Functional Characterization of SERRATE Interacting Proteins

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Summary

Plant microRNAs (miRNAs) regulate many aspects of plant development including hormone responses, floral development and phyllotaxy. Mature miRNAs associate with ARGONAUTE (AGO) proteins to bind and regulate target mRNAs. Mature miRNAs are released from longer primary-miRNAs (pri-miRNA) by the RNaseIII-like enzyme DICER-LIKE 1 (DCL1). Additional RNA-binding proteins including SERRATE (SE), HYPONASTIC LEAVES1 (HYL1), TOUGH (TGH) and the CAP BINDING COMPLEX (CBC) facilitate efficient and precise processing of pri-miRNA transcripts. SE and the CBC are of particular interest, because they fulfill an additional function in pre-messanger RNA (pre-mRNA) splicing. This leads to the hypothesis that SE and CBC build a platform, which recruits different RNA processing factors to Polymerase II derived transcripts.

I could show by using physical and genetic interaction studies as well as molecular analyses that SE and the CBC form a higher-order complex. Each component is equally required for accurate pri-miRNA processing and splicing. In order to identify new components, which might be recruited by the SE/CBC complex, we conducted a yeast to hybrid screen using SE as bait. The eukaryotic WD40 repeat containing scaffold protein RECEPTOR FOR ACTIVATED C KINASE (RACK1) was discovered as a putative SE interacting protein and I analyzed the function of RACK1 in respect to RNA processing.

The Arabidopsis genome encodes three *RACK1* genes (*A*, *B*, *C*), which control plant development throughout the life cycle. Molecular, biochemical and phenotypic analyses of *rack1* and known miRNA mutants lead me to the conclusion that RACK1 affects multiple steps of the miRNA pathway. In *rack1* mutants, mature miRNAs accumulate only to low levels and several miRNAs were not accurately diced from their respective pri-miRNAs. In addition, some pri-miRNA transcripts are elevated in *rack1* mutants implying that the RACK1/SERRATE interaction is important for early steps of the miRNA biogenesis.

In line with the observation that RACK1 is a novel miRNA factor, I found that miRNA targeted mRNAs are misregulated in *rack1* mutants and that *rack1* mutants exhibit typical phenotypic alteration that are often associated with reduced miRNA levels (e.g ABA hypersensitivity or phyllotaxy defects). However, vegetative phase change, which is tightly controlled by an interwoven network with miR156 as a central player, is not accelerated as in other miRNA mutants, but delayed in *rack1* mutants. Further analyses suggested that *RACK1* influences the expression miR156 targeted *SPL* transcription factors independently of its function in miR156 maturation.

Taken together, my results revealed that the SERRATE interacting scaffold protein RACK1 is a novel component of the plant miRNA pathway and that RACK1 affects plant development through miRNA-dependent and independent mechanisms.

Zusammenfassung

MicroRNAs (miRNA) spielen eine bedeutende Rolle in der pflanzlichen Entwicklung. Sie regulieren dabei wichtige Prozesse wie Hormonantworten, Blatt- und Blütenentwicklung sowie die Phyllotaxis. miRNAs sind negative Regulatoren der Genexpression und wirken auf post-transkriptioneller Ebene. Dabei binden ARGONAUTE Effektorproteine reife miRNAs, welche sequenzspezifisch Ziel-mRNAs erkennen und regulieren. Reife miRNAs werden aus längeren primary-miRNA-Transkripten (pri-miRNA) durch das RNaseIII-ähnliche Enzym DICER-LIKE 1 (DCL1) generiert. Weitere Proteine unterstützen DCL1 bei der effizienten und genauen Prozessierung der pri-miRNAs, unter anderem die RNA-bindenden Proteine SERRATE (SE), HYPONASTIC LEAVES1 (HYL1), TOUGH (TGH) und der CAP BINDING COMPLEX (CBC). SE und der CBC sind von besonderem Interesse, da sie eine zusätzlich Rolle beim Spleißen von pre-messenger RNAs (pre-mRNAs) spielen. Dies führte zur Annahme, dass SE und der CBC ein Gerüst bilden, das unterschiedliche RNA-Prozessierungsfaktoren zu verschiedenen RNA-Spezies rekrutiert.

Mit Hilfe genetischer und physischer Interaktionsstudien sowie molekularer Analysen konnte ich zeigen, dass SE in der Tat mit dem CBC interagiert. Eine Hefe-Zweihybrid Sichtung, in der SE als Köder eingesetzt wurde, führte zur Identifikation neuer putativer Interaktionspartner. Darunter befand sich das eukaryotische Gerüstprotein RECEPTOR FOR ACTIVATED C KINASE (RACK1), welches näher auf seine Funktion in der RNA-Prozessierung hin untersucht wurde.

Das Genom von Arabidopsis kodiert für drei *RACK1* Gene (*A*, *B*, *C*), welche essentiell für die pflanzliche Entwicklung sind. Durch molekulare, biochemische und phänotypische Analysen von *rack1*-Mutanten konnte ich zeigen, dass *RACK1* mehrere Schritte der miRNA-Biogenese beeinflusst. *rack1*-Mutanten akkumulieren geringere Mengen reifer miRNAs, was charakteristisch für bekannte miRNA-Biogenesemutanten wie *se* oder *hyl1* ist. Einige pri-miRNA-Transkripte werden zudem schlechter in *rack1*-Mutanten prozessiert, was darauf hindeutet, dass die SE/RACK1-Interaktion eine wichtige Funktion in der frühen miRNA-Biogenese spielt.

Eine fehlerhafte miRNA-Biogenese beeinflusst die durch miRNA regulierten Ziel-mRNAs. Verschiedene dieser miRNA-Zielgene sind in *rack1*-Mutanten misreguliert und die damit einhergehenden phänotypischen Veränderungen wie z. B. ABA-Hypersensitivität und phyllotaktische Defekte, konnten in *rack1*-Mutanten beobachtet werden. Interessanterweise zeigen *rack1* Mutanten nicht alle durch miRNAs ausgelöste Defekte, wie zum Beispiel die Regulation des Wechsels von der juvenilen zur adulten Phase. Meine Ergebnisse deuten darauf hin, dass RACK1 Entwicklungsprozesse durch miRNA-abhängige und miRNA-unabhängige Wirkmechanismen beeinflusst.

Zusammenfassend konnte ich in meiner Doktorarbeit zeigen, dass SE mit einem neuen miRNA-Faktor, RACK1, interagiert, der verschiedene Funktionen bei der Reifung von miRNAs ausübt und so einige miRNA-gesteuerte pflanzliche Entwicklungsprozesse beeinflusst.

Chapter I: Introduction into miRNA-mediated gene silencing in Arabidopsis

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Gene expression operates according to the central dogma of molecular biology: a gene encoded by the DNA is transcribed into its messenger RNA (mRNA), which then is translated into the corresponding protein, the executing product of the gene. A tight regulation of gene expression is crucial for plant developmental programs or adaptation to different environmental cues. Gene expression can be regulated on DNA, mRNA or protein level by influencing the production, the stability and function of mRNAs and proteins. 20 years ago, microRNAs (miRNA), a class of non-coding small RNAs, have been discovered in *C. elegans* and were shown to regulate gene expression at the post-transcriptional level (Lee *et al.* 1993). Today, we know about the fundamental regulatory function of miRNAs in animals and plants. MiRNAs regulate many aspects of plant development and stress responses, like leaf, root and flower development, flowering time and senescence, plant immunity and adaptation to abiotic stresses (Ruiz-Ferrer and Voinnet 2009, Sarwat *et al.* 2013, Sun 2012). The basic principles of miRNA biogenesis and action are remarkably similar in plants and animals. Mature miRNAs are released from a longer RNA precursor by a DICER protein and subsequently loaded in ARGONAUTE effector complexes to induce miRNA-mediated silencing of target mRNAs (Krol *et al.* 2010, Rogers and Chen 2013). However, several years of research in plants and animals have broadened this simplified perspective. MiRNA biogenesis and action build a complex regulatory network, which is influenced by many different factors and stimuli to guarantee accurate regulation of gene expression.

This introduction provides a detailed overview about miRNA-mediated gene silencing in Arabidopsis, focusing on *MIRNA* transcription and processing, the effector complex maturation and miRNA action, the subcellular localization of those processes and, finally describes regulatory mechanisms, influencing the output of the miRNA pathway.

MIRNA transcription and processing

Arabidopsis produces almost 300 different miRNAs (mirbase.org). The majority of *MIRNA* genes are located in intergenic regions forming a distinct transcriptional unit (Reinhart *et al.* 2002). *MIRNA* genes are transcribed by RNA Polymerase II (Pol II) like canonical protein-coding genes and the resulting transcript is modified with a 5' 7-methyl guanosine (7mG) cap and a poly(A)-tail (Kim *et al.* 2011, Xie *et al.* 2005, Zhang *et al.* 2005a). Some pri-miRNA transcripts contain introns, which are thought to be spliced by the canonical spliceosome (Laubinger *et al.* 2008, Mica *et al.* 2009, Rogers and Chen 2013, Szarzynska *et al.* 2009). These similarities between protein-coding genes and *MIRNA* genes indicated that some regulatory components like promoter structure or proteins involved in transcription might be shared by both classes of genes. Indeed, *MIRNA* promoters comprise a TATA-box, a core *cis*-regulatory element, to which general transcription factors bind in order to initiate transcription (Xie *et al.* 2005). Additionally, the mediator complex, a conserved eukaryotic transcriptional co-activator, is essential for *MIRNA* transcription by recruiting Pol II to *MIRNA* promoters (Kim *et al.* 2011; Figure 1).

In plants, the processing of non-coding small RNAs is performed by DICER-LIKE (DCL) proteins, a class of RNaseIII-like enzymes (Margis *et al.* 2006). The Arabidopsis genome encodes four different DCL proteins, each of them is involved in the biogenesis of a different classes of small RNAs (Vazquez 2006). DCL proteins use double-stranded RNA or single stranded RNA with a hairpin structure as substrate to generate 20 to 24 nucleotide (nt) long RNA duplexes with an 2-nt overhang at the 3' ends (Bernstein *et al.* 2001, Margis *et al.* 2006). Pri-miRNA transcripts form a hairpin-like structure, in which the stem comprises the miRNA (Reinhart *et al.* 2002). DCL1 performs the processing of pri-miRNA transcripts in a sequential manner. The first cut at the base of the stem region releases the precursor-miRNA (pre-miRNA) and this is followed by a second cut in the loop region, which leads to the production of a 21 nt long miRNA duplex (miRNA/miRNA*) (Liu *et al.* 2012, Park *et al.* 2005, Reinhart *et al.* 2002). DCL1 needs some structural determinants within the hairpin to release the correct miRNA duplex. Studies with modified or randomly mutagenized transcripts revealed that, in general, a loop distal to the miRNA duplex and a 15 base pairs (bp) stem followed by a junction with single stranded RNA proximal to the miRNA duplex are important for accurate processing (Mateos *et al.* 2010, Song *et al.* 2010, Werner *et al.* 2010).

A couple of other proteins have been shown to assist DCL during miRNA biogenesis with respect to transcript stability, processing efficiency and accuracy (Figure 1). DAWDLE (DDL), a nuclear forkhead-associated domain containing protein, is associated with DCL1 in a phosphorylation dependent manner and binds pri-miRNAs transcripts to influence their stability (Machida and Yuan 2013, Yu *et al.* 2008). TOUGH (TGH), another RNA-binding

protein, affects the processing efficiency of pri-miRNA transcripts (Ren *et al.* 2012). Additionally, the zinc-finger protein SERRATE (SE) and the double strand RNA-binding protein HYPONASTIC LEAVES 1 (HYL1), both binding to pri-miRNAs, act by ensuring accurate and efficient processing (Dong *et al.* 2008, Lobbes *et al.* 2006, Vazquez *et al.* 2004, Yang *et al.* 2006). Because physical interactions have been reported between DCL1, SE, HYL1 and TGH, it has been suggested that these proteins build the general pri-miRNA processing complex necessary for releasing mature miRNA duplexes (Fang and Spector 2007, Hiraguri *et al.* 2005, Ren *et al.* 2012, Yang *et al.* 2006). The CAP-BINDING COMPLEX (CBC), a hetero dimer consisting of CAP-BINDING PROTEIN 80 (CBP80) and CAP-BINDING PROTEIN 20 (CBP20), is well-known from its function in mRNA metabolism and binds also to the 7mG-cap of pri-miRNAs, thereby promoting the processing of the bound transcripts (Kim *et al.* 2008, Laubinger *et al.* 2008). Interestingly, the RNA-binding protein MODIFIER OF SNC1 (MOS2) affects miRNA processing, but does not interact with the canonical processing components like DCL1, HYL1, SE or TGH. Instead, MOS2 binds pri-miRNAs and influences the association of HYL1 with those transcripts, suggesting a supporting function in recruitment of pri-miRNAs to the processing complex (Wu *et al.* 2013). Other proteins, like NEGATIVE ON TATA less2 (NOT2) and CELL CYCLE DEPENDENT 5 (CDC5), which have been previously associated with transcriptional regulation and mRNA metabolism, physically associate with Pol II and components of the miRNA processing machinery, thereby influencing *MIRNA* transcription and processing (Wang *et al.* 2013, Zhang *et al.* 2013). This supports the idea, that *MIRNA* transcription and processing are connected processes and processing may be performed in a co-transcriptional manner.

Some proteins, which are important for miRNA processing, additionally affect splicing of pre-mRNA and pri-miRNA transcripts. This includes SE, the CBC, SICKLE (SIC) and STABILIZED 1 (STA1), indicating that there might be a functional overlap between miRNA processing and splicing (Ben Chaabane *et al.* 2013, Laubinger *et al.* 2008, Zhan *et al.* 2012).

AGO1 effector complex: miRNA loading and miRNA action

All small RNAs act only in concert with ARGONAUTE (AGO) effector proteins. Together they form the executing unit of small RNA-mediated processes, thereby regulating transcriptional and post-transcriptional gene silencing (Meister 2013, Vaucheret 2008). Arabidopsis expresses 10 different AGO proteins, having redundant and distinct functions in small RNA-mediated processes. Among them, AGO1 is the major player in the miRNA pathway (Vaucheret 2008; Figure 1).

It is assumed that AGO1 effector complexes pass through a maturation process to become fully active. This involves miRNA duplex (miRNA/miRNA*) loading and removal of the passenger strand (miRNA*), which may be promoted by conformational changes as well as

changes in associated proteins. The general chaperones, HEAT-SHOCK PROTEIN 90 (HSP90) and HEAT-SHOCK PROTEIN 70 (HSP70) as well as the *cis-trans*-peptidylprolyl isomerase SQUINT (SQN) are transiently associated with AGO1 effector proteins to ensure proper miRNA duplex loading in an ATP-dependent manner (Earley and Poethig 2011, Iki *et al.* 2012, Iki *et al.* 2010). The dissociation of the three proteins is accompanied by the removal of the passenger strand (miRNA*) and leads in the end to a mature AGO1 effector complex associated with the guide strand (miRNA) (Iki *et al.* 2010; Figure 1). There are hints that additional proteins might be involved in this process. A beta importin, SAD2/EMA1, usually involved in nuclear-cytoplasmic trafficking, has been shown to negatively regulate miRNA accumulation in AGO1 effector complexes, indicating a function in miRNA loading (Wang *et al.* 2011).

Guide and passenger strand selection is dependent on several determinates. One issue is the 5' nucleotide and the other one is the thermo stability of the duplex at the 5' end. The majority of guide strands, which are bound by AGO1, have a 5' Uracil (Mi *et al.* 2008, Takeda *et al.* 2008) and a lower thermo stability at the 5' end compared to the 5' end of the passenger strand (Eamens *et al.* 2009). Strand selection assays with artificial miRNAs demonstrated that the thermo stability of the duplex is the more important selection criteria (Eamens *et al.* 2009). Studies performed with *hyl1* mutants indicated a function for HYL1 in strand selection. The authors suggested a mechanism, by that HYL1 may bind to the more thermodynamically stable strand helping AGO1 to select the correct strand (Eamens *et al.* 2009). Also, mutations in *C-terminal domain phosphatase-like 1* (*CPL1*), which negatively regulates HYL1 function, lead to defects in strand selection (Manavella *et al.* 2012). This implies that there might be a connection between miRNA processing and miRNA loading into the effector complex mediated by the RNA binding protein HYL1.

MiRNAs regulate target mRNA transcripts at the post-transcriptional level by guiding AGO1 effector proteins to complementary target mRNAs in a sequence specific manner. This leads to cleavage and/or to translational repression of the bound target mRNA, resulting in a reduction of protein accumulation in both cases (Rogers and Chen 2013; Figure 1). AGO1 possesses a catalytically active RNaseH-like domain, which enables miRNA-mediated cleavage of target mRNAs (Baumberger and Baulcombe 2005, Mi *et al.* 2008, Qi *et al.* 2005). This process depends on the complementarities between miRNA and target mRNA sequence (Iwakawa and Tomari 2013, Mallory *et al.* 2004, Schwab *et al.* 2005). In contrast to animals, plant miRNAs are highly complementary to their target mRNAs, which might be one reason that most target mRNAs are regulated by cleavage (Addo-Quaye *et al.* 2008, Brodersen *et al.* 2008, German *et al.* 2008). Nevertheless, a couple of reports showing miRNA-mediated translational repression in plants, for example miR156, miR172, miR398

and their corresponding targets AP2, SPL3, CSD1/2, CCS (Aukerman and Sakai 2003, Beauclair *et al.* 2010, Brodersen *et al.* 2008, Dugas and Bartel 2008, Gandikota *et al.* 2007).

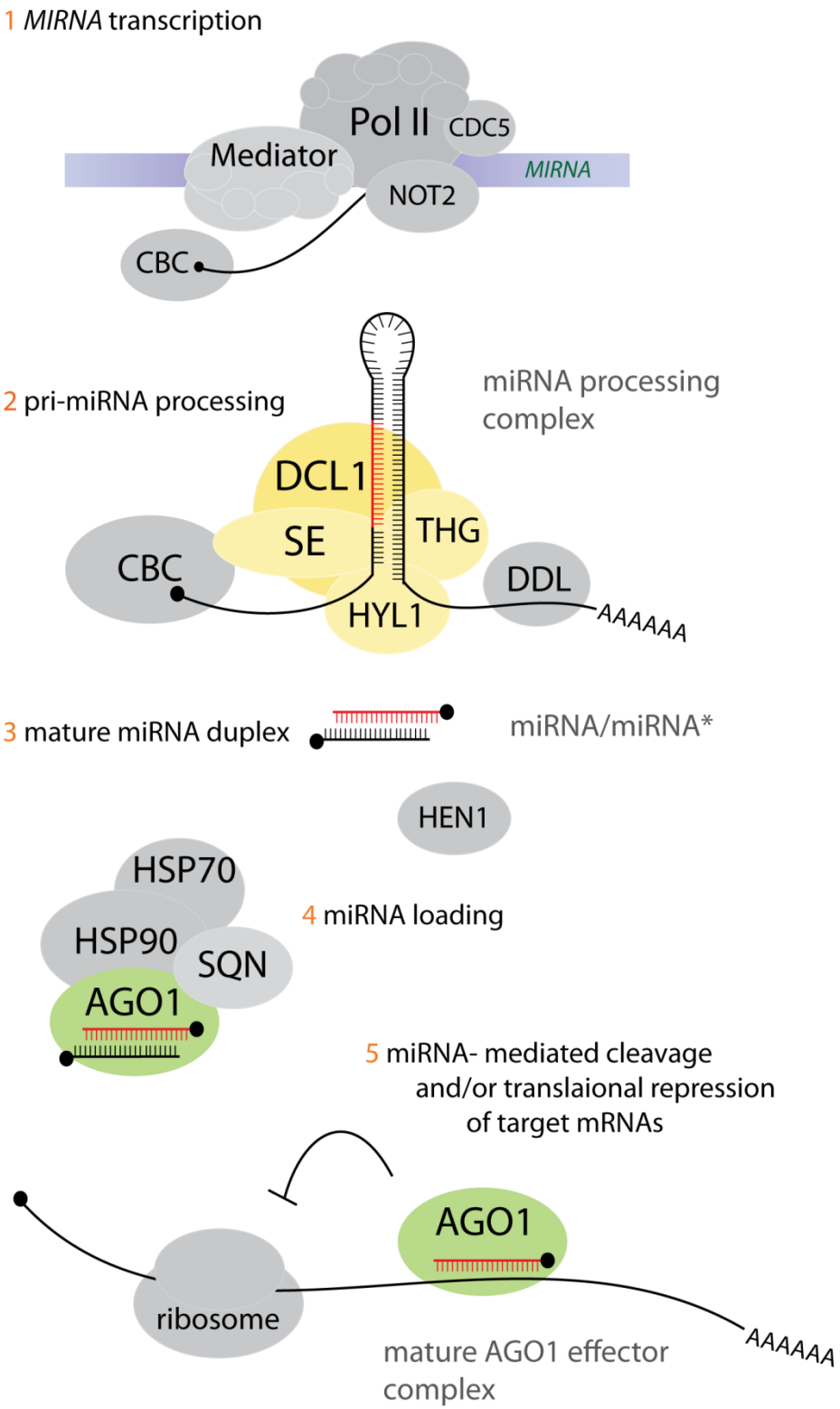

Figure 1: Schematic model of miRNA biogenesis and action

(Figure legend see page 6)

Figure 1: Schematic model of miRNA biogenesis and action

(1) The Mediator complex recruits Pol II to *MIRNA* promoter regions leading to transcription of the *MIRNA* loci. CDC5 and NOT2 are essential for *MIRNA* gene transcription. The CBC binds to the 7mG cap of the nascent pri-miRNA transcript. **(2/3)** Likely in a co-transcriptional manner, the DCL1 processing complex (yellow) releases the mature miRNA duplex (miRNA/miRNA*, red/black) from the pri-miRNA transcript. **(3)** The mature mRNA complex receives a 2-OH-methylation at the 3' end of each strand by HEN1 which protects it from degradation. **(4)** The miRNA duplex is loaded into the AGO1 effector protein with the assistance of HSP90, HSP70 and SQN. **(4/5)** The dissociation of the auxiliary proteins leads to the removal of the passenger strand (miRNA*) resulting in a mature AGO1 effector complex, which regulate complementary target mRNAs by translational repression and cleavage.

Mechanistic insights into miRNA-mediated translational repression were obtained by a couple of elaborated biochemical experiments. These studies demonstrate that miRNA/AGO1 complexes are associated with polysomes likely blocking translational elongation and inhibit translation initiation by preventing the formation of the 80s ribosome (Iwakawa and Tomari 2013, Lanet *et al.* 2009).

Subcellular localization of miRNA biogenesis and action

MiRNA processing components, like DCL1, HYL1, SE and the CBC colocalize and physically interact to some extend in subnuclear foci (Fang and Spector 2007, Raczynska *et al.* 2013, Song *et al.* 2007). Those nuclear speckles are distinct from cajal bodies, which are loci of small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA) processing (Fang and Spector 2007). Because the miRNA processing component form separate speckles, they were named dicing bodies (d-bodies) (Fang and Spector 2007) and assumed to be the place of pri-miRNA processing.

After processing, the miRNA duplex is loaded into the AGO1 effector protein. Different studies reported a nuclear as well as cytoplasmic localization for AGO1 (Fang and Spector 2007, Li *et al.* 2013, Pontes *et al.* 2013, Speth *et al.* 2013). However, HSP90/HSP70 and SQN, components essential for miRNA loading, are assumed to be cytoplasmic (Krishna and Gloor 2001, Prunet *et al.* 2008). Loading of AGO4 with small-interfering RNAs (siRNAs) requires the assistance of those chaperones and takes place in the cytoplasm. But AGO4 function is exclusively restricted to the nucleus (Ye *et al.* 2012). Therefore, it is most likely that miRNA loading into AGO1 effector proteins is a cytoplasmic process.

