, usually resulting in one mature miRNA duplex of 20-22 nt size, containing guiding strand (miRNA) and the complementary passenger strand (miRNA*), the latter is preferentially removed afterwards (Bologna & Voinnet, 2014).

The mature miRNA-RISC complex silences the complementary target mRNA either through the cleavage or translational repression. Animal miRNAs usually bind to the 3'UTR of the target mRNA, allowing mismatches in the miRNA-mRNA duplex, and inhibit its translation (Iwakawa & Tomari, 2022). In contrast, plant miRNAs through perfect base-pairing with coding region guide the cleavage of the target mRNA, although the cases of translational repression have been reported (Vaucheret, 2015). In addition to guiding primary PTGS, several plant miRNAs are able to trigger the production of secondary siRNAs which in turn guide the silencing of their target genes (Borges & Martienssen, 2015; de Felippes, 2019) or to direct DNA methylation (Bao *et al*, 2004). It has been observed in many organisms, that miRNAs have tissue- or developmental stage specific expression pattern, supporting its particular role in the regulation of vital processes (Bartel, 2004).

- **piRNAs**

There is a special class of sRNAs which associate with PIWI proteins, named PIWI-associated RNAs (piRNAs). PIWI proteins, which are found only in animals, comprise a distinct clade of Argonaute protein family. In complex with piRNAs they regulate the activity of the retrotransposons and other mobile elements in germline cells. In contrast to siRNAs and miRNAs, piRNAs are longer (26-32 nt) and derive from long single-stranded RNAs (ssRNA), transcribed from piRNA clusters. Instead of Dicer, piRNAs are sliced from their precursors by Zucchini endonuclease, specific for ssRNA. Mature piRNAs then associate with PIWI proteins and guide them to the degradation of complementary transposon transcripts (Meister, 2013; Olina *et al*, 2018).

1.1.2 Initiation step of RNA silencing

The induction of RNA silencing occurs when RNase III-type endonucleases of the Dicer family recognize dsRNA molecules and process them into perfect or near-perfect short RNA duplexes. The number of Dicer genes varies in different organisms. Drosophila, for example, encodes two Dicers: Dcr-1 and Dcr-2, specialized for miRNA and siRNA processing, respectively (Tomari *et al*, 2007). Whereas, the only human Dicer protein, located in the cytoplasm, is responsible for the biogenesis of both siRNAs and mature miRNAs from precursor miRNAs (pre-miRNA). The cleavage step of pri-miRNA to pre-miRNA in animal is performed by another RNase III endonuclease DROSHA in the nucleus (Bologna & Voinnet, 2014; Cullen, 2004; Lee *et al*, 2004).

Arabidopsis possesses 4 DCL proteins, having different affinities towards dsRNA substrates and giving rise to distinct sRNA species. Nuclear DCL1 specifically recognizes stem-loop structure of pri-miRNA and through two sequential cuts releases a single miRNA, most often of 21 nt size. DCL2 and DCL4, mainly cytoplasmic, usually act on the long dsRNA transcripts and are responsible for the accumulation of PTGS-related 22- and 21 nt long siRNAs, respectively. DCL3 in turn prefers shorter substrates, generated from heterochromatic regions of the genome and processes them into TGS-related 24 nt hc-siRNAs in the nucleus (Bologna & Voinnet, 2014; Henderson *et al*, 2006).

Numerous studies in Arabidopsis and tobacco have demonstrated that DCL proteins have hierarchical relationship and partially redundant functions. For example, DCL4 is the primary antiviral Dicer, however, when its function is compromised, DCL2 can substitute it. When DCL4 and DCL2 become saturated with the substrates, DCL3 also gets access to viral dsRNA and produces 24 nt siRNAs leading to the shift from PTGS to TGS. DCL2 and DCL4 in turn are able to replace DCL3 in RdDM pathway (W. Chen *et al*, 2018; Erdmann & Picard, 2020; Henderson *et al*, 2006; Wassenegger & Krczal, 2006).

Accurate processing of dsRNA usually requires additional co-factors including dsRNA binding proteins (DRBs) which specifically partner with distinct Dicers. In many organisms Dicers together with DRBs directly interact with effector proteins promoting the formation of RISC (Martínez de Alba *et al*, 2013; Meister, 2013). In addition, sRNAs undergo methylation at 3’ end by methyltransferase HEN1, conferring their protection from degradation (Bologna & Voinnet, 2014).

1.1.3 Effector step of RNA silencing

The main effectors of RNA silencing are proteins of AGO family, which together with sRNAs form RISC complexes to exert their function in one of several ways (Fang & Qi, 2016; Olina *et al*, 2018). Usually, eukaryotes encode multiple AGO proteins diversified in their preferences towards sRNA structural properties and the mode of action (Mi *et al*, 2008; Tomari *et al*, 2007). The core protein of the initiation step, Dicer, and its partner DRB protein often interact with specific AGO and participate in the selection of sRNA’s guide strand and its loading onto RISC, thereby they play crucial role in the sorting of sRNAs and defining their functions (Meister, 2013; Tomari *et al*, 2007).

Each AGO protein contains four domains: N-terminal, PAZ, MID and PIWI, with the last two of them thought to be responsible for diverse functions of the RISC complexes. The specificity of sRNA selection is often determined by MID domain, which accommodates 5’- terminal nt of sRNA guide strand. As a rule, 5’ end of the guide strand is thermodynamically less stable (Schwarz *et al*, 2003). PIWI domain possesses RNase activity and is responsible for target RNA cleavage and/or sRNA passenger strand removal. Some AGOs are catalytically inactive, however they still recognize the target and act

through recruiting of other components of RNA silencing machinery (Iwakawa & Tomari, 2022; Olina *et al*, 2018).

In Arabidopsis sRNAs are sorted between 10 AGOs, usually based on their length and 5'-terminal nt identity of the sRNA guide strand (Mi *et al*, 2008). AGO1 associates selectively with miRNAs and 21/22 nt siRNAs initiating with 5' uracil residue (U) and serves as a primary PTGS effector mostly through slicing of target mRNAs. Therefore, it is not surprising that plant miRNAs have evolved a strong bias towards 5' U (Mi *et al*, 2008). AGO5 and AGO2 also bind 21/22 nt sRNAs, but preferentially harboring 5' cytosine (C) or adenine (A), respectively. Whereas most miRNAs in plants bind to AGO1, miR390 has been found to specifically bind AGO7 and through double targeting of non-coding *TAS3* transcript trigger the generation of tasiRNAs, which in turn regulate the auxin-signaling pathway (Adenot *et al*, 2006; Axtell *et al*, 2006; Bologna & Voinnet, 2014; Jouannet *et al*, 2012; Montgomery *et al*, 2008). Apart from the nature of favored sRNAs, the different expression patterns of AGOs also contribute to the functional diversification of these proteins. AGO10 has been shown to bind miR165 and miR166 almost exclusively, which can also associate with AGO1. In contrast to AGO1, which accumulates in all plant tissues and directly silences the targets, expression of AGO10 is restricted to a few cell types including shoot apical meristem cells, where it functions by sequestering miRNAs from AGO1, thereby positively regulating their targets and is involved in shoot apical meristem development (Bologna & Voinnet, 2014; Meister, 2013; Zhu *et al*, 2011). Among TGS-associated AGOs, AGO4 is the major effector protein, which binds DCL3-dependent 24 nt long hc-siRNAs with a 5' A bias and is ubiquitously expressed. AGO6 and AGO9 have partially overlapping functions with AGO4 in RdDM and TGS and present specific expression patterns (Meister, 2013). In addition, AGO3 that arose from the recent duplication of AGO2 gene, has been shown to function redundantly with AGO4 in the epigenetic RNA silencing pathway by binding 5' A 24 nt siRNAs and to regulate gene expression in siliques (Jullien *et al*, 2020; Zhang *et al*, 2016).

Downstream of the RISC formation, AGO does not solely with sRNA define the outcome for the target, but rather strongly depend on the interaction partners present in close proximity. In plants, the recruitment of other components of RNA silencing, or competing for the substrate mRNA decay or translational machinery can lead either to the exonucleolytic degradation of the target, or entering the second round of RNA silencing (Hung & Slotkin, 2021; Iwakawa & Tomari, 2015, 2022). Recent study on the expression and subcellular localization of AGO proteins in Arabidopsis gametes and early embryos has uncovered a high specialization for distinct AGOs and strong asymmetry between cell types, although the significance of these differences for the most of AGOs remains to be investigated (Jullien *et al*, 2022). Altogether, AGOs' repertoires in different cell types, their subcellular localization and preferences towards specific sRNAs underlie the broad range of AGOs' functions (Voinnet, 2022).

1.1.4 Amplification of RNA silencing

In plants, nematodes and fungi RNA silencing has an additional layer of complexity regarding the action of the cellular RDR proteins, which are able to amplify the substrates for Dicer proteins, thereby enabling the second round of RNA silencing (Baulcombe, 2022; de Felippes & Waterhouse, 2020; Meister, 2013; Wassenegger & Krczal, 2006). Some RDR proteins can mediate both PTGS and RNA-mediated heterochromatin formation as in the case of *Schizosaccharomyces pombe* single RDR, Rdp1. In plants,

however, these functions are divided between multiple RDRs, though sometimes they may substitute each other.

Three out of six RDR proteins in Arabidopsis have assigned functions in antiviral RNA silencing: RDR6 and RDR1 in the case of RNA viruses, whereas RDR6 is responsible for the amplification of PTGS, RDR2 contributes to combating DNA viruses and is the key player in the TGS pathway (Baulcombe, 2004; Baulcombe, 2022). During virus infection, RDRs can have a role in both the initiation and the amplification steps. To initiate RNA silencing, RNA viruses have a highly effective substrate for DCLs in the form of dsRNA replication intermediate. DNA viruses can provide dsRNA through bidirectional transcription or via overlapping ORFs. In addition, viruses may generate aberrant RNAs derived from read-through transcription, which are considered as the predominant templates for RDRs (Ramesh *et al*, 2017; Wassenegger & Krczal, 2006). The characteristics of aberrant RNAs include the lack of 5'-cap structure or poly(A) tail or the accumulation over a certain threshold as in the case of transgenes expressed under the strong promoter (Wassenegger & Krczal, 2006). Viral RNA fragments resulting from the slicing by RISC also harbor such features and therefore serve as effective templates for RDRs, providing positive feedback loop for RNA silencing machinery and thereby reinforcing antiviral response.

It has been proved that the amplification of antiviral PTGS relies on the action of AGO1 in complex with DCL2-produced 22 nt viral siRNAs, that not only slice target RNAs but also recruit RDR6 and dsRNA binding protein SGS3. SGS3 specifically attaches to siRNA-target RNA duplex stabilizing it and probably protecting it from degradation. dsRNA generated by RDR6 is in turn processed by DCLs – predominantly DCL4 – into secondary siRNAs (sec-siRNAs), mostly 21 nt in size. As a result, siRNAs from the sequences flanking the primary target are generated. Such spread of RNA silencing, which has also been reported for worms, is called transitivity (Baulcombe, 2022; Wassenegger & Krczal, 2006).

It was initially thought that amplification of RNA silencing can be beneficial only in the context of combating virus infection, while transitive endogenous RNA silencing pathways can harm the organism through off-targeting essential genes. However, numerous studies so far have proved a crucial role of secondary siRNAs in expanding the action of primary regulatory sRNAs and conferring fast adaptation to various environmental stimuli (Bologna & Voinnet, 2014). Usually host genes targeted by 21 nt long miRNAs undergo slicing by RISC and subsequent degradation by XRN4 exoribonuclease and the exosome complex, resulting in the downregulation of a particular gene or several genes (Iwakawa & Tomari, 2015). However, the RISC loaded with 22 nt miRNA, upon slicing the target RNA, triggers the formation of dsRNA and subsequently the production of secondary siRNAs through SGS3/RDR6/DCL4 pathway. These siRNAs, mostly having phased pattern can silence in *cis*, reinforcing the initial silencing signal, or in *trans* targeting other genes, thereby creating regulatory cascade of a particular miRNA. An example of such regulatory network has been described in *Medicago truncatula*, showing that few miRNAs targeting conserved regions of some nucleotide-binding leucine-rich repeat (NB-LRR) resistance (R) genes regulate a larger number of R genes through the production of phasiRNAs that act in *cis* as well as in *trans* (de Felippes, 2019). This study also demonstrates that miRNA-pathway plays a role in plant innate immunity. Limiting the activity of R genes helps to keep the balance between growth and defense (Boccara *et al*, 2014; Cui *et al*, 2020; González *et al*, 2015; Zhai *et al*, 2011). In addition, RNA silencing amplification in plants may be initiated by endogenous

22 nt siRNAs processed by DCL2 from AGO1-RDR6-derived substrates including *TAS* and non-*TAS* transcripts. If normally DCL4 outcompetes DCL2 in the production of siRNAs from the endogenous substrates, various stresses including nitrogen starvation, high salinity and abscisic acid treatment induce the accumulation of DCL2-dependent 22 nt siRNAs. These siRNAs in complex with AGO1/SGS3/RDR6 cause the second wave of siRNAs via slicing-independent translational repression, both gene-specific and global, probably through stalling the ribosomes. These findings suggest an important role of transitive RNA silencing in mediating stress responses, reprogramming a plant from growth to defense (Wu *et al*, 2020).

In plants the action of RDR proteins is of special importance for RdDM – epigenetic RNA silencing pathway leading to TGS. The vast majority of TGS-related 24 nt hc-siRNAs derive from the transcription of heterochromatic regions by plant specific DNA dependent RNA polymerase IV (POL IV) into 30-45 nt single stranded RNAs (ssRNA) and their subsequent conversion into dsRNA by RDR2. After been processed by DCL3 and methylated by HEN1, hc-siRNA is exported to the cytoplasm where it associates with one of AGO4 clade proteins to form RISC. In AGO-bound state hc-siRNA returns to the nucleus where it guides the RISC onto the complementary part of RNAs transcribed by another plant specific RNA polymerase V (POL V) that recruits DNA methyltransferases to the cognate DNA sequence (Erdmann & Picard, 2020; Olina *et al*, 2018; Ye *et al*, 2012)). The major function of the canonical RdDM is to maintain and reinforce pre-existing DNA methylation thereby keeping the silent state of particular regions over the generations. However, novel TE insertions that are usually actively expressed or viruses and vast inserted transgenes can be also targeted by PTGS followed by *de novo* DNA methylation via non-canonical RdDM RDR6-DCL4-AGO1 pathway. Once the initial establishment of silent state occurs, the canonical RdDM through Pol IV-RDR2-DCL3 takes over the long-term maintenance of silencing. In addition, a recent study has demonstrated that siRNA-AGO4/POL V complex can establish *de novo* DNA methylation at cytosines in the region lacking pre-existing chromatin marks (Sigman *et al*, 2021).

1.1.5 Non-cell autonomous RNA silencing

Another fascinating aspect of RNA silencing in plants and some animals including nematodes, is its non-cell autonomous nature. When TGS or PTGS is induced in a single plant cell the mobile signal can travel intercellularly on short distances and systemically throughout the plant and initiate silencing in the recipient tissues. In plants most likely mobile signal molecules migrate symplastically: from cell to cell via plasmodesmata (PD) channels, and over long distances via phloem in a photosynthetic “source” to “sink” direction. However, other routes including vesicle-mediated, that is common for animal have been proposed for trans-species spread of silencing reported for several plant-pathogen interaction (Liu & Chen, 2018; Maizel *et al*, 2020).

Genetic requirements for short-range and systemic silencing as well as the precise nature and the structure of mobile signal have been widely studied over past few decades. Most recent studies have proved that all siRNAs and many miRNAs independently of size and sequence are capable to move between cells and over long distances (Devers *et al*, 2020). Unlike siRNAs that rather move by passive diffusion, miRNA mobility is thought to be more complicated and probably occurs via multiple parallel pathways. Moreover, along with mature miRNA, its precursors are also mobile and may exert their function at a distance (Brosnan *et al*, 2019). According to the latest model, sRNAs move in AGO-unbound duplex structure from the silencing inducing cells

to the silencing-receiving cells where one of the siRNA strands associates with AGO to form competent RISC and to silence the target (D. C. Baulcombe, 2022; Devers *et al*, 2020; Voinnet, 2022).

In the context of transgene silencing DCL4-dependent 21 nt siRNAs serve as a mobile signal that is found to be necessary and sufficient for the spread of PTGS over 10–15 cell layers. This short-range silencing spread is independent of the amplification step, however in some cases when RDR6/SGS3/DCL4 produce secondary siRNAs in the recipient cells – the process strongly enhanced by DCL2-dependent 22 nt siRNAs – silencing signal may spread further (10-15 cell layers). Such "extensive" short-range silencing spread can be observed in the upper leaves of GFP-transgenic *N. benthamiana* plants after agrobacterium-based induction of GFP silencing in the lower leaves (Himber, 2003; Kalantidis *et al*, 2008; Parent *et al*, 2015). Moreover, systemic silencing spread via reiterating cell-to-cell instead of phloem-dependent signal movement has been observed in Arabidopsis seedlings occurring against photoassimilates' flux (Liang *et al*, 2012). The short-range spread of transgene-induced silencing is reflected in the endogenous RNA silencing mediated by 21 nt miRNAs and ta-siRNAs. These sRNAs migrate from the site of their production over a similar distance as primary siRNAs do, gradually diluting and creating the expression gradients of target genes involved in developmental programs (Pyott & Molnar, 2015). Similarly, DCL3-dependent 24 nt siRNAs travel to the adjacent cells and in complex with AGO4 induce chromatin silencing of target genes. Moreover, it has been demonstrated in Arabidopsis that transgene-derived as well as endogenous 24 nt siRNAs are graft-transmissible and initiate epigenetic genome modification in the recipient cells, indicating that 24 nt siRNAs act also as systemic signal for TGS (Brosnan *et al*, 2021; Molnar *et al*, 2010). Interestingly, in the early studies of GFP transgene silencing in *Nicotiana benthamiana* system the abundance of 24 nt siRNAs in the silencing-inducing cells showed strong correlation with systemic spread of GFP silencing, suggesting a role as a mobile signal for PTGS (A. Hamilton, 2002). Although strong bias towards 24 nt siRNAs in the "sink" tissues of grafted Arabidopsis supported this idea (Molnar *et al*, 2010), recent studies showing distinct results have proposed an elegant explanation for this effect (Devers *et al*, 2020). Whether a particular sRNA reaches the target tissue strongly depends on the presence of specific AGO proteins in the traversed tissues, which tend to associate with sRNAs entering the cell in a size and 5' terminal nt-dependent manner. Because of significantly higher expression levels of AGO1 compared to AGO4 in most cell types, the higher abundance of 24 nt siRNAs in systemically silenced tissues can be explained by selective sequestering of 21/22 nt siRNAs with uracil at the 5' terminus by AGO1, preventing them from entering the phloem (Devers *et al*, 2020; Voinnet, 2022). Consistently, only miRNAs that are highly expressed or produced in cells close to the phloem have the potential to move and function at long distances (Brioudes *et al*, 2021; Brosnan *et al*, 2019). The functional relevance of systemic silencing spread is performed, for example, by miRNAs regulating physiological adaptation to environmental changes (Pyott & Molnar, 2015).

Whereas most of the endogenous genes tend to escape the amplification of silencing, in the case of transgenes, viruses and phasiRNA targets, an extensive systemic silencing spread may occur. Recent forward genetic screen on the transmission of silencing mutants in Arabidopsis has demonstrated that DCL2 is crucial for efficient systemic PTGS in both silencing inducing rootstock and signal receiving shoot, probably through stimulating RDR6-dependent sec-siRNA production, that increase the level of total siRNAs (Taochy *et al*, 2017). In parallel, research using transgenic *N. benthamiana* DCL

RNAi lines showed that suppression of DCL2 in the recipient tissues reduces the systemic spread of GFP silencing, supporting the role of DCL2 in RDR6-amplified systemic PTGS (W. Chen *et al*, 2018). In addition, several components of TGS including PolIV, RDR2, DCL3, 24 nt siRNAs and to some extent AGO4 are required for the reception of mobile signal in the systemic tissues and for further propagation of PTGS, probably by providing uncapped transcripts as substrates for RDR6-dependent amplification step (Brosnan *et al*, 2007). Interestingly, extensive spread of systemic silencing in plants is usually a rare event and is mostly associated with various stresses. Presumably due to the challenges of sessile life, plants have evolved this mechanism to efficiently react to environmental changes (Wu *et al*, 2020).

1.1.6 Suppressors of RNA silencing

Since RNA silencing has proved to be an important antiviral mechanism in eukaryotes it is not surprising that most if not all viruses have evolved suppressors to counteract this immune response. Viral suppressors of RNA silencing (VSR), often discovered as virulence factors, are usually multifunctional proteins that are highly diverse in the structure and their mode of action (Lopez-Gomollon & Baulcombe, 2022).

Many VSRs of plant viruses operate through direct binding of DCL product, like in the case of well-studied tombusviral P19 protein and potyviral helper component proteinase (HC-Pro) that prevent loading of the primary antiviral AGOs – AGO1/2 by selectively sequestering 21/22 nt siRNAs (Jin *et al*, 2022). Another strategy utilized by VSRs is to bind and block the activity of the core effectors of RNA silencing including AGO and RDR proteins. Moreover, there are examples of VSRs that affect multiple steps of the pathway. This is the case for Cauliflower mosaic virus (CMV) 2b protein, that in addition to binding siRNA duplexes, interacts with and blocks ribonuclease activity of AGO proteins and also affects the systemic spread of RNA silencing (Baulcombe, 2022; Jin *et al*, 2022). Since suppression of RNA silencing significantly benefits viruses it is not surprising that some of them evolved more than one VSR that may differ in their strength and the way of action as well. For example, Cocksfoot mottle virus (CfMV) studied by our group encodes two VSRs that function independently: the primary one – P1 protein – and the coat protein (CP) that in addition to structural function has also suppressor activity, although weaker than P1 (Olspert *et al*, 2014). The mechanism of action of these two sobemoviral suppressors has not been elucidated yet. Often the action of VSRs is not limited to inhibiting antiviral pathway but also contributes to the endogenous gene regulation via suppression of miRNA pathway and/or modulating hormone signaling and protein-based plant immunity, explaining the onset of various symptoms. In addition, some VSRs have evolved to hijack the host-encoded suppressors, that in natural conditions are aimed to restrict or fine-tune RNA silencing. For instance, potyviral suppressor HcPro induces the expression of and interacts with tobacco calmodulin-like protein, rgs-CaM – the first discovered endogenous suppressor of silencing in plants (Anandalakshmi *et al*, 2000; Lopez-Gomollon & Baulcombe, 2022).

Because of high amplification potential RNA silencing is not always appropriate RNA degradation mechanism and therefore it is a subject to the regulation by a cell itself. Among plant endogenous suppressors of RNA silencing identified so far there are multiple components of highly conserved 5’>3’ exoribonuclease (XRN) and 3’>5’ exosome RNA decay pathways. These include cytoplasmic XRN4, nuclear XRN2 and XRN3 enzymes and exosome co-factors SKI2, SKI3, SKI8 that degrade aberrant transcripts lacking 5’-cap structure and 3’-poly(A) tail, respectively (Gazzani *et al*, 2004; Gy *et al*, 2007; Yu *et al*,

2015). Since the prerequisite of RNA decay is deadenylation followed by decapping of the transcript, several proteins involved in these processes have also been found to inhibit RNA silencing (Hung & Slotkin, 2021; L. Liu & Chen, 2016). Another plant RNA silencing suppressor is RNase III-like 1 (RTL1) that degrades the substrates of DCLs, dsRNA, thereby repressing siRNA production (Shamandi *et al*, 2015). In addition, *Arabidopsis thaliana* ABCE2 (AtABCE2), has been shown by our group to suppress transgene RNA silencing and its long-distance spread in *N. benthamiana* plants (Sarmiento *et al*, 2006).

1.2 RNA Quality Control system

There are a number of mechanisms beyond RNA silencing that are responsible for the maintenance of RNA homeostasis in eukaryotic cell. Nonsense-mediated decay (NMD), No-go decay (NGD) and Non-stop decay (NSD) are highly conserved translation-coupled mRNA decay pathways collectively referred as RNA Quality Control (RQC) or mRNA surveillance system that inspect all mRNA species and selectively eliminate aberrant ones to prevent their accumulation and/or translation into dysfunctional proteins. These pathways are usually activated during the pilot round of translation when different features of mRNAs are recognized by specific effector complexes (L. Liu & Chen, 2016; Shoemaker & Green, 2012).

NMD degrades mRNAs containing premature termination codon (PTC) that can be discriminated from authentic stop codon by downstream NMD *cis*-elements including unusually long 3'-UTR and those containing introns. Inefficient translation termination caused by these mRNA features, recruits NMD complex that ultimately leads to the rapid decay of mRNA. In yeast and human NMD-associated mRNA decay starts from deadenylation followed by removal of 5'-cap structure and degradation from the ends by exosome and XRN exoribonucleases in 3'>5' and 5'>3' directions, respectively. Alternatively, in drosophila NMD induces the cleavage of targeted mRNA close to PTC followed by the decay of mRNA fragments without deadenylation and decapping (Kerényi *et al*, 2008). NMD has additional role in regulating stability of normal mRNAs that do not possess PTC, for example those resulting from alternative splicing or containing upstream ORF (uORF) (Shoemaker & Green, 2012). Moreover, RNA viruses that often contain internal termination codons and long 3' UTRs in their transcripts have been shown to induce NMD, indicating its role in the restriction of viral infection (Garcia *et al*, 2014).

NGD and NSD pathways are closely related since both target mRNAs that possess features causing ribosome stalling. NGD is induced by mRNAs that contain translation elongation inhibitory structure like stable stem-loop, rare codons, or long stretch of A residues characteristic for poly(A) tail, whereas NSD is responsible for the elimination of transcripts lacking in-frame stop codons. These NSD substrates are of two types: truncated stop codon-less transcripts and mRNAs lacking stop codon but polyadenylated (Szádeczky-Kardoss, Gál, *et al*, 2018). In the case of NSD ribosome runs to the 3' end of the template where stalls, however it has been also suggested for the latter type of non-stop mRNAs that ribosome stalling may occur during translation of poly(A) tail resembling the case of NGD. In both pathways mRNA undergoes endonuclease cleavage right upstream of the stalled ribosome, which is recognized by non-canonical ribosome release factors Pelota (Dom34 in yeast) and Hbs1, homologs of eukaryotic release factor 1 (eRF1) and eRF3, respectively. Together with ribosome recycling factor ABCE1, Pelota and Hbs1 mediate the dissociation from mRNA and recycling of stalled ribosomes (Pisareva

et al, 2011). Like NMD substrates, the fragments of cleaved mRNA or truncated non-stop mRNA are rapidly degraded through the exosome and XRN pathways (Szádeczky-Kardoss, Gál, *et al*, 2018). These two general pathways of mRNA decay are responsible for the exonucleolytic degradation of most functional as well as aberrant mRNAs in the cytoplasm. In plants cytoplasmic exosome operates in complex with co-factors SKI2, SKI3 and SKI8 and is responsible for the elimination of 5'-cleavage fragments (Lange & Gagliardi, 2022; L. Liu & Chen, 2016). Interestingly, in plants and Drosophila RISC-derived 5' cleavage fragments are substrates for NSD that may be targeted by exosome:SKI complex (Hashimoto *et al*, 2017; Szádeczky-Kardoss, Csorba, *et al*, 2018).

It has been long accepted that in plants RNA silencing is the primary RNA degradation mechanism for nucleic acids of exogenous origin, with rare exceptions for the endogenous transcripts, while RQC mainly drives the degradation of endogenous aberrant mRNAs. However, multiple research have shown that these pathways are linked functionally as well as spatially (reviewed by L. Liu & Chen, 2016). The findings, that siRNA/miRNA-guided RISC cleavage products are substrates not only for RDR6-dependent amplification of RNA silencing but also for NSD-mediated decay demonstrate that these systems are not independent but rather work cooperatively (Szádeczky-Kardoss, Csorba, *et al*, 2018). Another interesting fact is that core components of NMD complex have been found to localize in both: processing bodies (P-bodies) where mRNA decay occurs and siRNA bodies – the place of RDR6 and SGS3 localization. These cytoplasmic granules in turn often co-localize suggesting highly dynamic and competitive relationship between RQC and RNA silencing (Moreno *et al*, 2013). Finally, it has been demonstrated that impairment of core factors involved in mRNA decapping, deadenylation or exonucleolytic decay enhances RDR6-dependent PTGS response and causes developmental defects. According to the current model RNA silencing has access to aberrant RNAs only when RQC is saturated or impaired. This suggests that RQC system is the first line of defense against faulty mRNAs and restricts RNA silencing amplification (L. Liu & Chen, 2016).

1.3 Evolutionary highly conserved ABCE proteins

ATP-binding cassette (ABC) proteins comprise a large and diverse ATPase superfamily, which is represented mainly by transmembrane transporters, although there are also soluble enzymes that participate in other processes, including DNA repair and translation. ABCE proteins, members of sub-family E, are one of the most evolutionarily conserved proteins present in all archaea and eukaryotes, but not in bacteria (K.-P. Hopfner, 2012; Kerr, 2004). Most species have a single *ABCE* gene, usually named *ABCE1.* In all organisms tested so far inactivation of *ABCE1* gene leads to lethality at early developmental stage or to severe morphological abnormalities, indicating its crucial role for viability (Kispal *et al*, 2005; Navarro-Quiles *et al*, 2018; Petersen *et al*, 2004; Yu *et al*, 2023). However, many plants, including *A. thaliana*, and several animals possess two or more *ABCE* paralogs, that likely have arisen from duplication events, the significance of which remains yet unexplored (Gong & Wang, 2022; Navarro-Quiles *et al*, 2018).

ABCE1 is a soluble, mainly cytoplasmic protein that is implicated in diverse biological processes (Figure 1). Since ABCE1 lacks transmembrane domains (TMD), it performs functions other than transport of molecules across membranes. Initially, ABCE1 was described as RNase L inhibitor (RLI), a negative regulator of 2'-5' oligoadenylate synthase /RNase L system that is part of an antiviral interferon (IFN) response in mammals (Bisbal *et al*, 1995). Upon activation by long dsRNA RNase L destroys viral and cellular ssRNAs, thereby contributing to the suppression of viral replication as well as to the global protein

synthesis and cell proliferation (Le Roy *et al*, 2001; Salehzada *et al*, 2009). Through direct interaction with RNase L ABCE1 blocks its RNA degradation activity. However, in contrast to ABCE1 that is widely distributed in evolution, RNase L is found almost exclusively in tetrapods, therefore this function could not explain the significance of ABCE proteins (Braz *et al*, 2004).

Later, plant ABCE protein was found to suppress another dsRNA-induced RNA degradation mechanism that is conserved across eukaryotes, namely RNA silencing. The first connection to this process was observed as a slight but statistically significant increase in the expression of AtABCE2 (also named AtRLI2) in transgenic Arabidopsis plants exhibiting PTGS (Braz *et al*, 2004). Further research demonstrated that AtABCE2 reduced siRNA accumulation and systemic spread of GFP silencing when overexpressed in GFP-transgenic *N. benthamiana* plants (Sarmiento *et al*, 2006). The mechanism of its action remains elusive as far as it is not clear whether AtABCE2 interacts with components of RNA silencing machinery or acts indirectly through promoting other processes.