Because pri-miRNA processing is performed in the nucleus and miRNA loading in the cytoplasm, the miRNA duplex has to be transported from the nucleus into the cytoplasm. In animals, the shuttling and the stability of pre-miRNAs is influenced by EXPORTIN-5 and its homolog in Arabidopsis, HASTY (HST), indeed seems to affect the abundance of at least some of the Arabidopsis miRNAs, suggesting a function in the miRNA pathway (Park *et al.*

2005, Zeng and Cullen 2004). However, there are most likely unknown and HST-independent miRNA transport mechanisms.

Cytoplasmic processing bodies (p-bodies) are subcytoplasmic loci, which are connected to RNA metabolism mainly in respect to RNA degradation processes (Xu and Chua 2011). Proteins involved in miRNA-mediated mRNA regulation, like AGO1 and the 5'-3' exoribonuclease XRN4, which degrades 3' cleavage products of AGO1 effector complexes, co-localize with p-bodies, suggesting a role in miRNA target cleavage (Pomeranz *et al.* 2010, Souret *et al.* 2004). Also, SUO, a GW repeat containing protein, colocalize with proteins from p-bodies and is involved in translational repression of target mRNAs and (Yang *et al.* 2012). GW repeats are typical binding motives of AGO1 interactors, indicating that also miRNA-mediated translational repression takes place in p-bodies (Meister 2013, Yang *et al.* 2012). Translational repression has additionally been linked to the endoplasmatic reticulum (ER). *ALTERED MERISTEM PROGRAM1* (*AMP1*) is an integral membrane protein, which binds to AGO1 effector complexes at the ER and recruits target mRNAs, thereby influencing translational repression (Li *et al.* 2013).

MiRNA targets and components necessary for target mRNA metabolism are present in the cytosol. The majority of mature miRNAs accumulate in the nucleus and miRNAs are supposed to be transported into the cytoplasm after processing (Park *et al.* 2005). Therefore it has been assumed that miRNA-mediated target mRNA regulation happens in the cytoplasm (Jones-Rhoades *et al.* 2006). However, AGO1 proteins, which are mainly associated with miRNAs, can be found in the nucleus (Fang and Spector 2007, Pontes *et al.* 2013, Speth *et al.* 2013) and a rice miRNA was discovered to affect DNA methylation in the nucleus (Wu *et al.* 2010). Additionally, a global screen for intronic miRNA binding sites in Arabidopsis and rice discovered 40 and 1912 new sites, respectively (Meng *et al.* 2013), suggesting that AGO1 effector complexes also act in the nucleus.

Regulatory mechanisms modulating the miRNA pathway

MiRNAs regulate many aspects of plant development and adaptive responses; therefore, *MIRNA* expression has to be strictly controlled. This can be guaranteed by modulating *MIRNA* transcription, pri-miRNA processing, miRNA stability and action.

MIRNA expression is dynamically changed in a spatial, time and stimulus dependent manner (Hajheidari *et al.* 2012, Jung *et al.* 2012, Laubinger *et al.* 2010, Li *et al.* 2012, Reyes and Chua 2007), suggesting that *MIRNA* transcription may be regulated by transcription factors upon different cues. Confirming this hypothesis, computational analyses identified several known transcription factor binding sites overrepresented within *MIRNA* promoter regions

(Megraw *et al.* 2006, Zhao and Li 2013) and several transcription factors have been shown to influence expression of distinct *MIRNA* genes (Rogers and Chen 2013).

Efficient and precise pri-miRNA processing is performed by the DCL1 processing complex, which may be a target for modification. Regulatory mechanisms occurring during pri-miRNA processing can involve post-transcriptional and post-translational mechanisms. For instance, *DCL1* mRNA is post-transcriptionally regulated trough miR162, influencing miRNA biogenesis via a negative feed-back loop (Xie *et al.* 2003). The dsRNA-binding protein HYL1 needs to be in a hypophosphorylated state to be fully active. CPL1 phosphatases affect this status, thereby positively regulating HYL1 activity, which is important for accurate and efficient processing as well as strand selection (Manavella *et al.* 2012). Different studies showed, that processing of certain miRNAs as well as their tissue specific accumulation is not equally affected in miRNA processing mutants, indicating that there might be other factors influencing miRNA biogenesis, which still have to be discovered (Jung *et al.* 2012, Laubinger *et al.* 2010, Park *et al.* 2005).

The stability of miRNAs duplexes is guaranteed by a 2-OH methylation at each 3' end. The methyltransferase HUA ENHANCER 1 (HEN1) binds to miRNA duplexes and methylates their 3' ends (Park *et al.* 2002, Yu *et al.* 2005, Zhai *et al.* 2013). The methylation protects the duplex from 3'-5' truncation and uridylation which has been observed in *hen1* mutants (Zhai *et al.* 2013). SMALL RNA DEGARDING NUCLEASES (SDN), a family of 3'-5' exonuclease, are supposed to have a function in miRNA turnover, because they preferentially degrade mature single stranded small RNAs and *sdn* mutants accumulate mature miRNAs to higher extend (Ramachandran and Chen 2008). However, it is not known whether the SDNs are responsible for the 3' truncation observed in *hen1* mutants.

MiRNA action could also be influenced through changes of AGO1 mRNA and protein levels. The accumulation of *AGO1* mRNAs is indeed regulated by miR168 and the abundance of AGO1 proteins is affected by an F-box protein, F-BOX WITH WD-40 2 (FBW2), which promotes degradation AGO1 proteins (Earley *et al.* 2010, Vaucheret *et al.* 2006). In both cases, miRNA action can be modulated due to changes in the abundance of the essential AGO1 effector protein.

Closing remarks

The miRNA pathway is a complex and essential regulator of gene expression. 20 years of research shed light on miRNA biogenesis, action and function and extended our knowledge on the miRNA-mediated regulation of gene expression. However, this demonstrates at the same time that we are far away from fully understanding the intricacy of miRNA regulatory networks. In order to gain further insights into miRNA-mediated processes, future research

goals may focus on discovering new components of the pathway, thereby completing the model of miRNA biogenesis and action or analyzing the regulatory network of miRNA/target modules and their impact on plant development and adaption in detail.

Table 1: List of all described miRNA pathway components including their function, known interactors and subcellular localization.

miRNA component	**Function**		**Interactors**	**Subcellular localization**	**References**
	miRNA pathway	**other pathways**			
Pol II	*MIRNA* transcription	✓	Mediator, CDC5, NOT2	nucleus	(Kim *et al.* 2011, Wang *et al.* 2013, Zhang *et al.* 2013)
Mediator	*MIRNA* transcription	✓	Pol II	nucleus	(Kim *et al.* 2011)
CDC5	*MIRNA* transcription, pri-miRNA processing	✓	Pol II, DCL1, SE	nucleus	(Zhang *et al.* 2013)
NOT2	*MIRNA* transcription, pri-miRNA processing	✓	Pol II, DCL1, SE, CBC,	nucleus	(Wang *et al.* 2013)
MOS2	pri-miRNA processing	✓	-	nucleus	(Wu *et al.* 2013, Zhang *et al.* 2005b)
DCL1	pri-miRNA processing	-	HYL1, SE, DDL, NOT2, CDC5	nucleus	(Fang and Spector 2007, Hiraguri *et al.* 2005, Park *et al.* 2002, Wang *et al.* 2013, Yu *et al.* 2008, Zhang *et al.* 2013)
HYL1	pri-miRNA processing		DCL1, SE, CPL1	nucleus	(Hiraguri *et al.* 2005, Manavella *et al.* 2012, Vazquez *et al.* 2004, Yang *et al.* 2006)
CPL1	positive regulator of HYL1 activity	✓	SE, HYL1	nucleus	(Manavella *et al.* 2012)
SE	pri-miRNA processing	✓	DCL1, HYL1, CBC, CPL1	nucleus	(Fang and Spector 2007, Lobbes *et al.* 2006, Manavella *et al.* 2012, Raczynska *et al.* 2013, Wang *et al.* 2013, Yang *et al.* 2006)
THG	pri-miRNA processing		DCL1, HYL1, SE	nucleus	(Ren *et al.* 2012)
CBC	pri-miRNA processing	✓	SE	nucleus	(Laubinger *et al.* 2008, Raczynska *et al.* 2013, Wang *et al.* 2013)
DDL	pri-miRNA stability	-	DCL1		(Yu *et al.* 2008)
SIC	pri-miRNA processing	✓	-	nucleus	(Zhan *et al.* 2012)
STA1	pri-miRNA processing	✓	-	-	(Ben Chaabane *et al.* 2013)

miRNA component	Function		Interactors	Subcellular localization	References
	miRNA pathway	**other pathways**			
HST	miRNA export	-	-	nucleus, cytoplasm	(Bollman *et al.* 2003, Park *et al.* 2005)
HEN1	miRNA methylation	-	-	nucleus, cytoplasm	(Fang and Spector 2007, Yu *et al.* 2005)
SDN	miRNA degradation	-	-	-	(Ramachandran and Chen 2008)
AGO1	cleavage and/or translational repression of target mRNAs	-	HSP90, HSP70, SQN, AMP1	nucleus, cytoplasm	(Earley and Poethig 2011, Fang and Spector 2007, Iki *et al.* 2012, Iki *et al.* 2010, Li *et al.* 2013, Speth *et al.* 2013, Vaucheret *et al.* 2004)
HSP90/HSP70	miRNA loading into AGO1	✓	AGO1	-	(Iki *et al.* 2010)
SQN	miRNA loading into AGO1	✓	AGO1	-	(Earley and Poethig 2011, Iki *et al.* 2012)
SDA2/EMA1	miRNA loading into AGO1	-	-	-	(Wang *et al.* 2011)
FBW2	AGO1 stability	-	-	-	(Earley *et al.* 2010)
AMP1	translational repression of target mRNAs	-	AGO1	cytoplasm (ER)	(Li *et al.* 2013)
SUO	translational repression of target mRNAs	-	-	nucleus, cytoplasm (p-bodies)	(Yang *et al.* 2012)
XRN4	degradation of mRNA cleavage products	-	-	cytoplasm	(Kastenmayer and Green 2000, Souret *et al.* 2004)

Author Contribution

C.S. wrote the manuscript with contributions from S.L.

Objectives of this Work

The zinc-finger protein SERRATE (SE) and the CAP BINDING COMPLEX (CBC) attracted our attention, because these proteins have been implicated in two distinct RNA processing pathways, splicing and miRNA biogenesis. Mutations in *SE* or *CBC* lead to defects in splicing and pri-miRNA processing. This indicates that they may act together to ensure correct splicing and pri-miRNA processing. The CBC binds to the 5'-seven-methyl-guanosine (7mG) cap of mRNA and pri-miRNA transcripts. It has been shown that SE is a core component of the pri-miRNA processing machinery and it is most likely that SE is also part of other RNA processing complexes. This leads to the hypothesis that SE and the CBC may build a high-order complex, which binds to 7mG-capped transcripts, and recruit different RNA processing factors to specific RNA species.

The goal of this work is to gain insights into the functional relationship of SE and the CBC, and, subsequently, to identify new components, which interact with the SE-CBC complex. Afterwards, newly identified proteins are characterized in respect to their function in RNA processing.

I addressed the functional relationship between SE and the CBC by different genetic and molecular analysis. New SE interacting proteins were identified in a yeast two-hybrid screen using SE as bait. Among the putative SE interacting proteins, I analyzed the scaffold protein RECEPTOR FOR ACTIVATED C KINASE 1 (RACK1) and uncovered RACK1`s function in the miRNA pathway of Arabidopsis.

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Chapter II: The CAP-BINDING COMPLEX and SERRATE act together to ensure pri-miRNA processing and splicing

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Introduction

Plant microRNAs (miRNA) belong to the broad class of regulatory small RNAs and have been shown to act as negative regulators of gene expression at the post-transcriptional level (Rogers and Chen 2013, Voinnet 2009). Like protein-coding genes, *MIRNA* genes are transcribed by RNA polymerase II (Pol II) and the resulting primary-microRNA transcripts (pri-miRNA) are co-transcriptionally modified, including a 5'-seven-methyl-guanosine (7mG) cap and a poly(A)-tail (Kim *et al.* 2011, Xie *et al.* 2005, Zhang *et al.* 2005). Pri-miRNA transcripts form a hairpin-like structure, which is processed into a 21 nucleotide long mature miRNA duplexes by an RNaseIII-like enzyme, DICER-LIKE 1 (DCL1) (Park *et al.* 2002, Rogers and Chen 2013). Auxiliary proteins, like the RNA-binding protein TOUGH (TGH), the dsRNA-binding protein HYPONASTIC LEAVES 1 (HYL1) or the zinc-finger protein SERRATE (SE) assist DCL1 in respect to miRNA processing efficiency and the latter two additionally in processing accuracy (Dong *et al.* 2008, Kurihara *et al.* 2006, Lobbes *et al.* 2006, Ren *et al.* 2012, Vazquez *et al.* 2004, Yang *et al.* 2006).

As *MIRNA* genes are transcribed by Pol II also components necessary for transcription of protein-coding genes or general RNA metabolism, are essential for a functional miRNA pathway. The Mediator complex, a conserved eukaryotic transcriptional co-activator, is required for *MIRNA* transcription by recruiting Pol II to the *MIRNA* promoter (Kim *et al.* 2011). CELL CYCLE-DEPENDENT 5 (CDC5), a putative MYB-related transcription factor implicated in splicing and NEGATIVE ON TATA less2 (NOT2), a general regulator of RNA metabolism, are both associated with Pol II and components of the pri-miRNA processing complex. It has been assumed that CDC5 and NOT2 connect *MIRNA* transcription and pri-miRNA processing, thereby being essential for both processes (Wang *et al.* 2013, Zhang *et al.* 2013). Additionally, the CAP-BINDING COMPLEX (CBC), consisting of CAP-BINDING PROTEIN 80 (CBP80) and CAP-BINDING PROTEIN 20 (CBP20), binds to the 7mG-cap of mRNA and pri-miRNA transcripts in metazoans and plants after transcriptional initiation

(Gruber *et al.* 2009, Izaurralde *et al.* 1994, Kim *et al.* 2008, Topisirovic *et al.* 2011). In humans, the CBC was discovered as a component necessary for pre-mRNA splicing. It facilitates 5' splice site recognition by the U1 small nuclear ribonucleoprotein particle (snRNP), a component of the spliceosome (Izaurralde *et al.* 1994, Lewis *et al.* 1996). Further studies revealed a broader function for the CBC in mRNA metabolism, including RNA export into the cytosol and nonsense-mediated decay (Maquat 2004). In Arabidopsis, CBP80, CBP20 and SE, have overlapping functions in respect to pri-miRNA processing as well as splicing of some pre-mRNAs (Laubinger *et al.* 2008). The lack of a functional SE, CBP80 or CBP20 leads to reduced pri-miRNA processing and enhanced intron retention in some pre-mRNAs (Laubinger *et al.* 2008). This raises the question whether SE and the CBC may team up for RNA processing, thereby recruiting RNA processing machineries (e.g. spliceosome, DCL1 complex) to different 7mG-capped RNA species. In order to address this, we performed some physical and genetic interaction studies as well as molecular analyses with different *cbc* and *se* mutants.

Results

SE and the CBC form a higher-order complex

It has been noticed before that the mutant phenotypes of weak *se* mutants, like *se-1*, resemble the mutant phenotype of *cbp80* and *cbp20* mutants, all exhibiting leaf serrations (Grigg *et al.* 2005, Hugouvieux *et al.* 2001, Papp *et al.* 2004, Prigge and Wagner 2001). This led to the suggestion that the *CBC* and *SE* have overlapping functions. Indeed, SE and the CBC are important for proper pri-miRNA processing and pre-mRNA splicing (Laubinger *et al.* 2008). In order to find out if SE and the CBC act together, we performed epistasis analyses between the *se*, *cbp20* and *cbp80* mutants. The mutant phenotype of *se cbp20*, *se cbp80* and *cbp20 cbp80* double mutants were similar to the respective single mutants. All single and double mutant plants were a little smaller, exhibited a slight delay in development and showed the same leaf serrations compared to wild-type, suggesting a corporate function of SE and the CBC (Figure 1a).

Next, we sought to analyze direct protein-protein interactions between SE and the CBC, using the yeast two-hybrid assay. As a positive control we could show that CBP80 and CBP20 fused to the GAL4-activation domain (AD) and GAL4-DNA-binding domain (BD), are able to reconstitute yeast growth on selective media lacking WLH, demonstrating that both proteins interact. SE and CBP20, but not CBP80, fused to the DNA-binding or activation domain of the GAL4 transcription factor, respectively, also led to yeast growth on selective media (-WLH), suggesting that SE and the CBC build a complex, in which SE is associated with CBP20 but not with CBP80 (Figure 1b).

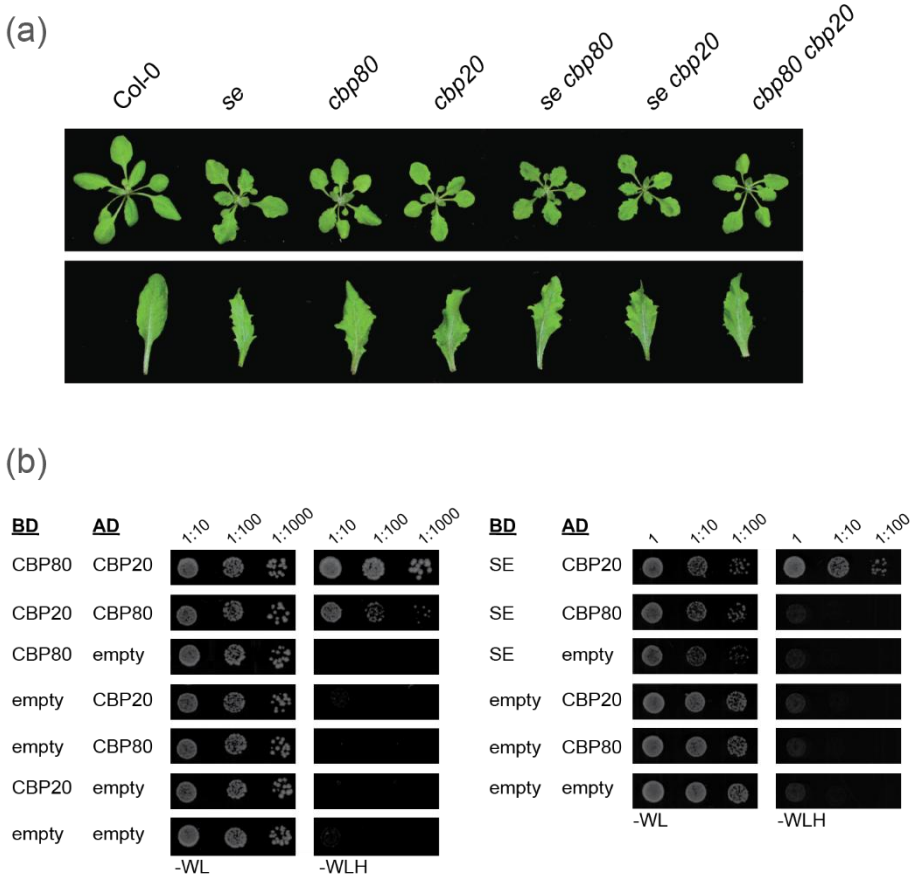

Figure 1: Genetic and physical interaction studies with SE and the CBC

(a) Gross morphology of wild-type (Col-0) and various *se*, *cbp80* and *cbp20* single and double mutants.
(b) Yeast two-hybrid interaction assay between CBP80, CBP20 and SE. CBP80, CBP20 and SE fused to the GAL4-DNA-binding domain (BD) or to the GAL4-activation domain were transformed and expressed in the yeast strain AH109. A serial 1:10 dilution of each transformed yeast was spotted on control (-WL) and selective media (-WLH). Yeast growth was monitored after 3 days incubation at 28 °C.

The CBC and SE team up to ensures correct miRNA processing and mRNA splicing

Our genetic and physical interaction studies indicate that SE and the CBC build a complex, which may function in the same pathway. Next, we wanted to know, if this is the case at molecular level, too. Because *se* and *cbc* single mutants have defects in the miRNA biogenesis and mRNA splicing, we sought to analyze pri-miRNA processing and pre-mRNA splicing in *se* and *cbc* single and double mutants.

Due to processing defects, miRNA processing mutants like *se* accumulate high levels of pri-miRNA transcripts and low levels of mature miRNAs. *cbc* single mutants have also elevated

pri-miRNA transcript and reduced miRNA levels, suggesting a function in miRNA processing (Laubinger *et al.* 2008).

First, we wanted to know, if miRNA accumulation is even more reduced in *se cbp80*, *se cbp20* and *cbp80 cbp20* double mutants when compared to each single mutant. Quantification of mature miRNAs revealed a reduced accumulation of all analyzed miRNAs in the *se cbc* double mutants that was similar to respective single mutants. These results suggest that pri-miRNA processing defects are not further increased and point again to a corporate function of SE and the CBC (Figure 2b).

In order to confirm this, we analyzed steady-state levels of some pri-miRNA transcripts. The quantification showed that in general every single and double mutant accumulates higher levels of all tested pri-miRNAs transcripts (Figure 2a).

Interestingly, *se* single and *se cbp80* double mutants accumulate higher levels for some pri-miRNA transcripts compared to the *cbc* single, *se cbp20* and *cbc* double mutants, indicating a more important role for SE in pri-miRNA processing than the CBC. In line with this, the mature miRNA levels are also stronger reduced in *se* and se *cbp80* mutants. Laubinger *et al.* already observed that *se* mutants accumulate higher amounts of pri-miRNA transcripts and lower amounts of mature miRNAs than *cbc* single mutants. They speculated that this may be an effect of the *cbc* mutant background and mechanistically this could include transcript destabilization, partial complementation by other cap-binding proteins or CBC independent processing of highly expressed transcripts (Laubinger *et al.* 2008).

Tiling array and PCR-based analyses revealed that some introns, mainly first introns of pre-mRNA transcripts, are retained in *se* and *cbc* single mutants (Laubinger *et al.* 2008). In order to analyze splicing efficiency in *se*, *cbp80*, *cbp20* single and double mutants, we quantified the decrease in correctly spliced mRNA transcripts of selected genes, which have already been shown to be retained in *cbc* and *se* mutants (Laubinger *et al.* 2008). Therefore quantitative real-time PCR was performed using forward oligonucleotides overspanning the 5’/3’ splice site and reverse oligonucleotides binding in the 3’ exon. By using such an oligonucleotide combination, only exact spliced mRNA transcripts can be amplified during PCR (Figure 2c). Interestingly, correctly spliced mRNAs accumulate to approximately 40 % in all mutants. This demonstrates an equal contribution of SE and the CBC in splicing efficiency of the analyzed introns (Figure 2c).

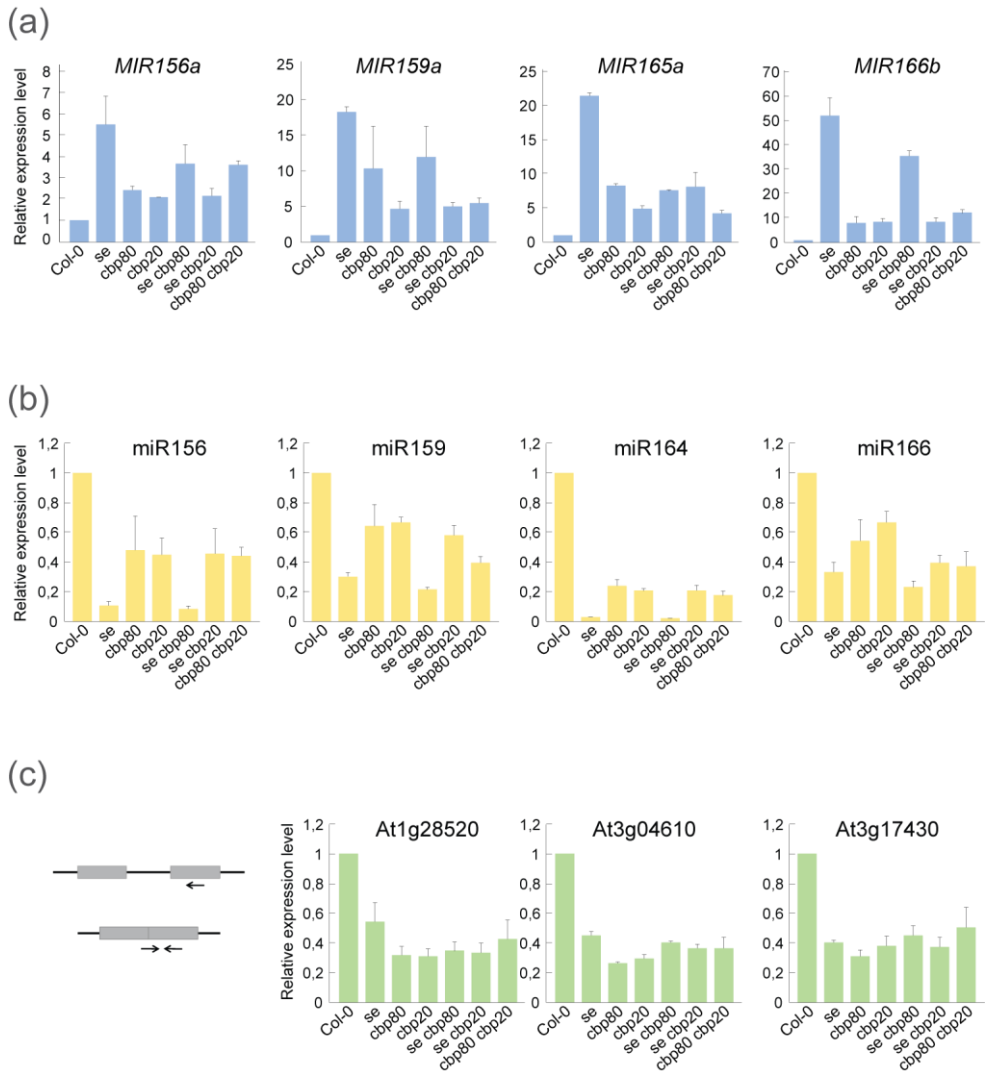

Figure 2: miRNA processing and mRNA splicing is affected in *se cbc* mutants

(a) Quantitative analysis of selected *MIRNA* transcripts in wild-type (Col-0) and various *se*, *cbp80* and *cbp20* single and double mutants. Error bars denote the range of two independent replicates.
(b) Quantitative analysis of selected mature miRNAs in wild-type (Col-0) and various *se*, *cbp80* and *cbp20* single and double mutants. Error bars denote the range of two independent replicates.
(c) Quantitative analysis of splicing deficiency in wild-type (Col-0) and various *se*, *cbp80* and *cbp20* single and double mutants. Error bars denote the range of two independent replicates. Forward primer over spanning the splice site and reverse primer in the exon were used for qPCR analysis resulting in a PCR product when splicing was performed.

Discussion

Our genetic and physical interaction studies as well as molecular analyses demonstrate that SE and the CBC physically and genetically act in concert to facilitate pri-miRNA processing and pre-mRNA splicing.

Using the yeast two-hybrid assay, we could confirm the already published interaction between Arabidopsis CBP20-CBP80 and discovered an interaction between SE-CBP20 (Kierzkowski *et al.* 2009, Raczynska *et al.* 2013, Wang *et al.* 2013). Our data lead us to the suggestion that SE and the CBC indeed build a complex and CBP20 bridges the interaction between SE and CBP80. Interestingly, two recent publications showed contradictory results with respect to the SE-CBP20 interaction *in planta*. Wang *et al.* could confirm our yeast interaction data, whereas Katarzyna *et al.* observed an interaction between SE and both proteins of the CBC. The contradictory data might be a result of the different experimental procedures used to analyze these interactions. Nevertheless, SE and the CBC build a high-order complex in Arabidopsis, which is an evidence for a mutual molecular function.

SE and the CBC act in concert to guarantee proper pri-miRNA processing and pre-mRNA splicing. This seems to be a partially conserved mechanism, as the human/fly CBC and ARSENITE-INSENSITIVE 2 (ARS2), the human/fly homolog of SE, are both required for pri-miRNA processing (Gruber *et al.* 2009, Sabin *et al.* 2009). Our molecular data indicate that SE has a more important function in processing of pri-miRNA transcripts than CBP80 or CBP20. This can be explained by the observation that *se* knock-outs are embryonically lethal compared to *cbc* mutants, which are still viable and there might be other cap-binding proteins like eIF4G, which act partially redundant (Hugouvieux *et al.* 2001, Marintchev and Wagner 2005, Papp *et al.* 2004, Prigge and Wagner 2001). Because the 5`-cap of an mRNA transcript is important for its stability, splicing and 3`end processing (Topisirovic *et al.* 2011), one can imagine that also the lack of *CBC* might lead to transcript destabilization. This can in addition contribute to the reduced pri-miRNA accumulation. But also SE-CBC protein complex stability might play a role. It has been shown that *cbp20* mutants still accumulate CBP80 protein but to a lower extend than WT and *cbp80* mutants additionally lack CBP20 proteins (Kierzkowski *et al.* 2009). SE and the CBC are part of the same complex and the lack of one or two of the three components may influence the integrity of the entire complex. Additionally, SE promotes not only processing efficiency but also processing accuracy of pri-miRNAs, which may contribute to the more sever defects in miRNA processing when compared to the CBC (Dong *et al.* 2008).

Taken together, transcript stability, processing efficiency and complex integrity may contribute to the observed molecular mutant phenotypes in *se*, *cbc* single and double

mutants. Uncovering the effect of each mechanism on pri-miRNA transcript processing might be a subject for future studies.

Some pre-miRNA transcripts require the CBC or SE to ensure accurate splicing of mainly the first intron (Laubinger *et al.* 2010). We could show that correct splicing of three of those pre-mRNA transcripts is equally dependent on the CBC and SE, demonstrating that they have overlapping functions and are mutually responsible for pre-mRNA splicing. Additionally, SE and the CBC have been implicated in alternative splicing (AS). RT-PCR based high-resolution AS analyses showed that the lack of a fully-active SE or the loss of CBC leads to missplicing of the same transcripts, preferentially affecting the 5' splice site of the first intron (Raczynska *et al.* 2010, Raczynska *et al.* 2013). This support the theory that SE and the CBC act together in pre-mRNA splicing.

In summary, SE and the CBC build a high-order complex, which has overlapping functions in several RNA metabolism pathways. This leads to the idea, that the SE-CBC complex may build a platform, which recruits different RNA processing machineries to bound RNA transcripts (Figure 3).

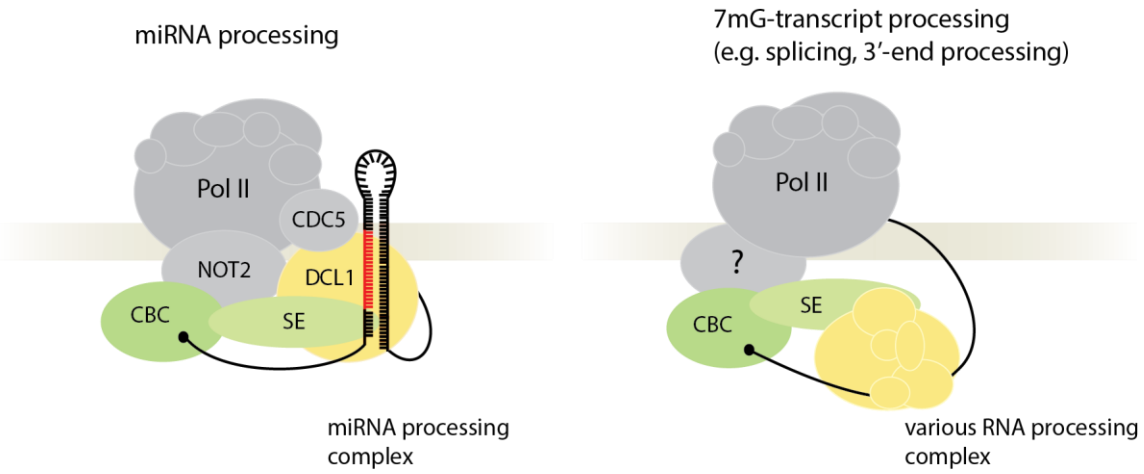

Figure 3: The function of the SE-CBC complex in RNA processing

The SE-CBC complex binds to nascent 7mG-transcripts recruiting various RNA processing components like the miRNA processing machinery or the spliceosome.

Many studies indicate that transcription and pre-RNA processing occurs in a co-transcriptional manner (Bentley 2005). NOT2, a protein involved in the Arabidopsis miRNA pathway, has been shown to interact with Pol II as well as the CBC, SE and DCL1 and CDC5, a putative transcription factor, interacts with Pol II, DCL1 and SE (Zhang *et al.* 2013, Wang *et al.* 2013). This suggests a bridging function between transcription and pri-miRNA processing (Zhang *et al.* 2013, Wang *et al.* 2013). Taken together, the SE-CBC complex interacts with components of the transcriptional and the pri-miRNA processing

machinery, which in turn may connect transcription and pri-miRNA processing by recruiting the miRNA processing complexes to nascent transcripts (Figure 3). In analogy to this scenario, the SE-CBC complex may recruit other RNA processing components to bound 7mG-transcripts like the spliceosome (Figure 3). Interestingly, recent publications confirmed this hypothesis. Besides the function in pri-miRNA processing, the human CBC and ARS2 bind to various capped non-coding and coding RNA species and promote their 3'-end processing by recruiting specific RNA processing proteins, affecting formation and expression of bound transcripts (Gruber *et al.* 2012, Hallais *et al.* 2013).

In order to find other RNA processing components, which might be recruited by the SE-CBC complex, Regina Kilian and Sascha Laubinger conducted a yeast two-hybrid screen using SE as a bait. During my PhD, I analyzed RECEPTOR FOR ACTIVATED C KINASE (RACK1), one of the putative SE interaction partners, with respect to its function in the miRNA pathway. This is addressed in chapter III, chapter IV and chapter IV.

Material and Methods

Plant material and growth conditions

All mutants plants, *se-1*, *cbp80* (*abh1-285*), *cbp20*, which were used in this study have been described before in other publications (Grigg *et al.* 2005, Laubinger *et al.* 2008, Papp *et al.* 2004, Prigge and Wagner 2001). All mutant plants are in the Colombia-0 (Col-0) background. For all experiments, plants were grown on ½ MS media for 10 days long day conditions (16 h light, 22 °C).

DNA constructs

cDNA was amplified from reverse transcribed cDNA using Phusion proof-reading polymerase (Thermo Fisher Scientific). Purified PCR products were cloned into the ENTRY vector pCR8-GW-TOPO (Life Technologies) according to the manufactures protocol. Using LR Clonase II (Life-Technologies) cDNAs were transferred into the pGADT7 and pGBKT7 destination vectors (Horak et al. 2008).

Yeast two hybrid assay

The yeast strain AH109 was transformed using standard LiAc based transformation as described in the Yeast Protocol Handbook (www.clonetech.com). After three days 5-10 colonies from each transformation were resuspended in 10 % (w/v) glycerol and adjusted to an OD_{600} of 1. We prepared a serial 1:10 dilution of the yeast cells and spotted them onto control (-WL) or selective media (-WLH) lacking the corresponding amino acids. The yeast growth was monitored after three days at 28°C.

Total RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted using TRIZOL (Life technologies) reagent. Up to 100 mg of ground plant material was resuspended in 1 ml TRIZOL reagent. Plant debris was collected by centrifugation. Supernatant was supplemented with 1/5 Vol of chloroform. Subsequently, the sample was mixed by vigorous vortexing and organic and aqueous phases were separated by centrifugation step (16.000g, 5 min, and 4°C). The upper aqueous phase containing total RNA was transferred to a new tube and washed several times with 500 µl chloroform until the interphase was clear. The upper phase was supplemented with 1 Vol of isopropanol and total RNA was precipitated during a 1 h incubation step at -80 °C and subsequently collected by centrifugation (16.000g, 30 min, and 4°C). After additional washing with 80 % (v/v) ethanol sedimented RNA was resuspended in 20 – 50 µl nuclease-free water. The integrity of 500 ng RNA was analyzed on a 1 % (w/v) agarose gel.

Between 250 ng and 2 µg total RNA were used DNase treatment in a total volume of 10 µl. The whole DNAse treated RNA was reverse transcribed with oligo-dT and a miRNA specific stem-loop oligonucleotide using the components of the First Aid cDNA synthesis Kit (Thermo Fisher) according to the method described by (Varkonyi-Gasic *et al.* 2007).

Quantitative real-time PCR (qPCR) was performed in the Bio-Rad CFX384 system using SYBR green containing 2xPCR Master-Mix (Thermo Fisher Scientific). All experiments were performed twice with two independent biological replicates. Amplification efficiency was calculated using a standard curve of amplification and *TUBULIN* was used for normalization. Relative expression levels were calculated using the ΔΔct-method.

All oligonucleotides are listed in Supplemental Table 1.

Author Contribution

C.S. and S.L. designed the research; C.S. performed research; C.S. wrote the manuscript with contributions from S.L.

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Supporting Information

Table S1: List of oligonucleotides.

	Oligonucleotide	Sequence	Figure
cDNA synthesis	miR156-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC	Fig. 2 b
	miR159a-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGAGC	Fig. 2 b
	miR164ab-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCACG	Fig. 2 b
	miR166-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGGAA	Fig. 2 b
qRT-PCR	miR156-F	GCGGCGGTGACAGAAGAGAGT	Fig. 2 b
	miR159-F	GCGGCGTTTGGATTGAAGGGA	Fig. 2 b
	miR164-F	AGGACATGGAGAAGCAGGGCA	Fig. 2 b
	miR166-F	TCGCTTCGGACCAGGCTTCA	Fig. 2 b
	universal miRNA-R	GTGCAGGGTCCGAGGT	Fig. 2 b
	pri-miR156a-F	GTTAAAACTCAGATCTAACACAAAG	Fig. 2 a
	pri-miR156a-R	GAGAACGAAGACAGGCCAAAG	Fig. 2 a
	pri-miR159a-F	GGTCTTTACAGTTTGCTTATG	Fig. 2 a
	pri-miR159a-R	AGAAGGTGAAAGAAGATGTAG	Fig. 2 a
	pri-miR165b-F	AGGCTATTTCTGTTGTGGGGAATGTTG	Fig. 2 a
	pri-miR165b-R	GGATGAAGCCTGGTCCGACGATAC	Fig. 2 a
	pri-miR166b-F	CCCGGGATCATTCTCTTCATCATCACCAC	Fig. 2 a
	pri-miR166b-R	CCCGGGATGGACAAATCTTCTTCGTTAATTCG	Fig. 2 a
	Atg128520-F	CACCCATTACTCTTCTTTTTATTTTCC	Fig. 2 c
	Atg128520-R	CGTCATAAGAAATCTCACCTC	Fig. 2 c
	At3g04610 -F	TTCAGCTTCAGCCATGACTAG	Fig. 2 c
	At3g04610-R	CACCCATTACTCTTCTTTTTATTTTCC	Fig. 2 c
	At3g17430 -F	CACCAAACTACTGTAGCTAGCTATCTC	Fig. 2 c
	At3g17430-R	CATCTTACTCGTCCCCTCTC	Fig. 2b
	Tubulin-F	GAGCCTTACAACGCTACTCTGTCTGTC	Fig. 2 a, b, c
	Tubulin-R	ACACCAGACATAGTAGCAGAAATCAAG	Fig. 2a, b, c

Chapter III: RACK1 scaffold proteins influence miRNA abundance in Arabidopsis

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Summary

MicroRNAs (miRNAs) regulate plant development by post-transcriptional regulation of target genes. In *Arabidopsis thaliana*, DCL1 processes precursors (pri-miRNAs) to miRNA duplexes, which associate with AGO1. Additional proteins act in concert with DCL1 (e.g. HYL1 and SERRATE) or AGO1 to facilitate efficient and precise pri-miRNA processing and miRNA loading, respectively. In this study, we show that the accumulation of plant microRNAs depends on RECEPTOR FOR ACTIVATED C KINASE 1 (RACK1), a scaffold protein that is found in all higher eukaryotes. miRNA levels are reduced in *rack1* mutants, and our data suggest that RACK1 affects the microRNA pathway via several distinct mechanisms involving direct interactions with known microRNA factors: RACK1 ensures the accumulation and processing of some pri-miRNAs, directly interacts with SERRATE and is part of an AGO1 complex. As a result, mutations in RACK1 lead to over-accumulation of miRNA target mRNAs, which are important for ABA responses and phyllotaxy, for example. In conclusion, our study identified complex functioning of RACK1 proteins in the Arabidopsis miRNA pathway; these proteins are important for miRNA production and therefore plant development.

Introduction

MicroRNAs (miRNAs) in both animals and plants are transcribed as longer primary microRNAs (pri-miRNAs) from which RNAse III-like DICER enzymes release miRNAs of 20-22 nt length. Mature miRNAs are incorporated into an ARGONAUTE (AGO) effector protein to create an RNA-induced silencing complex (RISC) that mainly regulates target mRNAs post-transcriptionally (Voinnet 2009). miRNA-mediated control of gene expression is essential for many aspects of plant development, including root and leaf development, hormone responses, developmental transitions and stress responses (Voinnet 2009).

In the model plant *Arabidopsis thaliana*, one of the four DICER-LIKE (DCL) proteins, DCL1, is mainly involved in the production of miRNAs (Fahlgren *et al.* 2009, Laubinger *et al.* 2010, Park *et al.* 2002). The proteins DAWDLE (DDL), TOUGH (TGH), HYPONASTIC LEAVES 1 (HYL1) and SERRATE (SE) interact with DCL1 and are responsible for precise and efficient miRNA production (Dong *et al.* 2008, Hiraguri *et al.* 2005, Kurihara *et al.* 2006, Lobbes *et al.* 2006, Ren *et al.* 2012, Vazquez *et al.* 2004, Yang *et al.* 2006, Yu *et al.* 2008). During miRNA production, long primary miRNA transcripts (pri-miRNAs) are cut to form precursor miRNAs (pre-miRNAs), from which miRNA duplexes are released. miRNAs are then methylated by HUA ENHANCER 1 (HEN1) and associate mainly with AGO1, one of the ten AGO proteins in Arabidopsis (Mi *et al.* 2008, Montgomery *et al.* 2008, Yu *et al.* 2005). HEAT SHOCK PROTEIN 90 (HSP90) and the cyclophilin-40 protein SQUINT (SQN) physically interact with AGO1 and ensure efficient miRNA loading (Earley and Poethig 2011, Iki *et al.* 2012, Iki *et al.* 2010, Smith *et al.* 2009). AGO1 removes the passenger strand (or miRNA*) of the miRNA duplex, allowing the miRNA guide strand to recognize complementary sequence regions within target RNAs (Mallory *et al.* 2008). Binding of AGO1 to target sequences results in mRNA cleavage within the miRNA binding site or translational inhibition occurring on the endoplasmic reticulum (Li *et al.* 2013, Voinnet 2009). Because miRNAs play such important roles in plant development, mutants impaired in the production of miRNAs or in their function exhibit a wide range of developmental defects, and null alleles of *DCL1* or *SE* are embryonically lethal (Lobbes *et al.* 2006, Schauer *et al.* 2002). Over recent years, additional small RNA (sRNA) factors have been identified that are not solely involved in sRNA metabolism, but possess pleiotropic functions in diverse biological pathways. Among them are RNA polymerase II, the MEDIATOR complex, CPL phosphatases, the importin β protein EMA1/SAD2, KATANIN and proteins involved in the isoprenoid biosynthesis pathway (Brodersen *et al.* 2008, Brodersen *et al.* 2012, Hajheidari *et al.* 2012, Kim *et al.* 2011, Manavella *et al.* 2012, Wang *et al.* 2011b). These examples indicate that some miRNA factors have already been reported to operate in diverse biological processes.

In order to identify additional components of the plant miRNA pathway, we performed a yeast two-hybrid screen using the pri-miRNA processing factor SE as bait, and identified RECEPTOR OF ACTIVATED C KINASE 1 (RACK1) as a potential interactor. RACK1 is present in all eukaryotic organisms studied, and possesses seven WD40-β-propellers, which mediate simultaneous interactions with multiple proteins (Adams *et al.* 2011, Nilsson *et al.* 2004, Ullah *et al.* 2008). RACK1 itself has no enzymatic activity, and instead acts as a bridge for interactions between proteins or competes with other proteins for binding pockets, thereby inhibiting specific interactions. RACK1 is often identified in proteomic approaches, suggesting that RACK1 is part of many complexes, in which it controls complex formation and stability, or creates a docking site for regulators (Gibson 2012). Several types of direct RACK1 interaction partners have been described (Nilsson *et al.* 2004). RACK1 is a core component of the eukaryotic 40S ribosomal subunit, and is thought to directly regulate translation in response to various stimuli (Adams *et al.* 2011, Nilsson *et al.* 2004). In the nucleus, RACK1 acts as adaptor to connect kinases with their substrates, and modulates transcription (He *et al.* 2010, Neasta *et al.* 2012, Nery *et al.* 2004, Wang *et al.* 2011a). Animal RACK1 has been shown to act in the miRNA pathway. The *Caenorhabditis elegans* Argonaute protein that is involved in miRNA action, ALG-1, directly binds to ribosome-associated RACK-1, implying that *C. elegans* RACK-1 may be an anchor point for ALG-1 on mRNAs that are being translated (Jannot *et al.* 2011). In another study using a liver tumor cell line, RACK1 was shown to affect loading of miRNAs into the effector complex and to regulate the localization of KH-TYPE SPLICING REGULATORY PROTEIN (KSRP), which promotes maturation of a small subset of pre-miRNAs (Otsuka *et al.* 2011). These contradictory results are further complicated by the observation that RACK1 knockdown in *C. elegans*, but not in human cells, results in higher miRNA levels (Jannot *et al.* 2011, Otsuka *et al.* 2011). More specific functions of RACK1 in the miRNA pathway may be obscured by its pleiotropic actions in many diverse cellular processes.

In contrast to animals and yeast, very little is known about RACK1 in plants including its function in the plant miRNA pathway. The Arabidopsis genome encodes three *RACK1* genes: *RACK1A*, *RACK1B* and *RACK1C* (Guo and Chen 2008). Previous analyses of single and multiple mutants revealed that *RACK1* genes act redundantly to control hormone signaling, leaf development and root growth (Chen *et al.* 2006, Guo and Chen 2008, Guo *et al.* 2009). *RACK1A* plays a predominant role: it is expressed most highly, and mutations in *RACK1A* cause slight phenotypic abnormalities, while *rack1b* and *rack1c* single mutants are indistinguishable from wild-type siblings (Chen *et al.* 2006, Guo and Chen 2008, Guo *et al.* 2009). *rack1* triple mutants exhibit strong pleiotropic defects and eventually die before producing seeds (Guo and Chen 2008).