Extensive research performed in archaea, yeast and animal has revealed a role for ABCE1 in such a fundamental process as mRNA translation. Through interaction with different translation factors and ribosomes ABCE1 is involved in multiple steps of protein synthesis in an ATP-dependent manner. It has been shown that *S. cerevisiae* ABCE1 (also named Rli1) and its human orthologue interact with 40S ribosomal subunit, polysomes and translation initiation factors eIF2, eIF3 and eIF5 promoting assembly of preinitiation complex. Consistently, depletion of ABCE1 resulted in significant decline in translation initiation rate and decrease in polysomal content and average size (Z. Chen *et al*, 2006; Dong *et al*, 2004; Toompuu *et al*, 2016). In addition, yeast ABCE1 has been found to participate in the biogenesis and transport of ribosomal subunits. These findings are consistent with ABCE1 partial localization in the nuclei of yeast and human cells (Kispal *et al*, 2005; Toompuu *et al*, 2016; Yarunin *et al*, 2005). Further, yeast ABCE1 was found to interact with translation termination factors eRF1 and eRF3 suggesting a role in the termination step (Khoshnevis *et al*, 2010). Interestingly, termination and subsequent ribosome recycling in bacterial translation are two separate steps with distinct factors involved. In contrast, these stages are combined in one release factor-mediated process in archaea and eukaryotes (Shoemaker & Green, 2011). Here, ABCE1 is recruited to the post-termination complex (PTC) and together with eRF1 (aRF1 in archaea) dissociates ribosomes into small 30S/40S and large 50S/60S subunits free for a new round of translation (Barthelme *et al*, 2011; Pisarev *et al*, 2010). The role of eRF3, that is absent in archaea, is assumed to prevent premature association of ABCE1 with PTC (Pisarev *et al*, 2010). Moreover, ABCE1 in complex with Pelota, paralog of eRF1, splits empty ribosomes and stalled ribosomal complexes, activating RQC pathways (Becker *et al*, 2012; Kashima *et al*, 2014; Pisareva *et al*, 2011). Notably, ABCE1 has been found to dissociate 80S-like ribosomes containing pre-40S subunit during ribosome maturation providing a link between ribosome recycling and biogenesis (Strunk *et al*, 2012). Altogether these finding indicate a universal function for ABCE1 as a ribosome recycling factor in both kingdoms and likely explain its exceptional conservation sharing more than 45% of amino acid sequence identity across species (Braz *et al*, 2004).

As ABCE1, several initiation factors have dual role in translation initiation and ribosome recycling. In particular, subunit j of eIF3 (eIF3j) acts as an accessory factor for ABCE1-mediated dissociation of 40S subunit from mRNA (Young & Guydosh, 2019). Recent structural research of mammalian translation initiation complex suggests that post-recycling 40S subunit enters a new round of translation in ABCE1-bound state.

ABCE1 remains associated to 40S subunit till the late-stage initiation phase after which elongation-competent complex is formed by joining 60S subunit. According to this model ABCE1 acts as a ribosomal subunit anti-association factor, which regulates the initiation step by preventing assembly of premature ribosomes (Simonetti *et al*, 2020).

ABCE1's involvement in translation has been reported for multiple organisms of different complexity including unicellular *Trypanosoma brucei*, worm *Caenorhabditis elegans* and human (Z. Chen *et al*, 2006; Estevez, 2004; Z. Zhao *et al*, 2004). ABCE1 mutants exhibit growth and cell division arrest and developmental alterations reminiscent of those caused by mutations in other components of translational machinery (Navarro-Quiles *et al*, 2018). In contrast to other lineages, plant ABCE proteins have been much less studied and only very recently their involvement in translation was confirmed (Navarro-Quiles *et al*, 2022).

AtABCE2, one of the two *A. thaliana ABCE* genes, is the one that is ubiquitously expressed during whole plant lifecycle and is therefore assumed to preserve the conserved functions of ABCE proteins (Braz *et al*, 2004; Navarro-Quiles *et al*, 2018). In contrast, AtABCE1 sharing 81% of amino acid sequence identity with AtABCE2, is present almost exclusively in generative organs like siliques and flowers. It was suggested that because ABCE1 evolves more rapidly, it has been partially defunctionalized although it is able to substitute AtABCE2 in rosette leaves if overexpressed (Navarro-Quiles *et al*, 2022).

Recently it was shown that as in other organisms, null mutant of AtABCE2 is lethal, while its hypomorphic mutant *Api7-1* caused pleiotropic morphological alterations in the vegetative part of the plant (Navarro-Quiles *et al*, 2022). The phenotype of *Api7-1* comprises short primary roots, main stem growth delay and small rosettes with altered venation pattern in leaves. These traits resemble auxin-related defects in the mutants of ribosomal proteins or ribosome biogenesis factors (Byrne, 2009; Rosado *et al*, 2012). Furthermore, for the first time, physical interactions with ribosomal proteins and translation factors – including eIF3j subunit – were confirmed for AtABCE2, strongly suggesting its conserved role in translation. In addition, perturbations in auxin metabolism and transport, that are important for leaf venation, were reported for *Api7-1* plants (Navarro-Quiles *et al*, 2022). Previously, similar auxin-associated defects were observed in ABCE2 mutant of *Cardamine hirsuta*, a close relative of Arabidopsis (Kougioumoutzi *et al*, 2013). Another recent research has demonstrated that the knock-down of AtABCE2 leads to the reduction in abundance of ribosomal subunits and downregulation of numerous auxin-related genes. Moreover, loss of function mutation of AtABCE2 impairs female gametophyte and embryo development that strongly depend on auxin signaling pathway (Yu *et al*, 2023).

Altogether, these findings suggest that plant ABCE proteins are involved in translation and influence development indirectly via effect on auxin homeostasis. However, it cannot be ruled out that other functions of ABCE proteins also contribute to the observed phenotypes in plants.

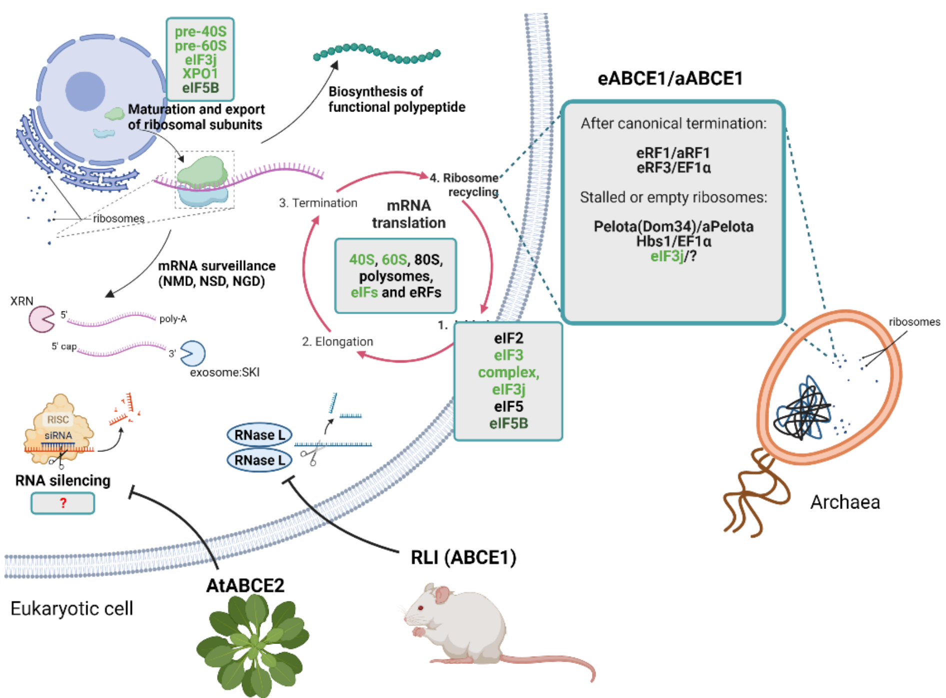

Figure 1. Functions of ABCE proteins. *ABCE proteins are involved in diverse processes associated with RNA homeostasis and protein synthesis (letters in bold). The most conserved function of ABCE proteins is in ribosome recycling: by splitting ribosomes into subunits ABCE1 assists biosynthesis of functional proteins as well as degradation of aberrant mRNAs through mRNA surveillance pathways. Additionally, in mammals ABCE1 interacts with RNase L and inhibits its RNA degradation activity, whereas plant AtABCE2 is recognized as RNA silencing suppressor. Molecular partners of ABCE1/2 that have annotated functions in the corresponding processes are listed in the boxes: interactors identified for Arabidopsis ABCE1/2 and for orthologous proteins (light green letters), only for AtABCE1/2 (dark green letters), or for other than plant ABCEs (black letters). The figure was created using BioRender.com.*

1.3.1 Structure and conformational dynamics of ABCE1

Typical ABC enzyme contains a pair of linked nucleotide binding domains (NBD) that form a catalytic core for ATP binding and hydrolysis and one or several TMDs that utilize ATP energy to translocate specific compounds across membranes. ABCE1 is an unusual member of ABC superfamily which along with twin-NBD cassette contains unique domains for ABC-ATPases: bipartite hinge domain and N-terminal iron-sulfur (FeS) cluster domain, required for its specific functions (Figure 2A, 2B).

The first insight into the architecture of ABCE proteins emerges from crystal structures obtained for several archaeal orthologues of ABCE1 (aABCE1) (Karcher *et al*, 2005). Regarding exceptionally high conservation in both amino acid sequence and domains' organization this knowledge can be extrapolated to their eukaryotic counterparts. Crystallization of P*yrococcus furious* (*P. furious*) ABCE1ΔFeS – ABCE1 lacking FeS cluster domain – in the presence of Mg^{2+}-ADP revealed that NBD1 and NBD2 are arranged in a head-to-tail orientation forming a V-shaped cavity with two composite active sites at the interface (Karcher *et al*, 2005).

Each NBD contains three strongly conserved sequences required for ATP binding and hydrolysis: Walker A (WA) and Walker B (WB) motifs, typical for many ATPase families, and ABC-specific signature (C-signature) motif (Karcher *et al*, 2005; Kerr, 2004). WA contains primary ATP and ADP binding P-loop, whereas WB is implicated in ATP hydrolysis coordinating Mg^{2+} ion and polarizing the attacking water molecule. Invariant LSGGQ sequence of C-signature has been shown to bind ATP-γ-phosphate thereby serving as a secondary binding site for a nucleotide. Both NBDs possess several additional motifs containing conserved key residues, namely H-loop, Q-loop and Y-loop (also named aromatic loop), that contribute to the catalytic activity of most ABC proteins (Braz *et al*, 2004; K. Hopfner, 2003) (Figure 2A).

Each active site is composed of WA, WB, and Q-loop from one NBD and C-signature from the opposite one. ATP binding in both active sites induces clamp-like motion of twin-NBD cassette which acquires a fully closed conformation with two occluded nucleotides; subsequent hydrolysis of ATP restores the V-shaped opened state. These catalytic cycle-mediated conformational rearrangements have been shown to pass to the associated domains and/or interaction partners (Karcher *et al*, 2005; Nürenberg & Tampé, 2013).

Despite overall similarity between NBD1 and NBD2, several lines of observations have demonstrated an unusual for ABC-ATPases structural and functional asymmetry of the two active sites. First, NBD1 but not NBD2 contains highly conserved helix-loop-helix (HLH) insertion, that often mediates interactions with nucleic acids in other proteins (Hopfner, 2012). Secondly, Y-loop that participates in nucleotide binding by accommodating adenine base is degenerated in NBD2 and this probably impacts ATP binding at active site II. Moreover, biochemical analysis of *Sulfolobus solfataricus* (*S. solfataricus*) ABCE1 catalytic activity has revealed that active sites I and II have different rates of ATP turnover. Accordingly, in the case of impaired ATP hydrolysis at site I ABCE1 has half lower efficiency, while equivalent mutations in active site II result in a 10-fold higher ATPase activity suggesting an essential regulatory function of active site II (Barthelme *et al*, 2011; Nürenberg-Goloub *et al*, 2018).

A significant role for structural architecture and dynamics of ABCE1 lies on the hinge domain that aligns twin-NBD cassette from the opposite side of active site cleft. Hinge domain is composed of hinge I and hinge II subdomains, two conserved regions located between NBDs and in the C-terminus, respectively. In the tertiary structure hinge I and hinge II are interconnected via hydrogen bond network formed by highly conserved arginine residues (R cluster). Through hydrophobic interactions hinge is tightly associated with both NBDs serving as a framework for proper twin-NBD cassette arrangement and is involved in ATP-driven clamp-like motion (Karcher *et al*, 2005; Nürenberg-Goloub *et al*, 2020).

The most fascinating feature of ABCE proteins is an unusual FeS cluster domain located in the cysteine-rich N-terminal region. FeS clusters constitute an ancient and versatile class of inorganic cofactors that engage in diverse cellular processes including metabolic reactions, photosynthesis, genome maintenance and protein synthesis. In many proteins FeS clusters mediate electron transfer in redox reactions, while in others they operate as sensors of environmental and intracellular cues or activate substrates; in rare cases FeS clusters simply fulfill a structural role (Johnson *et al*, 2005).

FeS cluster domain of ABCE1 is composed of two motifs with eight invariant cysteine residues that coordinate two non-equivalent diamagnetic $[4Fe\text{-}4S]^{2+}$ clusters. One, containing cysteine in positions 4-7, is of bacterial ferredoxin-type, while the other one

(cysteine in positions 1–3, 8) contains a helical insertion that is unique for ABCE1 (Barthelme *et al*, 2007; Karcher *et al*, 2008). Structural analysis of *S. solfataricus* ABCE1 coupled to the functional analysis in yeast have demonstrated that six of eight cysteine ligands are critical for the assembly of FeS clusters and for cell viability, indicating their significance for ABCE1 function. Notably, ABCE1 in all eukaryotes has an extra conserved cysteine residue in FeS cluster domain, that can substitute the missing or mutated ligand (Barthelme *et al*, 2007). Crystal structure resolved for *Pyrococcus abyssi* (*P. abyssi*) ABCE1 (pabABCE1), revealed that both clusters are situated in the core of FeS cluster domain shielded from the surrounding solvent by the hydrophobic cocoon, which is likely responsible for their stability under low redox potential. Accordingly, FeS cluster domain in ABCE1 is unlikely associated to electron transfer, having rather structural or regulatory function (Barthelme *et al*, 2007; Karcher *et al*, 2008).

In the open state, ABCE1 FeS cluster domain is positioned at the lateral opening of the active site cleft between NBDs and is directly linked to the outside of NBD1 via polar interactions with its Y-loop (Karcher *et al*, 2008). However, unlike hinge domain, which is inseparable from ATPase unit of ABCE1, FeS cluster domain is not required for folding and for structural integrity of the twin-NBD cassette. Moreover, depletion of FeS cluster domain in *S. solfataricus* ABCE1 das not influence its ATPase activity (Barthelme *et al*, 2011). Connected to NBD1 via flexible linker FeS cluster domain is able to undergo significant rotation and make contacts with NBD2 upon ATP binding, assuming therefore a role in mediating physical interactions with molecular partners in an ATP-dependent manner (Heuer *et al*, 2017; Karcher *et al*, 2008; Kiosze-Becker *et al*, 2016; Nürenberg-Goloub *et al*, 2020).

Structural snapshots during ribosome recycling together with strategic mutagenesis provided a model for molecular mechanism of ABCE1. It has been shown that ABCE1 in a semi-closed conformation interacts with either terminated or stalled ribosomes associated with release factor eRF1/aRF1 or its paralogue Pelota/aPelota, respectively, forming pre-splitting complex (pre-SC) (Becker *et al*, 2012; Nürenberg-Goloub *et al*, 2018; Preis *et al*, 2014). At this step one nucleotide is occluded in active site II, that is a prerequisite for ATP binding at site I and serves as checkpoint for proceeding to ribosome splitting. Subsequently, after several rounds of ATP binding/hydrolysis at active site I, NBD-dimer adopts a fully closed conformation with two occluded ATP molecules. This conformational switch forces FeS cluster domain to swing out from active site cleft into ribosome inter-subunit space causing their splitting. At the splitting stage, large ribosomal subunit and release factor dissociate, while ABCE1 remains in the closed conformation bound to the small subunit forming a post-splitting complex (post-SC). Thus, ATP binding is recognized as the motive force driving ribosome recycling, while ATP hydrolysis by ABCE1, resulting in the open conformation, is required for dissociation of post-SC which likely occurs in a late translation initiation stage with assistance of initiation factors (Simonetti *et al*, 2020). Notably, ATP hydrolysis at active site I has been shown to be not critical for this step, confirming the functional asymmetry of the two catalytic sites (Nürenberg-Goloub *et al*, 2018).

Structural studies of ABCE1-containing complexes at different steps of ribosome recycling determined the interactions of ABCE1 with release factors and ribosomal subunits. Cryo-electron microscopy (cryo-EM) reconstructions of yeast and archaeal pre-SCs on stalled ribosomes revealed that ABCE1 occupies the GTPase site of the ribosome and has extensive binding area: major contacts with small subunit are established via HLH motif and hinge domain, whereas NBD2 interacts with the large subunit.

Interestingly, FeS cluster domain in pre-SC is not involved in ribosome binding but mediates interactions with Pelota (Becker *et al*, 2012). The overall architecture and binding pattern of ABCE1 in the complex with terminated ribosome and eRF1 is remarkably similar to that in Pelota-containing pre-SC, supporting the common mechanism for ribosome recycling (Preis *et al*, 2014).

Upon transition to the post-SC, ABCE1 undergoes significant rearrangements which coincide with changes in its interaction pattern when compared to pre-SC. First, hinge I moves away from hinge II resulting in establishment of new contacts with the small subunit and opening up of hinge domain, allowing ABCE1 to adopt ATP-occluded state (Nürenberg-Goloub *et al*, 2020). HLH motif is also repositioned in post-SC, however, the most dramatic changes concern FeS cluster domain. Upon the closure of NBD-dimer, FeS cluster domain undergoes rotation of about 160° towards small subunit fitting between S12 protein and RNA helix 44 positioned in the inter-subunit space. Trajectory of this relocation suggests that FeS cluster domain collides with eRF1 which in turn pushes subunits apart (Heuer *et al*, 2017; Kiosze-Becker *et al*, 2016). Additional interactions with NBD1 and hinge I are established to stabilize FeS cluster domain in post-SC (Nürenberg-Goloub *et al*, 2020).

Recently, conformational dynamics of ABCE1 during ribosome recycling was assessed biophysically using single molecule FRET approach, which provided a refined model of its molecular mechanism. Instead of deterministic three state-model for NBD-dimer, active sites have been found to exist in distinct equilibria of open, intermediate and ATP-occluded states, whereas a ligand binding, such as ribosome or release factor, induces a shift towards the state with higher binding affinity, proposing an allosteric intra- and intermolecular cross-talk (Gouridis *et al*, 2019). Different dynamics in response to the availability of molecular partners as well as to FeS cluster domain movement, is in line with earlier described structural and functional asymmetry of catalytic sites (Barthelme *et al*, 2011; Gouridis *et al*, 2019; Nürenberg-Goloub *et al*, 2018). Altogether, these findings broaden the picture of ABCE1 behavior during ribosome recycling and suggest a general mode of action in the context of its diverse functions.

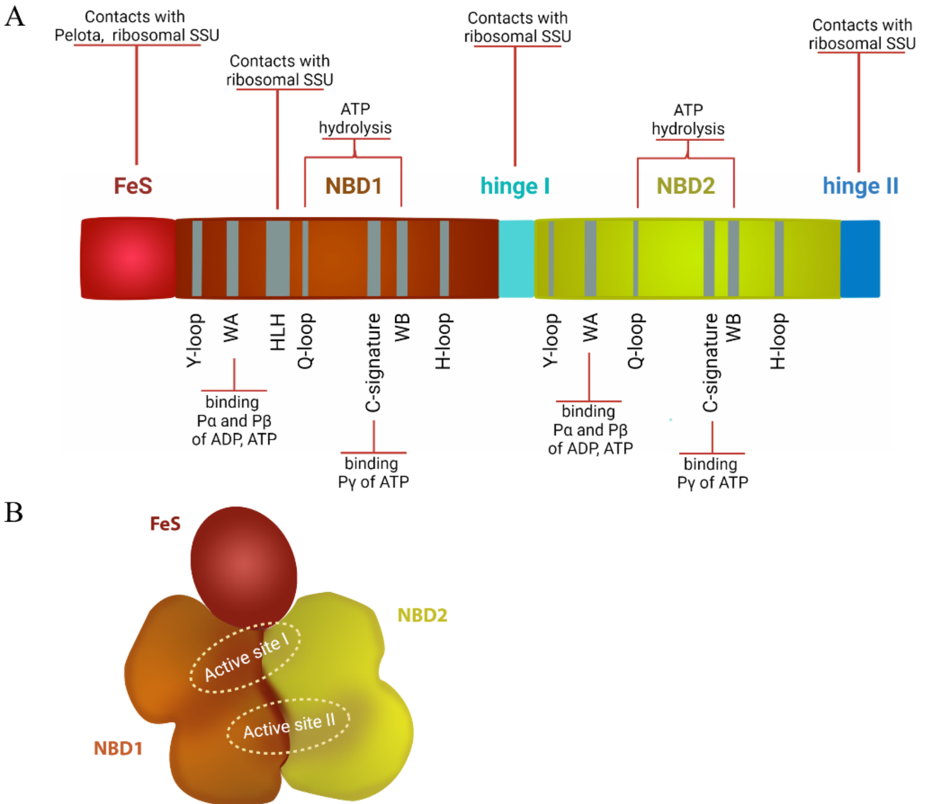

Figure 2. Structure of ABCE protein. *A) Linear structure of ABCE protein with assigned domains (colored) and conserved motifs (grey) that have annotated functions. Detailed description is in the main text. B) Schematic representation of ABCE1 tertiary structure. The figure was created using BioRender.com.*

2 Aims of the study

ABCE proteins, in most organisms represented by a sole member ABCE1, have been recognized as essential translational factors in yeast, animal and archaea, whereas data concerning plant ABCEs only recently started to emerge. The early studies in plants have found a link between ABCE proteins and RNA silencing and showed that at least AtABCE2 suppresses this highly conserved RNA degradation mechanism. Based on evolutionary conservation and importance of ABCE proteins in all organisms tested so far, the following aims for the current thesis were established:

- To investigate whether RNA silencing suppressor function of ABCE proteins in other than plant organisms is conserved.
- To study structural implications of AtABCE2 for its function as RNA silencing suppressor.
- To complement our research with phylogenetic analysis of plant *ABCE* genes.

3 Methods

Methods used in this study are described in detail in the indicated publications:

- Agroinfiltration assay and GFP imaging – Publication I, II
- Extraction of total RNA and Northern blot analysis of GFP mRNAs and siRNAs – Publication I, II
- Mammalian cell culture and transfection – Publication I
- Construction of expression vectors – Publication I
- RNAi inhibition assay in *C. elegans* – Publication I
- Western blot analysis and co-immunoprecipitation assay – Publication I
- Mass Spectrometry – Publication I
- *In vivo* GFP imaging – Publication I, II
- Cloning of AtABCE2 mutants – Publication II
- Protein structure modelling – Publication II
- Genomic and proteomic data acquisition – Publication III
- Protein and coding DNA sequence (CDS) alignments and construction of Maximum Likelihood trees – Publication III
- Assessing the impact of genomic parameters on *ABCE* gene copy number in a species using Linear regression analysis – Publication III
- Reconfirming the haplotypes of Arabidopsis ecotypes using PCR-amplification and Sanger sequencing – Publication III
- Generation of haplotype map using PopART – Publication III

4 Results and discussion

4.1 Suppression of RNA silencing is a conserved function of ABCE proteins in eukaryotes (Publication I)

In 2006, AtABCE2 was demonstrated by our group to function as a suppressor of RNA silencing (Sarmiento *et al*, 2006). Since ABCE proteins as well as RNA silencing process are strongly conserved across eukaryotes our first goal was to explore whether the suppressor function is common for ABCE proteins in other species. Therefore, we assessed suppressor activity of human ABCE1 (HsABCE1) in native and heterologous systems.

First, we tested HsABCE1 in a well-established plant system for RNA silencing-related studies–*N. benthamiana* plants, stably expressing GFP transgene (16c line). Using agroinfiltration assay we transiently overexpressed HsABCE1 together with an inducer of RNA silencing (additional copy of GFP gene) in the leaves and followed the establishment of local and systemic GFP silencing. Empty vector (pBin61) and AtABCE2 co-infiltrated with GFP were used as controls (Figure 3). The observation of the infiltrated leaf patches at five days post infiltration revealed that expression of HsABCE1, likewise AtABCE2, results in an enhanced GFP fluorescence intensity when compared to pBin61 (Publication I: Figure 1A, Supporting Figure S1.) Accordingly, Northern blot analysis of RNA from these samples showed accumulation of GFP mRNA and reduced GFP-specific siRNA levels in the case of HsABCE1 and AtABCE2 (Publication I: Figure 2A, B). Our results indicate that HsABCE1 is able to suppress local GFP RNA silencing in plants. Moreover, suppression was also confirmed when RNA silencing was enhanced by using a hairpin construct (GFFG) as an inducer (Publication I: Figure 2C, D), as well as in the context of a weaker RNA silencing response, where only ectopically expressed GFP was targeted for silencing in wild type *N. benthamiana* plants (Publication I: Figure 1B). To assess the suppression of systemic silencing we agroinfiltrated 16c *N. benthamiana* plants as described above and followed them during three weeks after infiltration. Systemic GFP silencing can be visualized under UV light as emerging red tissue in the distant leaves due to the disappearance of GFP fluorescence (A. Hamilton, 2002). At 21 dpi 78% of plants infiltrated with pBin61/GFP mixtures showed GFP silencing at the uppermost leaf, but only 31% and 41% in the case of AtABCE2 and HsABCE1, respectively (Publication I: Figure 1C). According to our results HsABCE1 suppressed systemic GFP RNA silencing in plants.

Altogether, the efficiency of HsABCE1 as a suppressor was comparable to its plant orthologue AtABCE2. The most pronounced effect of ABCEs was observed on the accumulation of 24 nt siRNAs in the infiltrated leaf patches and at systemic spread of silencing. Interestingly, early research demonstrated that the accumulation of DCL3-dependent 24 nt siRNAs at the initiation site correlated with systemic silencing, suggesting that this class of siRNAs was the primary mobile signal responsible for the spread of silencing (A. Hamilton, 2002). Therefore, we linked the strong influence of ABCE proteins on systemic silencing with a significant reduction of 24 nt siRNA levels in the infiltrated patches. However, later studies strongly supported the idea that DCL2, producing 22 nt siRNAs, through inducing RDR6-dependent secondary siRNA accumulation is responsible for efficient systemic PTGS. In contrast, DCL4 and DCL3 generating 21 and 24 nt siRNAs, respectively, have been shown to inhibit transmission of systemic silencing (W. Chen *et al*, 2018; Taochy *et al*, 2017). Considering these findings, ABCE proteins likely suppress RNA silencing at different steps and probably in distinct

subcellular compartments. Interestingly, in plants, 21 and 22 nt siRNAs generally act in the cytoplasmic PTGS pathway resulting in target mRNA degradation, while 24 nt siRNAs produced by nuclear-localized DCL3, guide chromatin methylation leading to TGS. The significant reduction of the latter class of siRNAs in the presence of ABCE protein might be due to its functioning as a suppressor in the nucleus. Partially nuclear localization of ABCE proteins was shown for yeast before our research (Kispal *et al*, 2005; Yarunin *et al*, 2005) and later also for human cells (Toompuu *et al*, 2016) and *A. thaliana* (Yu *et al*, 2023), which is in consistence with this hypothesis.

Although RNA silencing pathways in plants and animals share a common core mechanism, they still possess lineage-specific features and different potency. Therefore, our next aim was to explore whether HsABCE1 functions as endogenous suppressor in human cells. For that we co-transfected plasmids containing HsABCE1 (pABCE1-V5), human unc-51-like kinase 3 (pULK3FLAG) and ULK3-targeting RNAi construct in HEK293 cells. Tombusviral P19 protein, known to function as a suppressor of RNAi in human cells, was used as a positive control. We found that the expression of HsABCE1 led to the upregulation of ULK3 expression at both mRNA and protein levels, indicating the suppression of ULK3 RNAi (Publication I: Figure 3). Slight but statistically significant effect of HsABCE1 was comparable to the one of P19.

Next, it was of interest whether HsABCE1 is able to act as a suppressor in a heterologous animal system. Therefore, we developed an *in vivo* RNAi inhibition assay in *C. elegans*, a widely used model organism also for RNAi studies. Transgenic worms expressing GFP fused with nuclear localization signal under body wall muscle specific promoter (*unc-54::NLS::gfp*) were exposed to GFP-targeting RNAi via feeding. To test the effect of HsABCE1 on RNA silencing we generated double transgenic strains carrying, in addition to GFP, HsABCE1 sequence under body wall muscle specific promoter *myo-3*, or the known *C. elegans* RNAi inhibitor ERI-1 (Zhuang & Hunter, 2012) We found that the expression of HsABCE1 significantly enhanced GFP expression in the muscle cells when compared to the worms not expressing HsABCE1. The effect was similar to that of ERI-1 (Publication I: Figure 4). We also found that in the transgenic worms, which were not exposed to GFP RNAi, the expression of GFP was not influenced by the presence of either HsABCE1 or ERI-1. Our results indicate that HsABCE1 as well as ERI-1 suppress GFP RNAi in *C. elegans* body wall muscle cells.

Interestingly, the amplification step and non-cell autonomous nature of RNA silencing, common for plants, has been found in several animal species, including worms, but not in vertebrates. Furthermore, transmission routes of systemic silencing as well as assisting factors in plants and animals are different (Kalantidis *et al*, 2008; Zhuang & Hunter, 2012). Despite lineage-specific variations in this process, our study reveals that HsABCE1 acts as a suppressor of RNA silencing in plants, worms and human, suggesting that it targets a step that is conserved across plants and animals. However, in worms and plants HsABCE1 has shown strong suppressor activity, while in human cells the effect was weak. These results are hinting on the possibility that ABCE proteins might act at several steps of RNA silencing, one of them is likely related to the amplification and/or systemic level.

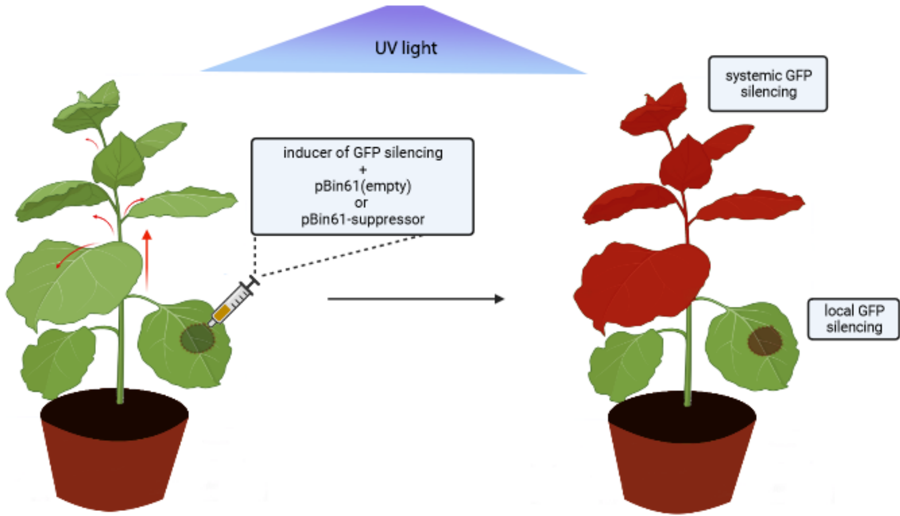

Figure 3. Schematic representation of agroinfiltration assay. *Agrobacterium tumefaciens harboring the inducer of RNA silencing (pBin61-GFP or pBin61-GFFG construct) was infiltrated together with A. tumefaciens strain containing pBin61-HsABCE1 or pBin61-AtABCE2 or pBin61 (empty vector) into leaves of GFP-transgenic N. benthamiana plants. Local GFP silencing and the spread of systemic GFP silencing were observed under UV light at 5 and 21 dpi, respectively. The figure was created using BioRender.com.*

4.2 Identification of HsABCE1 novel interaction partners (Publication I)

Multiple studies have shown that in yeast, fly and human ABCE1 protein interacts with a range of translational factors and ribosomal subunits, supporting its role in the fundamental process of protein synthesis (Z. Chen *et al*, 2006; Dong *et al*, 2004; Kispal *et al*, 2005; Yarunin *et al*, 2005). Since mechanism of RNA silencing suppression by ABCE proteins as well as binding partners related to this function were not evident, our next goal was to identify novel interacting proteins of HsABCE1.