Here, we show that Arabidopsis RACK1, unlike its homologs in animals, is important for miRNA accumulation. Our results show that Arabidopsis RACK1 controls correct pri-miRNA accumulation and interacts with known miRNA processing and effector complex components, suggesting that RACK1 fulfils more than one function in the plant miRNA pathway.

Results

RACK1, a SE interacting protein, is important for miRNA accumulation

We identified RACK1B as a potential SE interactor in a yeast two-hybrid screen. In a yeast interaction assay, all three Arabidopsis RACK1 proteins were capable of interacting with SE (Figure 1a). We subsequently determined whether RACK1 is associated with SE *in planta*. First, we performed co-immunoprecipitation experiments focusing on the major *RACK1* gene in Arabidopsis, *RACK1A*. A RACK1A–GFP fusion protein expressed under the control of endogenous *RACK1A* regulatory sequences (*RACK1A:RACK1A-GFP*) rescues the *rack1a* abscisic acid (ABA)-hypersensitive phenotype (Guo et al. 2009). Pull-down experiments using a GFP affinity matrix showed that SE co-purified with RACK1–GFP (Figure 1b). In contrast, SE was barely detectable in pellet fractions of immunoprecipitation experiments using wild-type extracts (Figure 1b). We further confirmed the SE–RACK1 interaction by bimolecular fluorescence complementation (BiFC). SE and RACK1 interacted in the nucleus, and were often concentrated in distinct foci (Figure 1c). This is in agreement with the fact that SE mainly localizes to nuclear D-bodies (Fang and Spector 2007), and that Arabidopsis RACK1 was present in the cytosol and nucleus *in planta* (Figure 1d). Some RACK1 targets have been reported to be stabilized or destabilized upon binding (Liu *et al.* 2007, Zhang *et al.* 2012), but we found that the amounts of SE remained unchanged in *rack1* mutants (Figure 1e). These results suggest that RACK1 does not influence SE protein levels.

Because RACK1 interacted with the miRNA factor SE, we tested whether miRNA levels were affected in *rack1* mutants. To do this, we quantified miR156, miR159, miR164 and miR166 by small RNA blot analyses and quantitative RT-PCR in *rack1* and the known miRNA-related mutants *se* and *ago1* (Figure 2a-e). *se* mutants are impaired in very early steps of miRNA biogenesis, and hence the levels of mature miRNAs were drastically decreased (Figure 2d). In *ago1* mutants, miRNAs were presumably not efficiently loaded and were therefore less stable (Figure 2d). We observed a drastic decrease in the miRNA levels in *rack1* triple mutants, in which miRNAs were reduced to 10–40% of the wild-type levels (Figure 2a, b). *rack1a* mutants also contained less miRNAs than the wild-type (Figure 2c, d). Analysis of *rack1* single and double mutants revealed that a functional *RACK1A* gene resulted in

wild-type miRNA levels under the conditions tested (Figure 2d), uggesting that *RACK1A* is the most important player controlling miRNA abundance.

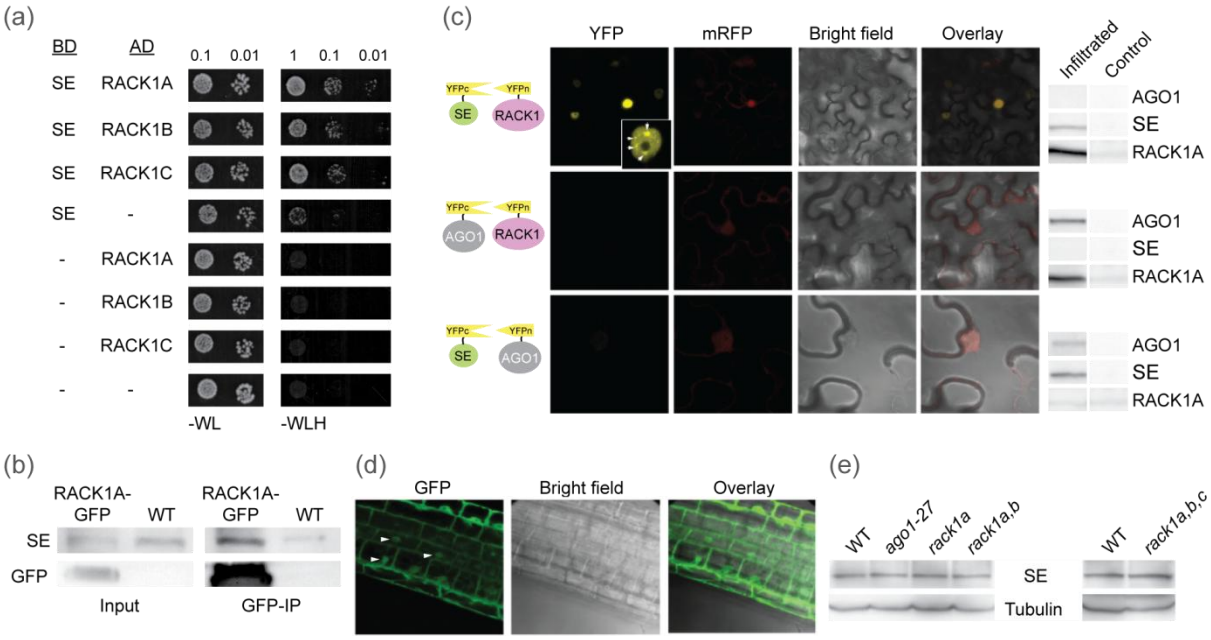

Figure 1: RACK1 interacts with SE

(a) Yeast two-hybrid interaction analysis between RACK1 and SE. A serial 1:10 dilution of yeast cells expressing SE fused to the GAL4 DNA-binding domain (BD) and RACK1 proteins fused to the GAL4 activation domain (AD), respectively, was grown for 4 days on control (–WL) and selective (–WLH) media.
(b) Co-immunoprecipitation with GFP antibodies from wild-type (WT) and *RACK1A:RACK1A-GFP rack1a* transgenic plants. Supernatant and pellet fractions were analyzed using GFP and SE-specific antibodies.
(c) Bimolecular fluorescence complementation experiments using SE–YFPc, YFPn–RACK1, YFPc–AGO1 and YFPn–AGO1. Fusion proteins were transiently expressed in *N. benthamiana* leaves and analyzed by confocal microscopy. monomeric RFP (mRFP) served as a transformation control. The presence of the respective fusion proteins was determined by immunoblot analyses.
(d) Analysis of the subcellular localization of RACK1A–GFP. Roots of *RACK1A:RACK1A-GFP rack1a* transgenic lines were analyzed by fluorescence microscopy.
(e) Immunoblot analysis of the SE protein in WT and various *rack1* mutants.

We also tested whether the accumulation of miRNA* species was affected in *rack1* mutants. We used *ago1* mutants as genetic controls for miRNA* quantification by real-time PCR. miRNA* species accumulate in *ago1* mutants because AGO1 activity is responsible for removal of the miRNA* strand after loading of the miRNA/miRNA* duplex (Eamens *et al.* 2009). As expected, the levels of some miRNA* species were increased in *ago1* mutants, but their abundance was low in *se* mutants (Figure 2e). We found that *rack1a* mutants possessed low miRNA* levels, as in *se-1* mutants. This effect was exaggerated in the *rack1a rack1b* double mutant (Figure 2e).

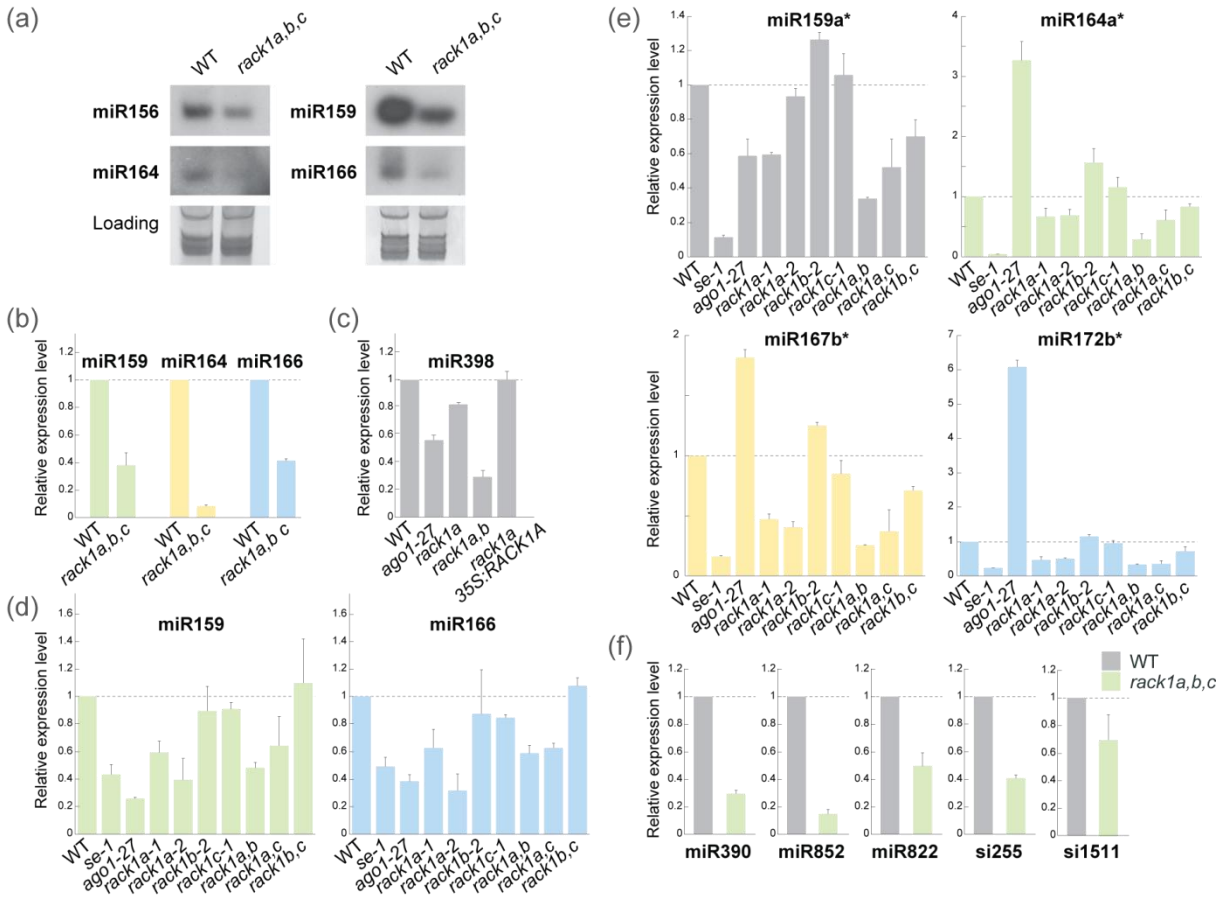

Figure 2: RACK1 regulates miRNA accumulation

(a) Small RNA blot analyses of miR156, miR159, miR164 and miR166 in wild-type (WT) and *rack1a,b,c* triple mutants.
(b) Quantitative real-time PCR analyses of selected miRNA and miRNA* species in WT, *se-1*, *ago1-27* and various *rack1* mutants. Error bars indicate the range for two independent biological experiments.

Additional expression studies demonstrated that RACK1 has no specificity for miRNAs that associate with AGO1, because the levels of miR390 and miR852, which mainly associate with AGO7 and AGO2, respectively, were strongly reduced in *rack1* triple mutants (Mi *et al.* 2008, Montgomery *et al.* 2008; Figure 2f). *rack1* mutants also accumulated low levels of miR822, the processing of which is accomplished by DCL4, suggesting that the function of RACK1 is not limited to DCL1-dependent miRNAs (Rajagopalan *et al.* 2006; Figure 2f). Probably due to impaired miRNA expression, we found that the levels of trans-acting small-interfering RNAs (tasiRNAs) were also reduced in *rack1* triple mutants (Figure 2f). Collectively, our findings demonstrate that the SE-interacting protein RACK1 is important for miRNA accumulation.

RACK1 affects accumulation and the processing precision of pri-miRNAs

To obtain further insights into the function of RACK1 in the plant miRNA pathway, we sequenced small RNA populations from two biological replicates of wild-type and *rack1* triple mutant seedlings using Illumina technology. After removal of reads smaller than 17 nt or larger than 27 nt and reads mapping to tRNAs and rRNAs, between 5 and 7 million reads remained for each sample. First, we performed an expression analysis to estimate the global effects of RACK1 on the miRNA transcriptome. A total of 209 known miRNAs were included in our analysis, of which 77 miRNAs were represented by at least five sequencing reads in all samples, which allows robust calculation of differential expression. Of these 77 miRNAs, 61 miRNAs were significantly less abundant in *rack1* triple mutants compared to wild-type (p-value < 0.05, FDR < 0.02, Figure 3a, Supplemental Figure 1). Interestingly, the levels of two miRNAs, miR827 and miR866-5p, were higher in *rack1* triple mutants, which may be explained by RACK1's pleiotropic functions. In general, our results suggest that RACK1 function is important for the accumulation of a large number of Arabidopsis miRNAs.

The low levels of miRNAs in *rack1* may be explained by low transcriptional levels of *MIRNA* genes (as in *mediator* mutants), less stable pri-miRNAs (as observed in *ddl* mutants) or less efficient or mis-processing of pri-miRNAs (as observed in *se* or *hyl1* mutants) (Dong *et al.* 2008, Kim *et al.* 2011, Manavella *et al.* 2012, Yu *et al.* 2008). In order to determine whether *rack1* mutants exhibit processing defects and accumulate non-canonical, unusually sized miRNAs, we mapped all sequenced 17–27 nt sRNAs to the annotated mature miRNAs. As exemplified by miR157a–c and miR319a/b species, we found that, in *rack1* mutants, most *MIRNA* loci released small RNAs with a similar size distribution to that in the wild-type (Figure 3b, Supplemental Figure 2). However, a small subset of *MIRNA* loci produced an array of aberrant non-canonical miRNAs, which were absent or less abundant in the wild-type. In the case of miR159b and miR167a/b, the production of 19 and 20 nt miRNA species was moderately increased in *rack1* mutants (Figure 3b). Similarly, the miR167d and miR393a species were more often 21 nt long in *rack1* mutants, compared to the predominant 22 nt long miRNA found in the wild-type (Figure 3b). These observations suggest a role for RACK1 in precise processing of some pri-miRNAs, similar to the known factors SE and HYL1. We also analyzed whether pri-miRNAs were inaccurately diced outside the miRNA duplex region in the *rack1* mutant background. To test this, we mapped all sequenced RNAs to the entire precursors of miR157a, miR159b and miR319a, and found that *rack1* triple mutants did not produce any major products resulting from mis-processing outside the miRNA/miRNA* region (Figure 3c).

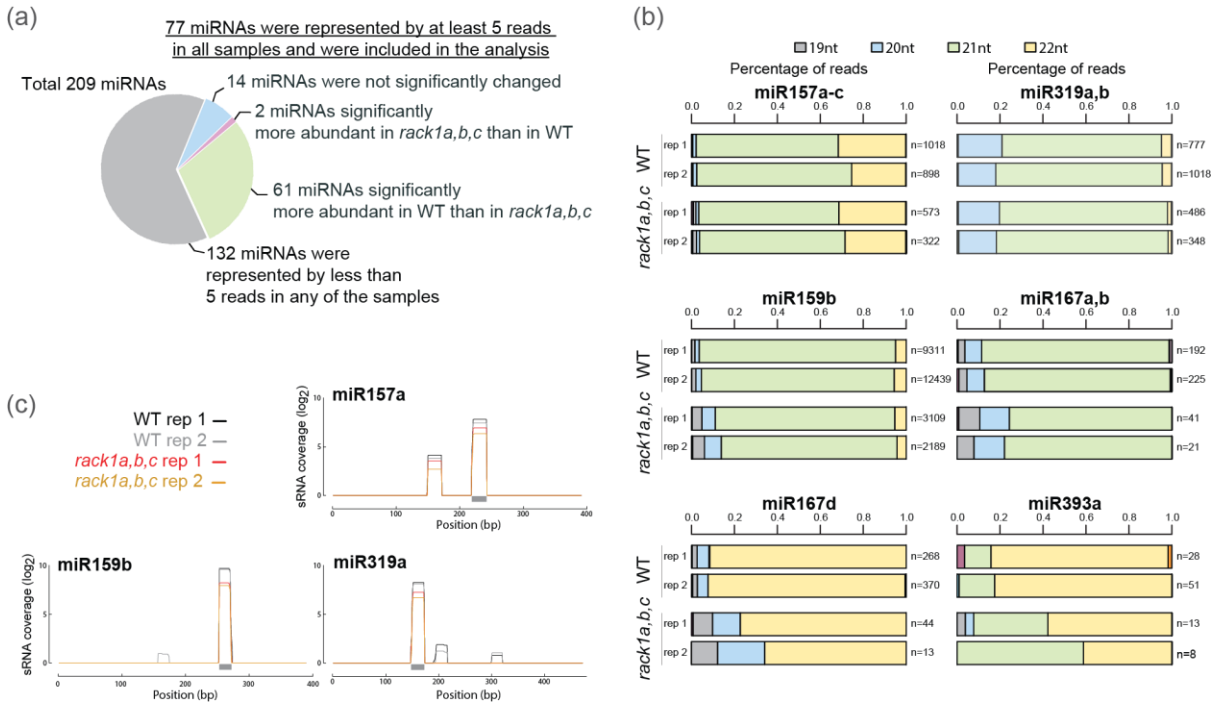

Figure 3: RACK1 plays a minor role in the accuracy of pri-miRNA processing

(a) Summary of the expression analysis of 209 miRNAs in wild-type (WT) and *rack1a,b,c* triple mutants. Between 5 and 7 million small RNAs in two biological replicates of wild-type and *rack1* triple mutant seedlings were sequenced and used for miRNA expression analysis (see Experimental procedures).
(b) Mis-processing analysis of various miRNAs in WT and *rack1a,b,c* triple mutants. Sequencing reads of 17–27 nt length were mapped to the respective mature miRNA sequence. Matches were counted in percentages, and *n* indicates the number of small RNAs that aligned with the respective miRNA.
(c) Coverage plot of the stem-loop region of *MIR157A*, *MIR159B* and *MIR319A*. All small sequencing reads from WT and *rack1* triple mutants (two biological replicates each) were aligned with the respective stem-loop region. Gray bars indicate the position of the mature miRNA. Additional peaks correspond to miRNA* species or secondary miRNAs that were not detectable in *rack1* triple mutants.

Next, we determined whether RACK1 influences pri-miRNA contents by analyzing the steady-state levels of various pri-miRNAs by quantitative PCR (Figure 4a-c). As in other pri-miRNA processing mutants such as *se-1*, we found that some pri-miRNAs (such as pri-miR393a) accumulated to higher levels in *rack1* triple mutants (Figure 4b, c). In contrast, *rack1* triple mutants also exhibited a reduction of several pri-miRNAs, suggesting that RACK1 affects *MIRNA* gene transcription and/or pri-miRNA stability (Figure 4b). Some pri-miRNAs were unaffected in the *rack1* triple mutants (Figure 4b), in agreement with the observation that RACK1 is not a general activator of gene expression (Guo *et al.* 2011).
In order to test whether RACK1 affects the processing of pre-miRNAs to mature miRNAs, we performed expression analysis using oligonucleotides specific for the pre-miRNA region. Priming the reverse transcription reaction using oligo(dT) oligonucleotides allows detection of pri-miRNAs only, while priming with random hexamers delivers information about pri-miRNAs

and pre-miRNAs (Figure 4c). We did not observe dramatic differences between oligo(dT)-primed and randomly primed cDNA samples (Figure 4c), suggesting that *rack1* mutants are not impaired in pre-miRNA processing.

In summary, our experiments show that RACK1 has pleiotropic functions in the miRNA pathway. RACK1 affects the processing and transcription/stability of certain pri-miRNAs, and plays a minor role in the precision of pri-miRNA processing.

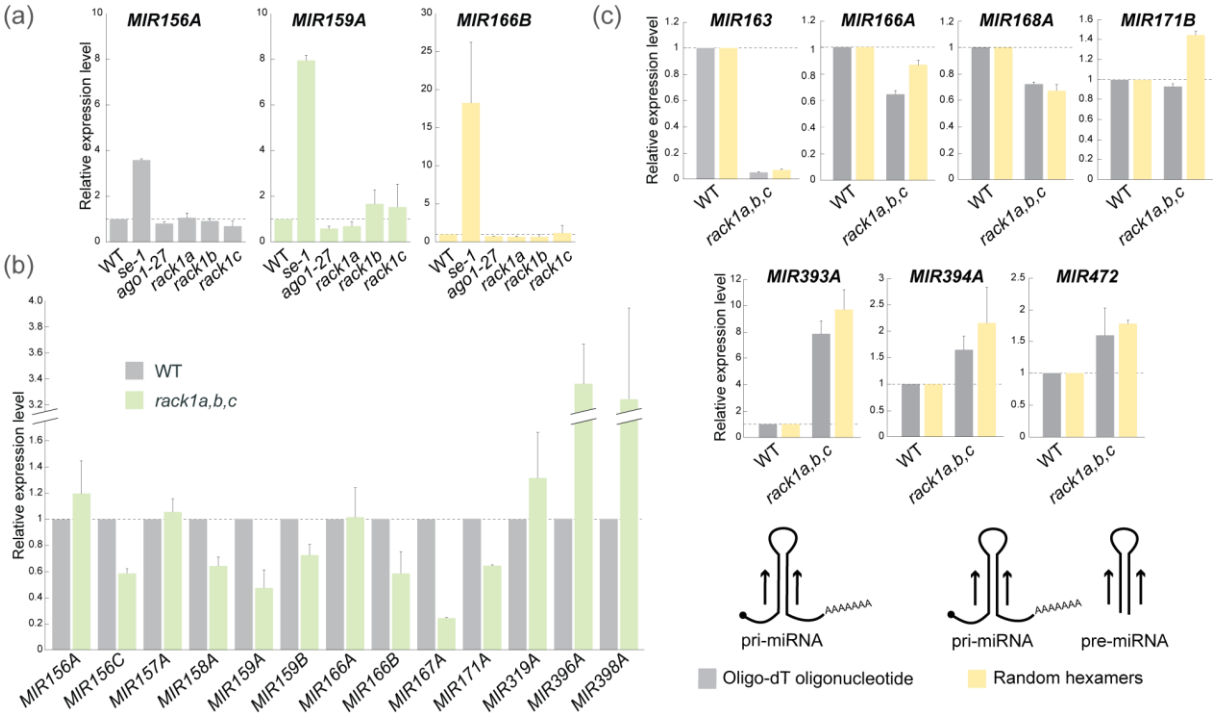

Figure 4*: Mutations in *RACK1* affect the accumulation of pri-miRNAs

(a), (b) pri-miRNA expression analyses in *rack1* mutants. The steady-state levels of up to ten pri-miRNAs were determined by quantitative PCR in wild-type (WT), *se-1*, *ago1-27* and various *rack1* mutants. Error bars indicate the range of two independent biological experiments.
(c) pri-miRNA and pre-miRNA expression analysis in *rack1a,b,c* mutants. cDNAs were prepared using oligo(dT) or random hexamer oligonucleotides. Steady-state levels of seven pri- and pre-miRNAs were determined by quantitative PCR in WT and *rack1a,b,c* mutants. Values are means ± SEM for three biological replicates.

Arabidopsis RACK1 is associated with AGO1-containing complexes

Our data provide evidence that Arabidopsis RACK1 is important for the production of the vast majority of miRNAs. In contrast to that, *rack-1*-deficient *C. elegans* strains contain higher amounts of miRNAs, and RACK-1 function is limited to bridging the interaction between the ribosome and the AGO miRNA effector protein (Jannot *et al.* 2011). Although our results suggest that Arabidopsis RACK1 functions in pri-miRNA transcription/stabilization and processing, the results of studies on human and worm RACK-1 prompted us to test whether

Arabidopsis RACK1 is associated with AGO1 (Jannot *et al.* 2011, Otsuka *et al.* 2011). Therefore, we performed immunoprecipitation experiments using plants expressing RACK1A–GFP. Immunoblotting experiments using an AGO1-specific antibody revealed that AGO1 co-precipitated with RACK1–GFP (Figure 5a). Therefore, we performed immunoprecipitation experiments using plants expressing RACK1A–GFP. Immunoblotting experiments using an AGO1-specific antibody revealed that AGO1 co-precipitated with RACK1–GFP (Figure 5a). Unlike in *C. elegans*, the interaction between RACK1 and AGO1 is likely to be indirect, as we did not detect a direct interaction between AGO1 and RACK1 in BiFC experiments (Figure 1c).

If AGO1 and RACK1 exist in a complex, they should co-localize in cells. In order to study the subcellular location of RACK1 and AGO1, we simultaneously expressed GFP–AGO1 and mRFP-RACK1 proteins in *Nicotiana benthamiana* cells. RACK1 and AGO1 co-localized in both the nucleus and the cytosol, further supporting the conclusion that RACK1 is part of an AGO1 complex (Figure 5b). In order to determine whether RACK1 and AGO1 exist in complexes of the same size, we performed gel filtration experiments followed by immunoblot analyses using RACK1- and AGO1-specific antibodies. Consistent with previous reports, we detected AGO1 complexes of 200–400 kDa (Azevedo *et al.* 2010, Csorba *et al.* 2010; Figure 5c). RACK1 was highly abundant in the same fractions, in agreement with the conclusion that RACK1 and AGO1 are part of a common complex (Figure 5c).

Because AGO1 is associated with polysomes, and RACK1 is part of the small ribosomal subunit, the interaction between AGO1 and RACK1 may be limited to ribosome-associated RACK1 and AGO1. To test whether AGO1 and RACK1 were also found in complexes other than the ribosome, we tested in which kind of complexes S14, a small ribosomal subunit, was detectable in our gel filtration experiments. The elution profile of AGO1 and RACK1 was largely distinct from that of S14-containing complexes (Figure 5c), suggesting that AGO1 and RACK1 are also present in complexes other than ribosomal complexes.

The exact function of RACK1 within AGO1 complexes remains to be elucidated, but it is important to note that RACK1 does not affect the overall integrity of AGO1-containing complexes. We tested this by performing gel filtration experiments in the *rack1a rack1b* double mutant, and obtained a very similar elution profile for AGO1 in wild-type and the *rack1* double mutant (Figure 5c). These results suggest that AGO1 complexes are assembled and remain stable in the absence of wild-type RACK1 levels.

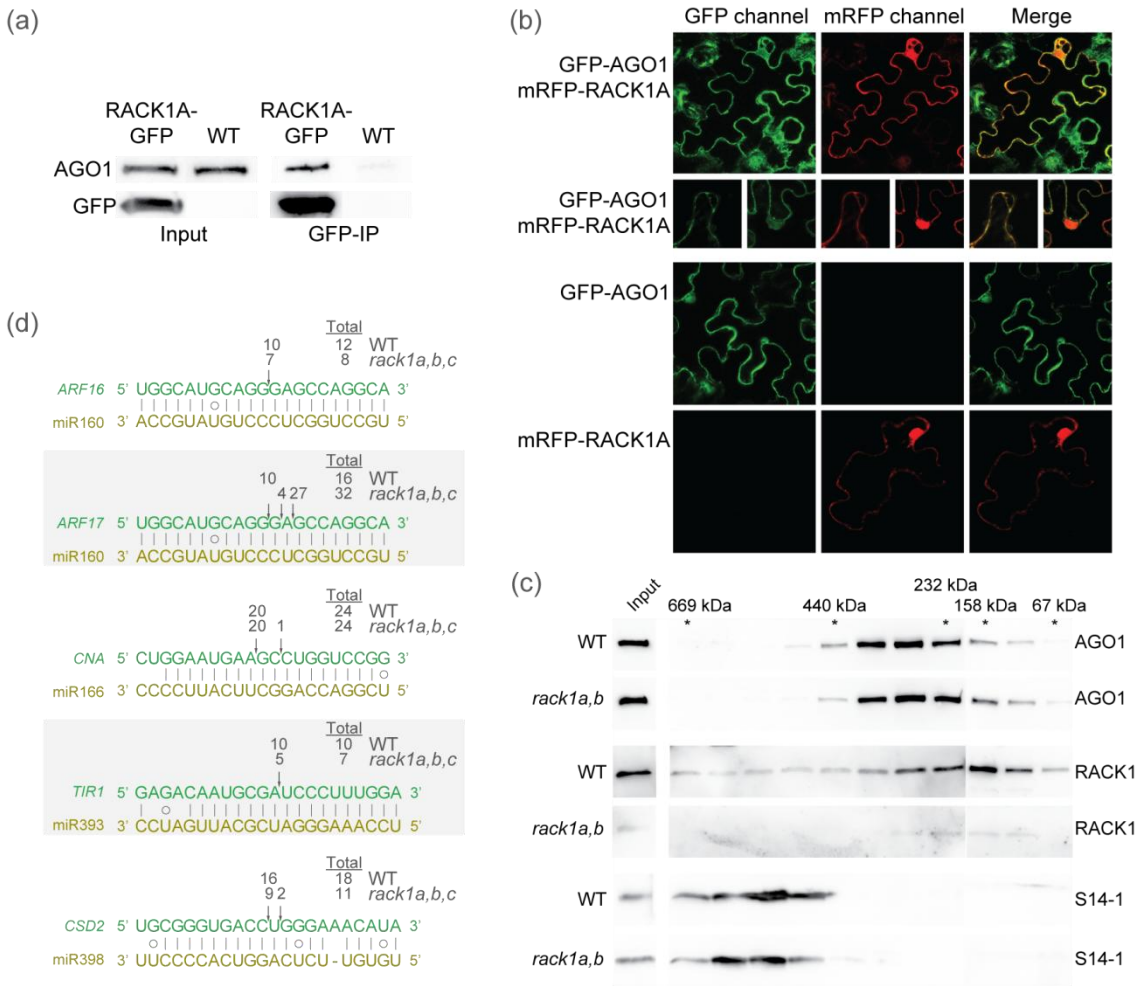

Figure 5: Interaction analyses between AGO1 and RACK1

(a) These results suggest that AGO1 complexes are assembled and remain stable in the absence of wild-type RACK1 levels.
(b) Co-localization experiments with mRFP-RACK1 and GFP–AGO1. Fusion proteins were transiently expressed in *N. benthamiana* leaves and analyzed by confocal microscopy.
(c) Protein complex analyses in wild-type (WT) and *rack1a rack1b* double mutants. Native complexes were passed through a Superdex 200 column, and 500 µl fractions were collected. Individual fractions were analyzed by immunoblotting using AGO1-, RACK1- and S14-1-specific antibodies.
(d) Analysis of the miRNA-mediated mRNA cleavage accuracy in WT and *rack1a,b,c*. 5′-RACE PCR products were cloned and sequenced. Vertical arrows indicate the 5′ position of RACE products. The frequency of the respective 5′ RACE product is shown as the number of all sequenced 5′ RACE products that match the target gene.

We also determined whether RACK1 affects the cleavage accuracy of AGO1 complexes. We found that most miRNA targets were cleaved at the expected positions, with the exception of the miR160 target *ARF17* (Figure 5d). In *rack1* triple mutants, the *ARF17* mRNA was mainly cleaved two nucleotides downstream of the main cleavage site found in wild-type. The impaired slicing accuracy is not due to mis-processing of miR160, because another miR160-targeted mRNA, *ARF16*, is cleaved at the correct position in *rack1* mutants (Figure 5d). aken together, these findings suggest that, in general, RACK1 does not affect the

accuracy of miRNA-guided target cleavage. However, the slicing of some targets, such as *ARF17*, may be either directly or indirectly influenced by RACK1.

RACK1 regulates miRNA-regulated gene expression

Mutants with defects in miRNA accumulation show deregulation of miRNA-mediated gene expression. Because *rack1* mutants are impaired in miRNA accumulation, we analyzed the steady-state levels of various known target transcripts. The levels of several miRNA-targeted mRNAs rose 1.5–13-fold in the *rack1* triple mutant, suggesting that *RACK1* is important for the regulation of miRNA target genes (Figure 6a). Most tested target mRNAs were not affected in *rack1* single mutants (exemplified by expression analysis of the miR164 target *CUC1*, Figure 6a), which may be explained by the genetic redundancy among the *RACK1* genes.

We also investigated the targets of miR398, which is known to act via transcript cleavage and translational inhibition (Bouche 2010, Dugas and Bartel 2008). *CSD1*, *CSD2* and *CCS* mRNA levels were increased even in *rack1a* single mutants, a phenotype that was completely reversed by introduction of a wild-type *RACK1A* copy (Figure 6b, c). Furthermore, the CSD1, CSD2 and CCS proteins were much more abundant in the *rack1a rack1b* double mutant, further indicating that *RACK1* controls miRNA-mediated gene regulation (Figure 6b, c). *rack1* triple mutants also accumulated more AGO1, the mRNA of which is targeted by miR168 (Vaucheret *et al.* 2004; Figure 6d).

Because miRNA-mediated gene expression is altered in *rack1* mutants, we determined whether *rack1* mutants share some common phenotypes with other miRNA-related mutants. Despite RACK1 having pleiotropic functions, *rack1a* single mutants exhibited an ABA hypersensitivity similar to that of *ago1-27* mutants (Guo *et al.* 2011; Figure 6e). With low penetrance, *rack1a rack1b* double mutants also showed phyllotaxy defects, a phenotype that is frequently observed in other miRNA-related mutants such as *se-1* (Prigge and Wagner 2001; Figure 6f). Taken together, our results show that *rack1* mutants accumulate miRNA-targeted mRNAs and exhibit phenotypic similarities to other miRNA mutants, suggesting that RACK1 is an important regulator of miRNA-mediated control of Arabidopsis development.

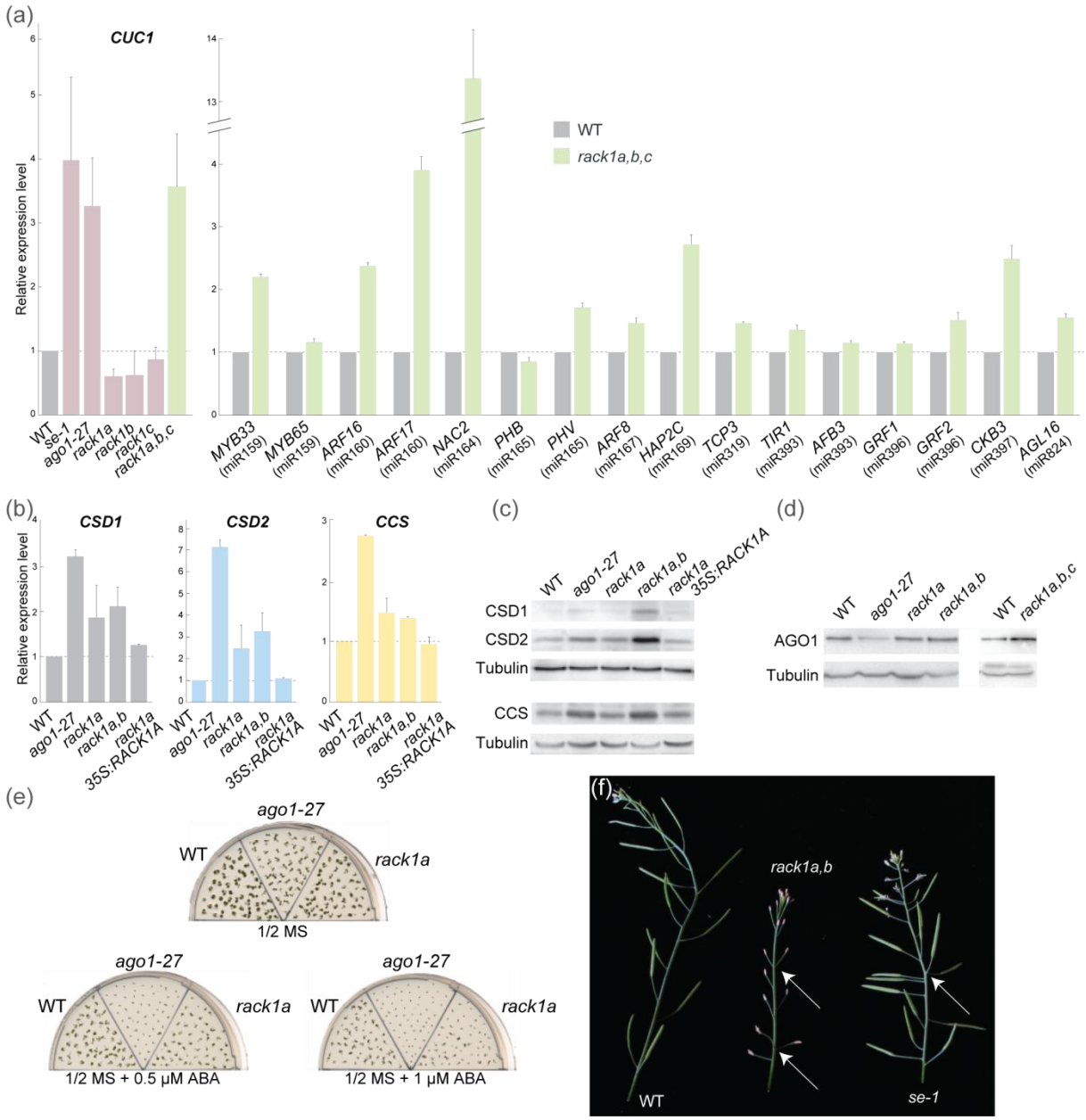

Figure 6*:* RACK1 influences miRNA-dependent physiology and development

(a), (b) Quantitative real-time PCR analyses of known miRNA-targeted mRNAs in wild-type (WT), *se-1*, *ago1-27* and various *rack1* mutants. Error bars indicate the range of two independent biological experiments.
(c), (d) Immunoblot analyses of CSD1, CSD2, CCS and AGO1 levels in WT, *ago1-27* and various *rack1* mutants.
(e) Comparison of ABA-mediated inhibition of seedling greening in WT, *ago1-27* and *rack1a* mutants.
(f) Shoot phenotypes of WT, *se-1* and *rack1a rack1b* mutants.

Discussion

Over a dozen proteins are known to be part of the miRNA pathway in plants, and specific functions such as RNA binding is assigned to most of them. Here, we have shown that a scaffold protein, RACK1, participates in the miRNA pathway.

Diverse functions of RACK1 scaffold proteins in different steps of the plant miRNA pathway

Although RACK1 plays essential roles in many cellular processes, which may obscure some of its specific functions during miRNA maturation, we were able to show that RACK1 function is essential for quantitative and qualitative accumulation of mature miRNAs. Some pri-miRNAs are less abundant in *rack1* mutants, suggesting that RACK1 controls *MIRNA* transcription and/or pri-miRNA stability. Mammalian RACK1 directly regulates transcription of the brain-derived neurotrophic factor (*BDNF*) gene by physical association with a distinct promoter region and induction of acetylation of histone H4 (He *et al.* 2010). RACK1 also indirectly influences transcription by interfering with the DNA binding capacity or reducing the stability of certain transcription factors (Liu *et al.* 2007, Okano *et al.* 2006, Zhang *et al.* 2012). Whether Arabidopsis RACK1 directly associates with certain *MIRNA* genes or whether RACK1 regulates the activity of transcription factors that bind to the promoters of *MIRNA* genes is an interesting subject for future research.

ur results showed that RACK1 not only affects the transcription/stabilization of pri-miRNAs, but also participates in later steps within the miRNA pathway. RACK1 interacts with SE, a factor that controls the efficiency and precision of pri-miRNA processing. This is in agreement with the observation that, similar to *se* mutants, some pri-miRNAs accumulate in *rack1* mutants. A small subset of pri-miRNAs was not accurately processed in the absence of RACK1, such as pri-miR167b, which is accurately diced only in the presence of SE (Dong *et al.* 2008). Together, these results imply that the RACK1–SE interaction is important for processing of a specific subset of pri-miRNAs. Several reports have suggested that some animal miRNA precursors require a specialized protein partner for optimal miRNA production (Paroo *et al.* 2009, Trabucchi *et al.* 2009, Viswanathan *et al.* 2008) and this is also likely to be the case in plants (Jung *et al.* 2012, Laubinger *et al.* 2010, Reyes and Chua 2007). The function of RACK1 may be to build a docking site for such factors, and hence pri-miRNAs and mis-processing products accumulate in the absence of RACK1 function. Because SE and its homologs in animals are not only involved in miRNA metabolism (Andreu-Agullo *et al.* 2012, Gruber *et al.* 2012, Laubinger *et al.* 2008), RACK1 may also control other RNA processing events in concert with SE.

In addition to its association with SE, we found that RACK1 is associated with an AGO1 complex. Defects in AGO1-related processes in the *rack1* mutant background may contribute to the strong reduction of miRNA levels, as observed in other AGO1-related mutants (Smith *et al.* 2009). While the exact molecular function of RACK1 within ARGONAUTE complexes in plants remains unknown, it may be hypothesized that either ribosomal or non-ribosomal RACK1 is important for mediating protein–protein interactions. For instances, Arabidopsis AGO1 associates with SQN and HSP90 only very transiently, and Iki *et al*. were only able to detect the interactions when they performed their experiments in the presence of a non-hydrolyzable ATP analog. A possible RACK1 function may be to bring components such as HSP90, SQN and an AGO1 complex together and transiently stabilize their interactions. Detailed studies on this possible function will require a sophisticated *in vitro* system such as that described by Iki *et al*., because higher levels of AGO1 in *rack1* mutants may have compensatory effects (Figure 6d).

In conclusion, we found that RACK1 function is important for the accumulation of many miRNAs, and that the reason for this is likely to be a combination of the role of RACK1 in *MIRNA* gene transcription/stabilization, pri-miRNA processing and as part of an AGO1 complex.

Differences between animal and plant RACK1 function in the miRNA pathway

Human RACK1 interacts with KSRP, a splicing factor that binds to terminal loops of pri- and pre-miRNAs featuring GGG triplets, but knock-down of human RACK1 does not affect miRNA levels (Otsuka *et al.* 2011, Trabucchi *et al.* 2009). Human RACK1 interacts with KSRP, a splicing factor that binds to terminal loops of pri- and pre-miRNAs featuring GGG triplets, but knock-down of human RACK1 does not affect miRNA levels (Jannot *et al.* 2011). This is in striking contrast to the results we obtained for Arabidopsis RACK1, as the levels of most miRNAs were reduced in Arabidopsis *rack1* mutants. Therefore, the function of Arabidopsis RACK1 appears to be much more general compared to its animal counterparts.

Down-regulation of RACK-1 by RNAi in *C. elegans* led to higher protein production from miRNA-targeted mRNAs, suggesting that animal RACK1 may be involved in miRNA-mediated translational repression (Jannot *et al.* 2011). The authors hypothesized that ribosomal RACK1 may guide the *C. elegans* ALG-1 protein to the mRNA, and, in agreement with this, ALG-1 recruitment to polysomes is reduced in *rack-1* RNAi lines (Jannot *et al.* 2011). To address the question of whether Arabidopsis RACK1 is also involved in miRNA-mediated translational repression, we investigated the targets of miR398, which is known to act via this mechanism (Bouche 2010, Brodersen *et al.* 2008, Dugas and Bartel 2008). Although we found that miR398-targeted mRNAs are not more abundant in *rack1a rack1b* double mutants compared with *rack1a* mutants, we observed a drastic increase in the

levels of the corresponding proteins (CSD1, CSD2 and CCS). However, these differences were accompanied by a strong reduction in miR398 levels in the *rack1a rack1b* double mutant, which may account for the increases in CSD1, CSD2 and CCS levels (compare Figure 2c with Figure 6b, c). As RACK1 plays an important role in the plant miRNA pathway upstream of AGO1, it will be challenging to decipher the role of plant RACK1 in miRNA-mediated translational repression in plants.

As the exact functions of RACK1 in the animal and plant miRNA pathways are difficult to study, because RACK1 acts pleiotropically and is involved in so many biological processes, we cannot entirely disregard the possibility that RACK1 controls translation of a specific miRNA factor, for example. However, in strong support of our hypothesis that RACK1 directly participates in the miRNA pathway, we found that RACK1 interacts with SE and is present within AGO1 complexes, making it rather unlikely that the miRNA defects observed in *rack1* mutants are indirect consequences. Most likely, RACK1 acts as an adaptor that enhances protein–protein interactions. We did not observe interactions between RACK1 and other known miRNA factors in yeast two-hybrid experiments (Corinna Speth and Sascha Laubinger, unpublished results). Although this negative result in yeast assays does not formally rule out such interactions, these observations imply that RACK1 could bridge the interaction between known miRNA components (SE or AGO1) and yet unknown miRNA players. The fact that *rack1* triple mutants are viable and still produced detectable amounts of miRNAs (Figure 2a) suggests that protein–protein interactions within the miRNA pathway also occur in the absence of RACK1, but presumably with much lower efficiency. Alternatively, additional scaffold proteins that are part of the plant miRNA pathway may compensate for the lack of RACK1 function to a certain extent.

Experimental Procedures

Plant material and growth conditions

The mutants and transgenic lines used in this study, i.e. the *se-1*, *ago1-27*, *rack1* mutants and *RACK1A:RACK1A-GFP rack1a* lines, have been described previously (Chen *et al.* 2006, Grigg *et al.* 2005, Guo and Chen 2008, Guo *et al.* 2009, Morel *et al.* 2002). Unless otherwise stated, the following *rack1* alleles were used for all experiments: *rack1a-1*, *rackb-2*, *rack1c-1*, *rack1a-1 rack1b-2*, *rack1a-1 rack1c-1*, *rack1a-1*/+ *rack1b-2 rack1c-1*, *35S:RACK1A rack1a-1* and *RACK1A:RACK1A-GFP rack1a-1*. Col-0 served as a wild-type control in all experiments.

Plants were grown on half-strength MS plates for 10 days or on soil for 21 days under long-day conditions (16 h light, 22°C). For all analyses involving *rack1a,b,c* triple mutants, plants were grown for 10 days on half-strength MS plates supplemented with 2% w/v sucrose.

DNA constructs

cDNAs of all genes were PCR-amplified from reverse-transcribed RNAs using Phusion polymerase (Thermo Fisher Scientific), and cloned into the GATEWAY®-compatible entry vectors pENTR1a® or pCR®8/GW/TOPO® (Life Technologies). LR Clonase™ II (Life Technologies) was used to transfer cDNAs into the destination vectors pGBKT7-DEST, pGADT7-DEST, pSPYNE-DEST, pSPYCE-DEST, pGWB652 and pGWB654 to generate in-frame fusions of AGO1, SE and RACK1A with the GAL4 DNA-binding domain (BD), the GAL4 activation domain (AD), YFPn (N-terminal part of YFP), YFPc (C-terminal part of YFP), mRFP or GFP (Horak *et al.* 2008, Nakamura *et al.* 2010, Walter *et al.* 2004).