Using co-immunoprecipitation assay we isolated V5-tagged HsABCE1 complexes with putative interactors from HEK293 cells and analyzed them by liquid chromatography combined with mass spectrometry (LC-MS/MS). From the generated list of interacting candidates, we filtered those, which have functions in RNA-associated cellular processes that are known to be interconnected with RNA silencing and thus might be of relevance to HsABCE1 suppressor function. The second criterion for the selection was the presence of homologues in *A. thaliana* and *C. elegans*. We then identified multiple putative interactors involved in transcription and epigenetic regulation, processes that are tightly linked to TGS pathway of RNA silencing. Since this study revealed strong effect of ABCE proteins on TGS-associated siRNA accumulation, it would be of interest to further assess these interactions. Several proteins related to mRNA surveillance system, that can compete in RNA degradation with RNA silencing, were also identified as putative interactors. Furthermore, components of pre-mRNA processing, that defines whether mature mRNA is translated or degraded, were found. Through these potential interactions ABCE proteins could indirectly affect RNA silencing. At that time, ABCE1 was recognized as a conserved ribosome recycling factor in archaea and eukaryotes (Nürenberg & Tampé, 2013), though in plants the involvement of ABCE proteins in

translation was beginning to emerge (Kougioumoutzi *et al*, 2013) and the specific functioning of ABCE at this step was not investigated yet. Ribosome recycling is the process that not only links translation termination with initiation, but also facilitates translation-associated mRNA surveillance pathways, including NSD, NGD NMD. ABCE1 has been shown to split ribosomes both after canonical translation termination and after Pelota-mediated stalled ribosome rescue (Becker *et al*, 2012; Pisarev *et al*, 2010; Pisareva *et al*, 2011). When our study was published, the functional involvement of fly ABCE1 in NSD was just demonstrated (Kashima *et al*, 2014) and later, it was found that the same protein is important for NMD (Hashimoto *et al*, 2017). Recently, the contribution to NMD has been observed also for HsABCE1 (Annibaldis *et al*, 2020). These discoveries supported our hypothesis concerning the indirect role of ABCE proteins as RNA silencing suppressors. Nonetheless, among the potential molecular partners of HsABCE1 we found the protein translin, a component of RNA silencing that is conserved across eukaryotes (Publication I: Figure 5). In a complex with its interaction partner TRAX, translin functions as an enhancer of RNA silencing by stimulating the degradation of siRNA passenger strand (Gupta *et al*, 2012). Further research is needed to define whether HsABCE1 suppresses RNA silencing directly through interaction with translin, or another not yet identified effector protein or indirectly via involvement in the competing RNA degradation pathways.

4.3 Mutational analysis of AtABCE2 shows that structural requirements for the suppressor function resemble those for ribosome recycling in archaea (Publication II)

To further understand how ABCE proteins suppress RNA silencing, we performed mutational analysis of AtABCE2 in the context of this function. First, we collected available data concerning structural requirements of ABCE proteins related to other roles, mainly from studies carried out in archaea and yeast (Barthelme *et al*, 2007, 2011; Karcher *et al*, 2005, 2008; Nürenberg-Goloub *et al*, 2018). As revealed from crystal structure of aABCE1, NBD1 and NBD2 are arranged by hinge domain in a head to tail orientation and contain two ATPase active sites (site I and site II), each bearing three strongly conserved motifs: WA and WB from one NBD and C signature from the opposing one (Karcher 2005, 2008). In ABC-type ATPases, WA serves as the primary ATP binding site, whereas C signature acts as secondary binding site responsible for ATP occlusion. WB motif plays a major role in ATP hydrolysis (Braz 2004). Substitution of key residues in these motifs, leads to loss of function in yeast (Karcher 2005). Moreover, functional analysis of the equivalent mutations in WB and C signature of aABCE1 confirmed the importance of these residues for ATPase activity (Barthelme 2011).

Considering high evolutionary conservation of ABCE proteins, we substituted the corresponding amino acid residues in AtABCE2 to investigate the requirement of ATP binding and hydrolysis for its suppressor function. In addition, the mutants lacking FeS cluster domain or HLH insertion in NBD1, known to be important sites for binding interaction partners, were verified in this study. To assess the effect of the chosen mutations on the ability of AtABCE2 to suppress GFP RNA silencing in plants, we employed previously described agroinfiltration assay in 16c *N. benthamiana* plants (Figure 3). Since differences in GFP mRNA levels were hardly detected for AtABCE2 mutants compared to AtABCE2 wt, local GFP silencing was evaluated as GFP siRNA accumulation and decline in GFP fluorescence intensity in the infiltrated leaf patches.

We found that AtABCE2 mutants with impaired ATP binding in active site I (WA1C2) or active site II (WA2C1) failed to suppress local and systemic GFP RNA silencing. Our results indicate that ATP binding is required for AtABCE2 suppressor function. However, when both active centers were mutated, unexpectedly AtABCE2 still exhibited suppressor activity at the systemic level (Publication II: Figures 2A, 2B, 3). This effect could not be explained in the framework of our study; however, one possible reason might be structural compensation, that may happen in response to the mutations, partially restoring ATP binding by AtABCE2.

Mutations in AtABCE2 disrupting ATP hydrolysis at active site I (WB1) and active site II (WB2) showed different impact on its suppressor function. WB2 failed to suppress GFP silencing, as double mutant (WB12) did. In contrast, WB1 was able to suppress both local and systemic GFP silencing only with slightly reduced efficiency (Publication II: Figures 3, 4A, 4B, 4C). It was of high importance, that similar functional dependence on ATP hydrolysis has been observed previously for aABCE1 in the context of ribosome recycling (Nürenberg-Goloub *et al*, 2018). In that study, ATP occlusion (C-signature) and ATP hydrolysis (WB) mutants of aABCE1 were tested at different steps of ribosome recycling. It was revealed that ATP binding at both sites is required for ABCE1 to accomplish the cycle of ribosome splitting. The separation of ribosomal subunits occurs when ABCE1 acquires fully closed conformation with two ATP molecules occluded and does not depend on ATP hydrolysis, as was also demonstrated for yeast ABCE1 (Heuer *et al*, 2017). However, ATPase activity is necessary for ABCE1 to release from post-splitting complex 30S/40S-ABCE1. Interestingly, ATP hydrolysis in site II is critical for this step, but not in site I (Nürenberg-Goloub *et al*, 2018). Based on our findings, demonstrating that only WB1 among selected ATP binding/hydrolysis mutants of AtABCE2 suppresses local and systemic GFP RNA silencing, we suggested that the mechanism of its action is similar to that of ABCE1 in ribosome recycling.

Further we explored whether FeS cluster domain and HLH insertion in NBD1 are important for the suppression of RNA silencing by AtABCE2. We have found that the mutant lacking FeS cluster domain was inactive as a suppressor. (Publication II: Figures 3, 5A, 5B). Based on the structural studies in yeast and archaea, FeS cluster domain of ABCE1 directly interacts with eRF1/Pelota release factors as well as ribosomal small subunit during ribosome recycling. Our results, showing its importance for suppressor function of AtABCE2 are in good agreement with the hypothesis, that AtABCE2 might indirectly affect RNA silencing through supporting mRNA surveillance pathways which strongly depend on ribosome recycling. However, the involvement of FeS cluster domain in suppressor function-specific interactions cannot be ruled out.

In turn, the deletion of HLH region did not ruin suppression activity of AtABCE2: the mutant was still able to suppress local GFP silencing, but not systemic (Publication II: Figures 3, 5A, 5C). In the context of ribosome recycling, HLH insertion together with Hinge domain have been found to make major contacts with 30S/40S ribosomal subunit, playing an important role in the formation of post-SC (Heuer *et al*, 2017; Kiosze-Becker *et al*, 2016). Limited effect of the deletion observed in our study suggested that HLH plays rather a supportive role for the suppressor function of AtABCE2, probably by enhancing specific interactions. Since full deletion of HLH region has not been previously studied in other systems, our results could contribute to better understand its role for the functioning of ABCE proteins.

Intriguingly, HLH deletion mutant and ATP binding double mutant (WA12C12) showed partial suppressor activity at the local and systemic levels, respectively. These observations

are tempting to assume that ABCE proteins, at least in plants, suppress RNA silencing by more than one mechanism and have different structural requirements. The recently demonstrated dual subcellular localization of AtABCE2 – nuclear and cytoplasmic – supports this idea (Yu *et al*, 2023).

Taken together, in this study we have found that structural requirements of AtABCE2 for the suppression of RNA silencing strongly resemble those for aABCE1 as ribosome recycling factor. We suggest that ABCE proteins might affect RNA silencing indirectly, via supporting ribosome recycling-dependent RNA degradation mechanisms, that compete for the substrate with RNA silencing. It is necessary to mention, that although involvement in translation has been confirmed for AtABCE2 (Navarro-Quiles *et al*, 2022; Yu *et al*, 2023), contribution to specifically ribosome recycling step remains to be investigated.

In plants, mRNA surveillance pathway NSD has been shown to eliminate 5'- fragments of miRNA-loaded RISC cleavage products, as well as of viral siRNA-guided RISC targets (Szádeczky-Kardoss, Csorba, *et al*, 2018). In addition, it has been shown that Arabidopsis Pelota1, an essential component of NSD and NGD pathways, limits the amplification of siRNAs from miRNA targeted transcripts through reducing ribosome stalling (Vigh *et al*, 2022). Exploring potential interactions of AtABCE2 with AtPelota1 and its involvement in NSD would help to clarify whether its suppressor activity is linked to the function in translation.

4.4 *ABCE* gene subfamily in plants is broader than in other lineages (Publication III)

ABCE proteins compose the smallest and the most conserved subfamily of ABC transporters superfamily. Whereas most of animals possess a single *ABCE* gene, previous studies have shown that in plants usually there are one to three *ABCE* genes in a species (Gong & Wang, 2022; Ofori *et al*, 2018).

Our next aim was to provide a comprehensive analysis of plant *ABCE* gene subfamily and to gain insight into its evolution. For this purpose, we retrieved from public databases ABCE sequences of 76 plant species, representing broad taxonomic sampling, including green algae, bryophytes and angiosperms. Using well-studied AtABCE2 protein sequence as a reference, we selected the queries sharing sequence identity with AtABCE2 over 33.5%. The second important criterion was the presence of all domains as well as functional motifs characteristic for ABCE proteins (Braz *et al*, 2004; Karcher *et al*, 2008). After removing incomplete or aberrant sequences, we identified a total of 152 ABCE protein sequences corresponding to the premises. The selected ABCE proteins shared at least 78% of amino acid sequence identity with AtABCE2 and contained complete set of essential structural elements and thus were considered as functional.

In addition to high sequence conservation observed for plant ABCEs, our analysis revealed that over 60% of the plant species involved in this study possess two or more ABCE proteins (Publication III: Figure 2A). Particularly high number of *ABCE* genes was observed in species from two agriculturally important families: *Brassicaceae* (mustard and cabbage family) and *Poaceae* (grasses). For instance, *Brassica napus* and *Triticum aestivum* encode eight, while *B. rapa* and *T. dicoccoides* encode five and four ABCE proteins, respectively (Publication III: TableS2). Thus, we propose that plant *ABCE* genes should be classified as low-copy instead of generally accepted single-copy gene subfamily. Expansion of *ABCE* gene family might be due to the complicated evolutionary history of plant kingdom encompassing at least 244 whole genome duplication (WGD)

events (also named polyploidization event) in addition to pervasive local duplications (Leebens-Mack, J. H., et. al., 2019) Using linear regression analysis, we explored the impact of genomic parameters on the number of *ABCE* genes in a species. We found slight positive effect of genome size and current ploidy level, but not of the WGD events occurred during the evolution, probably due to rapid loss of duplicates that often follows massive genome enlargement (Publication III: Figures 2B-E; (Sankoff *et al*, 2010).

In an attempt to understand the evolution of plant *ABCE* genes, we constructed Maximum Likelyhood trees of 152 full-length ABCE proteins and the corresponding CDS sequences. Both trees showed clustering of sequences according to the major taxonomic groupings, though CDS tree showed higher congruency with Tree of Life and proved to be more informative. We observed previously reported clustering of *Brassicaceae* ABCEs into ABCE1 and ABCE2 groups (Navarro-Quiles *et al*, 2022) and also showed that *Poaceae* ABCE proteins split into two groups. According to CDS tree, both families have separately undergone lineage-specific duplications of ancestral *ABCE* gene early in evolution. The members of *Pooideae*, the largest *Poaceae* subfamily, have gained additional *ABCE* gene copies through further duplications. Furthermore, in many other plant taxa *ABCE* gene copies have arisen from more recent duplication events, occurred independently from other lineages (Publication III: Figure 4).

Interestingly, all *Brassicaceae* species encode at least one ABCE2, while ABCE1 can be missing. In the phylogenetic trees, ABCE2 sequences have shorter branches compared to ABCE1, indicating fewer mutations. Altogether, these findings support the notion that AtABCE2 preserves the ancestral function (Navarro-Quiles *et al*, 2022).

Further, we explored natural variation in Arabidopsis *ABCE* genes. For this purpose, we analyzed the presence of single nucleotide polymorphisms (SNPs) among 1135 *A. thaliana* ecotypes from the 1001 Genomes Project (Weigel & Mott, 2009). We found 4 and 35 non-synonymous SNPs in the coding sequences of AtABCE2 and AtABCE1 genes, respectively. Our results, showing relatively low natural variation in AtABCE2 are in consistence with the essential roles of this protein (Navarro-Quiles *et al*, 2022; Petersen *et al*, 2004; Yu *et al*, 2023). Whereas non-synonymous SNPs in *AtABCE2* gene are at variable sites or cause substitution to a similar amino acid residue (Publication III: Figure 5C), in *AtABCE1* the point mutations occurred at highly conserved sites and could affect the protein's function, according to the structural studies in other organisms (Publication III: Figure 5A; (Karcher *et al*, 2005, 2008). This data is in agreement with the assumption that AtABCE1 is on its way to pseudogenization (Navarro-Quiles *et al*, 2022). Alternatively, AtABCE1 might be undergoing a process of sub-functionalization, which could result in gaining a specific role in generative organs.

Taken together, plant ABCE protein subfamily is highly conserved and is often represented by multiple members. According to our phylogenetic analysis *ABCE* genes in plants are prone to duplications, although the necessity of higher number of ABCE proteins in a species, as well as their functionality, remain to be explored. In addition, deeper analysis of *ABCE* genes involving also truncated and defunctionalized sequences could bring new insight into understanding their evolution.

5 Conclusions

The main goal of the present thesis was to investigate the function of the essential and exceptionally conserved translational factor ABCE as a suppressor of RNA silencing, in plants and other organisms. In addition, this research aimed to shed light on the evolution of the plant *ABCE* gene subfamily. The key findings of this work are as follows:

- HsABCE1, similarly to AtABCE2, suppresses GFP transgene RNA silencing in *N. benthamiana* plants at both local and systemic levels.
- HsABCE1 acts as a suppressor of RNA silencing in the native as well as heterologous animal systems.
- Among the putative interactors of HsABCE1 identified in this study, there are multiple components of RNA-associated processes, along with a direct effector of the RNA silencing machinery: translin.
- AtABCE2 mutants with impaired ATP binding at active site I or active site II fail to suppress GFP RNA silencing in *N. benthamiana*.
- ATP hydrolysis in active site II, but not in active site I, is important for AtABCE2 suppressor activity.
- Deletion of FeS cluster domain, but not of HLH insertion in NBD1, ruins the suppressor function of AtABCE2.
- The structural requirements of AtABCE2 suppressor function resemble those of aABCE1 as a ribosome recycling factor.
- Plant *ABCE* genes should be classified as low-copy gene subfamily instead of single-copy gene subfamily.
- In many plant species *ABCE*, genes have undergone duplications during evolution and have likely been retained to fulfill specific functions.
- Among the two Arabidopsis *ABCE* genes, *AtABCE2* is less prone to mutations, consistently with its essential roles.

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Abstract

ABCE Proteins' Role in RNA Silencing Suppression and their Evolution

ATP-binding cassette subfamily E (ABCE) proteins are exceptionally conserved across archaea and eukaryotes and have been proved to be critical for the viability of all organisms tested so far. Structurally, ABCE proteins represent ATPase core with two active sites, linked to the unique iron-sulfur (FeS) cluster domain, essential for interactions. Through ATP binding and hydrolysis cycle, ABCE proteins mediate interactions with various molecular partners. Studies in yeast and animals have demonstrated that a single member of ABCE subfamily, namely ABCE1, is involved in multiple steps of protein synthesis, including ribosome biogenesis and transport, translational initiation and termination. However, the central role of ABCE proteins in archaea and eukaryotes is recognized in mediating ribosome recycling, the step of translation associated with RNA degradation pathways of RNA Quality Control system. In addition, *Arabidopsis thaliana* ABCE2 (AtABCE2), one of two paralogs, has been described as a suppressor of RNA silencing, a complex RNA degradation mechanism. RNA silencing machinery is highly conserved across eukaryotes, sharing similar set of effectors and probably also negative regulators. However, while data concerning ABCE proteins' functions in translation is rapidly accumulating, their role in the suppression of RNA silencing has not been studied in other than plant organisms. Furthermore, the precise mechanism of AtABCE2 suppressor activity remained unclear. Notably, in contrast to animals, multiple plant species have been reported to encode more than one ABCE protein, although the significance of additional *ABCE* gene copies has not been explored yet.

The results of this thesis proved that human ABCE1 (HsABCE1) can suppress GFP RNA silencing in GFP-transgenic *Nicotiana benthamiana* plants as AtABCE2 does. Furthermore, HsABCE1 showed suppression activity in nematodes and in mammalian cells, suggesting that this function is conserved in eukaryotes. Multiple putative interaction partners of HsABCE1, that could potentially contribute to its suppressor function were identified.

In order to shed light on the molecular mechanism of RNA silencing suppression by ABCE proteins, mutational analysis of AtABCE2 was performed using the previously mentioned plant system. The effect of mutations disrupting ATP binding or hydrolysis, as well as the deletion of the essential FeS cluster domain or of the helix-loop-helix motif was tested. The observed structural requirements for the suppression activity of AtABCE2 strongly resemble those for mediating ribosome recycling by archaeal ABCE1, suggesting that ABCE proteins might suppress RNA silencing through supporting ribosome recycling-dependent RNA degradation pathways.

Finally, using available whole-genome sequencing data and bioinformatics tools, a comprehensive analysis of plant *ABCE* gene subfamily variance and phylogeny was carried out. It was found that plant ABCEs are highly conserved, sharing more than 78% of amino acid sequence identity. According to the phylogenetic analysis, many plants have gained additional *ABCE* gene copies during evolution through whole genome or local duplication events. Over 60% of 76 plant species from broad range of taxa were reported to have two to eight *ABCE* genes. This means that in plants *ABCE* genes are prone to duplicate and can be classified as a low-copy gene subfamily instead of single-copy gene subfamily.

In conclusion, the results of this thesis demonstrate that in eukaryotes, ABCE proteins likely suppress RNA silencing by functioning as ribosome recycling factors. These findings provide insight into the complex relationship between distinct RNA degradation pathways and translation. Elucidating the phylogeny and size variation of the plant *ABCE* gene subfamily encourages further research into the functional diversification of ABCE proteins.

Lühikokkuvõte

ABCE valkude roll RNA vaigistamise supressioonis ja nende evolutsioon

ABCE (ATP-*binding cassette subfamily* E) valgud on kõrgelt konserveerunud nii arhedes kui ka eukarüootides ning on määrava tähtsusega elujõu tagamisel kõigis seni uuritud organismides. ABCE valgud on struktuurilt kahe aktiivsaidiga ATPaasid, mis on seotud unikaalse, interaktsioonide vahendamiseks vajaliku raud-väävel (FeS) klastri domeeniga. ATP sidumise ja hüdrolüüsi tsükli kaudu vahendavad ABCE valgud interaktsioone erinevate molekulaarsete partneritega. Uuringud pärmides ja loomades näitavad, et ABCE alamperekonna ainus liige – ABCE1 – osaleb mitmes valgusünteesi etapis, sealhulgas ribosoomide biogeneesis ja transpordis, translatsiooni initsiatsioonis ning terminatsioonis. ABCE valkude keskseks rolliks arhedes ja eukarüootides peetakse siiski ribosoomide taaskasutust translatsiooni selles etapis, mis on seotud RNA kvaliteedikontrolli süsteemi kuuluvate RNA lagundamise radadega. Lisaks, *Arabidopsis thaliana* ABCE2 (AtABCE2) paraloogi on kirjeldatud ka kui RNA vaigistamise supressorit, mis on osa keerulisest RNA degradatsiooni mehhanismist. RNA vaigistamise süsteem on eukarüootides väga konserveerunud, hõlmates sarnaseid efektormolekule ning tõenäoliselt ka negatiivseid regulaatoreid. Kuigi informatsiooni ABCE valkude funktsioneerimise kohta translatsioonis tekib juurde kiiresti ja pidevalt, on nende rolli RNA vaigistamise supressioonis uuritud seni siiski vaid taimedes. AtABCE2 täpne toimemehhanism RNA vaigistamise supressorina on aga jäänud ebaselgeks. Märkimisväärne on asjaolu, et erinevalt loomadest kodeerivad mitmed taimeliigid rohkem kui ühte ABCE valku. *ABCE* geenide lisakoopiate tähtsust ei ole aga senini uuritud.

Käesoleva töö tulemused näitavad, et sarnaselt AtABCE2 valgule on ka inimese ABCE1 (HsABCE1) võimeline maha suruma GFP RNA vaigistamist GFP-transgeensetes *Nicotiana benthamiana* taimedes. Lisaks leiti, et HsABCE1 toimib vaigistamise supressorina ka nematoodides ning imetajate rakkudes, mis viitab sellele, et tegu on eukarüootides konserveerunud funktsiooniga. Tuvastati mitu potentsiaalset HsABCE1 interaktsioonipartnerit, mis võiksid toetada selle valgu toimimist supressorina.

ABCE valkude RNA vaigistamise supressiooni molekulaarse mehhanismi uurimiseks viidi läbi AtABCE2 mutatsioonianalüüs, kasutades eelnevalt mainitud süsteemi GFP-transgeensetes taimedes. Selleks testiti ATP sidumist või hüdrolüüsi häirivate mutatsioonide ning FeS klastri domeeni või heeliks-luup-heeliks motiivi deletsiooni mõju AtABCE2 funktsioneerimisele RNA vaigistamise supressorina. Katsete tulemused näitasid, et struktuursed nõuded AtABCE2 valgule RNA vaigistamise supressori aktiivsuse jaoks on võrreldavad sellega, mida vajab arhede ABCE valk, et funktsioneerida ribosoomide taaskasutuses. Leitud tähelepanek viitab sellele, et ABCE valgud võivad RNA vaigistamist supresseerida, toetades ribosoomide taaskasutusega seotud RNA lagundamise radasid.

Töö viimase osana viidi läbi taimede *ABCE* geenide alamperekonna varieeruvuse ja fülogeneesi põhjalik analüüs, kasutades olemasolevaid terve genoomi sekveneerimise andmeid ja bioinformaatika vahendeid. Leiti, et taimedes on ABCE valgud väga konserveerunud ja nende aminohappeline järjestus on enam kui 78% identne. Vastavalt fülogeneetilisele analüüsile on paljud taimed kas terve genoomi või lokaalsete duplikatsioonide kaudu saanud evolutsiooni käigus täiendavaid *ABCE* geeni koopiaid. Üle 60% 76-st uuritud taimeliigist sisaldavad kaht kuni kaheksat *ABCE* geeni.

See tähendab, et *ABCE* geenidel on tendents taimedes duplitseeruda ja neid võiks liigitada ühe geenikoopiaga alamperekonna asemel madala koopiaarvuga alamperekonda.

Kokkuvõttes näitavad käesoleva väitekirja tulemused, et ABCE valgud supresseerivad eukarüootides RNA vaigistamist tõenäoliselt ribosoomi taaskasutuse vahendamise kaudu. Tehtud tähelepanekud heidavad uut valgust seostele erinevate RNA degradatsiooni radade ja translatsiooni vahel. Taimede *ABCE* geenide alamperekonna fülogeneesi ja suuruse varieeruvuse selgitamine julgustab teostama edasisi uuringuid ABCE valkude funktsionaalse mitmekesisuse kohta.

Appendix 1

Publication I
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RESEARCH ARTICLE

ABCE1 Is a Highly Conserved RNA Silencing Suppressor

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Abstract

ATP-binding cassette sub-family E member 1 (ABCE1) is a highly conserved protein among eukaryotes and archaea. Recent studies have identified ABCE1 as a ribosome-recycling factor important for translation termination in mammalian cells, yeast and also archaea. Here we report another conserved function of ABCE1. We have previously described AtRLI2, the homolog of ABCE1 in the plant *Arabidopsis thaliana*, as an endogenous suppressor of RNA silencing. In this study we show that this function is conserved: human ABCE1 is able to suppress RNA silencing in *Nicotiana benthamiana* plants, in mammalian HEK293 cells and in the worm *Caenorhabditis elegans*. Using co-immunoprecipitation and mass spectrometry, we found a number of potential ABCE1-interacting proteins that might support its function as an endogenous suppressor of RNA interference. The interactor candidates are associated with epigenetic regulation, transcription, RNA processing and mRNA surveillance. In addition, one of the identified proteins is translin, which together with its binding partner TRAX supports RNA interference.

Introduction

RNA silencing is a conserved sequence-specific mechanism regulating the gene expression in eukaryotes. This pathway functions as an innate immune response aimed at combating invading nucleic acids. In addition, RNA silencing is also a complex gene regulation pathway that controls cell differentiation and developmental processes by acting both at the transcriptional and post-transcriptional level [1,2].

Plant viruses and also some animal viruses encode suppressor proteins that are able to inhibit RNA silencing in host cells [3]. Most of the viral suppressors identified to date are highly diverse multifunctional proteins that are able to target one or more key steps of the RNA interference (RNAi) pathway [4].

and analysis, decision to publish, or preparation of the manuscript.

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In addition to viral suppressors, genomes themselves encode endogenous silencing suppressors. Very little is known about these proteins and so far endogenous regulators have been identified only in plants, *Caenorhabditis elegans* and *Dictyostelium* [5–8]. One of these proteins is RNase L inhibitor of *Arabidopsis thaliana* (AtRLI2). AtRLI2 suppresses silencing in GFP-transgenic *Nicotiana benthamiana* plants at the local as well as at the systemic level. It does not bind siRNAs and the way it suppresses RNA silencing remains unknown [9].

AtRLI2 is the plant ortholog of human ABCE1. ABCE1—also known as RNase L inhibitor (Rli1 in yeast), Pixie in *Drosophila melanogaster* and host protein 68 kDa (HP68)—belongs to the ABCE subfamily of ABC proteins that contain two nucleotide-binding domains and two N-terminal iron-sulfur clusters. Unlike most ABC domain proteins, members of this subfamily do not contain the membrane-spanning domains and are therefore not likely to be transporter proteins [10]. ABCE1 was initially identified as a negative regulator of the interferon-induced 2–5A antiviral pathway, where it functions by blocking RNase L, an enzyme responsible for the degradation of mRNA and single-stranded RNA in virus infected cells [11,12]. ABCE1 is highly conserved in archaea and eukaryotes [10,13] and has been described as essential for the viability of several organisms [14–16]. By contrast, RNase L is found only in vertebrates and therefore the question of the ABCE1 role in the rest of eukaryotes remained unanswered for almost a decade.

Recent years have brought many breakthroughs in discovering the core functions of ABCE1. This conserved protein is involved in the regulation of translation and in ribosome biogenesis through interacting with different translation initiation factors, release factors and also with ribosomal subunits in yeast, *Drosophila* and mammalian cells [17–22]. Although ABCE1 seems to be important for translation initiation, it is not well understood if its role at this stage is merely a consequence of its need for ribosomal recycling. Moreover, ABCE1 splits ribosomes not only when translation terminates but also during ribosome biogenesis and in mRNA surveillance pathways on stalled ribosomes [22–26]. Interestingly, ABCE1 is able to shuttle between nucleus and cytoplasm and is essential for nuclear export of 60S and 40S subunits in yeast [17–19].

The vast majority of recent research has focused on the central function of ABCE1 in translation and no discoveries have been made concerning the ABCE1 role in RNA silencing. As ABCE1 is a very well conserved protein and we have shown that its plant homolog, AtRLI2, acts as an endogenous suppressor of RNA silencing, we were tempted to test the role of human ABCE1 as RNA silencing suppressor.

In the current study we demonstrate that human ABCE1 is able to suppress RNA silencing in *N. benthamiana* plants, mammalian HEK293 cells and in the worm *C. elegans*. Furthermore, we identify several potential interactors which might support ABCE1 functioning as an endogenous suppressor of RNA silencing, among them translin, a protein involved in the activation of the RNA-induced silencing complex (RISC) [27].

Materials and Methods

Expression constructs

Binary vectors used for agroinfiltration assays were constructed as follows: pBin61 [28] was linearized with restriction enzyme *Sma*I and subsequently dephosphorylated. *AtRLI2* cDNA was cut out from the ABRC clone 232A23T7 (GeneBank Accession No. N65784) with restriction enzymes *Sma*I and *Eco105*I. Human *ABCE1* coding region was cut out from pcDNA3/RLIΔ3 (kindly provided by C. Bisbal) with restriction enzymes *Pst*I and *Not*I. The protruding ends were subsequently filled with Klenow enzyme. Ligation reaction was then performed with dephosphorylated vector and blunt ended inserts to attain pBin61-AtRLI2 and pBin61-ABCE1,

respectively. pBin61 vector harboring the *GFP* gene (named here pBin-GFP) was kindly provided by D. Baulcombe and pBin61 comprising 2/3 of GFP sequence from 5' end as inverted repeat (IR) was kindly provided by J. Burgyan and named here pBin-GFFG.

The coding regions of *ABCE1* and *Tomato bushy stunt virus* (TBSV) *P19* were PCR amplified using respectively pBin61-ABCE1 and pBin61-P19 as templates and cloned into pcDNA3.1/V5-His mammalian expression vector according to the pcDNA 3.1 Directional TOPO Expression Kit (Invitrogen) protocol. pBin61-P19 stands here for pBin61 coding for P19, a construct kindly provided by D. Baulcombe. The primers used for the generation of expression constructs were as follows: 5`-CACCATGGCAGACAAGTTAA–3`and 5`-ATCATC CAAGAAAAAGTAGTTTCC–3`for ABCE1, 5`-CACCATGGAACGAGCTATAC–3`and 5`-CTCGCTTTCTTTTTCGAAGGT–3`for P19. The resulting plasmids pABCE1-V5 and pP19-V5 contain C-terminal V5 and His tags. The expression constructs were verified by sequencing and *in vitro* transcription-translation assay (Promega).

pULK3FLAG and siRNA1pSUPER constructs—here renamed as siRNA(ULK3)—are described in [29] and [30], respectively. Construct siRNA(Fu)pSUPER—here renamed as siRNA (X)—is described in [31]. Empty vectors pSUPER (OligoEngine) and pcDNA3.1/myc-His (Invitrogen) were used as controls.

To create constructs pAS1 and pCS1 expressing *C. elegans* ERI-1 and human ABCE1, respectively, under the control of the *C. elegans myo-3* promoter, *C. elegans eri-1* cDNA and human *ABCE1* cDNA were inserted into pPD96.52 [32]. pPD96.02 [32] was used to express *C. elegans unc-54*::*NLS*::*gfp*. To generate the GFP-specific construct for RNAi by feeding, *GFP* coding sequence was inserted into the L4440 backbone [32].