Yeast two hybrid assay

The yeast strain AH109 (James *et al.* 1996) was transformed using standard LiAc-based transformation according to the manufacturer's protocol (Clonetech, www.clonetech.com). After 3 days, 5–10 colonies from each transformation were resuspended in 10% w/v glycerol, and adjusted to an OD_{600} of 1. We prepared a serial 1:10 dilution of the yeast cells, spotted them onto control (–WL) or selective (–WLH) media lacking the corresponding amino acids mentioned in brackets, and grew the colonies for 4 days at 28°C.

Bimolecular fluorescence complementation (BiFC), co-localization and microscopy

AGO1, SE and RACK1A fusion proteins were transiently co-expressed in 2–3-week-old *Nicotiana benthamiana* leaves using standard methods (de Felippes and Weigel 2010). After 36 hours, leaf discs were analyzed by confocal microscopy (Leica TCS SP2). The expression of the transformed fusion proteins was analyzed by immunodetection using GFP-specific antibodies (Abcam). For analysis of the subcellular localization of RACK1, roots of *RACK1A:RACK1A-GFP rack1a* transgenic lines were analyzed by confocal microscopy as described above.

RNA isolation and analyses

Total RNA was extracted from plants using TRIZOL® (Life Technologies). For small RNA blot analysis, 10 µg total RNA was resolved by urea PAGE, blotted onto nylon membranes (Hybond-N+, Amersham), and hybridized with 5'-radiolabeled oligonucleotides (Supplemental Table 1).

For quantitative RT-PCR analyses, 200 ng to 2 µg of total RNA was treated with DNase and reverse-transcribed using a RevertAid™ first-strand cDNA synthesis kit (Thermo Fisher Scientific). Oligo(dT) primers and specific stem-loop primers were added for reverse transcription of mRNAs and miRNAs, respectively (Varkonyi-Gasic *et al.* 2007; Supplemental table 1). Quantitative PCR was performed in reactions containing SYBR Green (Thermo

Fisher Scientific) on a CFX384 system (Bio-Rad). All measurements were repeated twice with at least two biological replicates and in the presence of a standard curve of amplification. *TUBULIN* served as a normalization control for all experiments. All oligonucleotides are listed in Supplemental Table 1.

RACE experiments were performed using Firstchoice® RLM-RACE (Life Technologies). Briefly, 1 µg total RNA or 75 ng polyA RNA was ligated to the 5′-RNA adaptor, and, after reverse transcription, PCR reactions using adaptor- and gene-specific oligonucleotides (Supplemental Table 1) were performed using Phusion® polymerase or DreamTaq (Thermo Fisher Scientific). The resulting PCR products were cloned into a TOPO-TA cloning vector (Life Technologies), and 7–32 individual clones were sequenced.

Small RNA libraries were generated from total RNA of wild-type and *rack1* triple mutants. Construction of libraries and sequencing using the HiSeq 2000 Illumina system was performed by GATC Biotech (Konstanz, Germany). After adaptor trimming, reads between 17 and 27 bp length in each sRNA Illumina library were mapped without mismatches against the TAIR10 genome sequence (www.arabidopsis.org) using genomemapper (Schneeberger *et al.* 2009). Mapped reads were annotated according to the TAIR10 genome annotation, and reads mapping to tRNA and rRNA loci were removed. The remaining reads were mapped with no mismatches against known mature miRNA sequences (http://mpss.udel.edu/common/web/starExamples.php?SITE=at_pare). miRNA expression counts were normalized to reads per million (RPM) with the total number of reads mapping to the genome. If a read mapped to more than one miRNA from the same miRNA family, the count was divided by the number of different miRNA. All analyses were performed using custom Perl scripts, which are available upon request. Differential expression analysis was performed using the EdgeR package (Robinson *et al.* 2010). Sequencing data were deposited at GEO Omnibus (GSE40579).

Protein co-immunoprecipitation, gel filtration and immuno blot analyses

For protein extraction, plant material was ground in liquid nitrogen, resuspended in protein extraction buffer [50 mm Tris pH 7.5, 150 mm NaCl, 10% v/v glycerol, 1 mm dithiothreitol and Complete protease inhibitor (Roche) , and lysates were cleared by centrifugation at 16 000 *g*, 4°C, for 30 min. For co-immunoprecipitation, plant material was ground in liquid nitrogen, resuspended in binding buffer [50 mm Tris pH 7.5, 100 mm NaCl, 10% v/v glycerol, 100 nm MG132 (Life Sensors); and Complete protease inhibitor (Roche)] and Complete protease inhibitor (Roche)] (Bio-Rad). Each co-immunoprecipitation was performed using 5 mg of total protein according to published protocols with minor modifications (Isono and Schwechheimer 2010). Briefly, lysates was pre-cleared using 50 µl Agarose A beads (Roche) for 20 min at 4°C on a rotator. After removal of the beads, the lysates were incubated with 30 µl GFP–

TRAP (Chromotek) for 3 h under gentle rotation. The beads were washed three times with 1 ml of cold washing buffer (binding buffer + 0.05% v/v Triton X-100), each step 10 min at 4°C, and were resuspended in 25 µl of 2× Laemmli buffer (4% w/v SDS, 100 mM Tris pH 7.5, 20% v/v glycerol, 200 mM DTT, 0.4 mg/ml bromophenol blue).

For gel filtration experiments, plant material was resuspended in 50 mm Tris/HCl pH 7.5, 200 mm NaCl, 5% v/v glycerol), and cleared by centrifugation (16 000 ***g***, 4°C, 30 min). Total protein (500 µg) was loaded onto a Superdex 200 column (GE Healthcare), and 500 µl fractions were collected. Protein samples were concentrated using 10 µl StrataClean resin (Stratagene), and subjected to immunoblot analysis.

All protein samples were resolved by SDS–PAGE and transferred onto nitrocellulose or poly(vinylidene difluoride) membranes (GE Healthcare). Standard immunodetection was performed using protein-specific antibodies against AGO1, CCS, CSD1/2, S14, SE or RACK1 (all Agrisera), GFP (Abcam) or tubulin (Sigma-Aldrich), followed by visualization using enhanced chemiluminescence and a chemiluminescence imaging system (Chemi-Smart 5000, Peqlab).

Conflict of interests

The authors declare that they have no conflict of interest.

Author Contribution

C.S. and S.L. designed the research; C.S., S.R. and S.L. performed research; E.-M.W. and K.S. analyzed data; C.S. and S.L. wrote the paper with contributions from all authors.

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Supporting Information

Figure S1: Expression analysis of annotated miRNAs:

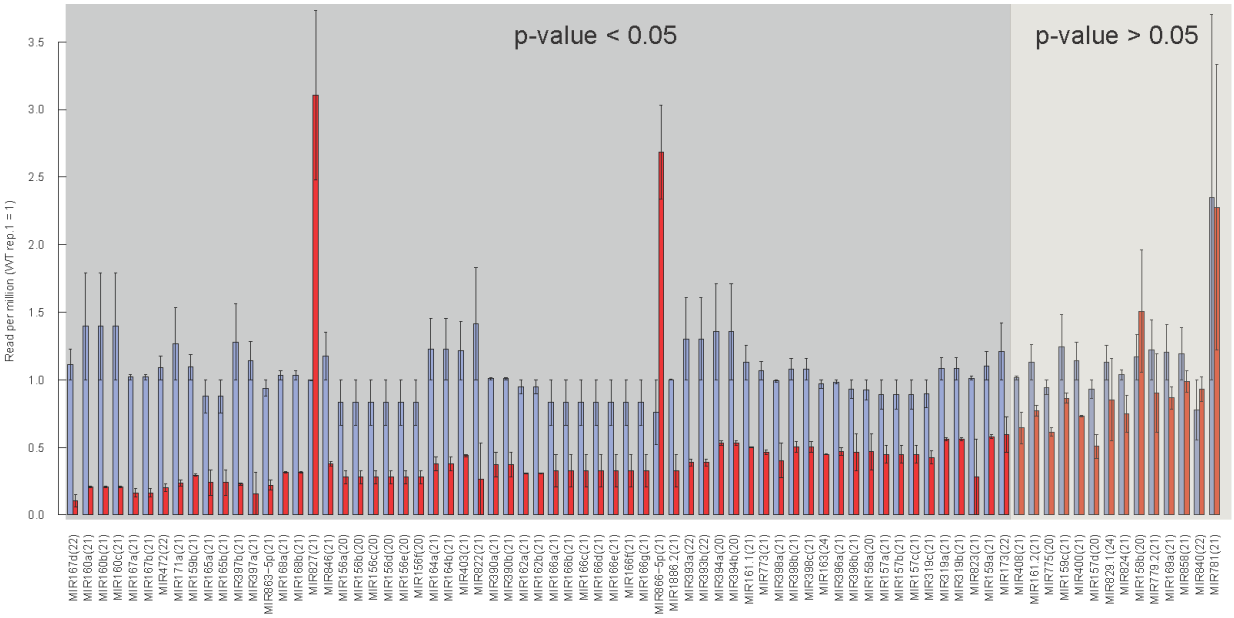

Figure S1: Expression analysis of annotated miRNAs.

Small RNAs from two biological replicates of WT and *rack1* triple mutant seedlings were sequenced and subjected to miRNA expression analyses. Figure S2 depict miRNAs that are significantly changed in *rack1a,b,c* triple mutants compared to WT. See Material and Methods for further information.

Figure S2: Pri-miRNA misprocessing analysis:

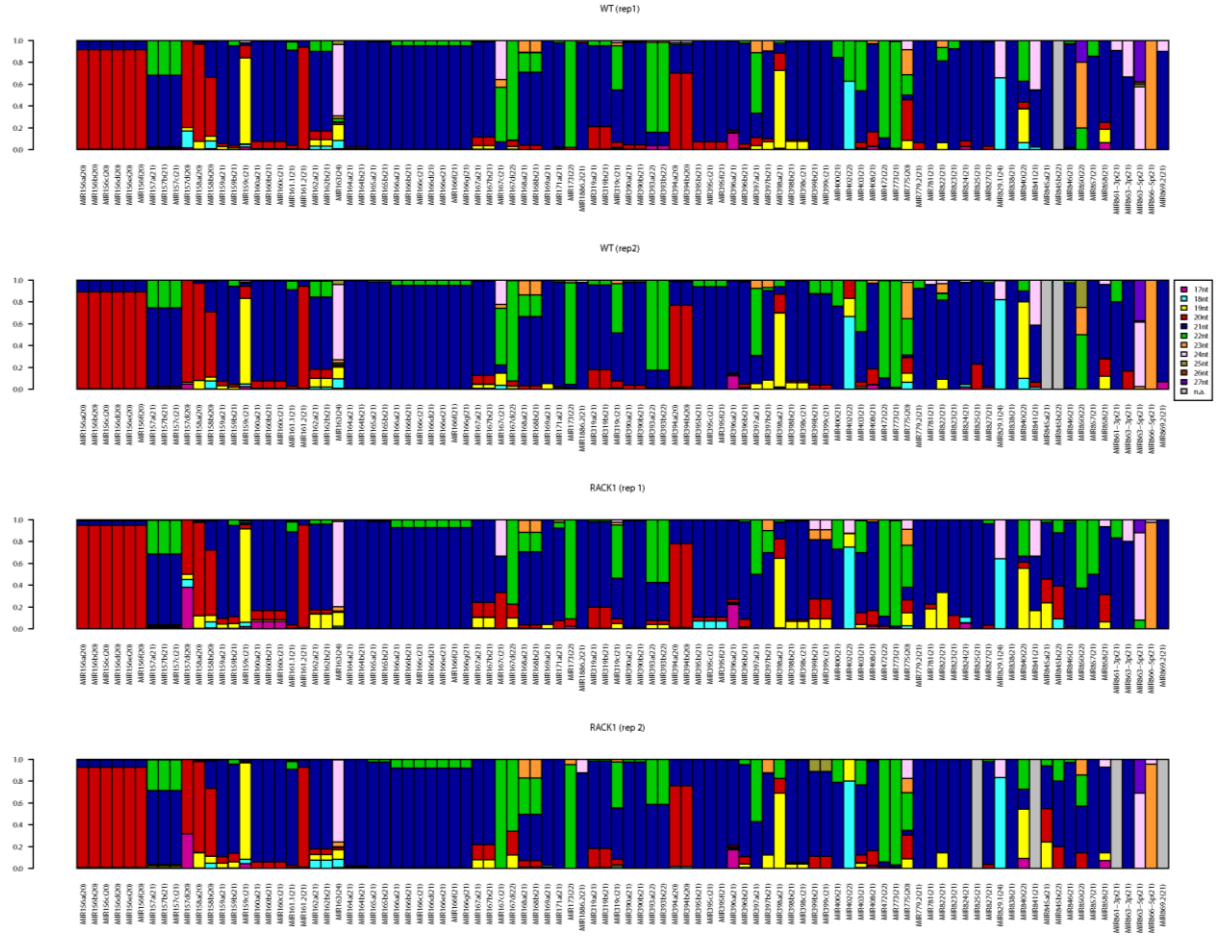

Figure S2: Pri-miRNA misprocessing analysis.

Misprocessing analysis of various miRNAs in WT and *rack1a,b,c* triple mutants (as performed in Figure 3).Sequencing reads of 17 to 27 nt length were mapped to the respective mature miRNA sequence. Matches were counted in percent.

Different colour represent different size classes. miR157a-c, miR319a,b, miR159b, miR167a,b, miR167d and miR393 are shown as examples in Figure 3b.

Table S1: List of oligonucleotides.

	Oligonucleotide	Sequence	Figure
cDNA synthesis	miR159a*-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGTTTG	Fig. 2 e
	miR159a-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGAGC	Fig. 2 b
	miR164ab*-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGWTGG	Fig. 2 e
	miR164ab-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCACG	Fig. 2 b
	miR166-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGGAA	Fig. 2 b
	miR167ab*-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACRGTGA	Fig. 2 e
	miR172ab*-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGTGAA	Fig. 2 e
	miR390-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCGCT	Fig. S1
	miR398-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTGGGG	Fig. 2 c
	miR822a-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCATGTG	Fig. 2 f
	miR852-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGAA	Fig. 2 f
	si1511-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGTAT	Fig. 2 f
	si255-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACGCT	Fig. 2 f
qRT-PCR	miR159ab*-F	GCT TCC GAG CTC CTT RAA GTT	Fig. 2 e
	miR159-F	GCGGCGTTTGGATTGAAGGGA	Fig. 2 b, d
	miR164a*-F	GCA CCG CAC GTA CTT AAC TTC	Fig. 2 e
	miR164-F	AGGACATGGAGAAGCAGGGCA	Fig. 2 b
	miR166-F	TCGCTTCGGACCAGGCTTCA	Fig. 2 b, d
	miR167b*-F	GTT GGA GGT CAT GCT CTG ACA	Fig. 2 e
	miR172b*-F	GGA AGG GCA GCA CCA TTA AGA	Fig. 2 e
	miR390-F	GAAGAGAAGCTCAGGAGGGAT	Fig. 2 f
	miR398-F	GGGCAGTGTGTTCTCAGGTCA	Fig. 2 c
	miR822-F	TGGACATGCGGGAAGCATTTG	Fig. 2 f
	miR852-F	CCACCCAAGATAAGCGCCTTA	Fig. S1
	si1511-F	GGTCGTCCAAGCGAATGATG	Fig. 2 f
	si255-F	CCGTCGTTCTAAGTCCAACAT	Fig. 2 f
	pri-miR156a-F	GTTAAAACTCAGATCTAACACAAAG	Fig. 4 a, b
	pri-miR156a-R	GAGAACGAAGACAGGCCAAAG	Fig. 4 a, b
	pri-miR157a-F	GAGAGGCATTGATAGTGTTGACAGAAG	Fig. 4 b
	pri-miR157a-R	AAAGGTGATGACAGAAGGCTAGAGAG	Fig. 4 b
	pri-miR158a-F	TGCAGAAGAAGAGTAACACGTCATCTC	Fig. 4 b
	pri-miR158a-R	AATCTCCACGACATCATCACGGTATTG	Fig. 4 b
	pri-miR159a-F	GGTCTTTACAGTTTGCTTATG	Fig. 4 a, b
	pri-miR159a-R	AGAAGGTGAAAGAAGATGTAG	Fig. 4 a, b
	pri-miR159b-F	TAGGTTAGATGCATGTAACTG	Fig. 4 b
	pri-miR159b-R	GATAGGGATGGAGAGATGAAG	Fig. 4 b
	pri-miR163-F	CCTCTTCAACGACAACGATTTCAACAC	Fig. 4 c
	pri-miR163-R	TGATATGGACTCACTCTCAGGAACCG	Fig. 4 c
	pri-miR166a-F	GACTCTGGCTCGCTCTATTCATGTTG	Fig. 4 b
	pri-miR166a-R	GCCTGGTCCGAAGACGCTAAAAC	Fig. 4 b
	pri-miR166b-F	CCCGGGATCATTCTCTTCATCATCACCAC	Fig. 4 a, b
	pri-miR166b-R	CCCGGGATGGACAAATCTTCTTCGTTAATTCG	Fig. 4 a, b
	pri-miR168a-F	CGGGAACCAATTCGGCTGACAC	Fig. 4 c

	Oligonucleotide	Sequence	Figure
qRT-PCR	pri-miR168a-R	CCAATCCCTGCTCACAAACCAATAAAG	Fig. 4 c
	pri-miR171a-F	TTCTCACTTCTCCTCCTCACACTTCAC	Fig. 4 c
	pri-miR171a-R	GCCAATATCAAAGGGACTCTCTCATGC	Fig. 4 c
	pri-miR319a-F	GAGAGCTTCCTTGAGTCCATTCACAG	Fig. 4 b
	pri-miR319a-R	CCGCATCATTCATTCATTTAACGAGTC	Fig. 4 b
	pri-miR393a-F	GGGATCGCATTGATCCTAATTAAGGTG	Fig. 4 c
	pri-miR393a-R	TCCAAAGAGATAGCATGATCCAAAACC	Fig. 4 c
	pri-miR394a-F	TGGCATTCTGTCCACCTCCTTCTATAC	Fig. 4 c
	pri-miR394a-R	CCCACCTCCTTCTTTCACACGAAAC	Fig. 4 c
	pri-miR398a-F	TGAAATTTCAAAGGAGTGGCATG	Fig. 4 b
	pri-miR398a-R	GGGAGATTCAAAGGGGTGACC	Fig. 4 b
	pri-miR472-F	TTTTGGTGTTTGCAAGTGTTGAG	Fig. 4 c
	pri-miR472-R	GGGCGGAGTAGGAAAAATCTTAC	Fig. 4 c
	AFB3-F	AGCTCGAGATGCTTTCGATAGCTTTTG	Fig. 6 a
	AFB3-R	TCATTCTGTTCCATCCCATTATTCTCA	Fig. 6 a
	AGL16-F	TCTCGTTCACCAAGAGAATT	Fig. 6 a
	AGL16-R	GTTCGAGGTATCTCTCATGT	Fig. 6 a
	ARF16-F	GATCAATTCGATTCCAGTACCT	Fig. 6 a
	ARF16-R	CAAACCTGATGCATCATGAAC	Fig. 6 a
	ARF17-F	AGCACCTGATCCAAGTCCTTCTATG	Fig. 6 a
	ARF17-R	TGGTGAATAGCTGGGGAGGATTTC	Fig. 6 a
	ARF8-F	ATTGGACTCCTTGCTGCTGCTG	Fig. 6 a
	ARF8-R	GTACCTGCGGACACTCGACTCT	Fig. 6 a
	CCS-F	CCACAGCTGGTGTATCAACG	Fig. 6 b
	CCS-R	CATCGGTCTTGTACACCACG	Fig. 6 b
	CKB3-F	ATGTACAAGGAACGTAGTGG	Fig. 6 a
	CKB3-R	CTAGATGTGGTGGTGGAAGT	Fig. 6 a
	CSD1-F	CTCAAGCACTTGATTCTTTCC	Fig. 6 b
	CSD1-R	AGACATGCAACCGTTAGTGG	Fig. 6 b
	CSD2-F	CTAACAACATGACACACGGAG	Fig. 6 b
	CSD2-R	GAGGTCATCCTTAAGCTCGTG	Fig. 6 b
	CUC1-F	GAAGAGTTGTTGGGTCATGC	Fig. 6 a
	CUC1-R	CGAAATCAATCTGTCCCGATG	Fig. 6 a
	GRF1-F	ACATGGATCCCGAGCCAGGG	Fig. 6 a
	GRF1-R	ATTATCTGATCCACGCATCG	Fig. 6 a
	GRF2-F	GCAGAACAGATGGGAAGAAA	Fig. 6 a
	GRF2-R	GCTGCGGTTGCTTGATGCAC	Fig. 6 a
	HAP2C-F	AACTCCGATAAACCGAGCC	Fig. 6 a
	HAP2C-R	TCTTGGTCGTTCTTGTGATGTC	Fig. 6 a
	MYB33-F	GACATTCACC TGTTATGATT	Fig. 6 a
	MYB33-R	TGGAGACTGA ATGTAAGTAT	Fig. 6 a
	MYB65-F	GATGGTTCCTGATAGCCATACAGTTAC	Fig. 6 a
	MYB65-R	TAGGCATCAACAGAGTCAAGGAGATC	Fig. 6 a
	NAC2-F	GTACAAAGGTTCCAATGTCA	Fig. 6 a
	NAC2-R	GGACTCGTGGACAAGTCTTT	Fig. 6 a
	PHB-F	GCTAACAACCCAGCAGGACT	Fig. 6 a

	Oligonucleotide	Sequence	Figure
qRT-PCR	PHB-R	TCGGAGCCAAGATGGACGAT	Fig. 6 a
	PHV-F	ATCAGCATCCTCAGCGTGAT	Fig. 6 a
	PHV-R	TTCCACTGCAGTTGCGTGAA	Fig. 6 a
	TCP3-F	CATCCAGTTTATAGCCAAAG	Fig. 6 a
	TCP3-R	ATGGCGAGAATCGGATGAAG	Fig. 6 a
	TIR1-F	GCCTCTCTCTATCTGGCCTCTTGAC	Fig. 6 a
	TIR1-R	AGGGCAGCTCTCTGGTCTCGAGTCC	Fig. 6 a
	Tubulin-F	GAGCCTTACAACGCTACTCTGTCTGTC	Fig. 2 b, c, d, e; 4 a, b; 6 a, b
	Tubulin-R	ACACCAGACATAGTAGCAGAAATCAAG	Fig. 2 b, c, d, e; 4 a, b; 6 a, b
Northern analysis	miR156	GTGCTCACTCTCTTCTGTCA	Fig. 2 a
	miR159	TAGAGCTCCCTTCAATCCAAA	Fig. 2 a
	miR164	TCGACGTGCCCTGCTTCTCCA	Fig. 2 a
	miR166	GGGGAATGAAGCCTGGTCCGA	Fig. 2 a
5`RACE PCR	ARF16 outer	TGCGTTTTCTCTATGTTTGCGGT	Fig. 5 d
	ARF16 inner	TTGGTATCATTGCATGGTGTTG	Fig. 5 d
	CSD2- outer	CCAATCACACCACATGCCAATCTC	Fig. 5 d
	CSD2 inner	AACCACAAAGGCTCTTCCAACAA	Fig. 5 d
	TIR1 outer	GCCTGGAAAACCTCATTGTTG	Fig. 5 d
	TIR1 inner	CAGGCATGTCAAATCGAGGAC	Fig. 5 d
	CNA outer	GCCGCCATTGTTGTCTTCTGTGCAA	Fig. 5 d
	ARF17 outer	GGGAGCTAGAACCTGCGTTGCTGTT	Fig. 5 d

Chapter IV: Mini-Review: RACK1 and the microRNA pathway: Is it a déjà-vu all over again?

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Abstract

MicroRNAs (miRNAs) control many aspects of development and adaption in plants and in animals by post-transcriptional control of mRNA stability and translatability. Over the last years numerous proteins have been identified in the miRNA pathway. The versatile scaffold protein RACK1 has been associated with efficient miRNA production and function in plants and metazoans. Here, we briefly summarize the differences of RACK1 function in the plant and animal miRNA pathways and discuss putative mechanisms and functional roles of RACK1 in miRNA biogenesis and action.

MiRNA biogenesis in animals and plants

Although the plant and animal miRNA pathways differ in respect to the processing location, the involvement of auxiliary proteins, and the main mode of action of the mature miRNA, some key steps are strikingly similar (Krol *et al.* 2010, Rogers and Chen 2013). In animals and plants, mature miRNA duplexes are released from longer primary-microRNA (pri-miRNA) transcripts with hairpin-like structures by the activity of RNaseIII-like proteins. Animal pri-miRNAs are first processed in the nucleus into precursor-miRNAs (pre-miRNAs) by DROSHA and an auxiliary protein, PASHA/ DIGEORGE SYNDROME CRITICAL REGION 8 (DGCR8) (Kim 2005, Lee *et al.* 2003). Pre-miRNAs are then transported into the cytoplasm and further processed by DICER and partners (e.g., *TRANS*-ACTIVATION RESPONSE RNA BINDING PROTEIN (TRBP)) to release mature miRNA duplexes (Haase

et al. 2005, Kim 2005). In plants, processing of pri-miRNAs into mature miRNA duplexes is performed exclusively in the nucleus by DICER-LIKE 1 (DCL1) which is assisted by other RNA-binding proteins like SERRATE (SE) or HYPONASTIC LEAVES 1 (HYL1) (Dong *et al.* 2008, Fahlgren *et al.* 2009, Laubinger *et al.* 2010, Lobbes *et al.* 2006, Park *et al.* 2002, Vazquez *et al.* 2004, Yang *et al.* 2006). Animal and plant mature miRNA duplexes are loaded into ARGONAUTE (AGO) effector complexes facilitated by the chaperones HEAT-SHOCK-PROTEIN 90 and 70 (HSP90 and HSP70) (Earley and Poethig 2011, Iki *et al.* 2012, Iki *et al.* 2010, Iwasaki *et al.* 2010). The AGO complex guided by the miRNAs binds and regulates target mRNAs (Krol *et al.* 2010, Rogers and Chen 2013).

The scaffold protein RACK1

In the beginning of the 1990s RECEPTOR FOR ACTIVATED C KINASE (RACK1) has been discovered as an interactor of an active form of PROTEIN KINASE C (PKC) (Ron *et al.* 1994). RACK1 is present exclusively in eukaryotes and structural analyses of yeast, human and Arabidopsis RACK1 revealed that RACK1 proteins form a seven bladed ß-propeller structure with each blade containing one WD40 repeat (Coyle *et al.* 2009, Ruiz Carrillo *et al.* 2012, Ullah *et al.* 2008). RACK1 proteins from different species share 43–73% identical amino acids, demonstrating the high conservation of RACK1 across kingdoms (Adams *et al.* 2011). RACK1 scaffold proteins have no catalytic activity; instead, they bridge interactions between proteins, they influence the stability and activity of interactors or their cellular localization (Adams *et al.* 2011). A vast diversity of RACK1 binding partners have been reported in yeast, animals and plants ranging from, kinases, phosphatases, channels, receptors, transcription factors to the eukaryotic 40s ribosomal subunit (Adams *et al.* 2011, Kundu *et al.* 2013, McCahill *et al.* 2002, Nilsson *et al.* 2004). Due to its plethora of interaction partners, RACK1 is believed to act as signal integrator, which interconnects distinct signaling pathways to control essential cellular processes such as transcription and translation, cell proliferation and growth as well as cell spreading and cell-cell interactions (Adams *et al.* 2011, McCahill *et al.* 2002, Nilsson *et al.* 2004).

Animal and plant RACK1 affects the miRNA pathway

Three reports described the functions of RACK1 scaffold proteins in the miRNA pathway of *C. elegans*, humans, and Arabidopsis (Jannot *et al.* 2011, Otsuka *et al.* 2011, Speth *et al.* 2013). Although RACK1 is an important factor in the miRNA pathway, the exact functions of RACK1 seem to vary in all organisms (Figure1a, Table 1).

In Arabidopsis, we found RACK1 to be interacting with the miRNA processing component SERRATE, suggesting a role for plant RACK1 in miRNA processing (Speth *et al.* 2013;

Figure 1a). Indeed, molecular analyses of *rack1* mutant plants show that miRNA accumulation is globally decreased like in other well-studied miRNA mutants (Fahlgren *et al.* 2009, Liu *et al.* 2012, Ren *et al.* 2012, Speth *et al.* 2013). In line with this observation, miRNA-mediated repression of target mRNAs is released in *rack1* mutants. In addition, RACK1 is found in complexes with the Arabidopsis AGO protein involved in miRNA action, AGO1, which provides a hint that plant RACK1 may also function during later steps of the plant miRNA pathway (Figure 1a).

Also the processing of metazoan pri-miRNAs is aided by auxiliary protein, among them KH-TYPE SPLICING REGULATORY PROTEIN (KSRP), which has been shown to interact with RACK1 in humans (Figure 1a). KSRP is a single-strand nucleic acid binding protein, which binds to the terminal loop of a subset of animal pre-miRNAs and promotes processing of the bound pre-miRNA by interacting with the nuclear DROSHA and the cytoplasmic DICER complexes (Trabucchi *et al.* 2009). However, the amounts of KSRP dependent mature miRNAs remain unchanged in *rack1* knock-down cells, indicating RACK1 is not involved in processing of KSRP dependent miRNAs. Because artificially delivered mature miRNA are fully functional in *rack1* knock down cell lines, the authors hypothesized RACK1 promotes the transfer and recruitment of miRNAs from processing complexes into AGO complexes (Otsuka *et al.* 2011).

Table 1: Summary of RACK1 functions in the miRNA pathway

	C. elegans	**human**	**Arabidopsis**
Early miRNA biogenesis	-	-	✓
AGO complex maturation	-	✓	?
AGO function	✓	-	?
RACK1 interactions with miRNA components	AGL1	KSRP AGO2	SE AGO1
References	(Jannot *et al.* 2011)	(Otsuka *et al.* 2011)	(Speth *et al.* 2013)

✓: proven function; ?: possible function; - : unknown

In a third publication, the authors identified RACK1 as a direct binding partner of the *C.elegans* AGO, AGL-1 (Jannot *et al.* 2011). RACK1 is important for miRNA-mediated silencing of target mRNAs probably by recruitment of AGO complexes to the translating

mRNA by ribosomal RACK1 (Jannot *et al.* 2011; Figure 1a). In agreement with this hypothesis, AGO occupancy to polysomes is reduced in *rack1* deficient cell lines (Jannot *et al.* 2011).

In summary, animal and plant RACK1 seem to influence the miRNA pathway via several distinct mechanisms, including early steps of the miRNA biogenesis as well as AGO dependent processes. Puzzling out RACK1's molecular mode of action in the miRNA pathway will be an important issue of future research.

Possible mechanisms and functions of RACK1 in the miRNA pathway

Protein-protein interactions play key roles in the progression and the regulation of the plant and animal miRNA pathway. As RACK1 acts as scaffold protein, it could function as a molecular glue to tighten interactions between key miRNA factors or to direct regulatory factors to the miRNA pathway. Post-translational modifications of miRNA factors are key regulatory events in animal and plant miRNA production and function. Phosphorylation of the RNA-binding proteins HYL1 and TRBP, co-factors of DCL1 and DICER, respectively, has been shown to influence miRNA biogenesis in plants and animals (Manavella *et al.* 2012, Paroo *et al.* 2009). In humans, the nuclear localization of DROSHA is dependent on its phosphorylation status and also a phosphorylated peptide of the plant DCL1 protein has been discovered (Engelsberger and Schulze 2012, Tang *et al.* 2010). A variety of post-translational modifications have been reported for animal and plant AGO proteins: hydroxylation and ubiquitination influence AGO stability, polyADP-ribosylalation inhibits miRNA-mediated target regulation upon stress and phosphorylation affects AGO localization and miRNA binding (Derrien *et al.* 2012, Earley *et al.* 2010, Meister 2013). As RACK1 binds a variety of phosphatases, kinases and has a role in ubiquitination (Adams *et al.* 2011, Liu *et al.* 2007), RACK1 scaffold proteins might transiently recruit regulatory factors to ensure efficient miRNA production and function.

A possible biological role of RACK1 in the miRNA pathway comes from the analogy of RACK1's function as part of the 40S ribosomal subunit. There, RACK1 is thought to act as a signal integrator that transfers environmental cues such as stress to the translating ribosome (Nilsson *et al.* 2004). Also the production, the stability and function of mature miRNAs is influenced in a spatial, time, and stimuli dependent manner (Jung *et al.* 2012, Laubinger *et al.* 2010, Michlewski *et al.* 2008, Paroo *et al.* 2009, Reyes and Chua 2007, Viswanathan *et al.* 2008). Several studies revealed that RACK1 expression is dynamically changed upon different exogenous and endogenous signals (Adams *et al.* 2011, Fennell *et al.* 2012, Guo *et al.* 2009, Guo *et al.* 2011, Islas-Flores *et al.* 2012, Otsuka *et al.* 2011). Also post-transcriptional modifications, including phosphorylations, have been reported to influence RACK1`s ability to modulate the function of interacting proteins (Adams *et al.*

2011). Hence, changes in RACK1 levels or post-translational modifications could contribute to shape miRNA expression patterns in response to environmental cues (Figure 1b).

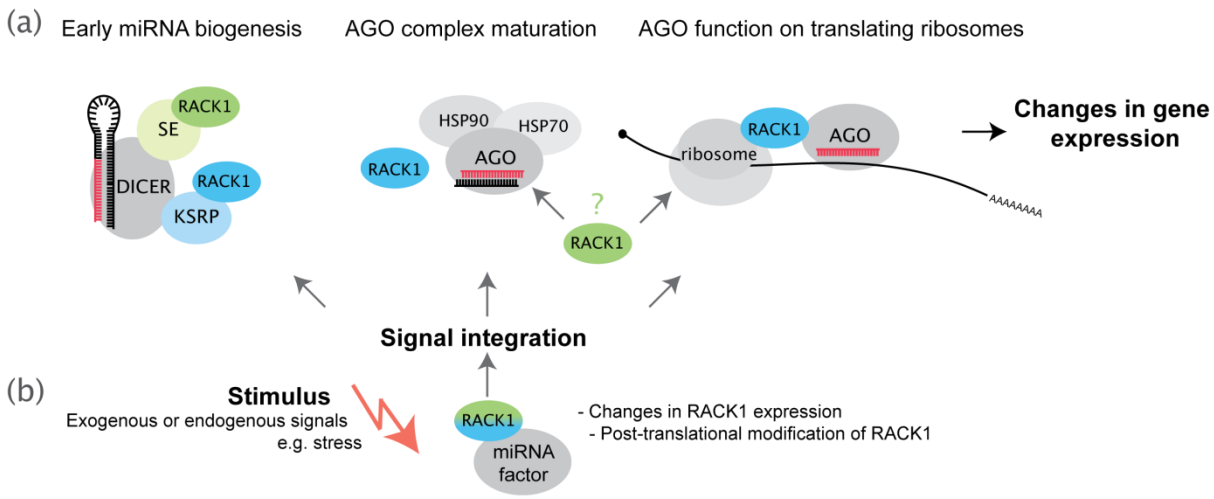

Figure 1: Model for RACK1 function in the plant and metazoan miRNA pathways

(a) RACK1 influences different steps of miRNA biogenesis and action. Green = plant proteins, Blue = Metazoan proteins, Grey = Common factors.
(b) Exogenous or endogenous signals may affect the miRNA pathway by either modulating RACK1 abundance or function. This may lead to changes in miRNA expression and function in response to different stimuli.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contribution

C.S. wrote the manuscript with contributions from S.L.

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Chapter V: RACK1 influences miR156 dependent and independent regulation of *SPL* expression

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Introduction

Arabidopsis produces almost 300 different miRNAs and the miRNA/target mRNA modules have important regulatory functions during plant development and adaption (www.mirbase.org, Mallory and Vaucheret 2006, Voinnet 2009). Among them miR156/157 and their targets, the SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) transcription factors, are a well-studied miRNA/target module with a huge impact in diverse developmental processes (Chen *et al.* 2010). SPLs are plant specific transcription factors. They have been found in all plant linages from monocots and dicots down to mosses and the single cell algae *Clamydomonas* (Guo *et al.* 2008). One characteristic feature of this family is its SQUAMOSA PROMOTER-BINDING PROTEIN (SBP)-box, which is a highly conserved ~79 amino acid long domain and exhibit a novel zinc-finger motive for DNA binding (Yamasaki *et al.* 2004). The first SPB-box protein was found in *Anthirrinum majus* (Snapdragon) and was shown to bind to the promoter region of the meristem identity gene *SQUAMOSA* (Klein *et al.* 1996). The Arabidopsis genome encodes 16 *SPL* genes (Cardon *et al.* 1999). The gene structure of *SPLs* is diverse, but they can be grouped in long and short *SPLs* comprising 6 and 10 members, respectively and the expression of the short *SPL*s is post-transcriptionally regulated by miRNA156 and miR157 (Guo *et al.* 2008, Rhoades *et al.* 2002, Schwab *et al.* 2005). MiR156 and miR157 are highly conserved in various plant species (Zhang *et al.* 2006). Arabidopsis encodes ten *MIR156* and four *MIR157* loci (www.mirbase.org). As the mature miR156 and miR157 have almost the same sequence and over expression leads to the same mutant phenotype, it has been assumed that miR156 and miR157 regulate the same targets (Schwab *et al.* 2005, Shikata *et al.* 2012, Wu and Poethig 2006).

SPL proteins are expressed in different tissues throughout the lifecycle, thereby regulating many different developmental processes (Chen *et al.* 2010). SPLs can be divided in a

miRNA-regulated and a miRNA independent group (Chen *et al.* 2010). SPL6, SPL7 and SPL8 are well studied members of the miRN156/mir157 independent group. SPL8 is important for fertility by influencing mircro- and mega sporogenesis as well as Gibberellin (GA) biosynthesis and signaling in a tissue specific manner (Unte *et al.* 2003, Zhang *et al.* 2007). SPL7 controls transcription of several *MIRNA* genes, like *MIR398*, *MIR408*, *MIR857* and *MIR397*, in order to regulate copper homeostasis (Yamasaki *et al.* 2009). Tobacco and Arabidopsis SPL6 are important for the resistance against tobacco mosaic virus and *Pseudomonas syringae*, respectively by binding to NB-LRRs thereby positively promoting immune response (Padmanabhan *et al.* 2013).

The miR156/miR157 dependent clades SPL3/4/5, SPL9/15 and SPL2/10/12 regulate all steps of phase transition in a miRNA dependent manner, including vegetative phase change, the transition from juvenile to adult growth phase as well as the transition from the vegetative to the reproductive growth followed by flower and fruit development (Cardon *et al.* 1997, Klein *et al.* 1996, Manning *et al.* 2006, Shikata *et al.* 2009, Wang *et al.* 2009, Wu and Poethig 2006, Yamaguchi *et al.* 2009). During the phase transitions, SPLs have overlapping but also distinct functions (Chen *et al.* 2010). For instance, during vegetative phase change SPL3 mainly mediates changes in epidermal patterning, whereas SPL9 and SPL10 additionally influence the laminar shape of the leaf (Wu *et al.* 2009).

It is well known that *MIR156* regulates the developmental timing of the plant by gradually decreasing its expression with proceeding age and this in turn leads to an increase in SPLs (Wang *et al.* 2009, Wu and Poethig 2006). For a long time it was not clear, which signal induces the decrease of miR156. But recently, it has been shown that increasing sucrose levels, which accumulate during plant aging, are responsible for the decrease of at least *MIR156A* and *MIR156C* (Yu *et al.* 2013).

MiRNA mutants have reduced levels of miR156, which is due to impaired pri-miRNA processing, miRNA destabilization or defects in miRNA activity. Therefore miRNA mutants accumulate high amounts of SPLs. This leads to misregulation of SPL-mediated processes causing typical miRNA mutant phenotypes such as acceleration of phase transition (Chen *et al.* 2010, Smith *et al.* 2009). Recent studies in metazoans and plants discovered a eukaryotic scaffold protein, RECEPTOR FOR ACTIVATED C KINASE (RACK1), which influences the miRNA pathway at several steps, including miRNA biogenesis and miRNA action (Jannot *et al.* 2011, Otsuka *et al.* 2011, Speth *et al.* 2013). Interestingly, we discovered that the typical vegetative phase change phenotype of miRNA mutants is delayed in the newly identified miRNA mutant *rack1*, even though *rack1* mutants accumulate low levels of mature miRNAs (Speth *et al.* 2013). Moreover, the central players of vegetative phase change, the SPLs, are unexpectedly misregulated in *rack1* mutants, suggesting that RACK1 scaffold proteins influence SPL-mediated developmental changes by a miRNA independent mechanism.

Results

RACK1, a newly identified miRNA component positively influences miRNA abundance via several distinct mechanisms, thereby regulating miRNA-mediated gene expression (Speth *et al.* 2013). In order to gain insights into the function of RACK1 in the miRNA pathway, we phenotyped *rack1* mutants for some characteristic miRNA regulated traits like ABA hypersensitivity, leaf serrations, phyllotaxy defects and phase change. For the latter, we observed some unexpected differences in *rack1* mutants.

SPL mediated developmental processes are positively affected by RACK1

Phase change, the transition from the juvenile to adult growth phase, is regulated by the miR156/SPL module (Chen *et al.* 2010). In young seedlings *MIR156* expression is high and therefore *SPL* levels are low. But, proceeding age of the plant leads to a decrease in miR156 expression, which results in *SPL* accumulation. These changes in *SPL* levels are associated with altered leave morphology and the appearance of abaxial trichomes. Therefore, juvenile leaves lack abaxial tichomes and shaped rounded, whereas adult leaves have abaxial trichomes and a lanceolate laminar shape. MiRNA mutants, like *ago1-27*, accumulate high levels of SPL from the beginning on due to low miR156 levels, leading to an adult leaf phenotype from leaf two or three onwards compared to wild-type, which starts its adult stage with leaf number five to six (Figure 1a). Unexpectedly, the appearance of abaxial trichomes is delayed in *rack1a* single and *rack1ab* double by 1.73 and 2.77 plastochrons, respectively (Figure 1a), even though *rack1* mutants accumulate low levels of mature miR156 like *ago1-27* mutants (Figure 2b).

SPLs have been shown to negatively regulate anthocyanin production in the stem in an agropetal manner by directly affecting transcription of anthocyanin/flavonoid biosynthesis genes (Gou *et al.* 2011). Transgenic lines over expressing *MIR156* have low levels of SPL transcription factors. Those lines accumulate high levels of anthocyanins at the stem near the inflorescences, which results in a purple stain at those regions (Gou *et al.* 2011). Interestingly, *rack1a* single mutants also exhibit a purple stain at the stem of the inflorescences (Figure 1b).

These results demonstrate that although miR156 is down regulated, *rack1* mutants exhibit a delayed phase change and enhanced anthocyanin accumulation, which suggest an additional role for RACK1 in the miR156/SPL regulatory network besides RACK1’s function in the miRNA pathway.

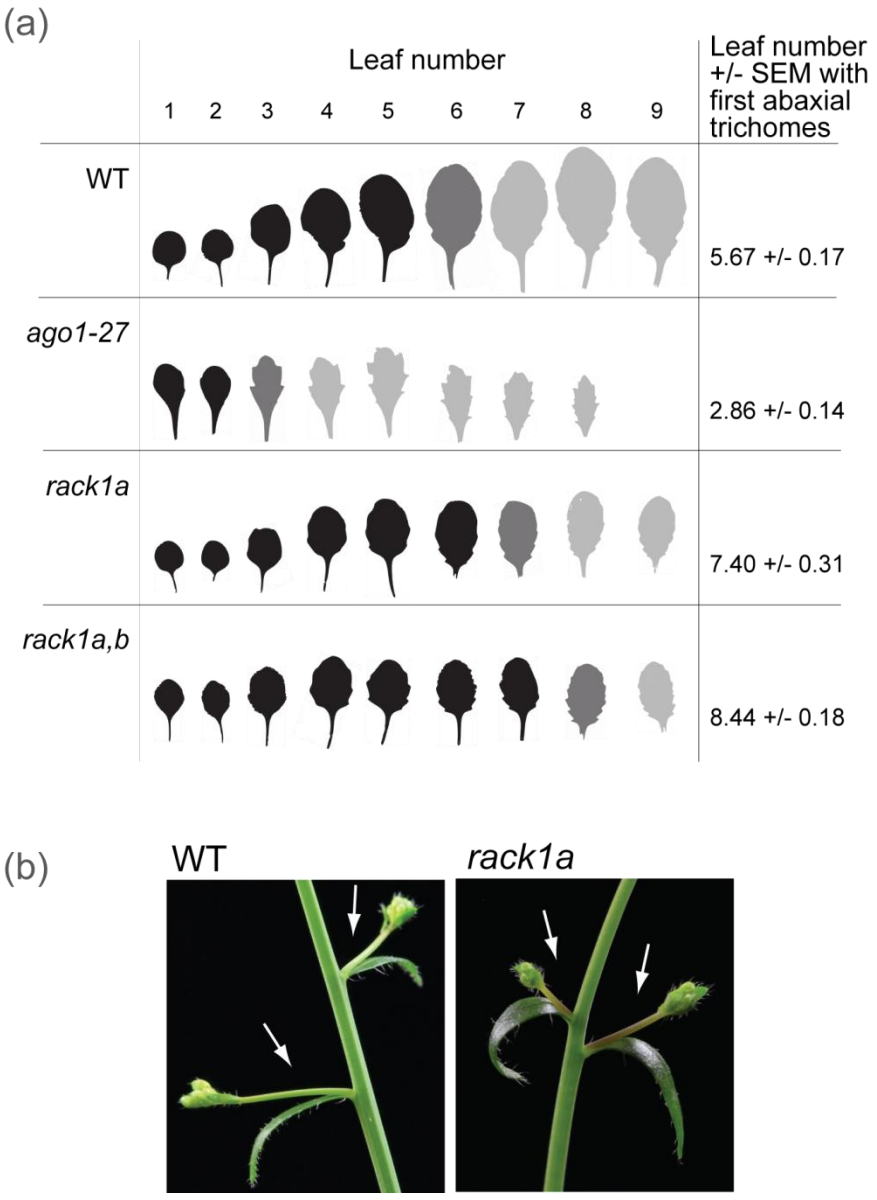

Figure 1: Phenotypic analyses of miR156-regulated processes in *rack1* mutants

(a) Phase change analysis of wild-type (WT), *ago1-27*, *rack1a* and *rack1ab* mutants as indicated in the right column. In the middle column, leaf shape of wild-type, *ago1-27* and *rack1* mutants are documented in a developmental ascending order. Juvenile leaves and adult leaves are indicated in black and light grey, respectively. The average first leaf with abaxial trichomes is colored in medium-grey. The right column shows the average number +/- SEM (n=10) of the first leaf exhibiting abaxial trichomes.
(b) Inflorescences of wild-type (WT) and *rack1a* mutants. Arrow heads marking the identical regions in wild-type and *rack1a* mutants where *rack1a* mutants have a purple stain.