Plant material and agroinfiltration

Wild-type *N. benthamiana* and *N. benthamiana* GFP-transgenic line 16c (kind gift of D. Baulcombe) were grown in a plant chamber at 22°C or 25°C with a 16-h photoperiod. 5-week old plants were used for agroinfiltration. All binary plasmids were transformed into *Agrobacterium tumefaciens* strain C58C1 harboring pCH32 [33]. Recombinant *A. tumefaciens* strains were incubated and infiltrated as described in [34] adjusting the final densities to $OD_{600} = 0.5$ with the exception of pBin61-GFFG that had a final density of $OD_{600} = 0.05$. *A. tumefaciens* carrying the inducer (pBin61-GFP or pBin61-GFFG) was mixed in 1:1 ratio with bacterium containing pBin61-AtRLI2 or pBin61-ABCE1 or the empty vector pBin61. Six independent experiments were carried out, each including 4–7 infiltrated plants for each mixture. GFP fluorescence was monitored using long-wave ultraviolet (UV) lamp (Black-Ray B-100AP, Ultraviolet Products). Plants were photographed with a Nikon p7000 camera using a yellow UV(O) filter (Tokina) and images were processed with Adobe Photoshop CS5.

Mammalian cell culture and transfection

HEK293 cells (ATCC Number: CRL-1573) were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all purchased from PAA) at 37°C and 5% CO_2. For the *ULK3* RNAi assays, cells were co-transfected with expression constructs pULK3FLAG encoding FLAG-tagged ULK3, siRNA (ULK3) encoding *ULK3*-specific shRNA and pABCE1-V5 or pP19-V5 using polyethylenimine (PEI) (Inbio). Constructs pULK3FLAG, siRNA(ULK3), pABCE1-V5/ pP19-V5 or their respective empty vectors were used in ratio 0.05:0.5:1.5. Approximately 0.25 μg of DNA per 1 cm^2 of plate surface area was used. DNA and PEI were diluted in 100 μl of MEM in mass ratio 1:2 and incubated for 10 min at room temperature (RT). The cells were washed with phosphate buffered saline (PBS) prior to transfection, and DNA/PEI complex was added to the cells in growth

medium without supplements for 3 h. The medium was changed for normal growth medium, and the cells were propagated for an additional 30 h prior to lysis. For immunoprecipitations (IP), HEK293 cells were transfected with 20 μg of pABCE1-V5 or empty vector on 10-cm plates using PEI with DNA to reagent ratio 1:2.

C. elegans transgenic strains and RNAi inhibition assays

C. elegans strains and genotypes (in brackets) used in this study are as follows, whereby N2 designates a wild-type background: OE4201 (N2; ofEx851 [*unc-54*::*NLS*::*gfp; rol-6(su1006)*]; AS2 (N2; asEx2 [*myo-3*::*eri-1*; *myo-2*::*dsRed*]); AS3 (N2; asEx3 [*myo-3*::*eri-1*; *myo-2*::*dsRed*]); AS4 (N2; asEx4 [*myo-3*::*ABCE1*; *unc-122*::*dsRed*]); AS5 (N2; ofEx851; asEx2); AS6 (N2; ofEx851; asEx3); AS7 (N2; ofEx851; asEx4). The *unc-54*::*NLS*::*gfp* reporter was created by injection of the following mix: 5 ng/μl pPD96.02, 20 ng/μl pRF6 [35]. For lines expressing RNAi suppressors, 10 ng/μl of *myo-3*::*eri-1* (pAS1) were co-injected with *myo-2*::*dsRed*, and 15 ng/μl of *myo-3*::*ABCE1* (pCS1) were co-injected with *unc-122*::*dsRed*. In all cases, the DNA concentration in the injection mixes was brought to 100 ng/μl using digested yeast genomic DNA.

E. coli strain HT-115, freshly transformed with GFP-L4440 or L4440 plasmids was used to seed Nematode Growth Medium (NGM) plates supplemented with tetracycline and ampicillin at standard working concentrations. Double transgenic worms were picked onto a separate NGM plate, allowed to lay eggs and progeny expressing the *rol-6(su1006)* marker at the L3 stage was placed on the *GFP* RNAi plates for 24 hours at 20°C. At this point, worms expressing both the *rol-6* and the red fluorescent markers, *i.e.* containing the *myo-3*::*eri-1* or the *myo-3*::*ABCE1* transgenes, were designated as "double transgenic" and imaged as the test group, whereas *rol-6*-only worms were the control group. At the same time, *rol-6*-only worms on *GFP* RNAi were used as a general control for RNAi efficacy of each batch of the RNAi plates. The L4440 plasmid was used as a control in this case.

Live worms were anesthetized with 15 mM NaN_3 and imaged using Axioplan (Zeiss) microscope equipped with an FITC filter and an LCD camera (Hamamatsu). For each experiment all worms were imaged using identical camera and microscope settings. The GFP intensity was quantified using ImageJ [36]. The average GFP intensity in GFP-positive muscle cell nuclei was measured as the average intensity along a segmented line drawn inside each nucleus. At least 10 worms were examined per strain per condition, and at least 10 nuclei per animal. Two independent transgenic lines were tested for ERI-1 expression and one for ABCE1 expression, in at least two experiments involving independent RNAi plate batches. The statistical analysis of the GFP intensity data was performed using R [37].

RNA extraction and northern blot analysis

Total RNA was extracted from infiltrated leaf patches as described previously [38] and 10 μg was used for GFP mRNA northern blot analysis as reported in [9]. For the detection of GFP siRNAs, RNA extraction was performed with TRIzol Reagent (Invitrogen) following manufacturer's instructions. 30 μg of total RNA was analyzed according to [39]. Radioactive signals were scanned and analyzed by Personal Molecular Imager FX (BioRad) after 30 min for mRNA and 24 h for siRNA.

Western blot analysis

Mammalian cells were lysed in RIPA buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate) containing ProteoBlock Protease Inhibitor Cocktail (Thermo Scientific) and incubated on ice for 10 min. Cell lysates were centrifuged for 3 min at 4°C and 15,000 g. Supernatants were mixed with Laemmli Sample Buffer and

denaturated for 5 min at 96°C. Proteins were separated on 10% polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% non-fat dry milk (AppliChem) in PBS containing 0.1% Tween 20 (Sigma-Aldrich). The antibodies were diluted in PBS containing 0.1% Tween 20 and 1% non-fat dry milk as follows: mouse monoclonal anti-V5 (1:2000, Invitrogen, catalog #R960–25), mouse monoclonal anti-FLAG M2-Peroxidase (1:5000, Sigma-Aldrich, catalog #A8592), rabbit polyclonal anti-actin (1:1000, Santa Cruz Biotechnology, catalog #sc-7210), HRP-conjugated goat anti-mouse IgG (1:3000, Thermo Scientific, product #32430) and HRP-conjugated goat anti-rabbit IgG (1:3000, Thermo Scientific, product #32460). The proteins were visualized using SuperSignal West Femto Chemiluminescense Substrate kit (Thermo Scientific).

ULK3FLAG western blot images were quantified with ImageJ software [36] and the data was expressed as mean of three independent experiments ± standard deviations. Statistical analysis was carried out using Microsoft Excel and JMP 10.0 software. Data was analyzed by mean centering and autoscaling as suggested in [40]. Two-tailed p-values were calculated using ANOVA with Dunnett's post-hoc comparison.

Co-immunoprecipitation

HEK293 cells were collected 28 h post-transfection and lysed with buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5% glycerol, protease inhibitors cocktail Complete (Roche)). Lysates were centrifuged for 25 min at 4°C and 13,400 g, and the supernatants were used for IP. Supernatants were incubated with 2 μg mouse monoclonal anti-V5 antibody (Invitrogen, catalog #R960–25) for 1 h at 4°C with gentle agitation. The protein-antibody complexes were incubated with ethanolamine-blocked protein G sepharose beads (GE Healthcare) overnight at 4°C with gentle agitation. After IP the beads were washed three times with ice cold PBS and the precipitated immune complexes were analyzed by mass spectrometry.

Mass spectrometry

For mass spectrometry analysis, three different sample preparation methods were used—in-gel digestion (experiment was carried out twice), in-solution digestion and Filter Aided Sample Preparation (FASP). For in-gel digestion, precipitated proteins were eluted with LDS sample buffer (Invitrogen) and separated by SDS-PAGE using a 4–12% NuPAGE Bis-Tris gel system (Invitrogen). The gel was stained with SimplyBlue SafeStain (Invitrogen) for 1 h at RT and washed in distilled H_2O prior to excision of equal slices. Gel pieces were destained in 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) and dehydrated with 100% acetonitrile. Proteins were reduced and alkylated, first by the addition of 10 mM dithiothreitol (DTT), and then by the addition of 50 mM iodoacetamide. After alkylation, proteins were digested with 2.5 ng/μl trypsin overnight at 37°C. Peptides were extracted from gel pieces using 5% formic acid/acetonitrile (1:2, v/v) and sample volume was reduced in SpeedVac to about 25% of the starting volume. 1/10 of 5% acetic acid was added to the sample and peptides were purified on StageTip columns as described in [41]. For in-solution digestion, immunocomplexes were eluted with 1% SDS and precipitated with methanol and chloroform. Precipitated proteins were resuspended in 7 M urea/2 M thiourea solution and reduction/alkylation step was performed using DTT and iodoacetamide. Proteins were first digested with Lys-C for 3 h at RT and then with trypsin overnight at RT. 1/10 of 10% trifluoroacetic acid was added to the sample and peptides were purified on StageTip columns. For FASP, precipitated proteins were eluted with 1% SDS, out-dilution of SDS with urea and protein digestions were performed with 10 k filter as described in [42]. Peptides were again purified on StageTip columns.

Purified peptides were resuspended in 0.5% acetic acid and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of two technical replicates was performed using an Agilent 1200 series nanoflow system (Agilent Technologies) connected to a LTQ Orbitrap mass-spectrometer (Thermo Scientific) equipped with a nanoelectronspray ion source (Proxeon) as described in [43].

Raw data files were analyzed with the MaxQuant software package (version 1.2.7.4) [44]. Generated peak lists were searched using the Andromeda search engine (built into MaxQuant) against the UniProt human database. MaxQuant search was performed with full tryptic specificity, a maximum of two missed cleavages and a mass tolerance of 0.5 Da for fragment ions. Carbamidomethylation of cysteine was set as a fixed modification and methionine oxidation and protein N-terminal acetylation were set as variable modification. The required false discovery rate was set to 1% both for peptide and protein levels and the minimum required peptide length was set to six amino acids. Candidate interacting proteins were those present in at least two experimental samples with experimental sample and control sample intensity ratio cut-off set at > 2. Proteins that could be linked to transcriptional (TGS) or post-transcriptional silencing (PTGS) and that have putative homologs in *A. thaliana* and in *C. elegans* (according to [45]) were chosen as ABCE1 potential binding partners.

Results

ABCE1 suppresses RNAi of *GFP* in *Nicotiana benthamiana* plants

To investigate whether human ABCE1 is able to suppress RNA silencing in plants, we used a transient expression system in *N. benthamiana* harboring a stably expressing GFP transgene (16c line). Leaves were co-infiltrated with *A. tumefaciens* carrying pBin61-GFP (*GFP* gene as silencing inducer) and pBin61-ABCE1. As controls, we co-infiltrated *A. tumefaciens* containing pBin61-GFP and pBin61-AtRLI2 or the empty vector pBin61. The suppression activity was assessed according to [9]. At 5 dpi the patches infiltrated with pBin61-GFP/pBin61 mixture displayed a weak GFP fluorescence due to RNA silencing. In contrast, tissues infiltrated with pBin61-GFP/pBin61-ABCE1 mixture showed high intensity of GFP fluorescence, similarly to pBin61-GFP/pBin61-AtRLI2, indicating that ABCE1, as well as AtRLI2, suppresses local *GFP* RNAi (Fig. 1A). Using an *in vivo* imaging system we quantified the GFP fluorescence in the infiltrated patches and verified that the presence of either ABCE1 or AtRLI2 enhanced the expression of GFP (S1 Fig.). Thereafter, we also examined ABCE1 as a suppressor in wild type *N. benthamiana* plants, considered a weak silencing system, since RNA silencing in this case targets only ectopically expressed GFP. We found that the level of GFP expression was likewise enhanced in the presence of ABCE1, as well as in the presence of AtRLI2, compared to the empty plasmid control (Fig. 1B).

Further, we explored if ABCE1 is able to suppress systemic RNA silencing. For this reason, we infiltrated 16c *N. benthamiana* plants as described above and followed the spread of silencing for three weeks. Systemic silencing of GFP can be clearly observed under UV light as emerging red tissue in the leaves above the infiltrated ones. At 21 dpi, plants co-infiltrated with pBin61-GFP and pBin61-ABCE1 displayed significantly less silenced tissue compared to empty plasmid pBin61 (Fig. 1C). Only 41% and 31% of the plants infiltrated with pBin61-GFP/pBin61-ABCE1 and pBin61-GFP/pBin61-AtRLI2 mixtures, respectively, were silenced at the uppermost leaf compared to 78% in the case of pBin61-GFP/pBin61. Thus, ABCE1 suppressed *GFP* RNAi at the systemic level.

To confirm the suppression activity of ABCE1, we analyzed by northern blot GFP mRNA levels and the accumulation of GFP-specific siRNAs—indicators of RNA silencing—in the infiltrated patches. We found that GFP mRNA levels were increased in the patches infiltrated

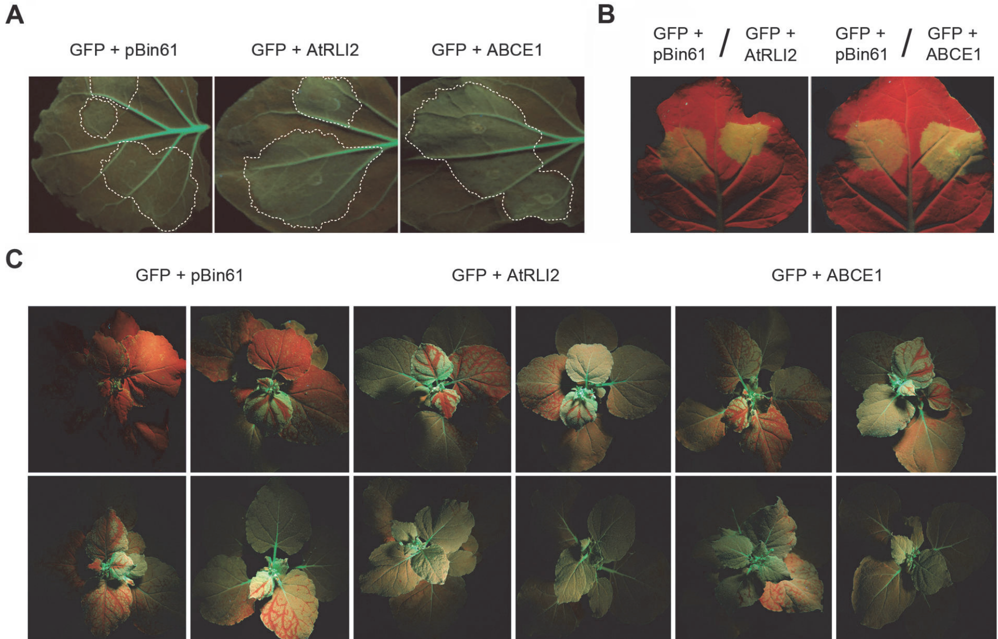

Fig 1. ABCE1 suppresses RNAi of *GFP* in *N. benthamiana*. 5-week old GFP-transgenic *N. benthamiana* 16c and wild-type *N. benthamiana* plants were co-infiltrated with *A. tumefaciens* harboring the RNA silencing inducer (pBin61-GFP) and pBin61-AtRLI2 or pBin61-ABCE1 or pBin61 as control. **(A)** Representative infiltrated GFP-transgenic *N. benthamiana* 16c leaves were photographed under UV light at 5 dpi. Strong GFP fluorescence in the patches infiltrated with pBin61-GFP/pBin61-AtRLI2 (indicated as "GFP + AtRLI2") and pBin61-GFP/pBin61-ABCE1 (indicated as "GFP + ABCE1") mixtures revealed the suppression of local GFP silencing. Control leaf infiltrated with pBin-GFP/pBin61 mixture (indicated as "GFP + pBin61") displayed weak GFP fluorescence. Infiltrated patches are circled in white. **(B)** Representative infiltrated wild-type *N. benthamiana* leaves were photographed under UV light at 5 dpi. Strong GFP fluorescence in the patches infiltrated with pBin61-GFP/pBin61-ABCE1 or pBin61-GFP/pBin61-AtRLI2 indicates the suppression of local GFP silencing. **(C)** *N. benthamiana* 16c plants showing systemic GFP silencing in the uppermost leaves. Representative plants were photographed under UV light at 21 dpi. Plants infiltrated with pBin61-AtRLI2 or pBin61-ABCE1 displayed less silenced tissue compared to the control (pBin61).

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with either pBin61-GFP/pBin61-ABCE1 or pBin61-GFP/pBin61-AtRLI2 mixtures with respect to pBin61-GFP/pBin61 (Fig. 2A). Accordingly, accumulation of GFP siRNAs was high in the case of empty plasmid, but was significantly reduced, particularly 24 nt long siRNAs, in the presence of ABCE1 or AtRLI2 (Fig. 2B). In addition, we challenged the suppressor efficiency of ABCE1 using conditions that favor strong GFP silencing: higher temperature and an IR construct as inducer [46,47]. A pBin61-GFFG construct comprising partial sequence of GFP, was agroinfiltrated together with pBin61-ABCE1 or pBin61-AtRLI2 into 16c *N. benthamiana* plants kept at 25°C. RNA analysis demonstrated that ABCE1 reduced the degradation of GFP mRNA and strongly affected the accumulation of GFP siRNAs in this system (Fig. 2C and 2D). Overall, these experiments show that ABCE1 suppresses local and systemic RNA silencing in plants by reducing siRNA accumulation.

ABCE1 suppresses RNAi mediated silencing of *ULK3* in mammalian HEK293 cells

To examine whether ABCE1 is able to suppress RNA silencing in mammalian cells, we overexpressed ABCE1 together with ULK3 (Unc-51-like serine/threonine kinase controlling Gli

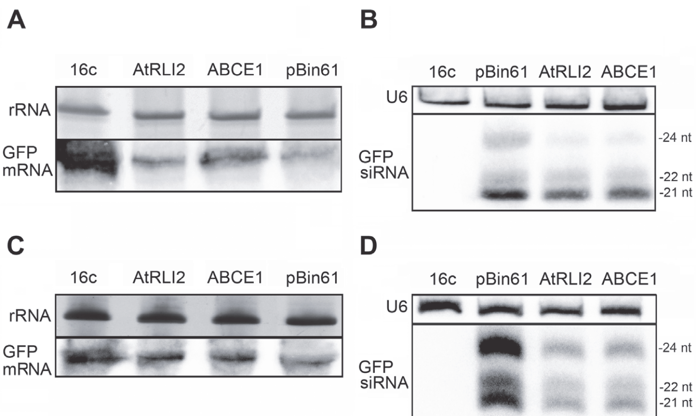

Fig 2. Northern blot analysis showing the suppression of *GFP* RNA silencing in *N. benthamiana* by ABCE1. (A) GFP-transgenic *N. benthamiana* 16c leaves were infiltrated with *A. tumefaciens* harboring pBin61-GFP together with *A. tumefaciens* carrying pBin61-AtRLI2 or pBin61-ABCE1 or empty vector (pBin61), as indicated on the upper part of the panel. Total RNA was extracted from the infiltrated patches and analyzed by northern blot. Levels of GFP mRNA in the patches infiltrated with pBin61-GFP/pBin61-AtRLI2 or pBin61-GFP/pBin61-ABCE1 were higher than in the case of pBin61. 16c indicates non-infiltrated leaf. GFP mRNAs were detected using [α-^{32}P] UTP-labeled antisense GFP transcripts. Ethidium bromide staining of rRNA was used as loading control. **(B)** Total RNA was extracted from the infiltrated patches and analyzed by northern blot as indicated before. GFP siRNA levels were reduced in the presence of AtRLI2 or ABCE1 compared to the control (pBin61). 16c indicates non-infiltrated leaf. For the detection of GFP siRNAs [γ-^{32}P] ATP end-labeled GF-probe was used. U6 stands for U6 snRNA used as loading control. **(C)** pBin61-GFFG was infiltrated as RNA silencing inducer instead of pBin61-GFP. The northern blot analysis of GFP mRNAs was performed as in (A) and shows higher levels in the presence of AtRLI2 or ABCE1 than in the case of the empty vector. **(D)** The accumulation of GFP siRNAs in the patches infiltrated with pBin61-GFFG/pBin61-AtRLI2, pBin61-GFFG/pBin61-ABCE1 or pBin61-GFFG/pBin61 was analyzed as in (B). GFP siRNA levels were reduced in the presence of AtRLI2 or ABCE1 as compared to the control (pBin61).

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proteins in the Sonic hedgehog pathway [29]) and suppressed ULK3 expression by RNAi. For this, we co-transfected pABCE1-V5, pULK3FLAG and siRNA(ULK3) constructs in HEK293 cells and analyzed ULK3 protein levels with western blot 30 h post-transfection. TBSV P19, which has been shown to function effectively in mammalian cells [48,49], was used as the positive control for RNAi suppression. Expression of V5-tagged ABCE1 and P19 was detected with anti-V5 antibody (S2 Fig.) while expression of FLAG-tagged ULK3 was detected using anti-FLAG antibody. The experiment was repeated three times, and the results of a representative experiment are shown in Fig. 3A. ULK3 overexpressed protein levels in the three independent experiments were quantified with ImageJ software (Fig. 3B). Cells expressing ABCE1-V5 showed higher ULK3 transient expression level than mock-transfected cells (Fig. 3A and B). The effect of ABCE1 on *ULK3* RNAi was comparable to the effect of P19 (Fig. 3A and B). While the expression of both proteins resulted only in a minor increase of ULK3 expression levels, the results were reproducible and statistically significant (Fig. 3B).

In order to exclude the possibility that ABCE1 induces ULK3 expression levels despite silencing, we transfected the examined protein expression constructs or empty vector together with pULK3FLAG and siRNA(X), a plasmid generating scrambled siRNAs. No significant changes in ULK3 expression levels were observed (S3A Fig.). Furthermore, we included an additional reporter, Firefly luciferase, to our test system. Cells were transfected with pABCE1-V5, pP19-V5 or pcDNA3.1 together with pULK3FLAG, siRNA(X) and pFLuc, a plasmid encoding

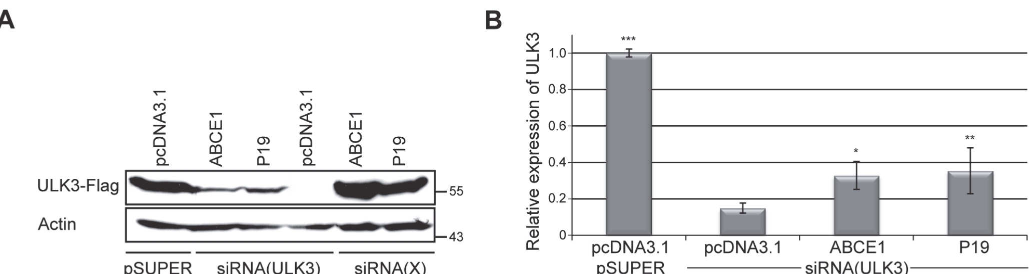

Fig 3. ABCE1 suppresses RNAi mediated silencing of *ULK3* in HEK293 cells. FLAG-tagged ULK3 was expressed in HEK293 cells in combination with empty vectors pcDNA3.1 and pSUPER or with siRNA(ULK3) or scrambled siRNA(X) and plasmids encoding either ABCE1 or P19 proteins. Cells were analyzed 30 h post-transfection. **(A)** FLAG-tagged ULK3 and actin (loading control) were detected by western blotting. The blot shown is representative for three independent experiments. ABCE1 and P19 were able to increase ULK3 expression levels in silenced cells. ABCE1 and P19 did not have any significant effect on ULK3 expression level in siRNA(X) transfected (non-silenced) cells. Molecular masses (in kDa) are shown on the right. **(B)** Quantification of relative ULK3 expression levels derived from three independent experiments. ULK3 expression levels were normalized to actin expression levels. The means relative to the levels of non-silenced ULK3 (cells transfected with empty vectors) are shown. Error bars indicate standard deviations. ABCE1 and P19 rescued ULK3 expression level significantly (*p = 0.0835, **p = 0.0461).

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Firefly luciferase. Neither ULK3 expression analysis nor luminescence measurements showed any significant changes in reporter gene translation (S3B and C Fig.). We therefore conclude that ABCE1 suppresses RNAi of *ULK3* in HEK293 cells.

ABCE1 suppresses RNAi of *GFP* in the worm *C*. *elegans*

To address whether human ABCE1 can function as a *bona fide* RNAi inhibitor in a heterologous animal context, we tested if it can inhibit RNAi in the worm *C. elegans*, as this model organism has a robust and well characterized RNAi pathway and has frequently been used for studies of RNAi [50,51].

We developed an *in vivo* RNAi inhibition assay, whereby GFP fused to the nuclear localization signal (NLS) from SV40, expressed under the control of a body wall muscle specific promoter *unc-54* (*unc-54*::*NLS*::*gfp*), was used as a reporter (Fig. 4A). 24 h after the worms had been subjected to GFP-specific RNAi by feeding, the GFP expression was effectively silenced (Fig. 4B). To test the functionality of the assay, we asked whether the known *C. elegans* endogenous RNAi inhibitor ERI-1 can suppress the *GFP*-specific RNAi [52]. We constructed a double transgenic strain, expressing ERI-1 under the control of a body wall muscle specific *myo-3* promoter, and the *unc-54*::*NLS*::*gfp* reporter on separate extrachromosomal arrays. Upon expression of ERI-1, the GFP signal in worms subjected to *GFP*-specific RNAi was significantly stronger than in worms not expressing ERI-1 (Fig. 4C and E). Similarly, expression of human ABCE1 in *C. elegans* body wall muscles lead to increased GFP levels of the *unc-54*::*NLS*::*gfp* reporter in worms subject to *GFP*-specific RNAi, compared to worms not expressing human ABCE1 (Fig. 4D and E). In both cases, the expression level of the *unc-54*::*NLS*::*gfp* reporter in worms not subject to *GFP*-specific RNAi was the same irrespective of the presence of the extrachromosomal array carrying either the ERI-1 or the ABCE1 expressing construct (data not shown). Taken together, our results indicate that both *C. elegans* ERI-1 and human ABCE1 inhibited *GFP*-specific RNAi similarly, when expressed in body wall muscle cells in *C. elegans*.

Identification of potential ABCE1-interacting proteins

To date, it is known that human ABCE1 or its orthologs interact with different ribosomal proteins, translation initiation and termination factors [17–21,23]. In order to identify novel

Fig 4. ABCE1 suppresses RNAi of *GFP* in *C. elegans*. (A) Schematic representation of the *in vivo* RNAi inhibition assay—the transgenes expressed and the GFP status are indicated. (**B)** Representative photomicrographs of the posterior part of animals expressing the NLS::GFP reporter in body wall muscles, treated with *GFP*-specific RNAi for 24 h at 20°C; control RNAi—empty vector. (**C)** Representative photomicrographs of the posterior part of animals expressing *C. elegans* ERI-1 and the NLS::GFP reporter in body wall muscles, treated with *GFP*-specific RNAi for 24 h at 20°C; ERI-1(+)—animals carrying the *myo-3*::*eri-1* transgene; ERI-1(-)—animals not carrying the *myo-3*::*eri-1* transgene. (**D)** Representative photomicrographs of the posterior part of animals expressing ABCE1 and the NLS::GFP reporter in body wall muscles, treated with *GFP*-specific RNAi for 24 h at 20°C. ABCE1(+)—animals carrying the *myo-3*::*ABCE1* transgene; ABCE1(-)—animals not carrying the *myo-3*::*ABCE1* transgene. (**E)** Relative GFP fluorescence intensity of the NLS::GFP reporter in worms expressing ERI-1 and ABCE1 and treated with *GFP*-specific RNAi for 24 h at 20°C. The ratio of the GFP signal intensity in worms expressing ERI-1 or ABCE1 and the NLS::GFP reporter compared to reporter alone is presented. Results from a representative experiment are shown ($n > 100$). Error bars represent the 95% confidence interval for the mean. Asterisks denote a statistically significant increase of the GFP signal intensity in worms expressing ERI-1 or ABCE1 and the NLS::GFP reporter as compared to the reporter alone ($p < 0.0001$ by two-tailed Student's *t*-test), showing that ERI-1 and ABCE1 are able to suppress *GFP* RNAi.

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ABCE1-associated proteins, V5-tagged ABCE1 was overexpressed in HEK293 cells and immunoprecipitated using anti-V5 antibody. As a negative control we immunoprecipitated pcDNA3.1 transfected cells with anti-V5 antibody. The protein content of the obtained immune complexes was analyzed using LC-MS/MS. The candidate ABCE1-interacting proteins present in at least two experimental samples are presented in S1 Table. Mass-spectrometry analysis identified a number of proteins (with putative homologs in *A. thaliana* and *C. elegans*) that could be linked to TGS or PTGS and might support ABCE1 functioning as an endogenous suppressor of RNAi (S2 Table). These potential binding partners were grouped according to their biological functions: epigenetic regulation, transcription/transcription regulation, RNA processing, mRNA surveillance and RNA silencing (Fig. 5). One of the identified proteins was translin, which together with its binding partner TRAX forms the C3PO (component 3 promoter of RISC) complex that is known to activate RISC [27].

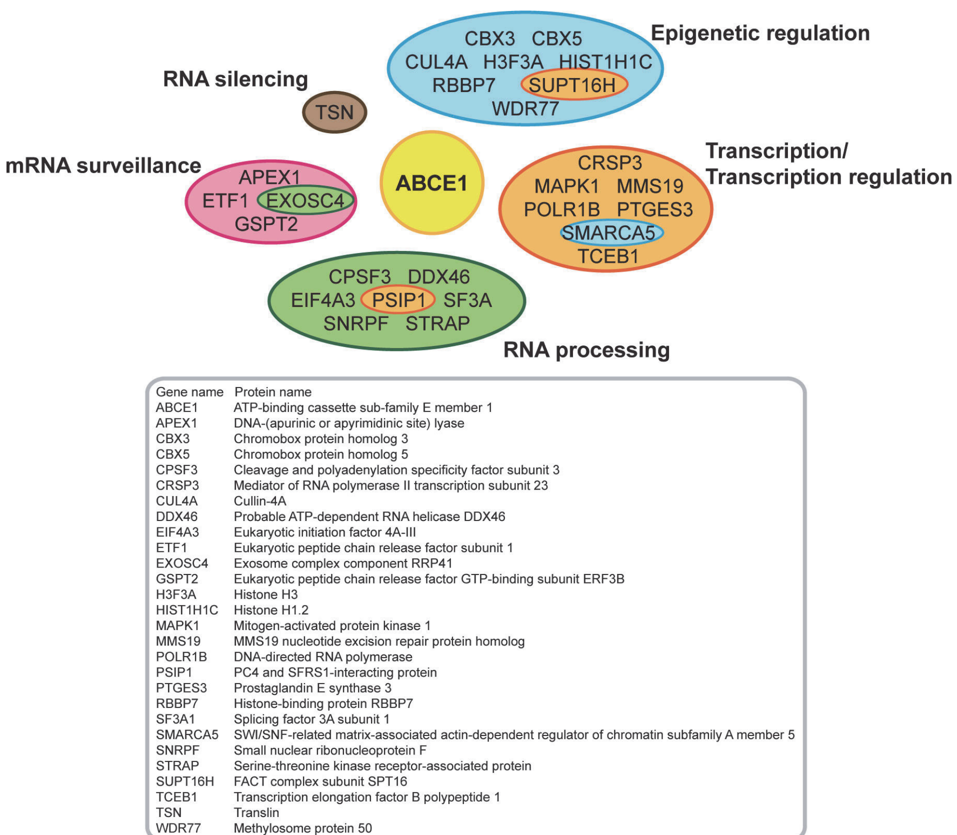
Discussion

ABCE1 is a very well conserved protein in eukaryotes and archaea and is considered to be a multifunctional protein essential for the viability of several organisms [14–16]. Initially described as a negative regulator of the 2–5A antiviral pathway, human ABCE1 is at present known to play an important role in several steps of translation, in ribosome biogenesis and recycling. It is assumed that these functions are conserved among eukaryotes and archaea, as they have also been described for some ABCE1 orthologs [13,53]. However, up to now there is no evidence that an ABCE1 plant ortholog is involved in translation. We propose here that another possible common role of ABCE1 across kingdoms is linked to RNA silencing, since we have reported that the ABCE1 plant ortholog AtRLI2 is an endogenous suppressor of RNAi [9].