RACK1 positively influences SPL accumulation in a miRNA independent manner

Next, we sought to determine the reason for this unexpected function of RACK1 in miR156-regulated phase change and anthocyanin accumulation. Therefore we quantified steady-state levels of two miR156-targeted mRNAs, *SPL3* and *SPL9*, which are central players of phase change and anthocyanin accumulation (Chen *et al.* 2010, Gou *et al.* 2011). Surprisingly, *SPL3* transcripts accumulate to lower extend in *rack1a* and *rack1a,b*, mutants as well as *SPL3* and *SPL9* in *rack1a,b,c* mutants, whereas *ago1-27* effector mutants accumulate higher levels of *SPL3* transcripts (Figure 1b,c). SPL3 protein was undetectable in *rack1a* single or *rack1ab*, *rack1ac* double mutants which may be the result of reduced *SPL3* transcript levels (Figure 1c,d). In *ago1-27* mutants, SPL3 protein accumulates to higher extend, correlating with the increase in *SPL3* transcript in this mutant background (Figure 1d). The reduced accumulation of miRNA targets in *rack1* mutants might be the reason for the delay of vegetative phase change and the accumulation of purple stain at the stem in *rack1* mutants.

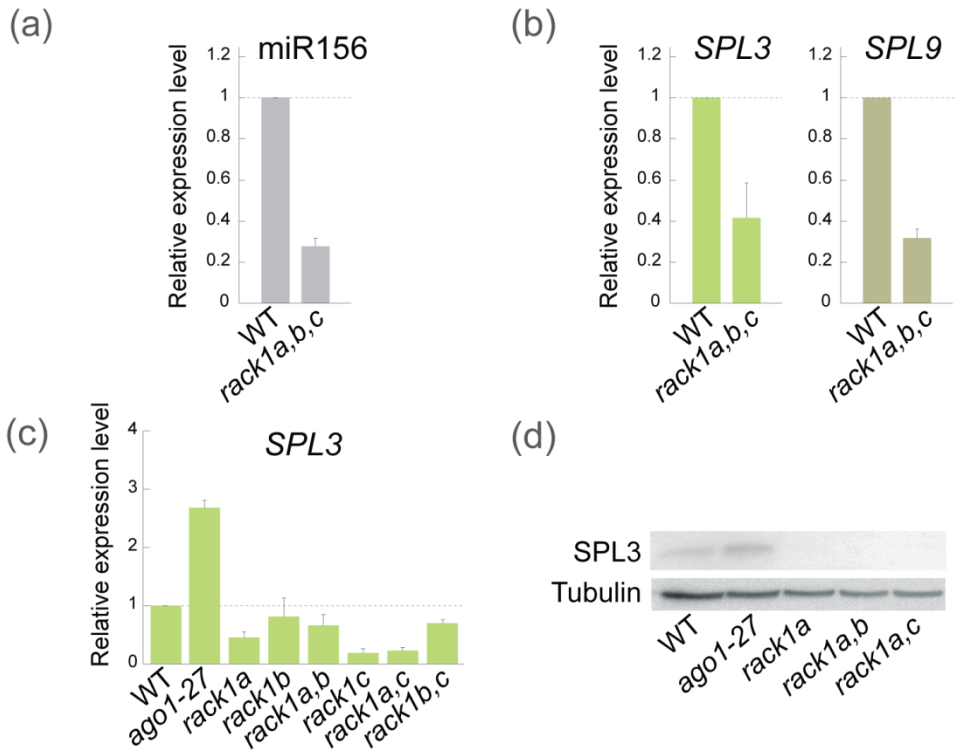

Figure 2: Expression analyses of the miR156/SPL module in *rack1* mutants

(a) - (c) Quantitative real-time PCR of miR156, SPL3 and SPL9 in wild-type (WT), *ago1-27* and various *rack1* mutants. Error bars indicate the range of two independent biological replicates. **(d)** Immunoblot analysis of SPL3 and TUBULIN levels in wild-type (WT), *ago1-27* and various *rack1* mutants.
Plants grown on plates and on soil were used for experiments (a, b) and (c, d), respectively.

Previous studies showed that all Arabidopsis *RACK1 (A, B, C)* genes are ubiquitously expressed in all organs and during every developmental stage, whereas *RACK1A* is the most and *RACK1C* the least abundant (Guo and Chen 2008, Guo *et al.* 2009). Furthermore, they act redundantly in a dosage dependent manner. Therefore, the intensity of the Arabidopsis *rack1* mutant phenotype correlates with the amount of expressed *RACK1* (Guo and Chen 2008, Guo *et al.* 2009). Interestingly, quantification of *SPL3* in different *rack1* mutant backgrounds suggested a RACK1 dose dependent reduction of the *SPL3* transcript. Only *rack1a*, but not *rack1b* and *rack1c* single mutants, have reduced *SPL3* levels and this is enhanced in the *rack1ab* and *rack1ac* but not in the *rack1bc* double mutant background (Figure 1c). This is in line with the observation that vegetative phase change is even more delayed in the *rack1a,b* double mutants when compared to the *rack1a* single mutants, which supports the idea of a RACK1 dosage dependent effect also in the regulation of phase change.

In summary, RACK1 may affect SPL-mediated processes in Arabidopsis by a miRNA dependent and miRNA independent mechanism.

Discussion

Molecular analyses revealed that loss of *RACK1* leads to a reduced expression of the transcription factors *SPL3* and *SPL9*, which are both regulated by miR156. But the reduction seems to be miR156-independent, as mature miR156 also accumulated to lower extend in *rack1* mutants. The molecular phenotype observed in *rack1* mutants is reflected in the developmental phenotype leading to misregulation of SPL-mediated processes like vegetative phase change and anthocyanin production in the stem.

How could RACK1 scaffold proteins influence SPL transcript levels on the molecular level? Some miRNA target mRNAs accumulate to higher extend in *rack1* mutants, therefore low levels of miRNA targets are not a general effect caused by mutations in *RACK1* (Speth *et al.* 2013). This suggests a more specific function for RACK1 in *SPL* regulation. Reduced steady-state levels of SPL transcription factors in *rack1* mutants may be caused by impaired transcriptional regulation or reduced transcript stability. In metazoans it has been shown that RACK1 influences transcriptional changes in different ways. It mediates chromatin remodeling in the promoter region of the *BRAIN-DERIVED NEUROTROPHIC FACTOR* (*BDNF*) gene, thereby inducing transcription of a specific exon of *BDNF* (He *et al.* 2010). Many reports demonstrate that RACK1 scaffold proteins associate with various transcription factors, thereby indirectly affecting transcription. Binding of RACK1 to transcription factors modulates their ability to suppress or activate transcription by several mechanisms including DNA binding, protein degradation or changes in subcellular localization (Liu *et al.* 2007, Neasta *et al.* 2012, Okano *et al.* 2006, Robles *et al.* 2010, Zhang *et al.* 2012). Recently, it

has been proposed that RACK1 may function in regulating transcription and/or transcript stability of some *MIRNA* transcripts (Speth *et al.* 2013). As *MIRNA* genes are transcribed by the DNA-dependent RNA-polymerase II machinery like protein coding genes and both transcripts share some down-stream RNA-binding and processing components (Rogers and Chen 2013), one could imagine that RACK1 influences transcription and/or transcript stability of *SPL* transcripts similar to *MIRNA* genes. If RACK1 is associated with *SPL* promoter regions or has the potential to bind to *SPL* transcripts and influence there stability, could be an interesting subject for future studies. This may provide some insights into the mechanism of RACK1 dependent regulation of *SPL* expression.

Arabidopsis encodes 16 different *SPL* transcription factors which are grouped in 7 different clades based on their SBP-domain amino acid sequences (Guo *et al.* 2008). It has been shown that the members of the different clades have overlapping and distinct functions in plant development (Chen *et al.* 2010). As we have only studied *SPL3* and *SPL9* gene expression, it might be interesting for future studies to analyze to what extent other SPL transcription factors are affected by RACK1 and whether this has an impact on other SPL-mediated processes. This can be addressed by quantifying other *SPL* transcripts in *rack1* mutant background and by screening *rack1* mutants for other mutant phenotypes caused by misregulation of *SPL* expression like changes in flowering time and leaf development, impaired GA or copper responses (Chen *et al.* 2010, Wang *et al.* 2009, Wu and Poethig 2006, Yamaguchi *et al.* 2009, Yamasaki *et al.* 2009, Yu *et al.* 2012, Zhang *et al.* 2007). It has been shown, that *rack1* mutants indeed flower late and act hypersensitive towards GA (Chen *et al.* 2006). Whether these mutant phenotypes are caused by misexpression of *SPLs* or other RACK1-mediated processes remains elusive.

RACK1 positively influences miRNA abundance via several mechanisms (Speth *et al.* 2013) and in addition, positively affects *SPL* accumulation. Hence, RACK1 modulates the miR156/SPL regulatory network by two independent steps: RACK1 influences the abundance of mature miR156 and thereby indirectly *SPL* accumulation. Additionally, RACK1 affects *SPL* accumulation via a miRNA-independent mechanism, which could be transcriptional regulation or transcript stability (Figure 3).

In order to distinguish between both processes, the miRNA-dependent and independent, a system is needed, which uncouples RACK1's effect on the miR156 biogenesis and RACK1's effect on the *SPL* transcription or transcript stabilization. Expressing different *SPLs* under the control of a constitutive promoter (e. g. 35s) may compensate RACK1's influence on *SPL* transcription and transcript stabilization. This would allow the analyses of miR156-dependent effects on *SPL* expression. Using a *mir156* knock-out line would be the perfect tool to analyze RACK1's impact on miR156-independent regulation of *SPL*. But there are 10 different *MIR156* loci and 4 *MIR157* loci (mirbase.org), which probably act in a redundant

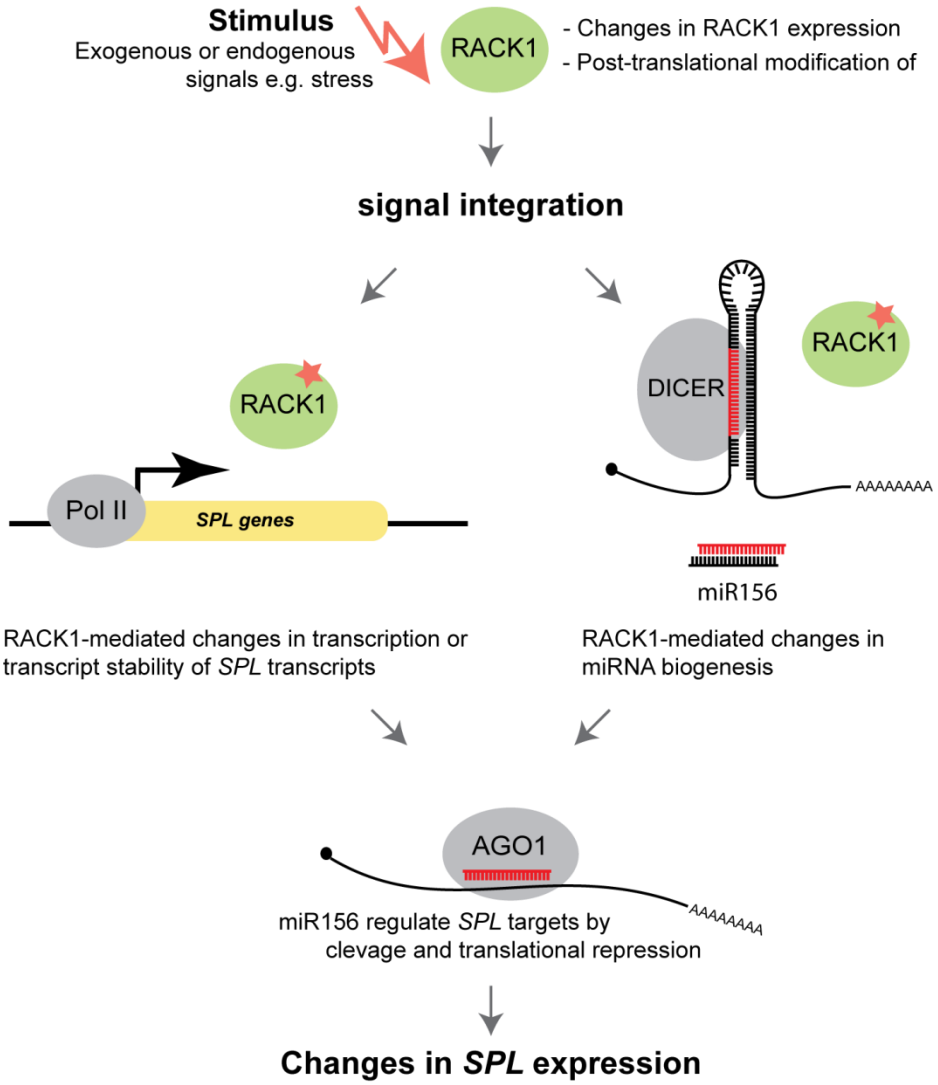

Figure 3: Putative mechanism by which RACK1 influences the miR156/SPL regulatory module

Exogenous or endogenous signals may affect RACK1-mediated changes in SPL accumulation and/or changes in miR156 biogenesis and /or action. Finally, both regulatory mechanisms affect SPL expression.

manner (Schwab *et al.* 2005, Shikata *et al.* 2009, Wu and Poethig 2006, Yu *et al.* 2013). Nevertheless, miRNA activity can be reduced by expressing miRNA target mimicries, which sequester mature miRNAs. MiRNA target mimicries are RNA transcript with have a miRNA binding site with a bulge in the middle, so that miRNAs can associate to the miRNA target mimicry but not cleave anymore (Franco-Zorrilla *et al.* 2007). Due to the sequestering, the miRNA it is not able to regulate its endogenous target mRNA. Such a target mimicry for miR156 could reduce the impact of miR156 regulation on *SPL* expression, allowing the analyses of the miRNA-independent influences of RACK1 on *SPL* expression. Analyzing

miR156 target mimicries or *SPL* over expression in wild-type and *rack1* mutant backgrounds may help to uncover RACK1's impact on the miR156/SPL regulatory network.

RACK1 scaffold proteins are thought to act as signaling integrators in different pathways, promoting adaptive processes in response to exogenous or endogenous signals (Adams *et al.* 2011, McCahill *et al.* 2002, Nilsson *et al.* 2004). We proposed a model, in which RACK1 acts as a signal integrator, thereby influencing miRNA-mediated gene expression amongst others on transcriptional level (Speth and Laubinger 2014). In analogy to this, RACK1-mediated SPL accumulation may be as well dynamically regulated in this way (Figure 3). One challenge for future studies is to decipher how RACK1, as a signaling hub, integrates different exogenous and endogenous signals to influence the miR156/SPL regulatory module.

Material and Methods

Plant material and growth conditions

All mutants plants used in this study were described before in other publications (Chen *et al.* 2006, Grigg *et al.* 2005, Guo and Chen 2008, Morel *et al.* 2002). The following *rack1* alleles were used in this study for all experiments: *rack1a-1*, *rack1b-2*, *rack1c-1*, *rack1a-1 rack1b-2*, *rack1a-1 rack1c-1*, *rack1b-2 rack1c-1*, *rack1a-1 racl1b-2 rack1c-1*.

Plants were grown on soil for 21 days or on ½ MS media supplemented with 2 % (w/v) sucrose for 10 days both under long day conditions (16 h light, 22 °C).

Total RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted using TRIZOL (Life technologies) reagent. Up to 100 mg of ground plant material was resuspended in 1 ml TRIZOL reagent. Plant debris was collected by centrifugation. Supernatant was supplemented with 1/5 Vol of chloroform. Subsequently, the sample was mixed by vigorous vortexing and organic and aqueous phases were separated by centrifugation step (16.000g, 5 min, and 4°C). The upper aqueous phase containing total RNA was transferred to a new tube and washed several times with 500 µl chloroform until the interphase was clear. The upper phase was supplemented with 1 Vol of isopropanol and total RNA was precipitated during a 1 h incubation step at -80 °C and subsequently collected by centrifugation (16.000g, 30 min, and 4°C). After additional washing with 80 % (v/v) ethanol sedimented RNA was resuspended in 20 – 50 µl nuclease-free water. The integrity of 500 ng RNA was analyzed on a 1 % (w/v) agarose gel.

Between 250 ng and 2 µg total RNA were used DNase treatment in a total volume of 10 µl. The whole DNAse treated RNA was reverse transcribed with oligo-dT and a miRNA specific

stem-loop oligonucleotide using the components of the First Aid cDNA synthesis Kit (Thermo Fisher) according to the method described by (Varkonyi-Gasic *et al.* 2007).

Quantitative real-time PCR (qPCR) was performed in the Bio-Rad CFX384 system using SYBR green containing 2xPCR Master-Mix (Thermo Fisher Scientific). All experiments were performed twice with two independent biological replicates. Amplification efficiency was calculated using a standard curve of amplification and *TUBULIN* was used for normalization. Relative expression levels were calculated using the ΔΔct-method.

All oligonucleotides are listed in Supplemental Table 1.

Immunoblot analysis

Total proteins were extracted by resuspending ground plant material in protein extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 % (w/v) glycerol, 1 mM EDTA, 1 mM DTT, Complete protease inhibitor (Roche)). Cell debris was collected by centrifugation (16.000g; 30 min, 4 °C) and the supernatant was used for further analyses.

Protein concentration was measured using Bio-Rad protein assay (Bio-Rad) according to the manufactures protocol. 20 µg total proteins for each sample was separated on a 15 % SDS-PAGE and transferred to a nitrocellulose membrane (2h, 200mA, RT) using tank blot system. Nitrocellulose membrane was blocked for 1h using 1x Roti-Block (Roth) followed by 1.5 h incubation with the primary antibodies in 5 % (w/v) milk powered resuspended in 1x PBS (xx) detecting TUBULIN (Sigma) or SPL3 (xx). After three times washing with 1xPBS, 0.1 % (v/v) Tween-20 membranes were incubated for 1 h with corresponding secondary antibodies coupled to horse-reddish-peroxidase. Signals were detected using luminol based substrate (GE Healthcare) and a CCD-camera.

Phase change analyses

Single plants were grown under long day conditions on soil (16h light, 22 °C). Leaves were numbered in a developmental ascending order. Before plants start bolting abaxial trichome appearance was documented by counting the leaf with the first abaxial trichomes. For each genotype 10 plants were analyzed. In order to get an image file of the leaf shape, single leaves were scanned and further processed using an image processing application (Adobe Photoshop). The color information was replaced by a single color mode to obtain only the leaf shape. Wounded regions which have no impact on the overall shape were repaired by coloring.

Author Contribution

C.S. and S.L. designed the research; C.S. performed research; C.S. wrote the manuscript with contributions from S.L.

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Supporting Information

Table S1: List of oligonucleotides.

	Oligonucleotide	Sequence	Figure
cDNA synthesis	miR156-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC	Fig. 2 a
qRT-PCR	miR156-F	GCGGCGGTGACAGAAGAGAGT	Fig. 2 a
	universal miRNA-R	GTGCAGGGTCCGAGGT	Fig. 2 a
	SPL3-F	ACGCTTAGCTGGACACAACGAGAGAAG	Fig. 2 b, c
	SPL3-R	TGGAGAAACAGACAGAGACACAGAGGA	Fig. 2 b, c
	SPL9-F	GGAACCAAGAGATAGGATGG	Fig. 2 b
	SPL9-R	TTGTCATGTGGTTGATGTGG	Fig. 2 b

Appendix

Curriculum vitae

Personal information	Surname, name	Speth, Corinna
	Date of birth	16 July 1983
	Place of birth	Bietigheim-Bissingen, Germany
Education	2010-03 – today	ZMBP Pflanzenphysiologie,
	PhD student	Eberhard-KarlsUniversität Tübingen
	2009-05 – 2009-08	ZMBP Pflanzenphysiologie,
	student assistant	Eberhard-KarlsUniversität Tübingen
	2008-05 – 2009-04	ZMBP Pflanzenphysiologie,
	Diploma thesis	Eberhard-KarlsUniversität Tübingen
	2003-10 – 2009-09	Eberhard-Karls-Universität Tübingen
	Diploma, Biology	
	2003-07 – 1995-08	Herzog-Christoph-Gymnasium Beilstein
	Abitur	

List of Publication

Schwab, R., Speth, C., Laubinger, S., and Voinnet, O. (2013) Enhanced microRNA accumulation through stemloop-adjacent introns ***EMBO Reports*** 14, 615-621

Speth, C., Willing, E.M., Rausch, S., Schneeberger, K. and Laubinger, S. (2013) RACK1 scaffold proteins influence miRNA abundance in Arabidopsis ***The Plant Journal : for cell and molecular biology***, 76, 433-445

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Submitted Manuscripts

Speth, C., Laubinger, S. (2013) Rapid and parallel quantification of small and large RNA species ***submitted to Methods in Molecular Biology***

Speth, C., Toledo, L., Laubinger, S. (2013) Immunoprecipitation-based analysis of protein-protein interactions ***submitted to Methods in Molecular Biology***

Oral Presentations

Speth, C., Laubinger, S. (2011) A core component of the ribosome functions in miRNA mediated gene regulation in *Arabidopsis thaliana* ***Keystone Meeting: RNA Silencing****, Monterey*

Speth, C., Laubinger, S. (2012) RACK1 – a new component of the miRNA pathway ***PhD Summer Academy****, Freudenstadt*

Speth, C., Willing, E.M., Rausch, S., Schneeberger, K. and Laubinger, S. (2014) RACK1 scaffold proteins influence miRNA abundance in Arabidopsis ***Keystone Meeting: RNA Silencing****, Seattle*

Speth, C., Willing, E.M., Rausch, S., Schneeberger, K. and Laubinger, S. (2014) RACK1 scaffold proteins influence miRNA abundance in Arabidopsis ***27. Tagung Molekularbiologie der Pflanzen****, Dabringhausen*

Poster Presentations

Speth, C., Laubinger, S. (2010) The function of SERRATE and the nuclear CAP-BINDING COMPLEX in RNA processing ***TNAM****, Salzburg*

Speth, C., Laubinger, S. (2011) A core component of the ribosome functions in miRNA mediated gene regulation in *Arabidopsis thaliana* ***Keystone Meeting: RNA Silencing****, Monterey*

Speth, C., Laubinger, S. (2011) RACK1 - a new component of the miRNA pathway ***Botanikertagung****, Berlin*

Speth, C., Willing, E.M., Schneeberger, K. and Laubinger, S. (2012) RACK1 a novel component of the miRNA pathway in *Arabidopsis thaliana* ***Congress on Plant Molecular Biology****, Jeju*

Speth, C., Willing, E.M., Rausch, S., Schneeberger, K. and Laubinger, S. (2013) RACK1 scaffold proteins influence miRNA abundance in Arabidopsis ***Botanikertagung****, Tübingen*

Speth, C., Willing, E.M., Rausch, S., Schneeberger, K. and Laubinger, S. (2014) RACK1 scaffold proteins influence miRNA abundance in Arabidopsis ***Keystone Meeting: RNA Silencing****, Seattle*

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