To evaluate the hypothesis that functioning as an endogenous suppressor is conserved for ABCE1, we analyzed human ABCE1 effect on RNA silencing in *N. benthamiana* plants. Firstly, we observed that ABCE1 is able to suppress GFP-induced silencing in *N. benthamiana* at the local and at the systemic level. Furthermore, we found that the effect of human ABCE1 is comparable to the effect of AtRLI2, its plant ortholog. RNA analysis showed that the expression of ABCE1 leads to the accumulation of GFP mRNA and reduction of GFP siRNAs. Interestingly, the reduction in 24 nt siRNA levels was most significant. When using a stronger silencing system (IR construct as inducer and higher temperature), the siRNA levels decreased similarly in the presence of ABCE1. Again we observed that the levels of 24 nt siRNAs were most affected. 24 nt siRNAs are associated with systemic spread of silencing and are candidates for the long-range phloem entry signal because viral proteins that block systemic silencing also prevent accumulation of the 24 nt siRNAs [34,54]. We indeed observed a strong effect of ABCE1 at the systemic silencing level. Moreover, this class of siRNAs is the only mobile small RNA species that is active in RNA-dependent DNA methylation (RdDM) and TGS [55].

To test whether the ABCE1 ability to suppress RNA silencing is not only plant specific, we analyzed the effect of ABCE1 on exogenous ULK3 silencing in mammalian HEK293 cells. ABCE1 expression resulted in a reproducible and statistically significant upregulation of ULK3 protein level. Moreover, we compared ABCE1 with tombusvirus P19, a well-characterized viral RNA silencing suppressor which has been shown to function effectively in HeLa and HepG2 cells [48,49], and found the ABCE1 effect to be only slightly weaker.

After showing that ABCE1 is able to function as an endogenous RNA silencing suppressor in plants and mammalian cells, whereby the effectiveness seems to differ in the tested systems, we analyzed ABCE1 suppressor function in the worm *C. elegans*. We found that the expression of ABCE1 leads to increased GFP levels in worms subjected to *GFP*-specific RNAi.



Gene name	Protein name
ABCE1	ATP-binding cassette sub-family E member 1
APEX1	DNA-(apurinic or apyrimidinic site) lyase
CBX3	Chromobox protein homolog 3
CBX5	Chromobox protein homolog 5
CPSF3	Cleavage and polyadenylation specificity factor subunit 3
CRSP3	Mediator of RNA polymerase II transcription subunit 23
CUL4A	Cullin-4A
DDX46	Probable ATP-dependent RNA helicase DDX46
EIF4A3	Eukaryotic initiation factor 4A-III
ETF1	Eukaryotic peptide chain release factor subunit 1
EXOSC4	Exosome complex component RRP41
GSPT2	Eukaryotic peptide chain release factor GTP-binding subunit ERF3B
H3F3A	Histone H3
HIST1H1C	Histone H1.2
MAPK1	Mitogen-activated protein kinase 1
MMS19	MMS19 nucleotide excision repair protein homolog
POLR1B	DNA-directed RNA polymerase
PSIP1	PC4 and SFRS1-interacting protein
PTGES3	Prostaglandin E synthase 3
RBBP7	Histone-binding protein RBBP7
SF3A1	Splicing factor 3A subunit 1
SMARCA5	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5
SNRPF	Small nuclear ribonucleoprotein F
STRAP	Serine-threonine kinase receptor-associated protein
SUPT16H	FACT complex subunit SPT16
TCEB1	Transcription elongation factor B polypeptide 1
TSN	Translin
WDR77	Methylosome protein 50

Fig 5. Candidate ABCE1-interacting proteins. HEK293 cells expressing V5-tagged ABCE1 and mock-transfected cells were subjected to IP with anti-V5 antibody. The protein content of the obtained immune complexes was analyzed using LC-MS/MS. Potential ABCE1 binding partners (proteins present in at least two experimental samples with experimental and control sample intensity ratio > 2, cf. S1 Table) that could be linked to RNA silencing and have putative homologs in *A. thaliana* and *C. elegans* (cf. S2 Table) were grouped according to their biological functions: epigenetic regulation (blue), transcription/transcription regulation (orange), RNA processing (green), mRNA surveillance (pink) and RNA silencing (brown). Four proteins were categorized into two groups and are therefore marked differently: EXOSC4—epigenetic regulation and transcription/transcription regulation, PSIP1—RNA processing and transcription/transcription regulation, SMARCA5—transcription/transcription regulation and epigenetic regulation, SUPT16H—epigenetic regulation and transcription/transcription regulation. ABCE1 is depicted in yellow.

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Additionally, we observed that ABCE1 ability to rescue GFP levels is comparable to ERI-1, a well-described *C. elegans* endogenous RNA silencing suppressor that forms a complex with Dicer [8].

It is noteworthy that in *C. elegans* ABCE1 seems to act as a strong silencing suppressor and the same appears to be the case for *N. benthamiana*, at least at the systemic level. This might indicate that ABCE1 predominantly affects a step in the RNAi pathway that is similar in plants and *C. elegans*, for instance the ability to amplify siRNAs. In nematodes and plants, but not in mammals, RNA-dependent RNA polymerases (RdRPs) are required for RNA silencing pathways acting in the cytoplasm and at the chromatin level. As a result of the RdRP-mediated mechanisms, from a single aberrant RNA species (only in the case of plants) or primary siRNA

molecules, many dsRNAs/secondary siRNAs can be generated and these are then able to silence even more target molecules. In addition, in plants and *C. elegans* the silencing signal is able to spread systematically through the organism [54]. Thus, we speculate that ABCE1 is involved to some extent in siRNA amplification and/or systemic movement of the silencing signal.

We next aimed to understand the mechanisms underlying ABCE1 role in RNA silencing pathway. In the case of AtRLI2, we have previously shown that it does not bind siRNAs [9]. Hence, the mechanism how ABCE1 and its orthologs work as negative regulators of RNAi might be through interaction with other proteins that function in different steps of RNAi pathways. To address this speculation, we co-immunoprecipitated V5-tagged ABCE1 in mammalian HEK293 cells and analyzed the immune complexes with mass spectrometry. From the obtained list of proteins we selected the ones that could be linked to RNA silencing and have putative homologs in *A. thaliana* and *C. elegans*. We then grouped these potential binding partners according to their biological functions. Keeping in mind that different RNAi pathways are interconnected by competing for substrates, effector proteins and by cross-regulating each other, we firstly chose proteins associated with transcription and epigenetic regulation. These putative binding partners might be linked to TGS as in different organisms TGS is associated with RdDM, nucleosomal histone tail modifications, heterochromatin formation and transcription inhibition [56]. Recent years have brought growing evidence suggesting that different RNAi pathways interact with RNA surveillance and processing [57,58]. Due to these possible connections between different RNA-associated pathways, we secondly selected proteins related to RNA processing and mRNA surveillance for putative ABCE1-interacting proteins. In addition, we identified one protein, which is directly involved in PTGS, namely translin. Together with its binding partner TRAX, translin has been purified as part of C3PO from *Drosophila* and human cells. C3PO is a Mg^{2+}-dependent RNA-specific endonuclease complex that activates RISC by degrading the Ago2-nicked passenger strand of the siRNA duplex. It has been shown that translin and C3PO can bind ss-DNA and ss-siRNA, but barely interact with ds-siRNA [27,59].

Interestingly, although nuclear localization has not been reported for mammalian ABCE1, we were able to identify several nuclear proteins with co-IP experiments. This finding may indicate that similarly to yeast Rli1, the majority of ABCE1 might reside in the cytoplasm, while a minor fraction is able to enter the nucleus [17–19].

In summary, our results show that human ABCE1 is able to act as a negative regulator of RNAi in the plant *N. benthamiana*, in mammalian HEK293 cells and in the worm *C. elegans*. Therefore we have identified the first human endogenous RNA silencing suppressor. Significant reduction in 24 nt *GFP* siRNA levels in *N. benthamiana* and co-immunoprecipitated potential binding partners in HEK293 cells indicate that ABCE1 might function not only in PTGS but also in TGS. It will be important to determine whether mammalian ABCE1 localizes to the nucleus and its potential role in nuclear RNAi pathways. In addition, ABCE1-translin interaction and its functional significance needs further studies.

Supporting Information

S1 Fig. GFP fluorescence is enhanced in the presence of ABCE1 and AtRLI2.
(PDF)

S2 Fig. Expression of V5-tagged ABCE1 and P19 in HEK293 cells.
(PDF)

S3 Fig. ABCE1 has no significant effect on reporter gene translation.
(PDF)

S1 Table. Putative ABCE1-interacting proteins. HEK293 cells transfected with pABCE1-V5 and mock-transfected cells were subjected to co-IP with anti-V5 antibody. Three different sample preparation methods were used—in-gel digestion (experiment was carried out twice), in-solution digestion and FASP. The protein content of the obtained immune complexes was analyzed using LC-MS/MS. For candidate ABCE1-interacting proteins we chose those present in at least two experimental samples with experimental and control sample intensity ratio cut-off set at > 2.
(XLSX)

S2 Table. Candidate ABCE1-interacting proteins that might support its function as an endogenous suppressor of RNAi.
(XLSX)

S1 Text. Supporting Materials and Methods.
(DOCX)

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Author Contributions

Conceived and designed the experiments: KK JG AP AS PS ET CS. Performed the experiments: KK JG LN AP AS KV CS. Analyzed the data: KK JG AP AS PK KE PS ET CS. Wrote the paper: KK AS PS ET CS.

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Appendix 2

Publication II

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ORIGINAL ARTICLE

Mutational analysis of *Arabidopsis thaliana* ABCE2 identifies important motifs for its RNA silencing suppressor function

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Keywords
ABCE1; ABCE2; AtRLI2; RNA silencing; suppression of RNA silencing; Arabidopsis thaliana.

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ABSTRACT

- ATP-binding cassette sub-family E member 1 (ABCE1) is recognized as a strongly conserved ribosome recycling factor, indispensable for translation in archaea and eukaryotes, however, its role in plants remains largely unidentified. *Arabidopsis thaliana* encodes two paralogous ABCE proteins (AtABCE1 and AtABCE2), sharing 81% identity. We previously reported that AtABCE2 functions as a suppressor of RNA silencing and that its gene is ubiquitously expressed. Here we describe the structural requirements of AtABCE2 for its suppressor function.
- Using agroinfiltration assays, we transiently overexpressed mutated versions of AtABCE2 together with GFP, to induce silencing in GFP transgenic *Nicotiana benthamiana* leaves. The influence of mutations was analysed at both local and systemic levels by *in vivo* imaging of GFP, Northern blot analysis of GFP siRNAs and observation of plants under UV light.
- Mutants of AtABCE2 with impaired ATP binding in either active site I or II failed to suppress GFP RNA silencing. Mutations disrupting ATP hydrolysis influenced the suppression of silencing differently at active site I or II. We also found that the N-terminal iron–sulphur cluster domain of AtABCE2 is crucial for its suppressor function.
- Meaningfully, the observed structural requirements of AtABCE2 for RNA silencing suppression were found to be similar to those of archaeal ABCE1 needed for ribosome recycling. AtABCE2 might therefore suppress RNA silencing *via* supporting the competing RNA degradation mechanisms associated with ribosome recycling.

INTRODUCTION

ATP-binding cassette sub-family E member 1 (ABCE1) is a multi-functional protein, which is highly conserved among eukaryotes and archaea. Inactivation of the *ABCE1* gene in all organisms tested so far leads to lethality or to severe morphological alterations (Petersen *et al.*, 2004; Kispal *et al.*, 2005; Navarro-Quiles *et al.*, 2018). Initially, ABCE1 was described in mammals as RNase L Inhibitor (RLI), a component of the 2-5A system, an antiviral pathway induced by interferons (Bisbal *et al.*, 1995). Blocking RNase L, ABCE1 inhibits viral mRNA degradation and regulates cellular mRNA stability in mammalian cells (Le Roy *et al.*, 2001; Salehzada *et al.*, 2009). Extensive studies in recent years have demonstrated that ABCE1 is associated with different stages of protein synthesis in eukaryotes (Navarro-Quiles *et al.*, 2018). In yeast, ABCE1 interacts with eukaryotic initiation factors eIF5, eIF2 and eIF3 and promotes the assembly of the pre-initiation complex (Dong *et al.*, 2004; Chen *et al.*, 2006; Toompuu *et al.*, 2016). Moreover, ABCE1 is involved in ribosome biogenesis and transport in yeast. Indeed, it was demonstrated that in yeast and mammalian cells ABCE1 is localized in both cytoplasm and nucleus (Kispal *et al.*, 2005; Yarunin *et al.*, 2005; Toompuu *et al.*, 2016). However, the central role of ABCE1 in eukaryotes and archaea is in ribosome recycling, the process linking translation termination with initiation. Together with release factor 1 (eRF1/aRF1), ABCE1 promotes the dissociation of the post-termination complex, enabling translation re-initiation (Pisarev *et al.*, 2010; Barthelme *et al.*, 2011; Nürenberg & Tampé, 2013; Nürenberg-Goloub *et al.*, 2018). Besides its role in canonical translation termination, ABCE1 together with eRF1/eRF3 paralogues Pelota and Hbs1 splits stalled ribosomes, activating mRNA surveillance pathways, namely, nonstop and no-go decay. These RNA decay mechanisms degrade aberrant mRNAs, lacking stop codons or containing elongation inhibiting features (Pisareva *et al.*, 2011; Kashima *et al.*, 2014; Hashimoto *et al.*, 2017; Navarro-Quiles *et al.*, 2018). Thus, data suggest a pivotal role of ABCE1 in RNA homeostasis.

Although, most genomes encode a single ABCE protein, in some species two or more *ABCE* genes were identified (Navarro-Quiles *et al.*, 2018). *Arabidopsis thaliana* encodes two highly similar *ABCE* paralogues: *AtABCE1* and *AtABCE2*. According to the transcriptome map, based on RNA-seq (Klepikova *et al.*, 2016), *AtABCE1* expression is restricted to generative organs, while *AtABCE2* is expressed in all tissues, suggesting its global role throughout the plant's life. We have previously reported that AtABCE2, also named AtRLI2, as well as its human orthologue ABCE1 function as suppressors of RNA silencing, another RNA degradation mechanism, which is conserved among eukaryotes (Sarmiento *et al.*, 2006; Kärblane *et al.*, 2015). Although there is growing data concerning the role of ABCE1 in translation and mRNA surveillance, there is no information about the precise activity of ABCE proteins in RNA silencing suppression.

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RNA silencing is triggered by double-stranded RNA (dsRNA). In plants, dsRNA is processed by Dicer-like enzymes into small RNA (sRNA) duplexes, ranging from 21 to 24 bp in size. Loaded into an Argonaute (AGO) containing RNA-induced silencing complex (RISC), sRNA guide RISC for degradation of complementary RNA or inhibition of its translation (Hammond *et al.*, 2000; Hamilton *et al.*, 2002; Baulcombe, 2004; Brodersen & Voinnet, 2006). RNA silencing can be amplified in plants through the action of RNA-dependent RNA polymerase (RDR), giving rise to secondary small interfering RNAs (siRNA). Moreover, RNA silencing may spread from cell to cell *via* plasmodesmata, or systemically through the phloem beyond the initiation site and guide the target degradation in distant tissues (Tang *et al.*, 2003; Brosnan *et al.*, 2007; Pyott & Molnár, 2015; Zhang *et al.*, 2019). Through different pathways, RNA silencing mediates developmental gene regulation, and biotic and abiotic stress responses (Ding & Voinnet, 2007; Sunkar *et al.*, 2007). Furthermore, this mechanism plays a major role in antiviral immunity in plants. In this context, amplification and spread of RNA silencing are beneficial for the host and, thus, it is not surprising that viruses encode RNA silencing suppressors (Baulcombe, 2004; Burgyán & Havelda, 2011). In addition, plants also encode suppressors to regulate the expression of endogenous genes. Only a few of them, including AtABCE2, have been identified so far (Gazzani *et al.*, 2004; Sarmiento *et al.*, 2006; Gy *et al.*, 2007; Yu *et al.*, 2015; Zhang *et al.*, 2015; Li *et al.*, 2017; Huang *et al.*, 2019). Interestingly, essential components of mRNA surveillance pathways were found among plant endogenous RNA silencing suppressors, supporting an obvious connection between these RNA degradation mechanisms (Gazzani *et al.*, 2004; Gy *et al.*, 2007; Yu *et al.*, 2015; Zhang *et al.*, 2015). We previously demonstrated that AtABCE2 affects the accumulation of siRNAs when overexpressed (Sarmiento *et al.*, 2006). In this research, we aimed to assess the structural requirements for AtABCE2 suppressor function.

The ABCE proteins belong to the large ATP-binding cassette (ABC) protein superfamily. They contain two nucleotide binding domains (NBD1 and NBD2) characteristic for ABC proteins, which compose the core for ATP binding and hydrolysis (Braz *et al.*, 2004). In addition, ABCE proteins possess several specific domains important for their diverse functions, including the 'hinge' domain and N-terminal iron-sulphur (FeS) cluster domain. The crystal structure of archaeal ABCE1 (aABCE1) shows that both NBDs, arranged by 'hinge' in a head-to-tail orientation, compose the V-like cavity with two ATP-binding sites in the interface. Each active site is composed of highly conserved Walker A (WA) and Walker B (WB) motifs from one NBD and C-signature from the opposite one. The WA motif from one NBD binds α- and β-phosphates of the ATP molecule, while C-signature from the other NBD binds γ-phosphate of the same ATP molecule. Consequently, ATP hydrolysis relies on the WB located to the same NBD as the WA motif (Braz *et al.*, 2004; Karcher *et al.*, 2005). Because of high amino acid sequence conservation among all ABCE proteins, the structures of eukaryotic ABCEs can be deduced from those of aABCE1 (Navarro-Quiles *et al.*, 2018). It was suggested that ATP binding and hydrolysis in aABCE1 induces tweezer-like motion of the NBD-dimer, which in turn induces conformational changes in the associated domains that are transmitted to the interaction partners (Karcher *et al.*, 2005; Karcher *et al.*, 2008). Recent research on aABCE1 as ribosome recycling factor confirmed the proposed model and described the action of aABCE1 in detail. According to the results, ABCE1 binds to the post-termination complex in a semi-closed state with one ATP molecule occluded in the second ATP-binding site (site II). The occlusion of both ATP molecules causes radical relocation of the FeS cluster domain towards the cleft between ribosomal subunits, and this leads to ribosome splitting. After formation of the post-splitting complex and recruitment of initiation factors, aABCE1 dissociates from the small ribosomal subunit upon hydrolysis of ATP. Interestingly, hydrolysis of ATP at site II, but not at site I, is crucial at this step (Nürenberg-Goloub *et al.*, 2018). In addition, it was previously demonstrated that mutants of the catalytic site I display reduced ATPase activity, while inactivation of site II makes aABCE1 hyperactive. These studies revealed the functional asymmetry of the two ATP binding sites in ATP hydrolysis, ribosome binding and splitting (Barthelme *et al.*, 2011; Nürenberg-Goloub *et al.*, 2018). In addition, helix-loop-helix (HLH) insertion in the NBD1, with earlier unclear function, was shown to be an important site for interaction with the ribosomal small subunit in the post-splitting complex (40S/30S·ABCE1) (Kiosze-Becker *et al.*, 2016; Heuer *et al.*, 2017; Nürenberg-Goloub *et al.*, 2020). ABCE1 can remain bound to the small ribosomal subunit until the final step of translation initiation, preventing it from re-association with the large ribosomal subunit (Simonetti *et al.*, 2020).

In order to understand how AtABCE2 suppresses RNA silencing, we carried out mutational analysis of this protein and tested it in GFP-transgenic *Nicotiana benthamiana* plants. Mutants impaired in ATP binding or ATP hydrolysis or lacking potential interaction sites (FeS cluster domain or HLH) were assessed for their ability to suppress GFP transgene RNA silencing at the local and systemic levels.

MATERIAL AND METHODS

Cloning of AtABCE2 mutants

To obtain AtABCE2 mutants the construct pBin61-AtABCE2 (named in a previous publication as pBin61-AtRLI2) was used as the primary template (Kärblane *et al.*, 2015). Mutants bearing single amino acid substitutions (WB1, WB2, WA1 and WA2) and that carrying the HLH motif deletion (AtABCE2ΔHLH) were generated by two-step PCR amplification. In the first step, two fragments of AtABCE2 were amplified by Phusion polymerase and subsequently ligated by T4 DNA ligase (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. The first fragment was produced using 5′-AtABCE2 and 3′-PR1 primers. The second fragment was obtained with primers 5′-PR2 and 3′-AtABCE2. Finally, using 5′-AtABCE2 and 3′-AtABCE2 primers, the full-length AtABCE2 mutated cDNAs were obtained. Since AtABCE2ΔFeS implies the deletion of the 5′ terminus of AtABCE2 cDNA, mutated cDNA was obtained by one-step PCR amplification. All mentioned mutants were cloned into Invitrogen™ pcDNA3.1/V5-His TOPO vector according to the manufacturer's manual (Thermo Fisher Scientific) and transformed into TOP10 *E. coli* cells. In parallel, the AtABCE2 sequence

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was also cloned into pcDNA3.1/V5-His TOPO vector. WA1C2, WA2C1 and WB1/2 were generated with QuikChange II XL Site-Directed Mutagenesis Kit (SDM) (Agilent Technologies, Santa Clara, CA, USA), using as templates pcDNA3.1-WA1-V5, pcDNA3.1-WA2-V5 and pcDNA3.1-WB1-V5 constructs, respectively, following the manufacturer's instructions. To produce the WA1/2C1/2 mutant, point mutations in WA2 and C-signature 1 were introduced into pcDNA3.1-WA1C2-V5 by two subsequent SDM procedures. Primers used in mutagenesis are listed in Table 1.

For agroinfiltration assays, sequences of AtABCE2 wild type (wt) and its mutants, all containing the V5-His-tag, were excised from pcDNA3.1/V5-His TOPO vectors with *Kpn*I and *Mss*I restriction enzymes and cloned into the binary plasmid pBin61 downstream of Cauliflower mosaic virus (CaMV) 35S promoter as described (Kärblane *et al.,* 2015). The obtained constructs were sequenced and transformed into *Agrobacterium tumefaciens* strain C58C1 harbouring pCH32 (Hamilton *et al.,* 1996).

Plant material and growth conditions

Seeds of *N. benthamiana* stably expressing the GFP gene (16c line) were kindly provided by David Baulcombe. Plants were grown in a plant chamber at 25 °C with a 16-h photoperiod. Plants used for agroinfiltration were 4–5 weeks old.

Agroinfiltration assay and GFP imaging

All recombinant *A. tumefaciens* cultures were prepared for infiltration as described in Hamilton *et al.* (2002) with several modifications. Overnight cultures were used to inoculate 20 ml of the final medium: LB broth medium supplemented with kanamycin (50 μg ml^{-1}), tetracycline (5 μg ml^{-1}), 10 mM 2-(N-morpholino)ethanesulfonic acid (MES, pH 6.3) and 20 μM acetosyringone. After overnight incubation (29 °C, 180 rpm), cells were collected by centrifugation (3,000 *g* for 5 min at room temperature), washed once with distilled water (3,000 *g* for 5 min at room temperature) and resuspended in the infiltration buffer (10 mM MES, 150 μM acetosyringone and 10 mM $MgCl_2$). All suspensions were adjusted to a final OD_{600} of 0.5 and incubated with slow shaking for 3 h. *A. tumefaciens* carrying the inducer of GFP RNA silencing (GFP gene cloned into pBin61) was mixed in 1:1 ratio with *A. tumefaciens* harbouring the empty vector pBin61 or the vector containing either AtABCE2 wt or one of its mutated sequences. The agrobacteria mixtures were named GFP/pBin61, GFP/AtABCE2 and GFP/AtABCE2-mutant, respectively. The pBin61 vector and agrobacteria containing GFP in pBin61 were kindly provided by D. Baulcombe.

For local silencing suppression assays, leaves of 16c *N. benthamiana* were infiltrated with GFP/pBin61 and GFP/AtABCE2 or with GFP/AtABCE2-mutant mixtures into opposite sides of the leaf midrib. Because of high variability in GFP fluorescence intensity between leaves, the levels of GFP expression were always compared separately between infiltrated patches in each leaf. At 5 dpi, GFP fluorescence was quantified and analysed using IVIS Lumina II (Caliper Life Sciences, Waltham, MA, USA) and Living Image (version 4.1) software as described in Stephan *et al.* (2011) including the modifications reported in Kärblane *et al.* (2015). For the local silencing suppression assay, we measured radiant efficiency of GFP in agroinfiltrated leaves and analysed the difference between two patches in the same leaf using a paired two-tail *t*-test. To assess suppression of systemic

Table 1. List of primers used to generate AtABCE2 wild type and AtABCE2 mutated sequences. Substituted nucleotides are underlined in the case of mutagenizing primers.

primer name	primer sequence (5′–3′)	cloning of construct(s)	mutation(s) obtained
3′-PR1WA1	CTC TCC AAT ACC ATT GGT TCC A	WA1	K116E
5′-PR2WA1	TCA ACT GCT CTG AAA ATT		
3′-PR1WA2	TCT CCC CTG TAC CAT TCT	WA2	K387E
5′-PR2WA2	CAA CAT TTA TTC GGA TGC T		
3′-PR1WB1	ATC GAG GTA ACT AGA TGG TTG AT	WB1	E242Q
5′-PR2WB1	GTT AAG CAA AGA CTC AAA GCT		
3′-PR1WB2	CGA GAT ATG CAC TTG GCT GAT	WB2	E493Q
5′-PR2WB2	ATT CTG AGC AAC GTA TTG TT		
3′-PR1ΔHLH	GGA GGA CTA GTG AAA CGG CCC AAA T	AtABCE2ΔHLH	ΔD139-L160
5′-PR2ΔHLH	AGA AGA TAA TCT TAA GGC CAT T		
5′-PR2ΔFeS	CAC CAT GAT CAA TCT TCC A	AtABCE2ΔFeS	ΔA2-I72
3′-AtABCE2	ATC ATC CAA GTA GTA GTA TGA G		
5′-AtABCE2	CAC CAT GGC AGA TCG ATT G	AtABCE2 wt and the above listed mutants	
3′-AtABCE2	ATC ATC CAA GTA GTA GTA TGA G		
SDM fwC2	TCA AGA AGT TGT CAA TCT CCG AGG AGG AGA ATT GCA AAG GG	WA1C2	K116G, S469R
SDM revC2	CCC TTT GCA ATT CTC CTC CTC GGA GAT TGA CAA CTT CTT GA		
SDM fwC1	CGT GAT GTT GAG AAT TTA CGT GGT GGT GAG CTG CAG AG	WA2C1	K387G, S218R
SDM revC1	CTC TGC AGC TCA CCA CCA CGT AAA TTC TCA ACA TCA CG		
SDM fw1C1	CGT GAT GTT GAG AAT TTA CGT GGT GGT GAG CTG CAG AG	WA12C12	K116G, S469R, K387G, S218R
SDM rev1C1	CTC TGC AGC TCA CCA CCA CGT AAA TTC TCA ACA TCA CG		
SDM fw2C2	TCA AGA AGT TGT CAA TCT CCG AGG AGG AGA ATT GCA AAG GG		
SDM rev2C2	CCC TTT GCA ATT CTC CTC CTC GGA GAT TGA CAA CTT CTT GA		
SDM fwWB2	GCC TGC GGA TAT ATA CCT GAT CGA TCA GCC AAG TGC ATA TCT CG	WB12	E242Q, E493Q
SDM revWB2	CGA GAT ATG CAC TTG GCT GAT CGA TCA GGT ATA TAT CCG CAG GC		

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silencing, five to seven plants were infiltrated with each agrobacteria mixture and the spread of GFP silencing to the upper leaves was followed over 3 weeks. GFP fluorescence was observed under long wavelength ultraviolet (UV) light (Black-Ray B-100AP, Ultraviolet Products , Analytik Jena US, Upland, CA, USA) at 14 and 21 dpi. GFP systemic silencing can be seen as the appearance of red tissues in new growing leaves of the infiltrated plants. If more than 90% of non-infiltrated leaves of a plant remained green, the suppression of systemic silencing was evidenced (Jing *et al.,* 2011). At 14 dpi, plants were photographed with a Nikon p7000 camera (Nikon, Tokyo, Japan) using a yellow filter (Tokina, Tokyo, Japan). The data from nine independent experiments (for each AtABCE2 mutant, four to six experiments were carried out) were pooled and analysed with a Chi-square test and Fisher's exact test as post-test (Tables S1, S2). AtABCE2 and its mutants were compared to pBin61 in a pairwise manner. In all tests $P \leq 0.05$ was considered statistically significant. Statistical analysis was carried out using JMP 14 software (SAS Institute, Cary, NC, USA).

Extraction of RNA and Northern blot analysis

Total RNA was isolated from infiltrated leaf patches, collected at 5 dpi, using Invitrogen TRIzol™ reagent (Thermo Fisher Scientific) following the manufacturer's instructions. A total of 25 µg of total RNA were resolved in 15% denaturing polyacrylamide gel (8 M urea) as previously described (Olspert *et al.,* 2014). After electrophoresis, the gel was cut in two: the lower part was used for analysis of GFP siRNA and the upper part for U6 snRNA, utilized as loading control. RNA was transferred to Amersham Hybond™-N + membrane (GE Healthcare, Chicago, IL, USA) by overnight electroblotting in 0.5 × TBE at constant current (450 mA) and fixed to the membrane by UV cross-linking twice at 1,200 mJ (Stratagene UV Stratalinker 1800). To detect GFP-specific siRNAs, the [α-^{32}P] UTP-labelled *in vitro* transcript corresponding to antisense GFP was synthesized (MAXIscript kit, Thermo Fisher Scientific) according to the manufacturer's protocol. For loading control, DNA oligos complementary to U6 snRNA were end-labelled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Thermo Fisher Scientific). Radioactive probes were purified through NICK Sephadex G-50 columns (GE Healthcare). Hybridization and washing conditions were carried out as previously described (Szittya *et al.,* 2002), with the following modifications for U6 detection: ULTRAhyb-Oligo buffer (Thermo Fisher Scientific) was used and an additional wash under more stringent conditions was performed. Radioactive signals were scanned by Personal Molecular Imager FX (BioRad, Hercules, CA, USA) after 30 min exposure for U6 and 24 h for siRNAs. Bands of interest (GFP siRNAs) were quantified by densitometry using ImageQuant TL software (GE Healthcare), normalized to U6 bands and always comparing each construct *versus* pBin61.

Protein structure modelling

The amino acid sequence of AtABCE2 was provided as input for the HHpred server (Zimmermann *et al.,* 2018) to search for models of homologous proteins. Default parameters were used. Three models (PDB IDs: 3BK7, 1YQT and 3OZX) were selected as templates and the corresponding alignment was submitted to MODELLER (Webb & Sali, 2016) for comparative modelling. Molecular graphics were produced in UCSF ChimeraX (Goddard *et al.,* 2018).

RESULTS

Suppression of GFP RNA silencing by WA1C2, WA2C1 and WA12C12 mutants

Based on mutational analysis carried out earlier in ABCE1 from archaea and yeast (Karcher *et al.,* 2008; Barthelme *et al.,* 2011), we generated AtABCE2 analogous mutants (Table 1). First, we questioned whether ATP binding by AtABCE2 is needed for the suppression of RNA silencing. Taking into account high conservation of ABCE proteins and numerous evidence on the structural requirements for ATP binding by ABC superfamily, as well as by aABCE1 proteins, we assume that the key residues play the same role for AtABCE2 (ter Beek *et al.,* 2014; Navarro-Quiles *et al.,* 2018; Nürenberg-Goloub *et al.,* 2018). The mutant with assumed affected ATP binding in the first active site (site I) contained substitutions K116E in the WA motif from NBD1 and S469R in the C-signature from NBD2 (from here on named WA1C2 mutant). The corresponding residues were mutated in WA2 and C1 motifs (substitutions K387E and S218R, respectively) to obtain the mutant WA2C1 with affected ATP binding in the second active site (site II). In addition, we generated the AtABCE2 mutant lacking ATP binding activity at both sites (named WA12C12). ATP binding by AtABCE2 is illustrated by the homology model based on published aABCE1 crystal structures (Fig. 1).

To evaluate the suppression activity, we transiently overexpressed the generated mutants together with an RNA silencing inducer (GFP gene) in the leaves of GFP-transgenic *N. benthamiana* plants (16c line). For local silencing suppression assays, we infiltrated *A. tumefaciens* carrying the GFP gene cloned into pBin61 together with *A. tumefaciens* harbouring empty vector pBin61 (GFP/pBin61) into one side of the leaf midrib and *A. tumefaciens* harbouring GFP together with *A. tumefaciens* carrying either AtABCE2 wt or the mutated sequence (GFP/AtABCE2 wt or GFP/AtABCE2 mutant) into the other side of the midrib. Normally, after 2 days post-infiltration (dpi), the GFP expression in the infiltrated patches started to decline due to the initiation of RNA silencing by the endogenous plant machinery. However, in the case of suppression, we observed elevated GFP expression levels also after 2 dpi. Using an *in vivo* imaging system, equipped with a GFP-specific filter set, we detected GFP fluorescence and measured its radiant efficiency in both infiltrated patches of each leaf at 5 dpi. GFP radiant efficiency values obtained from infiltrated patches were normalized to the background and compared in each leaf separately (Figure S1). As expected, in the presence of AtABCE2, GFP fluorescence levels were higher compared to the empty plasmid pBin61 ($P = 0.002$), confirming the suppressor activity. In contrast, there was no significant difference between any of the mutants (WA1C2, WA2C1 and WA12C12) and pBin61, indicating that these mutants are unable to suppress local silencing (Fig. 2a).

To confirm the results at the molecular level, we collected the infiltrated tissues and performed Northern blot analysis of GFP-specific siRNAs. In the patches infiltrated with GFP/pBin61 agrobacteria mixture, GFP siRNAs of all size classes

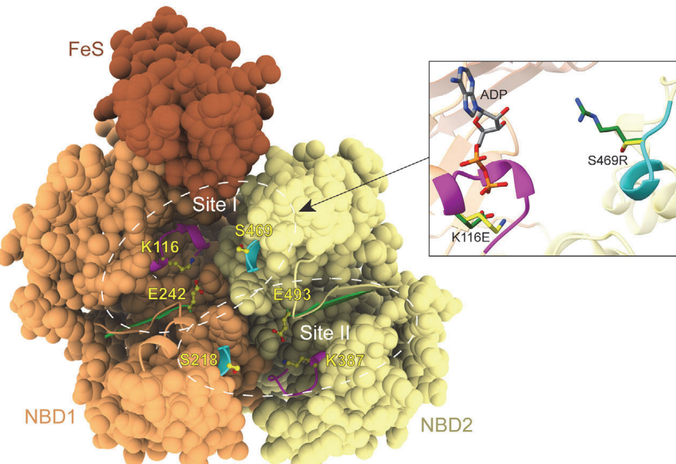

Fig. 1. Homology model of AtABCE2. The FeS cluster domain (FeS) and the two nucleotide-binding domains (NBD1 and NBD2) are rendered as space-filling models. Conserved motifs Walker A (WA, magenta), Walker B (WB, green) and C-signature (cyan) are highlighted as ribbons in both binding sites, with the residues of interest modelled as yellow balls and sticks. Inset: The mutations introduced to ATP binding site I in this study are illustrated using stick representation (wild type residues in yellow, mutant in green) with an ADP molecule aligned as it is bound in the crystal structure model of *Pyrococcus abysii* ABCE1 (PDB ID 3BK7). Oxygen atoms are in red, nitrogen in blue and phosphorus in orange.

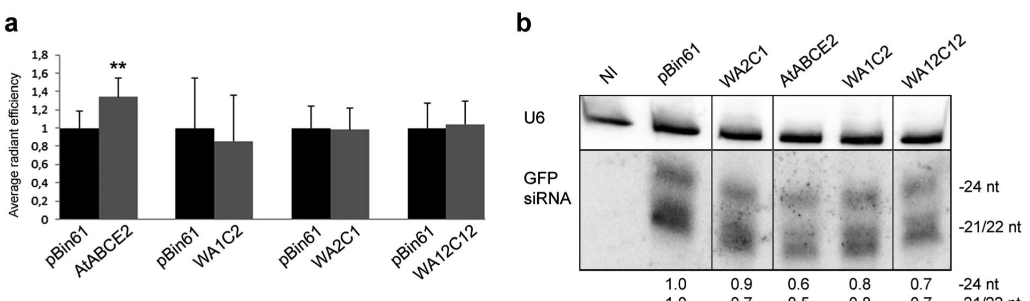

Fig. 2. Suppression of local GFP RNA silencing by WA1C2, WA2C1 and WA12C12 mutants. *Agrobacterium tumefaciens* harbouring pBin61-GFP was infiltrated together with *A. tumefaciens* carrying pBin61-AtABCE2 mutant or pBin61-AtABCE2 wt into one side of GFP transgenic *Nicotiana benthamiana* (16c) leaf midrib (indicated as WA1C2, WA2C1, WA12C12 and AtABCE2). The other half of the leaf was infiltrated with GFP/pBin61 agrobacteria mixture (indicated as pBin61). At 5 dpi, GFP fluorescence in the infiltrated patches was detected and quantified using an *in vivo* imaging system. (a) The means of GFP radiant efficiency were compared between pBin61 and AtABCE2 (n = 25), pBin61 and WA1C2 (n = 27), pBin61 and WA2C1 (n = 28), pBin61 and WA12C12 (n = 27). Statistical analysis was performed using a paired two-tail *t*-test (**$P < 0.01$). Standard errors are indicated as error bars. (b) Total RNA was extracted from agroinfiltrated patches and analysed by Northern blot. GFP-specific siRNAs were detected using [α-^{32}P] UTP-labelled antisense GFP transcript. As loading control, U6 snRNA was detected with [γ-^{32}P] ATP-labelled complementary DNA oligos. The levels of GFP siRNAs (24 and 21/22 nt long), quantified with ImageQuant TL, were normalized to U6 and compared with pBin61. NI indicates non-infiltrated leaf. For better visualisation, the lanes were cropped from the same blots. Intact membranes are presented in Figure S2a.

were abundant. AtABCE2, in turn, strongly reduced accumulation of siRNAs. In the case of WA1C2, WA2C1 and WA12C12 mutants, siRNA levels were higher compared to AtABCE2 wt. This is in agreement with the observed decline of GFP fluorescence levels in the infiltrated patches (Fig. 2b).

We further analysed the suppression of systemic GFP RNA silencing by the mutants. For this purpose, we infiltrated *N. benthamiana* leaves as indicated above and monitored the spread of GFP silencing in whole plants over the following 3 weeks. Systemic GFP silencing can be visualized under UV light as emerging red tissue in the new, growing leaves. The percentages of systemically silenced plants are shown in Table 2. In the case of AtABCE2 mutants, the percentages were calculated taking into account four to six independent experiments, each including five to seven plants (Table S1). Almost all plants infiltrated with GFP/pBin61 showed extensive spread of GFP systemic silencing (91% at 14 dpi and 95% at 21 dpi), while in the presence of AtABCE2, more than half of the plants had over 90% completely green, non-infiltrated leaves, indicating suppression of systemic silencing, as reported previously (Sarmiento *et al.*, 2006). At 14 dpi, 44% of the plants infiltrated with AtABCE2 wt were silenced and 1 week later the percentage rose to 47%. Over 70% of the plants infiltrated with the mutants WA1C2 or WA2C1 showed systemic silencing at 14 and 21 dpi, meaning that this mutations affected the suppressor function of AtABCE2. In contrast, the amount of plants infiltrated with GFP/WA12C12 displaying systemic silencing was less than that of the previous mutants (53–55%) and

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Table 2. Percentage of plants showing systemic silencing at two different time points. The number of plants displaying systemic silencing out of the total number of infiltrated plants is provided in brackets.

construct	14 dpi	21 dpi
GFP + pBin61	91% (50/55)	95% (52/55)
GFP + AtABCE2	44%*** (24/55)	47%*** (26/55)
GFP + WA1C2	71% (17/24)	79% (19/24)
GFP + WA2C1	75% (18/24)	83% (20/24)
GFP + WA12C12	53%** (20/38)	55%*** (21/38)
GFP + WB1	56%** (19/34)	56%*** (19/34)
GFP + WB2	71%* (24/34)	79% (27/34)
GFP + WB12	79% (19/24)	79% (19/24)
GFP + AtABCE2ΔFeS	76% (26/34)	79% (27/34)
GFP + AtABCE2ΔHLH	79% (23/29)	83% (24/29)

***Statistically significant difference with GFP + pBin61, used as a control group ($P < 0.0001$).
**Statistically significant difference with GFP + pBin61, used as a control group ($P < 0.001$).
*Statistically significant difference with GFP + pBin61, used as a control group ($P < 0.05$).

showed a statistically significant difference from the empty vector (Fig. 3, Table 2). Therefore, mutations in both WA and both C-signature motifs seem to retain the suppressor function of AtABCE2 at the systemic level.

Our results show that ATP binding at active centre I and II is necessary for the suppression activity of AtABCE2, at least at the local level.

Suppression of GFP RNA silencing by WB1, WB2 and WB12 mutants

Next, we explored how substitution of key residues in WB motifs (Fig. 1) shown to be involved in ATP hydrolysis in the case of aABCE1, affect the suppressor function of AtABCE2. The mutants WB1 (E242Q) and WB2 (E493Q), with putatively affected ATP hydrolysis in site I and site II, respectively, showed different suppression efficiency. GFP fluorescence intensity was significantly stronger in the leaf patches infiltrated with GFP/WB1 agrobacteria mixture compared to GFP/pBin61 ($P = 0.0271$) (Fig. 4a). This result was consistent with the detected reduced siRNA accumulation (Fig. 4b) and implies that WB1 suppresses local RNA silencing. Moreover, the WB1 mutant was able to interfere with the spread of systemic silencing, although less efficiently than AtABCE2 wt: 56% of plants infiltrated with this mutant were systemically silenced at 14 and 21 dpi, showing statistically significant difference from the empty vector (Table 2). In the leaf patches infiltrated with GFP/WB2 we detected low GFP fluorescence levels, consistent with abundant GFP siRNAs (Fig. 4a, b). In the case of the WB2 mutant, 71% and 79% of the infiltrated plants showed systemic silencing at 14 and 21 dpi, respectively. At 14 dpi, the difference between pBin61 and WB2 was significant ($P = 0.04301$) and the visible silencing (red tissue) was less extensive in the case of the mutant. However, at 21 dpi this statistical significance was lost (Table 2, Fig. 3). These results indicate that in contrast to WB1, the WB2 mutant failed to suppress GFP RNA silencing at both local and systemic levels. We also tested the double mutant, named WB12 (E242Q and E493Q), which possesses arrested closed conformation with both ATP molecules occluded in the interface between the NBDs. GFP expression was strongly reduced in the leaf patches infiltrated with WB12, and GFP siRNA levels were as high as in the case of pBin61 (Fig. 4a,c). Further, we observed the spread of systemic silencing in 79% of the plants infiltrated with GFP/WB12, showing that the simultaneous mutation of both WB motifs affects not only local but also systemic silencing suppression (Table 2).

Suppression of GFP RNA silencing by AtABCE2ΔFeS and AtABCE2ΔHLH mutants

Finally, we analysed mutants comprising deletions of AtABCE2 potential interaction sites, namely the FeS cluster domain (AtABCE2ΔFeS) and the HLH insertion in NBD1 (AtABCE2ΔHLH). We found that in the presence of the AtABCE2ΔFeS mutant, GFP expression levels in the infiltrated leaves were as low as in the case of pBin61, and this was in agreement with abundant levels of GFP siRNAs (Fig. 5a,b). In addition, AtABCE2ΔFeS was not able to suppress systemic silencing (76% and 79% of the plants were silenced at 14 and 21 dpi, respectively) (Table 2). Exploring the importance of the HLH insertion for AtABCE2 RNA silencing suppression capacity, we found that at the local level it does not play a role: GFP expression levels were significantly higher in the leaf patches infiltrated with GFP/AtABCE2ΔHLH compared to GFP/pBin61 ($P = 0.0304$), similar to AtABCE2 wt *versus* pBin61 (Fig. 5a). Consistently, GFP siRNA levels were strongly reduced in the presence of AtABCE2ΔHLH, supporting the suppression of local silencing (Fig. 5c). Although AtABCE2ΔHLH suppressed local GFP silencing, it was unable to do this at the systemic level: 79% and 83% of the plants were systemically silenced at 14 and 21 dpi, respectively (Table 2).

DISCUSSION

There have been many studies of ABCE1 as a translational and ribosome recycling factor in archaea, yeast and animals (Dong *et al.,* 2004; Kispal *et al.,* 2005; Yarunin *et al.,* 2005; Chen *et al.,* 2006; Pisarev *et al.,* 2010; Nürenberg & Tampé, 2013; Nürenberg-Goloub *et al.,* 2018; Gerovac & Tampé, 2019). However, little is known about ABCE proteins in plants. We have previously demonstrated that AtABCE2 and human ABCE1 act as RNA silencing suppressors, although the structural basis as well as the mechanism of suppression have remained elusive so far (Sarmiento *et al.,* 2006; Kärblane *et al.,* 2015). In this research, we report the structural requirements of AtABCE2 for the suppression of transgene RNA silencing in GFP-*N. benthamiana* plants. Within the framework of mutational analysis, we examined the importance of ATP binding and hydrolysis for the suppressor function of AtABCE2. Mutants lacking the potential

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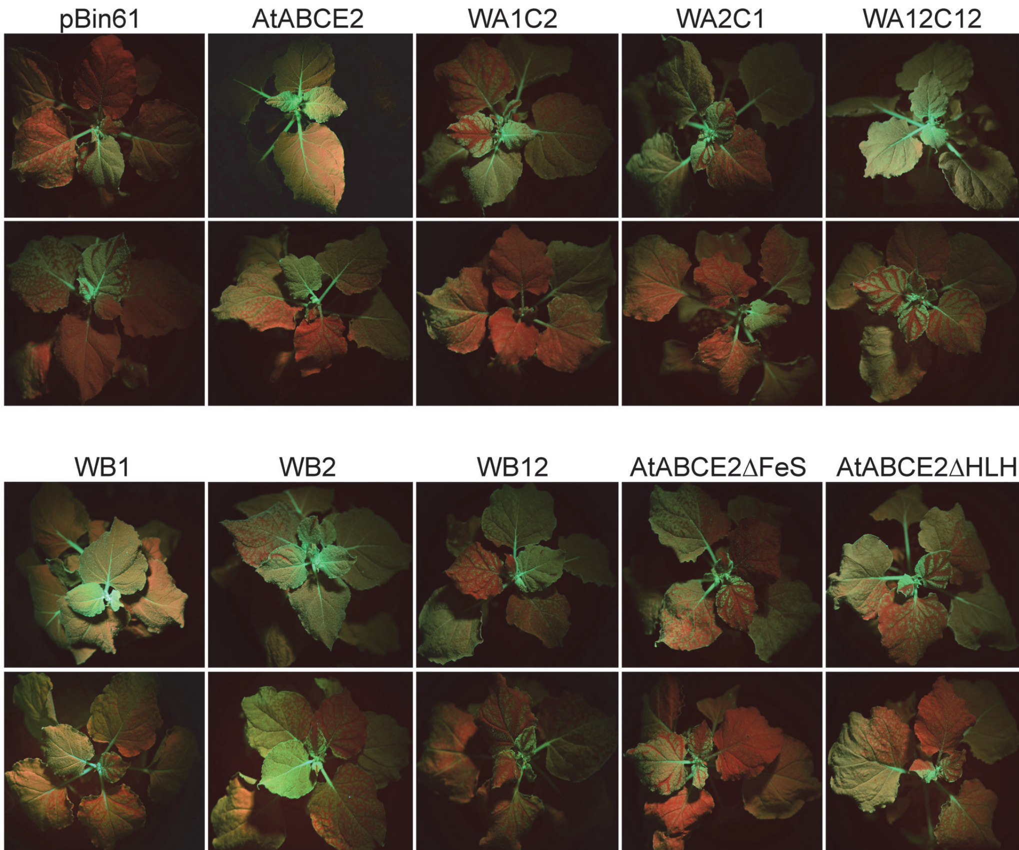

Fig. 3. Suppression of systemic GFP RNA silencing by AtABCE2 mutants. Four- to 5-week-old GFP-transgenic *Nicotiana benthamiana* plants were co-infiltrated with *Agrobacterium tumefaciens* harbouring pBin61-GFP and pBin61-AtABCE2 wt or pBin61-AtABCE2 mutant or pBin61 (indicated as AtABCE2, WA1C2, WA2C1, WA12C12, WB1, WB2, WB12, AtABCE2ΔFeS, AtABCE2ΔHLH and pBin61). Representative plants were photographed under UV light at 14 dpi. The red tissues in the leaves indicate systemic RNA silencing of GFP.

interaction sites, the FeS cluster domain and the HLH motif, were also explored.

We found that substitution of key residues involved in ATP binding at either the first (WA1C2 mutant) or the second (WA2C1) active centre strongly affects AtABCE2 suppression ability. At the local level, a higher accumulation of GFP siRNAs was observed in tissues expressing WA1C2 and WA2C1 compared to the AtABCE2 wt. These results are consistent with low GFP fluorescence levels, comparable with those observed in tissues transformed with the empty plasmid. Furthermore, the mutants could not suppress the spread of GFP silencing into the upper leaves. Based on these findings, we report that ATP binding at the first and the second active centre is necessary for the suppression activity of AtABCE2 at local as well as at systemic levels. Interestingly, WA12C12, harbouring simultaneously mutated ATP binding site I and site II, suppressed systemic GFP silencing but had reduced suppression activity at the local level. We cannot rule out the possibility that a disruption of key residues in both active sites leads to drastic conformational changes in the ATPase core that provides an alternative mode of ATP binding. This issue needs further investigation.

Next, we questioned whether ATP hydrolysis is necessary for AtABCE2 suppressor function. We found that an AtABCE2 mutant with affected ATP hydrolysis in site I (WB1) suppresses RNA silencing at the local level, similar to the AtABCE2 wt. WB1 is also able to suppress systemic silencing, however less efficiently than the AtABCE2 wt. In contrast, the WB2 mutant, defective in hydrolysis in site II, could not suppress both local and systemic GFP silencing. The loss of function was also observed in the case of the double mutant (WB12). These results indicate that ATP hydrolysis in site II is necessary and sufficient for AtABCE2 suppression activity, while ATP hydrolysis in site I does not seem to be critical for this function. The fact that the two ATP binding sites play distinct roles and have different ATPase activities was previously established for archaeal ABCE1 in the context of ribosome recycling (Barthelme

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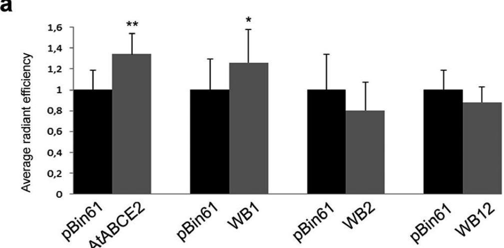

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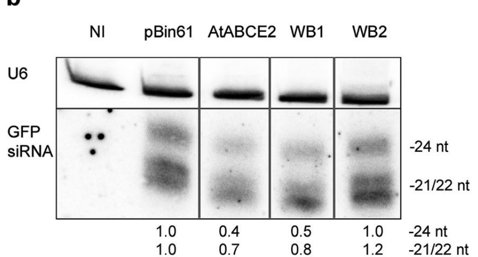

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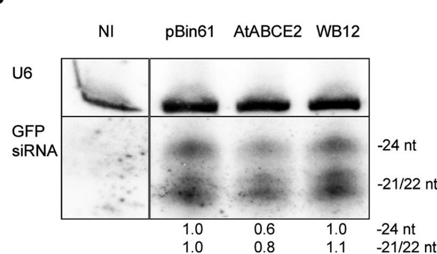

Fig. 4. Suppression of local GFP RNA silencing by WB1, WB2 and WB12 mutants of AtABCE2. (a) *Agrobacterium tumefaciens* harbouring pBin61-GFP was co-infiltrated with *A. tumefaciens* carrying the pBin61-AtABCE2 mutant or pBin61-AtABCE2 wt into one half of 16c *Nicotiana benthamiana* leaf blade (indicated as WB1, WB2, WB12 and AtABCE2). The other half was infiltrated with GFP/pBin61 agrobacteria mixture (indicated as pBin61). At 5 dpi, the leaves were detached and subjected to *in vivo* imaging of GFP. Average of fluorescent radiant efficiency was measured in the infiltrated patches and compared in each leaf separately. The means of GFP expression were compared between pBin61 and AtABCE2 (n = 25), pBin61 and WB1 (n = 24), pBin61 and WB2 (n = 28), pBin61 and WB12 (n = 24). Obtained results were statistically analysed with a paired two-tail *t*-test ($*P < 0.05$, $**P < 0.01$). Standard errors are indicated as error bars. (b), (c) Total RNA was extracted from agroinfiltrated patches and analysed by Northern blot. GFP-specific siRNAs were detected using [α-^{32}P] UTP-labelled antisense GFP transcript. U6 snRNA is shown as a loading control (indicated as U6). The levels of GFP siRNAs (24 and 21/22 nt long), quantified with ImageQuant TL, were normalized to U6 and compared with pBin61. NI indicates non-infiltrated leaf. The lanes were cropped from the same blots. Intact membranes are presented in Figure S2b,c.

et al., 2011; Nürenberg-Goloub *et al.*, 2018), and therefore it was not entirely surprising to observe a similar effect for another function of ABCE1. A recent article on the role of aABCE1 in ribosome recycling during canonical termination of translation provided data on timing and conformational changes in aABCE1 during each step (Nürenberg-Goloub *et al.*, 2018). There is an obvious parallel to our observations in the case of RNA silencing suppression by AtABCE2. To bind the post-termination complex, site II in aABCE1 must occlude ATP, thereby strongly stimulating ATP binding and hydrolysis in site I. ATP binding at site I is crucial for splitting the ribosomal subunits. After final and stable ATP binding in both sites, aABCE1 remains bound to the 40S subunit. For further dissociation from the ribosomal subunit, aABCE1 must hydrolyse ATP. At this step, ATP hydrolysis in site II is critical, but not in site I, according to the results published by Nürenberg-Goloub *et al.* (2018). In our research, we observed that only WB1, the mutant with affected ATP hydrolysis in site I, is able to suppress GFP RNA silencing at the local and systemic levels. The mutants lacking ATP binding activity at either site I or site II, as well as the mutants with impaired ATP hydrolysis at both sites or only at site II, have no suppression capacity.

Beside the ATPase core, ABCE1 possesses a unique (among ABC ATPases) FeS cluster domain and a no less intriguing HLH motif in NBD1. According to multiple research on ABCE1 in archaea, yeast and mammalian cells, the FeS cluster domain was found to be indispensable for ABCE1 function in translation and ribosome recycling. It was reported that the FeS cluster domain directly interacts with the ribosomal subunits and recycling factors eRF1 and Pelota, which are involved in canonical and mRNA surveillance-triggered termination of translation, respectively. During ribosome recycling, the FeS cluster domain undergoes extreme relocation upon ATP binding and thereby drives ribosome splitting (Pisareva *et al.*, 2011; Becker *et al.*, 2012; Kiosze-Becker *et al.*, 2016). In addition, through chemical cross-linking and mass spectrometry, multiple contacts were detected between the HLH motif and S24e ribosomal protein, as well as between the FeS cluster domain and S12 in the post-recycling complex (Kiosze-Becker *et al.*, 2016; Heuer *et al.*, 2017).

Our interest was to find out whether the FeS cluster domain or the HLH motif also contribute to the AtABCE2 suppressor function. We found that the AtABCE2ΔFeS mutant failed to suppress local as well as systemic silencing, indicating the indispensable role of this domain for AtABCE2 suppressor activity. Despite the fact that the FeS cluster domain forms an autonomous rigid entity, the complex of two NBDs directly regulates its relocation through an ATP binding/hydrolysis cycle (Kiosze-Becker *et al.*, 2016; Heuer *et al.*, 2017). According to our results, the FeS cluster domain, and likely its translocation, are necessary for the suppression activity of AtABCE2. The effect of HLH full deletion in other systems has not previously been studied. Here we demonstrate that deleting the entire HLH motif does not inhibit AtABCE2 suppressor function, since the AtABCE2ΔHLH mutant was able to suppress local GFP silencing. However, the deletion seems to affect the suppression of systemic silencing, indicating the somewhat supportive role of the HLH motif. One possible explanation might be that the HLH motif allows stronger interaction with factors affecting RNA silencing. Another possible interpretation would be to consider it as an important factor for ATPase core stability during the ATP binding/hydrolysis cycle.

Importantly, other RNA degradation mechanisms in eukaryotes, apart from RNA silencing, are required to provide RNA

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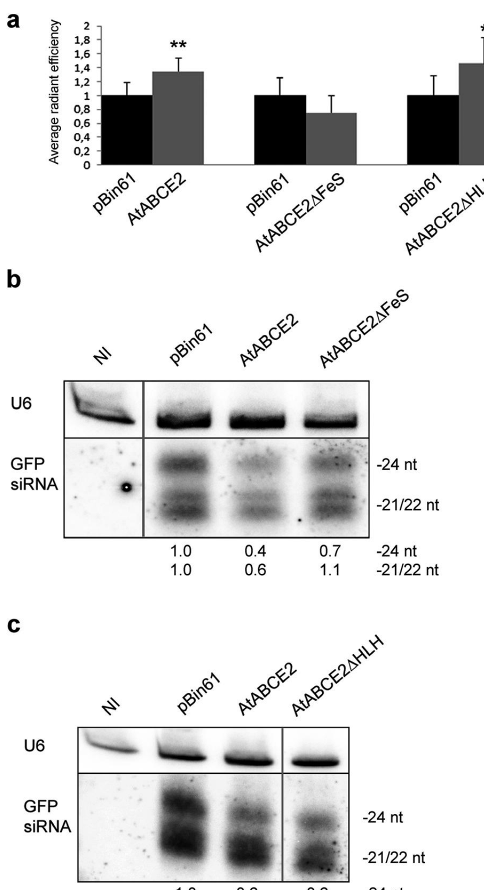

Fig. 5. Suppression of local GFP RNA silencing by AtABCE2ΔFeS and AtABCE2ΔHLH mutants. (a) *Agrobacterium tumefaciens* harbouring pBin61-GFP was co-infiltrated with *A. tumefaciens* carrying the pBin61-AtABCE2 mutant or pBin61-AtABCE2 wt into one side of 16c *Nicotiana benthamiana* leaf blade (indicated as AtABCE2ΔHLH, AtABCE2ΔFeS and AtABCE2), and the other side was infiltrated with *A. tumefaciens* harbouring pBin61-GFP together with *A. tumefaciens* carrying the empty vector (indicated as pBin61). At 5 dpi, the GFP fluorescence in the agroinfiltrated patches was measured with an *in vivo* imaging system. The values obtained from each leaf were compared separately. The data obtained from pBin61 and AtABCE2 (n = 25), pBin61 and AtABCE2ΔFeS (n = 27), pBin61 and AtABCE2ΔHLH (n = 24) pairs were statistically analysed with a paired two-tail *t*-test (*$P < 0.05$, **$P < 0.01$). Standard errors are indicated as error bars. (b), (c) Total RNA, extracted from agroinfiltrated patches was analysed by Northern blotting. The [α-^{32}P] UTP-labelled antisense GFP transcript was used for hybridisation of GFP siRNAs. U6 snRNA was detected as equal RNA loading control (indicated as U6). The levels of GFP siRNAs (24 and 21/22 nt long), quantified with ImageQuant TL, were normalized to U6 and compared with pBin61. NI indicates non-infiltrated leaf tissue. The lanes were cropped from the same blots. Intact membranes are presented in Figure S2d,e.

homeostasis in the cells. These are referred to as mRNA surveillance pathways: nonstop decay, no-go decay and nonsense-mediated decay. These processes are coupled with translation and strongly depend on the recycling of ribosomes (Graille & Séraphin, 2012). In *Drosophila*, mammalian cells and in yeast, ABCE1 mediates, in a concerted way with Pelota/Dom34 and Hbs1, the dissociation of ribosomes, which are stalled because of aberrant mRNAs. As a result, defective mRNAs can be eliminated by the Ski/exosome complex and Xrn1 exonuclease through either NSD, in the case of stop codon-less mRNA, or NGD, if mRNA contains elongation inhibiting features (Pisareva *et al.,* 2011; Kashima *et al.,* 2014; Hashimoto *et al.,* 2017; Navarro-Quiles *et al.,* 2018). In plants, the data concerning NSD and NGD are still largely missing. However, it was recently shown that *A. thaliana* encodes two Pelota paralogues: Pelota1 is a positive regulator of NSD and NGD, while Pelota2 suppresses both RNA decay pathways (Szádeczky-Kardoss *et al.,* 2018a; Szádeczky-Kardoss *et al.,* 2018b). It is not yet certain whether plant ABCE proteins interact with Pelota and function in ribosome recycling, although the high conservation of this process makes this very likely. Moreover, experiments involving *Cardamine hirsuta* ABCE2 (also named ChRLI2) revealed it as an important regulator of leaf growth and proposed, for the first time, a link between ABCE2 and ribosomes in plants (Kougioumoutzi *et al.,* 2013). Notably, mRNA surveillance pathways were found to compete with RNA silencing machinery for the substrate (Christie *et al.,* 2011; Liu & Chen, 2016; Szádeczky-Kardoss *et al.,* 2018a; Kim *et al.,* 2019). Because of the above results, we suggest that AtABCE2 might suppress RNA silencing *via* positive regulation of mRNA surveillance mechanisms. However, we cannot rule out another scenario, by which AtABCE2 directly interacts with components of RNA silencing pathways. We have previously identified a number of potential interactors for human ABCE1, which might contribute to its suppressor function and have putative homologues in *A. thaliana* (Kärblane *et al.,* 2015).

Taken together, mutational analysis results show that ATP binding and hydrolysis are important for AtABCE2 suppressor function. The FeS cluster domain likely mediates interactions with partners and is critical for the suppression of RNA silencing. High similarity between the structural requirements for the AtABCE2 suppressor function and those for aABCE1 role in ribosome recycling, crucial for mRNA surveillance, leads us to hypothesize that AtABCE2 could support RNA degradation pathways that compete with RNA silencing and therefore act as a negative regulator of silencing. The interactions of the AtABCE2 protein with the translational machinery, as well as with RNA silencing factors in plants, remain to be investigated.

AUTHOR CONTRIBUTIONS

Conceptualization, J.M., E.T. and C.S.; methodology, J.M., P.E. and C.S.; formal analysis, J.M. and C.S.; investigation, J.M., S.M. and C.S.; data curation, J.M.; writing of original draft preparation, J.M.; writing for review and editing, S.M., P.E., E.T. and C.S.; visualization, J.M.; supervision, C.S.; project administration, C.S.; funding acquisition, E.T and CS. All authors have read and agree to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

SUPPORTING INFORMATION

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

Figure S1. Representation of *in vivo* imaging of GFP.

Figure S2. The full-length Northern blots corresponding to the figures in the main text.

Table S1. Results of systemic silencing assay for nine independent experiments.

Table S2. Statistical analysis for the systemic silencing assay.

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Appendix 3

Publication III
Jakobson L*, **Mõttus J***, Suurväli J, Sõmera M, Tarassova J, Nigul L, Smolander O-P, Sarmiento C. (2024) Phylogenetic insight into ABCE gene subfamily in plants. Frontiers in Genetics, 15:1408665. doi: 10.3389/fgene.2024.1408665
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Phylogenetic insight into ABCE gene subfamily in plants

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ATP-BINDING CASSETTE SUBFAMILY E MEMBER (ABCE) proteins are one of the most conserved proteins across eukaryotes and archaea. Yeast and most animals possess a single *ABCE* gene encoding the critical translational factor ABCE1. In several plant species, including *Arabidopsis thaliana* and *Oryza sativa*, two or more *ABCE* gene copies have been identified, however information related to plant *ABCE* gene family is still missing. In this study we retrieved *ABCE* gene sequences of 76 plant species from public genome databases and comprehensively analyzed them with the reference to *A. thaliana ABCE2* gene (*AtABCE2*). Using bioinformatic approach we assessed the conservation and phylogeny of plant ABCEs. In addition, we performed haplotype analysis of *AtABCE2* and its paralogue *AtABCE1* using genomic sequences of 1,135 *A. thaliana* ecotypes. Plant ABCE proteins showed overall high sequence conservation, sharing at least 78% of amino acid sequence identity with AtABCE2. We found that over half of the selected species have two to eight *ABCE* genes, suggesting that in plants *ABCE* genes can be classified as a low-copy gene family, rather than a single-copy gene family. The phylogenetic trees of ABCE protein sequences and the corresponding coding sequences demonstrated that *Brassicaceae* and *Poaceae* families have independently undergone lineage-specific split of the ancestral *ABCE* gene. Other plant species have gained *ABCE* gene copies through more recent duplication events. We also noticed that ploidy level but not ancient whole genome duplications experienced by a species impacts *ABCE* gene family size. Deeper analysis of *AtABCE2* and *AtABCE1* from 1,135 *A. thaliana* ecotypes revealed four and 35 non-synonymous SNPs, respectively. The lower natural variation in *AtABCE2* compared to *AtABCE1* is in consistence with its crucial role for plant viability. Overall, while the sequence of the ABCE protein family is highly conserved in the plant kingdom, many plants have evolved to have more than one copy of this essential translational factor.

KEYWORDS

ABCE gene subfamily, ABCE, gene evolution, phylogenetics, natural variation

1 Introduction

Members of the ATP-BINDING CASSETTE (ABC) subfamily E (ABCE) belong to the superfamily of ABC proteins, which can be found in all living organisms studied to date and are regarded as highly essential in all eukaryotes. Most ABC proteins function as ATP-dependent membrane transporters. They possess transmembrane domains (TMDs) coupled with nucleotide-binding domains (NBD) otherwise known as ATP-binding

cassettes (Andolfo et al., 2015; Navarro-Quiles et al., 2018). ABCE [initially denoted RNASE L INHIBITOR (RLI)] proteins, in contrast, lack TMDs, but still have two NBDs associated with several specific domains and thus are soluble proteins.

In most species the ABCE subfamily is represented by a single member, ABCE1, which is involved in ribosome biogenesis and several stages of translation regulation (Yarunin et al., 2005; Andersen and Leevers, 2007; Barthelme et al., 2011; Mancera-Martínez et al., 2017; Navarro-Quiles et al., 2018). In accordance with its fundamental role, ABCE1 expression has been detected in most tissues and developmental stages of the species studied. In addition, loss-of-function of *ABCE1* genes results in a lethal phenotype in all studied species (Du et al., 2003; Zhao et al., 2004; Maeda et al., 2005; Sarmiento et al., 2006; Kougioumoutzi et al., 2013). ABCE1 has been found to participate in translational initiation and termination, however, its most conserved function is in the process linking these two stages of translation—ribosome recycling (Navarro-Quiles et al., 2018). During that process, ABCE1 splits the ribosome through direct interactions with ribosomal subunits and release factors, either after canonical stop codon-dependent termination or after recognition of stalled and vacant ribosomes. The latter is recognized during mRNA surveillance mechanisms such as no-go decay (NGD), non-stop decay (NSD), and non-functional 18S rRNA decay (18S-NRD) (Graille and Séraphin, 2012). Furthermore, ABCE1 dissociates the 80S-like complex during maturation of ribosomal subunits (Strunk et al., 2012). The role in ribosome biogenesis is supported by the nuclear accumulation of 40S and 60S ribosome subunits in the absence of ABCE1 (Kispal et al., 2005; Yarunin et al., 2005; Andersen and Leevers, 2007). Additionally, it has a key role in RNA silencing in both plants and animals (Sarmiento et al., 2006; Kärblane et al., 2015). Moreover, we have previously shown that human ABCE1 (HsABCE1) is directly or indirectly involved in histone biosynthesis and DNA replication (Toompuu et al., 2016).

The study of ABCE functions in plants has been mostly limited to the model plants *Arabidopsis thaliana*, *Nicotiana benthamiana*, *Nicotiana tabacum* and *Cardamine hirsuta* (Petersen et al., 2004; Sarmiento et al., 2006; Kougioumoutzi et al., 2013; Mõttus et al., 2021; Navarro-Quiles et al., 2022). In *A. thaliana* there are two genes encoding for paralogous ABCE proteins (AtABCE1 and AtABCE2, also referred to as AtRLI1 and AtRLI2, respectively), which share 80.8% identity (Mõttus et al., 2021; Navarro-Quiles et al., 2022). AtABCE2 is orthologous to HsABCE1 and is ubiquitously expressed in all plant organs (Sarmiento et al., 2006). Recently, AtABCE2 was found to interact with ribosomal proteins and translational factors, confirming its conserved ancestral function in translation that is coupled to general growth and vascular development, likely indirectly via auxin metabolism (Navarro-Quiles et al., 2022). Furthermore, through regulation of translation AtABCE2 is involved in the development of gametophyte and embryo (Yu, et al., 2023). In addition, AtABCE2 has been shown to suppress GFP transgene RNA silencing in heterologous system at the local and at the systemic levels by reducing accumulation of siRNAs (Sarmiento et al., 2006; Kärblane et al., 2015). Mutational analysis of AtABCE2 revealed that the structural requirements for RNA silencing suppression are similar to those needed for ribosome recycling in archaea (Mõttus et al., 2021). This indicates that AtABCE2 might suppress RNA silencing via supporting translation-associated RNA degradation mechanisms. The role of AtABCE1 in *A. thaliana*, which is expressed almost exclusively in generative organs (Navarro-Quiles et al., 2022; Yu et al., 2023), is yet to be studied.

Silencing of *ABCE* orthologues *(RLIh)* in *N. tabacum* resulted in a single viable transgenic plant exhibiting severe morphological alterations, supporting the important role of ABCE proteins at the whole-organism level. At that time, it remained unclear how many *RLIh* genes there are in tobacco species (Petersen et al., 2004). In *C. hirsuta,* a close relative of *A. thaliana* that has composite leaves, there is only one *ABCE* gene in the genome, named SIMPLE LEAF3 (SIL3, or ChRLI2) (Kougioumoutzi et al., 2013). Hypomorphic mutation Pro177Leu in the NBD1 domain of ChRLI2 affects the determination of leaf shape and regulation of auxin homeostasis (Kougioumoutzi et al., 2013). Interestingly, the expression of *ChRLI2* was not ubiquitous as in *A. thaliana*, but instead it was shown to be expressed in meristematic and vascular tissues of young developing leaves and in leaflet initiation sites (Kougioumoutzi et al., 2013).

It is commonly claimed that most eukaryotes only have one *ABCE* gene (Dermauw and Van Leeuwen, 2014). Exceptions to this have been detected in plants such as thale cress, rice, maize, potato and tomato, but also in animals such as catfish, cod and mosquitoes (Braz et al., 2004; Garcia et al., 2004; Verrier et al., 2008; Liu et al., 2013; Pang et al., 2013; Andolfo et al., 2015; Lu et al., 2016). Although some plant species have more than one *ABCE* gene, it is still the smallest and most conserved of all ABC subfamilies (Andolfo et al., 2015).

In this study we aimed to characterize the phylogenetic evolution of *ABCE* genes in plants in order to shed light on the possible functional diversification within ABCE protein family. Here we present the results of an extensive bioinformatics analysis of publicly available sequences for plant *ABCE* genes and corresponding proteins, together with haplotype analysis of *A. thaliana ABCE*s.

2 Methods

2.1 Construction of the phylogenetic diagram

The phylogenetic diagram of the studied plants species together with the bar chart of *ABCE* gene number was created based on NCBI taxonomy with phyloT and visualized with iTOL (Letunic and Bork, 2007; 2019; phylot.biobyte.de).

2.2 Genome and proteome data acquisition

ABCE sequence data for 55 species was downloaded from the online resource Phytozome portal https://phytozome.jgi.doe.gov/ (Goodstein et al., 2012). ABCE sequence data for additional 18 species was downloaded from Ensembl Plants (Howe et al., 2020). The genome data for *C. hirsuta* was accessed at http://bioinfo.mpipz.mpg.de/blast/ (Gan et al., 2016). Genome data for *N. tabacum* and *N. benthamiana* was downloaded from Sol Genomics Network http://solgenomics.net (Edwards et al., 2017;

Kourelis et al., 2019). We downloaded genomic, CDS and translated amino acid sequences for each plant *ABCE* gene used in the study.

The length of amino acid sequences was calculated with SeqinR package (version 3.6.1) in R 4.0.2 (Charif and Lobry, 2007). In order to calculate their similarities to AtABCE2, all 152 sequences were aligned using the online interface of MUSCLE with default Pearson/FASTA parameters provided by the European Bioinformatics Institute (EBI) (Madeira et al., 2019). Thereafter the percent identity scores were calculated with MUSCLE algorithm for aligned sequences in R 4.0.2 package Bio3D version 2.4-1 (Edgar, 2004; Grant et al., 2021).

Next, sequences aligned to AtABCE2 were inspected for the general protein structure, that is the presence and correct order of the domains, including iron-sulphur (FeS) cluster domain, NBD1, NBD2 and bipartite Hinge domain (Supplementary Figure S1). Sequences lacking critical motifs within these domains (Karcher et al., 2005; Barthelme et al., 2007; Nürenberg and Tampé, 2013; Nürenberg-Goloub et al., 2020) were filtered out from the analysis.

2.3 Evolutionary analysis by maximum likelihood method

Amino acid sequences were aligned with default parameters of the MUSCLE algorithm, as implemented in the MEGA software package (version 11.0.13) (Tamura et al., 2021). CDS sequences were aligned with the default parameters of MAFFT (version 7.4.9.0) (Katoh and Standley, 2013). As the alignments contained gaps, sites with no data for more than 10% of the sequences were removed with trimAl (version 1.4. rev22) (Capella-Gutiérrez et al., 2009), resulting in an amino acid alignment of 603 positions and a CDS alignment of 1,810 positions.

Phylogenetic trees of ABCE full length amino acid sequences and the corresponding CDS sequences were constructed by IQ-TREE (version 2.0.7) (Minh et al., 2020) with the Maximum Likelihood method and 10,000 rapid bootstrap replicates. Initial tests suggested that the best models to use would be JTTDCMut for amino acids and TIM2e for CDS sequences, both with five degrees of FreeRate heterogeneity (Soubrier et al., 2012). Tree calculation was performed 20 times independently for both CDS and amino acid input, with random seed values ranging from 1 to 20. TreeGraph (version 2.15.0–887) (Stöver and Müller, 2010) was used to root all trees on *Chlorophyta* and collapse any branches supported by bootstrap values of 50% or less. The trees were then all added to a single file in the order of their log likelihoods (highest to lowest) and annotated in FigTree (version 1.4.3; http://tree.bio.ed.ac.uk/software/figtree/). Final adjustments (fine-tuning the color scheme) were done in Adobe Illustrator.

Unrooted tree supporting suitability of *Chlorophyta* as an outgroup as well as separate trees based on the amino acid sequences of ABCE domains were constructed by MEGA software. Phylogenetic relationships were inferred using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992), selected for each data set based on the lowest BIC scores (Bayesian Information Criterion). A discrete gamma distribution was used to model evolutionary rate differences among sites. Bootstrap analysis was performed with 500 replicates. The trees with the highest log likelihood were published for each analysis.

2.4 Modelling the drivers of ABCE copy number

Two different approaches were used to test the impact of assembly size, ploidy level, and the number of known ancient whole genome duplications on ABCE copy numbers. A simple linear regression was performed with the lm function available in base R (version 4.3.2) (R Core Team, 2023). In an alternative approach, the R package taxize (version 0.9.100) (Pinheiro et al., 2023) was first used to extract phylogenetic relationships of all species involved from the NCBI databases. Next, the function gls (generalized least squares) from R package nlme (version 3.1–164) (Pinheiro and Bates, 2000; Pinheiro et al., 2023) was used to create additional regression models with phylogenetic signal included as a random effect. Ggpredict from the R package ggeffects (version 1.3.4) (Lüdecke, 2018) was used to obtain prediction intervals for the models. The results were plotted with the packages ggplot2 (version 3.4.2) (Wickham, 2016) and patchwork (version 1.2.0) (Pedersen, 2024), then adjusted in Adobe Illustrator.

2.5 Data acquisition for 1135 Arabidopsis ecotypes

Data (SNPs and indels) available for 1135 *A. thaliana* strains was downloaded from the 1,001 Genomes Project depository (Weigel and Mott, 2009).

2.6 Reconfirming the haplotypes of Arabidopsis ecotypes

Seeds of the selected 21 Arabidopsis ecotypes were acquired from the Nottingham Arabidopsis Stock Centre (NASC). Both *AtABCE1* and *AtABCE2* full coding sequences were PCR-amplified and sequenced by Sanger sequencing for ecotypes Can-0, Ei-2, IP-Car-1, Kia1 and Pra-6. For the other 15 ecotypes only *AtABCE1* full coding sequences was sequenced. Primer pairs used for the PCRs are shown in the Supplementary Table S1. For all the PCR reactions touchdown PCR method with the following conditions was used: 95°C for 15 min; 13 cycles of at 95°C for 15 s, at the gradually decreasing temperature from 60°C to 54°C (the temperature drops by 0.5°C per cycle) for 30 s, at 72°C for 70 s; 15 cycles of 95°C 15 s, 54°C 30 s, 72°C 70 s and the final extension at 72°C for 10 min. Amplified DNA fragments were purified from the agarose gel using GeneJET Gel Extraction Kit (Thermo Scientific) according to the manufacturer's instructions. Thereafter the purified DNA fragments were Sanger sequenced and aligned respectively to *AtABCE1* or *AtABCE2*. The final results were based on the sequencing of at least two plants for each ecotype. Columbia (Col-0) ecotype was used as a reference.

2.7 Generating haplotype map with PopART

For the haplotype analysis, first an alignment file with CDS sequences was created in Nexus format. Thereafter the multiple sequence alignment was analyzed with PopART version 1.7

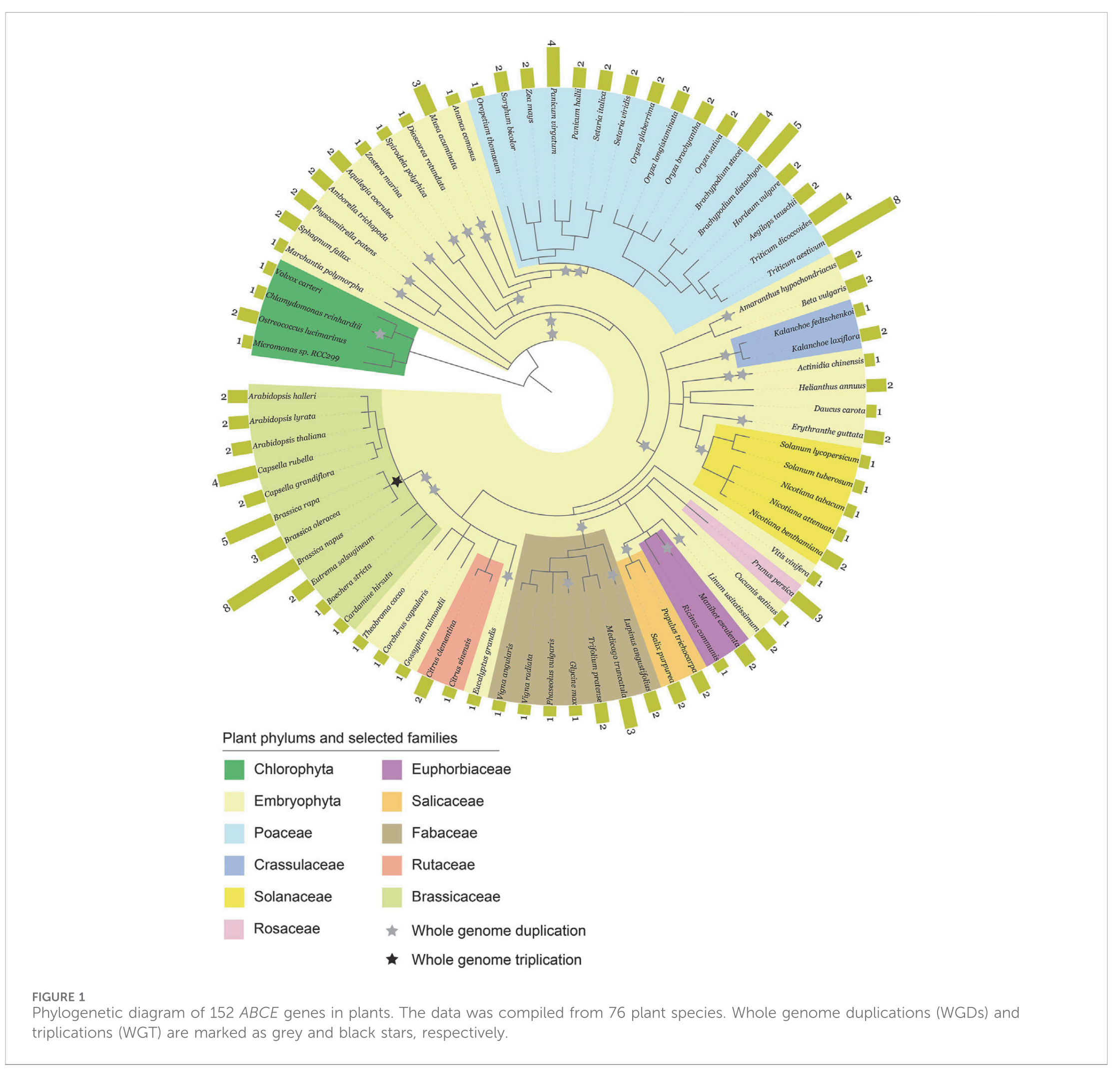

FIGURE 1
Phylogenetic diagram of 152 *ABCE* genes in plants. The data was compiled from 76 plant species. Whole genome duplications (WGDs) and triplications (WGT) are marked as grey and black stars, respectively.

(Population Analysis with Reticulate Trees) (Leigh and Bryant, 2015). The network was constructed with Median Joining Network algorithm (epsilon = 0).

3 Results

3.1 Variability of plant *ABCE* genes

To gain insight into the diversity of *ABCE* genes in plants, we compiled a selection of *ABCE* genes from 76 different plant species available in public databases. The selection criteria for including in further analysis was the presence of all known essential structural elements of ABCE proteins (Karcher et al., 2005; Barthelme et al., 2007; Nürenberg and Tampé, 2013; Nürenberg-Goloub et al., 2020). Truncated or aberrant sequences were discarded from further analysis. Altogether 152 plant *ABCE* genes were included in the study. We found that the amino acid sequence identity among the 152 proteins was 78% or higher when compared to *A. thaliana* AtABCE2. The selected species represented a wide range of plant groups, including unicellular algae such as *Chlamydomonas reinhardtii* and *Micromonas* sp. *RCC299*, monocots such as *Zea mays* and *Triticum aestivum*, *Solanum* species such as *Solanum tuberosum* and *N. benthamiana*, and *Brassicaceae* such as *Brassica napus* and *A. thaliana* (Figure 1; Supplementary Table S2). Our analysis revealed that plant species from the phylum *Chlorophyta* (green algae) usually possess only a single *ABCE* gene, except for *Ostreococcus lucimarinus*, which has two genes. In contrast, most of the analyzed species in the *Poaceae* family have at least two *ABCE* genes, and some have as many as eight genes in their genome, as is the case for *T. aestivum*. Another group of plant species with an above-average number of *ABCE* genes is the *Brassicaceae* family. For

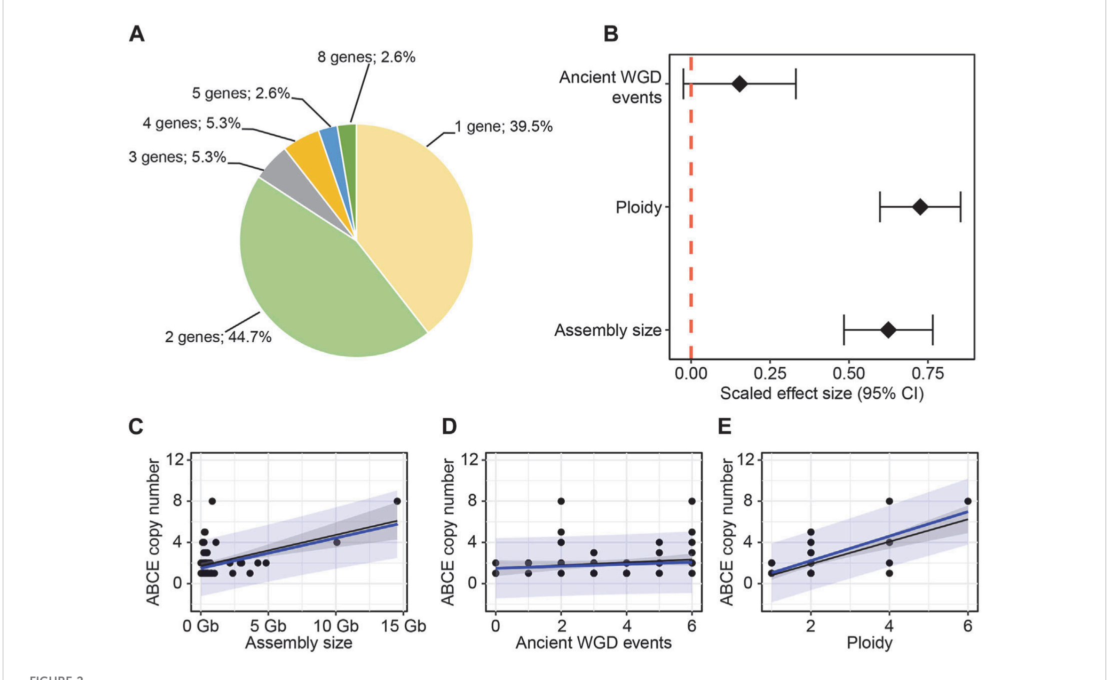

FIGURE 2
Relationship between *ABCE* copy number and genomic parameters. **(A)** *ABCE* gene copy number among the studied 76 plant species. **(B)** Both ploidy level and the size of the genome assembly have a positive effect on the number of *ABCE* genes. The number of ancient whole genome duplications has no clear effect. **(C–E)** Correlation between *ABCE* copy number and different predictor variables. Black: linear regression, with 95% confidence intervals. Blue: phylogenetic generalized least squared regression, with 95% prediction intervals.

example, *B. napus* has eight genes, *B. rapa* five genes, and *Capsella rubella* four genes. On the other hand, among *Brassicaceae*, *C. hirsuta* and *Boechera stricta* have only a single *ABCE* gene. Despite clustering of multi-gene-species in the *Poaceae* and *Brassicaceae* families, there was no visual segmentation between the number of *ABCE* genes and phylogenetic origin in other plant families (Figure 1). Interestingly, only 30 species out of 76 (39.5%) had a single *ABCE* gene. Similarly, there were 34 species (44.7%) possessing two *ABCE* genes (Figure 2A). This shows that despite some species having only single functional *ABCE* gene (containing full set of critical structural elements) *ABCE* genes in plants can be classified as a low-copy gene family instead of single-copy gene family.

Our next goal was to explore how genomic parameters contribute to *ABCE* gene copy number in plants. We tested the effect of ploidy level, genome size and WGD events during evolution on the *ABCE* gene family size of a species. WGD data was based on the data of 53 plant species published by the One Thousand Plant Transcriptomes Initiative (Leebens-Mack et al., 2019). The observation of WGT data in the common ancestor of *Brassica* species was based on the study of Wang and coworkers (Wang et al., 2011). Regression analysis showed positive effect of ploidy level and genome size on *ABCE* gene copy number. Among selected parameters a degree of ploidy is likely the most suitable as a prediction factor, although there were examples of tetraploid species with a single *ABCE* gene (e.g., *Nicotiana tabacum*) and diploids with five *ABCE* genes (e.g., *Brachypodium distachyon*, *B. rapa*) (Figures 2B,C,E; Supplementary Table S2). In contrast, there was no clear correlation between ancient WGD events experienced by a species and *ABCE* gene family size (Figures 2B,D; Supplementary Table S2). Interestingly, there are examples of species, which have encountered at least five WGDs in their evolutionary history, but still possess only a single *ABCE* gene, for example, *Actinidia chinensis* and *Glycine max* (Figure 1; Supplementary Table S3).

3.2 Plant *ABCE*s are highly conserved

ABCE proteins are composed of four domains: NBD1 and NBD2 forming the ATPase core, bipartite hinge domain that is tightly engaged in twin-NBD cassette arrangement and a unique N-terminal FeS cluster domain (Figure 3A). Additionally, ABCEs embody a helix–loop–helix (HLH) motif in NBD1 that distinguishes it from otherwise superimposable NBD2 (Karcher et al., 2005).

As many as 86.8% out of the 152 analyzed gene sequences encode ABCE of canonical protein length (600—609 amino acids) (Figure 3B). Along with exceptional conservation within functionally critical motifs, all analyzed ABCE sequences are highly similar to AtABCE2 sharing at least 78% of amino acid sequence identity (Figure 3C; Supplementary Table S4).

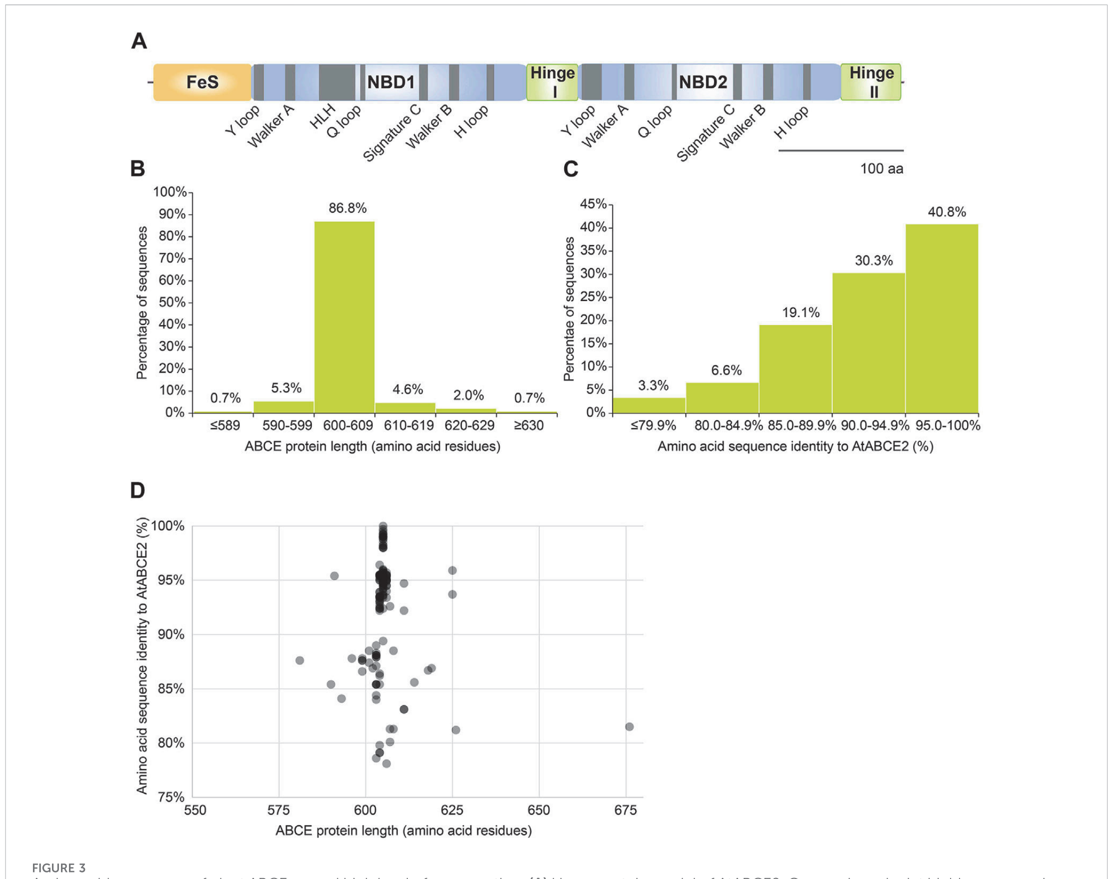

FIGURE 3
Amino acid sequences of plant ABCEs reveal high level of conservation. **(A)** Linear protein model of AtABCE2. Grey regions depict highly conserved motifs within AtABCE2. FeS—iron-sulphur cluster domain, NBD1—nucleotide-binding domain 1, NBD2—nucleotide-binding domain 2. **(B)** Histogram of protein sequence lengths of the studied 152 plant ABCEs. **(C)** Histogram of amino acid sequence identities of the studied 152 plant ABCEs, based on MUSCLE alignment. **(D)** The correlation between amino acid sequence identity and protein sequence length of the studied 152 plant ABCEs.

We also plotted amino acid sequence length to amino acid sequence identity for the studied 152 ABCE proteins. There was a clear clustering of proteins with the length of 605 amino acids (Figure 3D). Proteins with lower sequence identity did not cluster by protein length (Figure 3D). Interestingly, proteins with more than 90% identity to AtABCE2 could be as short as 591 amino acids and as long as 625 amino acids long (Figure 3D). Hence, despite some variance in amino acid sequence length and sequence identity to AtABCE2, the selection of amino acid sequences analyzed here is uniform and represents well the plant *ABCE* genes.

3.3 Phylogeny of plant *ABCE*s

To understand how ABCE proteins have evolved in the green plant lineage, we constructed 20 Maximum Likelihood (ML) trees of 152 full-length ABCE protein sequences and the corresponding coding DNA (CDS) sequences from 76 species (Figure 4; Supplementary Figures S2–S4). *Chlorophyta* (green algae), the earliest lineage to have split off from the rest of the green plants, was used as an outgroup for rooting the trees. In an unrooted tree the representatives of *Chlorophyta* formed a separate cluster with high bootstrap support (Supplementary Figure S5).

The CDS tree (Figure 4; Supplementary Figure S4) proved much more informative than the amino acid tree (Supplementary Figures S2, S3). Most internal branches in the amino acid tree are poorly supported by bootstrap values, likely resulting from the lack of phylogenetic signal in the highly conserved sequences. The CDS tree is more congruent with the known species tree (Figure 1). However, both show that the sequences cluster according to major taxonomic groupings, usually with high bootstrap support. For instance, all ABCEs from *Fabaceae* (legumes) form a single cluster, as is the case for *Solanaceae* (nightshades), *Poaceae* (grasses), *Brassicaceae* (mustard and cabbage family, including the thale cress *A. thaliana*), and others. These groupings appear older than any duplication events present in the ABCE family. For example, the *AtABCE1* and *AtABCE2* genes of *A. thaliana* are the result of a duplication that happened in *Brassicaceae*, whereas the multiple

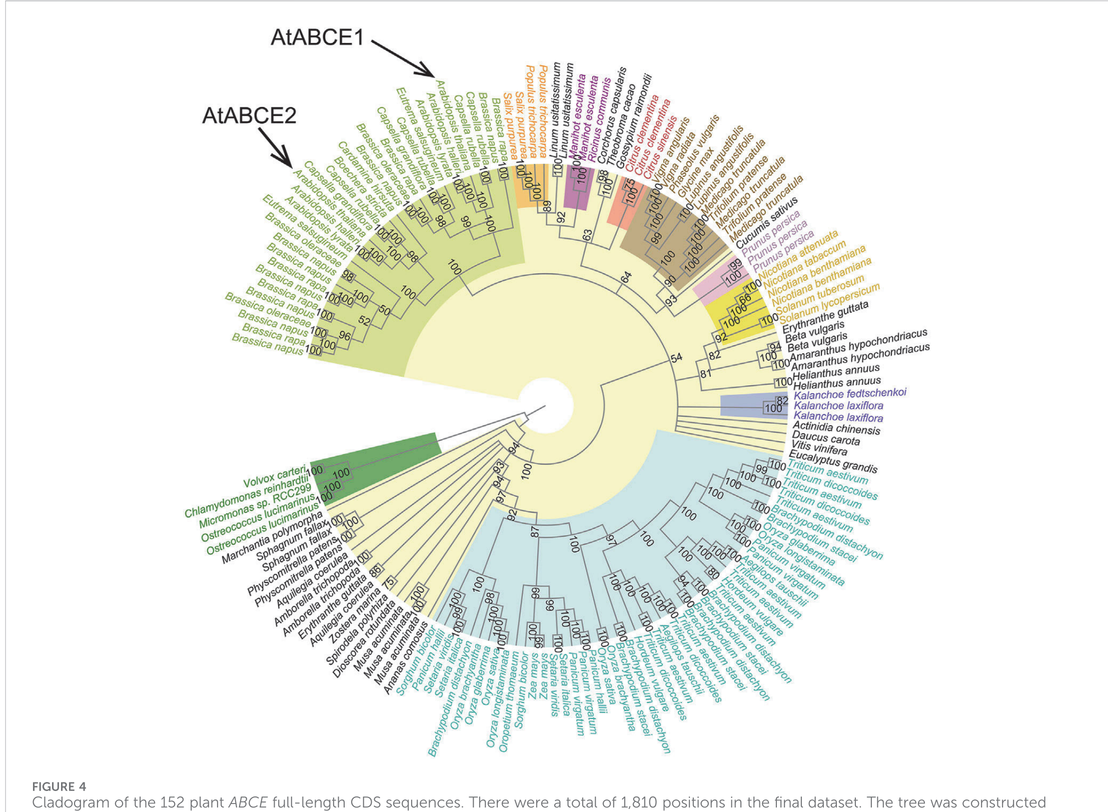

FIGURE 4
Cladogram of the 152 plant *ABCE* full-length CDS sequences. There were a total of 1,810 positions in the final dataset. The tree was constructed using the Maximum Likelihood method, TIM2e model with five categories of FreeRate heterogeneity and 10,000 rapid bootstrap replicates. All branches supported by bootstrap values of less than 50% were collapsed. Color-coding refers to affiliation with larger plant phyla or families as indicated in Figure 1. The tree is rooted on the phylum *Chlorophyta*.

copies seen in wheat and other members of *Poaceae* result from different duplication events. Further examination of the data revealed that all cases with more than one ABCE sequence in a given species can be broadly divided in two. Often the closest neighbor for one of the sequences was a different one from the same species, which is most likely reflective of recent duplication events. However, in other cases much older duplications were found, with two or more gene copies evolving independently across different species of the same plant family or order. This is well known for *ABCE* genes in *Brassicaceae* (Navarro-Quiles et al., 2022), and our analysis confirms that many of their family members including *A. thaliana* encode distinct ABCE1 and ABCE2 (Figure 4). In support of the notion that AtABCE2 preserves the ancestral function (Navarro-Quiles et al., 2022), we show that ABCE2 sequences have fewer mutations and shorter branch lengths compared to ABCE1s in the phylogenetic tree (Supplementary Figure S4). Notably, all *Brassicaceae* species have at least one ABCE2 protein while ABCE1 can be missing.

Similarly to the ABCE1 of *Brassicaceae*, *Poaceae* (grasses) also include one set of ABCEs that have acquired more mutations than the others. It is present in all analyzed species of rice (*Oryza*), in foxtails (*Setaria*), in sorghum and in the common grasses *Panicum hallii* and *Brachypodium distachyon*. All of those also have at least one slower evolving copy. Wheat and related species (*Triticum*) do not have a direct homolog of the fast-evolving ABCE copy, but they have multiple ABCEs regardless. In any case, the fast-evolving ABCE of *Poaceae* is not the direct homologue of the ABCE1s in *Brassicaceae*, and it is much closer to other ABCEs in *Poaceae* instead. Thus, both *Poaceae* and *Brassicaceae* have at least two distinct ABCE lineages that appeared in the ancestors of the respective families. In both cases there is evidence of rapid accumulation of mutations in one of the genes (long branches in the phylogeny), likely reflective of neo- or subfunctionalization. In amino acid trees (but not CDS trees) those two unrelated groups stemming from long branches are typically grouped together, which is likely due to long branch attraction. In most such cases, one of the three genes from the plum *Prunus persica* also tends to group together with those two sets of sequences (as seen from supplementary trees presented in the associated GitHub repository: https://github.com/jsuurvali/abce152). As expected, the plum gene originates from a longer branch than the other two genes in that species.

In contrast to *Brassicaceae* and *Poaceae*, based on our dataset no such ancestral subtype separation was found in other plant clades. For example, the model species *N. benthamiana* has two ABCEs, but some *Solanaceae* have only one, and all ABCEs in *Solanaceae* are

TABLE 1 Non-synonymous SNPs found in *AtABCE1* and *AtABCE2* among 21 *A. thaliana* ecotypes. All SNPs were verified by Sanger sequencing and whole-genome sequencing published in the 1,001 Genomes project (Weigel and Mott, 2009; Cao et al., 2011). SNP locations were numbered according to the position in the cDNA sequence starting from ATG. Change in amino acid sequence corresponding to the SNP is presented. Orange color depicts SNPs that are not present in 1,001 Genomes Project data but verified by Sanger sequencing within this study. Green color shows SNPs positioned in conserved arginine residues.

No	Ecotype	SNPs in *AtABCE1* cDNA	Amino acid change in AtABCE1	SNPs in *AtABCE2* cDNA	Amino acid change in AtABCE2	Origin
1	**IP-Ezc-2**	257G>A	Arg86Gln	None	None	Spain
		1682A>T	His561Leu			
2	**IP-Cot-0**	374G>A	Gly125Glu	None	None	Spain
		1682A>T	His561Leu			
3	**IP-Hoy-0**	374G>A	Gly125Glu	None	None	Spain
		1682A>T	His561Leu			
4	**IP-Loz-0**	374G>A	Gly125Glu	None	None	Spain
		1682A>T	His561Leu			
5	**IP-Vis-0**	878C>A	Pro293Gln	None	None	Spain
		1682A>T	His561Leu			
6	**IP-Moz-0**	967C>T	Arg323Cys	None	None	Spain
		1682A>T	His561Leu			
7	**Lebja-1**	1010C>G	Thr337Arg	None	None	Russia
		1682A>T	His561Leu			
8	**Leska-1-44**	1321G>A	Ala441Thr	None	None	Bulgaria
		1682A>T	His561Leu			
9	**Toufl-1**	1682A>T	His561Leu	None	None	Morocco
		1715G>T	Arg572Leu			
10	**Kly4**	1682A>T	His561Leu	None	None	Russia
		1762A>T	Lys588STOP			
11	**Et-0**	473G>A	Arg158Gln	None	None	France
		478G>A	Val160Ile			
		1682A>T	His561Leu			
12	**Cvi-0**	811G>A	Val271Ile	None	None	Cape Verde
		1321G>A	Ala441Thr			
		1682A>T	His561Leu			
13	**IP-Mdd-0**	386C>A	Pro129Gln	None	None	Spain
		415G>A	Asp139Asn			
		1321G>A	Ala441Thr			
		1682A>T	His561Leu			
14	**Grivo-1**	386C>A	Pro129Gln	None	None	Bulgaria
		757C>T	Leu253Phe			
		1321G>A	Ala441Thr			
		1682A>T	His561Leu			

(Continued on following page)

TABLE 1 (*Continued*) Non-synonymous SNPs found in *AtABCE1* and *AtABCE2* among 21 *A. thaliana* ecotypes. All SNPs were verified by Sanger sequencing and whole-genome sequencing published in the 1,001 Genomes project (Weigel and Mott, 2009; Cao et al., 2011). SNP locations were numbered according to the position in the cDNA sequence starting from ATG. Change in amino acid sequence corresponding to the SNP is presented. Orange color depicts SNPs that are not present in 1,001 Genomes Project data but verified by Sanger sequencing within this study. Green color shows SNPs positioned in conserved arginine residues.

No	Ecotype	SNPs in *AtABCE1* cDNA	Amino acid change in AtABCE1	SNPs in *AtABCE2* cDNA	Amino acid change in AtABCE2	Origin
15	**Lag1-7**	386C>A	Pro129Gln	None	None	Georgia
		1117G>C	Asp373His			
		1321G>A	Ala441Thr			
		1646C>G	Ala549Gly			
		1682A>T	His561Leu			
15	**Qar-8a**	386C>A	Pro129Gln	None	None	Lebanon
		1117G>C	Asp373His			
		1321G>A	Ala441Thr			
		1646C>G	Ala549Gly			
		1682A>T	His561Leu			
17	**Ei-2**	1682A>T	His561Leu	130G>A	Gly44Ser	Germany
18	**Kia 1**	1682A>T	His561Leu	130G>A	Gly44Ser	Sweden
19	**Pra-6**	1682A>T	His561Leu	130G>A	Gly44Ser	Spain
20	**IP-Car-1**	1682A>T	His561Leu	1136T>C	Met379Thr	Spain
21	**Can-0**	1682A>T	His561Leu	1214G>C	Gly405Ala	Spain

closer to each other than to any of the ones in *Brassicaceae* or *Poaceae* (Figure 4; Supplementary Figure S4). However, in CDS tree the placement of some groups in relation to each other could not be resolved from ABCE sequences alone and was both incongruent with their known positions in the Tree of Life and poorly supported by bootstrap analyses. This is the case for the relationship between *Brassicaceae* and other representatives of Rosids (including *Citrus* sp.). The placement of *ABCE* sequences from *Amborella trichopoda*, a single extant member of a sister lineage to all other angiosperms, did not match the species tree as well (Figure 4; Supplementary Figure S4). However, the effect of those artifacts was reduced by collapsing poorly supported branches in the tree, and showing then clearly that the exact relationship between *Brassicaceae* and other eudicots, or *A. trichopoda* and other angiosperms cannot be fully resolved based on the data. In the current version, those branches and groupings appear as part of polytomies in a multifurcating tree.

In addition, we separately analyzed the three main domains of ABCE proteins, FeS cluster domain, NBD1, and NBD2, by realigning the corresponding amino acid sequences and constructing a ML-tree for each (Supplementary Figures S6–S8). The topology of the resulting trees was different for each domain, but none of those were well supported by bootstrap analysis.

3.4 Natural variation of Arabidopsis *AtABCE1* and *AtABCE2* genes

Natural variation among *A. thaliana* ecotypes has been well documented by the 1,001 Genomes Project (Weigel and Mott, 2009). We analyzed the *ABCE* gene sequences of all 1135 *A. thaliana* ecotypes reported in that project and found 35 and four non-synonymous SNPs in *AtABCE1* and *AtABCE2*, respectively (Supplementary Table S5). Only four reported non-synonymous SNPs in *AtABCE2* indicate a low degree of natural variation, which is consistent with its fundamental, conserved role in growth and development. On the other hand, 35 non-synonymous SNPs annotated for *AtABCE1* show relatively higher natural variation. This finding is in agreement with the results from the transspecies phylogenetic analysis of *Brassicaceae* ABCE1 and ABCE2. From the previously reported SNPs in AtABCE1, we selected 18 that cause amino acid substitutions at conserved and important positions or that were present in combination with other SNPs of interest. Therefore, 21 ecotypes were included in the further study and resequencing, together with Col-0 (Table 1; Supplementary Table S5). Two SNPs causing amino acid substitution could not be detected by resequencing (Pro399Thr in Grivo-1 and Gly182Ser in IP-Cot-0). Instead, one SNP previously undocumented in the 1,001 Genomes Project database (Leu253Phe in Grivo-1) was identified. Figure 5A shows the positions of the amino acid substitutions caused by the 17 SNPs sequenced in the *AtABCE1* gene. In our resequencing analysis, the most frequent SNPs in *AtABCE1* caused the changes His561Leu and Ala441Thr (Table 1; Supplementary Table S5). Noteworthy, histidine at the position 561 seems to be characteristic to Col-0, as all the other analyzed ecotypes had leucine at this position. Next, we performed haplotype analysis with CDS sequences on the PopART platform and found that the most conserved sequence of *AtABCE1* is most probably the one identical to Ei-2, Kia1, Pra-6, IP-Car-1 and Can-0. Eight SNPs out

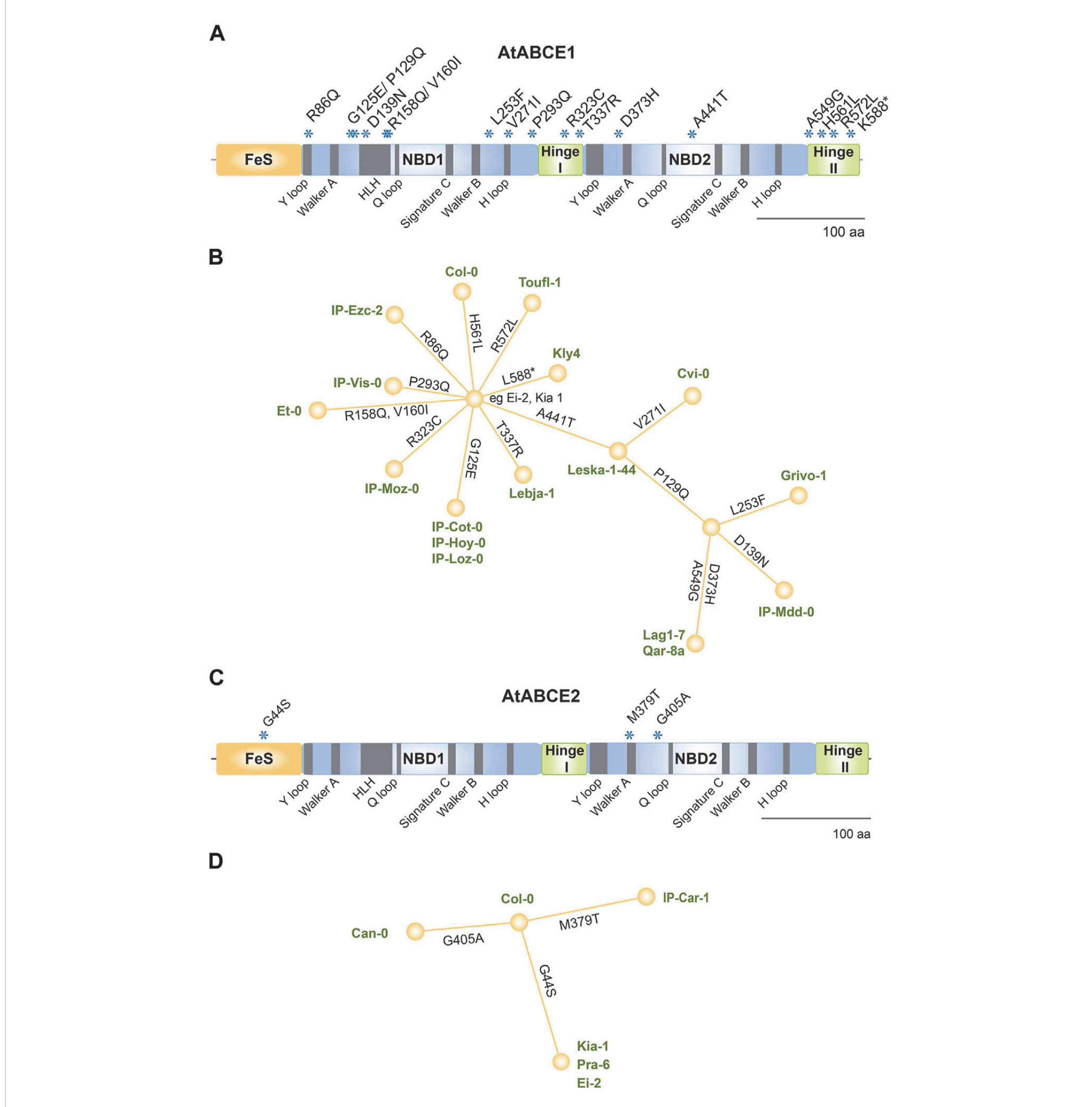

FIGURE 5
Analysis of non-synonymous SNPs in *AtABCE1* and *AtABCE2*. **(A)** Linear protein model of AtABCE1. Asterisks depict amino acid substitutions due to SNPs verified in different ecotypes. **(B)** Haplotype map of AtABCE1 detected among 22 Arabidopsis ecotypes. Branch length represents the number of mutations between sequences. For pairs of haplotypes whose distances on the tree are longer than the distances between the sequences, edges are added to shorten the distance. **(C)** Linear protein model of AtABCE2. Asterisks depict amino acid substitutions due to SNPs verified in different ecotypes. **(D)** Haplotype map of AtABCE2 detected among six Arabidopsis ecotypes. Branch length represents the number of mutations between sequences. For pairs of haplotypes whose distances on the tree are longer than the distances between the sequences, edges are added to shorten the distance.

of 17 appear as single SNPs in the *AtABCE1* of Kly4, Toufl-1, Col-0, IP-Ezc-2, IP-Vis-0, IP-Moz-0, IP-Hoy-0, IP-Loz-0, IP-Cot-0 and Lebja-1. Interestingly, a substitution of Ala441Thr can appear both as the consequence of a single SNP in Leska-1-44 and together with other SNPs such as in Cvi-0 or Qar-8a (Figure 5B). Some amino acid changes, like Pro129Gln, Ala549Gly and His561Leu, are always grouped (Table 1). Pro129Gln appears only together with at least two other SNPs, e.g., in Grivo-1 or Qar-8a (Figure 5B).

From the previously reported four non-synonymous SNPs in *AtABCE2*, we were able to reconfirm three SNPs causing amino acid changes (Gly44Ser, Met379Thr and Gly405Ala), which were located in FeS cluster domain and NBD2 at non-conserved positions (Figure 5C; Supplementary Table S5). Asp189Glu in AtABCE2 was not possible to reverify due to unavailable seed material. The verified SNPs were present in five different ecotypes (Table 1). The haplotype map of *AtABCE2* SNPs shows

that Col-0 has the most conserved sequence and the three different SNPs root from it (Figure 5D).

In this study we could not find any correlation between the presence of non-synonymous SNPs in *AtABCE* genes and the geographical origin of the ecotype (Table 1). Visual rosette phenotype of the studied ecotypes matched with characterization available in the public databases (Supplementary Figure S9).

In the case of 18 out of 21 ecotypes, all SNPs were confirmed as reported earlier. For three ecotypes only part of the SNPs was validated: Leska-1-44 did not exhibit Pro129Gln, IP-Cot-0 did not exhibit Gly182Ser and Grivo-1 did not exhibit Pro399Thr amino acid changes in AtABCE1 (Supplementary Table S5; Table 1). More interestingly, we verified Gly125Glu in IP-Cot-0 and Pro129Gln, Leu253Phe, Ala441Thr and His561Leu in Grivo-1. Leu253Phe had not been annotated in any *A. thaliana* ecotype in the 1,001 Genomes Project database (Table 1).

Surprisingly, we noticed some SNPs affecting highly conserved amino acid residues in AtABCE1. These include arginine residues from R cluster of Hinge domains (Arg323Cys and Arg572Leu of IP-Moz-0 and Toufl-1, respectively), and Arg86Gln of IP-Ezc-2 ecotype that locates to the Y-loop I (Figure 5A; Table 1). According to the 1,001 Genomes Project database the latter SNP is present in 19 *A. thaliana* ecotypes (Supplementary Table S4).

Although, the length of ABCE proteins in *A. thaliana* ecotypes is very conserved, in a single ecotype, namely, Kly-4, we found a SNP in *AtABCE1* causing premature stop codon that makes the protein 14 amino acid residues shorter. Despite this deletion, the cluster of arginine residues remains intact in Hinge II subdomain (Figures 5A,B; Supplementary Figure S1).

4 Discussion

The availability of high-quality plant genome sequences is growing day by day, which creates a completely new and underexploited repository. It has been recognized that the plant genome evolution has been very complex, including polyploidy, periods of rapid speciation and extinction (Leebens-Mack et al., 2019; Wang et al., 2021; Qiao et al., 2022). Interestingly, massive expansions of gene families took place before the origins of green plants, land plants and vascular plants. Whole genome duplications (WGDs) that have occurred at least 244 times throughout the evolution of plants and ferns increase ploidy of genomes and largely impact gene family size variation within different lineages. Apart from autopolyploidy, which results from intraspecies WGD events, there are also allopolyploid species, which originate from interspecies hybrids and render gene evolution tracking challenging (Leebens-Mack et al., 2019).

4.1 How many *ABCE* genes do plants have and need?

As was previously mentioned, in most animal and in yeast species the *ABCE* gene family is represented by a single gene that encodes the vital ABCE1 protein. In plant kingdom, *ABCE* gene family size across different lineages is more variable. Based on the data from the public databases and our analysis we were able to reconfirm the same number of *ABCE* genes for a selection of plant species. For example, there is a single gene in *C. hirsuta* (Kougioumoutzi et al., 2013), in *Chlamydomonas reinhardtii* (Li et al., 2022), in *Citrus sinensis* and in *Theobroma cacao* (Navarro-Quiles et al., 2022). Similarly to previous studies, we reverified two genes in *Z. mays* (Pang et al., 2013), in *Solanum lycopersicum* (Ofori et al., 2018), in *Oryza sativa*, and in *Populus trichocarpa* (Navarro-Quiles et al., 2022). The same was true for five *ABCE* genes from *Brassica rapa* (Navarro-Quiles et al., 2022). Intriguingly, Zhang and others found three *ABCE* genes in *Hordeum vulgare*, whereas our study identified only two fully intact ABCE sequences with all canonical subunits (604 and 611 amino acids long) (Zhang et al., 2020). Moreover, for *C. rubella* we identified four *ABCE* genes as opposed to two sequences analyzed earlier (Navarro-Quiles et al., 2022). These discrepancies might be due to the strict filtering of non-functional protein sequences performed in our study. In addition, fast-developing sequencing technologies and implementation of novel annotation tools contribute to higher accuracy of the newer genome versions. Taken together, plant species present in our dataset possess one to eight *ABCE* genes encoding complete proteins, suggesting that plant *ABCE* genes do not comprise a single-copy gene family, but rather should be classified as a low-copy gene family.

Most plant species have experienced at least two ancient WGD events, followed by additional rounds of genome doubling in many lineages (Qiao et al., 2022). Thus, we were tempted to speculate that WGD could impact *ABCE* family size variation. However, in this study we did not notice significant correlation between the number of ancestral WGD events obtained from the literature and *ABCE* gene copy number in a species. This might be due to the rapid genome downsizing following WGD event that is leading to chromosomal rearrangements and extensive loss of duplicated copies (Wang et al., 2021). In addition, the WGD-derived duplicates are often subject to relaxed selection, meaning rapid mutation resulting in defunctionalisation (Qiao et al., 2019). For example, *Glycine max* and *A. chinensis* retained a single functional *ABCE* gene after five documented WGD events. Interestingly, the overexpression of ABCE1 in yeast causes growth inhibition (Dong et al., 2004), meaning that the amount of *ABCE* present—and therefore probably also the number of hypothetical redundant genes—is critical for the well-functioning of translation, a crucial process.

However, when higher expression of a particular gene is beneficial, its duplicate might be retained in the genome. This could be the case for the two ABCE paralogues in *Z. mays* that are located close to each other in our phylogenetic analysis (Figure 4) and share the same expression pattern profiles (Pang et al., 2013). Alternatively, as a result of faster evolution, gene duplicates may obtain novel functions or specialized expression patterns (Prince and Pickett, 2002). In Arabidopsis, AtABCE1 and AtABCE2 exhibit partial functional redundancy. In contrast to AtABCE2, which is ubiquitously expressed, AtABCE1 is mostly present in generative organs and at relatively low levels (Klepikova et al., 2016; Navarro-Quiles et al., 2022; Yu et al., 2023). This could mean an ongoing process of pseudogenization or subfunctionalization, where the paralogues acquire specific roles. There is growing evidence regarding ribosomal heterogeneity and the existence of specialized cell-type-specific ribosomes (Xue and Barna, 2012; Barna et al., 2022), suggesting that AtABCE1 is involved in the

regulation of translation in generative tissues. Paralogous *ABCE* genes in plants may serve to provide specificity in fine-tuning translation and controlling cellular translatome (Gerst, 2018).

We also noticed slight positive effect of ploidy level on *ABCE* gene copy number in a species. In the future, the determinants of *ABCE* copy number can be further elucidated by including more species from diverse lineages and using statistical modelling that takes phylogenetic structuring of the data also into account. These models could also potentially incorporate other information from the species that was not used for the present study, such as whether the species is annual or perennial, their preferred mode of reproduction, or what kind of environments do they grow in.

4.2 In plants *ABCE* genes are prone to duplicate

In this study we analyzed 152 *ABCE* sequences from 76 plant species. This included the most well studied plant *ABCE* gene—AtABCE2, which is thought to preserve the ancestral functions of *ABCE* proteins (Navarro-Quiles et al., 2022). Phylogenetic trees of full-length *ABCE* sequences confirmed previously reported clustering into ABCE2 and ABCE1 groups for *Brassicaceae* (Navarro-Quiles et al., 2022) but also demonstrated that the multiple copies observed in several other plant lineages originate from separate duplication events. The results suggest that *Brassicaceae* and *Poaceae* families have undergone independent lineage-specific splits of the ancestral *ABCE* gene. *Pooideae*, the largest *Poaceae* subfamily that includes barley and wheat, appears to have had further duplication events and its members have additional *ABCE* genes. In addition to *Brassicaceae* and *Poaceae*, many other plant taxa have also gained *ABCE* gene copies, most likely because of more recent duplications. Interestingly, it was recently shown that one of the four *ABCE* gene copies from *Prunus dulcis*, a close relative of *P. persica*, originated from tandem duplication. *ABCE* genes share strong collinearity between these species, suggesting that this duplication preceded speciation event (Zhang et al., 2024). We can therefore postulate that in contrast to species which possess a single *ABCE* gene and are sensitive to copy number changes (Dong et al., 2004), many plants have gained additional *ABCE* copies through WGD or local duplication and likely have evolved to benefit from higher numbers of this essential translational factor. Gene copies may arise from different events including WGD, tandem- and transposon-related duplications, but the precise source of *ABCE* subfamily expansion in plants remains to be investigated.

4.3 Natural variation of *A. thaliana* ABCEs

Usually, essential genes are subject to strong evolutionary pressure and thus, non-synonymous SNPs in conserved regions of gene sequences are rare (Castle, 2011; Pang et al., 2016). In *AtABCE1* we found three SNPs that could potentially impact the protein's function (Table1; Figure 5A). SNPs causing the substitutions Arg323Cys (in IP-Moz-0) and Arg572Leu (in Toufl-1) located at the Hinge domain I and II, respectively, could be of importance, since these domains are essential for NBD-twin cassette assembly in the case of ABCE1 in other organisms (Karcher et al., 2005). In addition, AtABCE1 of IP-Ezc-2 ecotype contains an amino acid substitution at position Arg86Gln, which is an exceptionally conserved site across archaea as well as eukaryotes and locates to Y-loop I. In the context of Y-loop with consensus sequence H**R**YGVNAF, the arginine residue has been shown to mediate interaction between FeS cluster domain and NBD1 in the sole *ABCE1* gene of *Pyrococcus abyssi* (Karcher et al., 2008).

The His561Leu amino acid change was reported to be present in 997 out of 1135 *A. thaliana* ecotypes (Supplementary Table S5), which suggest that histidine at this position of *AtABCE1* might be characteristic only to a small subset of ecotypes including Col-0. Thus, it seems that leucine is the most conserved residue at position 561 in *AtABCE1* among Arabidopsis ecotypes.

As expected, in *AtABCE2* gene, known to be essential for the viability of an organism (Navarro-Quiles et al., 2022; Yu et al., 2023) only four non-synonymous SNPs residing in non-conserved regions were reported among 1135 *A. thaliana* ecotypes (Supplementary Table S5). Importantly, the *AtABCE2* gene seems to be hard to mutate, since up to now there is no T-DNA homozygous line available and only one viable, hypomorphic allele has been recently isolated after ethyl methanesulfonate mutagenesis (Navarro-Quiles et al., 2022). Interestingly, the only non-synonymous SNP present in more than one ecotype in the case of *AtABCE2* is leading to Gly44Ser substitution in the FeS domain. According to the 1,001 Genomes Project database this mutation is present in 54 ecotypes, three of them were confirmed in the current study (Table 1; Figure 5; Supplementary Table S5). The same position is able to incorporate 12 different amino acid residues among other plant ABCEs studied herein, alanine, glycine and serine being the most common ones. Moreover, the SNP M379T verified only in IP-Car-1 poses a promising material for further mutational analysis as this site is highly conserved among plant ABCEs with only leucine as a rare alternative (Figure 5D; 152_pepseq_MUSCLE.fa from the associated GitHub repository).

Taken together, this study has shown the surprisingly high number of *ABCE* genes among the plant kingdom. We hypothesize that plants have developed a number of specialized ABCEs with more specific functions compared to species carrying a single copy of *ABCE* gene such as humans, fruit fly or yeast.

Data availability statement

Data relevant to this study has been made publicly available in Supplementary Materials and Github 496 repository https://github.com/jsuurvali/abce152.

Author contributions

LJ: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Software, Validation, Writing—original draft. JM: Data curation, Investigation, Methodology, Software, Validation, Writing—original draft. JS: Data curation, Methodology, Software, Validation, Writing—review and editing. MS: Methodology, Software, Writing—review and editing. JT: Methodology, Writing—review and editing. LN: Methodology, Writing—review and editing. O-PS: Software, Writing—review and editing. CS: Conceptualization,

Funding acquisition, Investigation, Supervision, Validation, Writing—review and editing.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2024.1408665/full#supplementary-material

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Publications

Jakobson L*, **Mõttus J***, Suurväli J, Sõmera M, Tarassova J, Nigul L, Smolander O-P, Sarmiento C. (2024) Phylogenetic insight into ABCE gene subfamily in plants. Frontiers in Genetics, 15:1408665. doi: 10.3389/fgene.2024.1408665

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Publikatsioonid

Jakobson L*, **Mõttus J***, Suurväli J, Sõmera M, Tarassova J, Nigul L, Smolander O-P, Sarmiento C. (2024) Phylogenetic insight into ABCE gene subfamily in plants. Frontiers in Genetics, 15:1408665. doi: 10.3389/fgene.2024.1408665

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