Article type : Original Article

Accumulation of the coumarin scopolin under abiotic stress conditions is mediated by the *Arabidopsis thaliana* THO/TREX complex

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Running title: Döll et al. Scopolin accumulation mediated by THO1

Key words: scopolin, *THO1/HPR1/EMU* (*At5g09860*), *RDR6* (*AT3G49500*), RNA silencing, abiotic stress, cold stress, osmotic stress, RNA sequencing, *Arabidopsis thaliana*

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Summary

Secondary metabolites are involved in plant stress response. Among these are scopolin and its active form scopoletin, which are coumarin-derivatives associated with reactive oxygen species scavenging and pathogen defence. Here we show that scopolin accumulation can be induced in the root by osmotic stress and in the leaf by low temperature stress in *Arabidopsis thaliana*. A genetic screen for altered scopolin levels in *A. thaliana* revealed a mutant compromised in scopolin accumulation in response to stress; the lesion was present in a homologue of *THO1* coding for a subunit of the THO/TREX complex. The THO/TREX complex contributes to RNA silencing, supposedly by trafficking precursors of small RNAs. Mutants defective in *THO, AGO1, SDS3* and *RDR6* were impaired with respect to scopolin accumulation in response to stress, suggesting a mechanism based on RNA silencing like the transacting small interfering RNA pathway which requires THO/TREX function.

Introduction

The population of low molecular mass compounds referred to as secondary metabolites exceeds 100,000 in plant tissues. Most of these are considered as non-essential for basic metabolism, while many appear to have evolved as components of the plant's response to stress (Dixon, 2001). The phenylpropanoids comprise a major group of secondary metabolites involved in the plant's interaction with its environment. Among these are the coumarins, which feature in the defence response against pathogen attack and abiotic stress. Scopolin is produced along with its related aglycon scopoletin (the active form) in leaves as part of the host's rapid response to both pathogen attack (Sequeira, 1967; Spring et al., 1991; Elmodafar et al., 1995; Garcia et al., 1999), and abiotic stress (Koeppe et al., 1969). Its role in leaves is thought to combine antibiosis (resulting from the toxicity of the

phenolic moiety) with reactive oxygen species (ROS) scavenging. In roots, its function is much less clear although, unlike its induced production in leaves, its accumulation in roots appears to be constitutive in *A. thaliana* (Rohde et al., 2004; Kai et al., 2006). It may also indirectly influence certain hormone and enzyme functions (Sirois and Miller, 1972). Scopoletin and some of its derivatives also participate in the uptake of iron from calcareous soils (Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014).

The regulation of the biosynthesis pathway leading to scopolin and scopoletin is largely unknown. It is likely, however, that this branch of phenylpropanoid metabolism is as tightly controlled as the better understood parts in which appropriate compounds are produced only when and where required. Control of phenylpropanoid formation is largely exerted by transcription factors (TFs) which interact with the promoters of relevant synthesis genes (Quattrocchio et al., 2006) and can regulate pathway branches either positively or negatively. It was shown before that the accumulation of scopolin is controlled partially independent of *PAL,* the gene coding for the initial key enzyme of the pathway, phenylalanine ammonia-lyase. Not every activator of *PAL* necessarily enhances the level of scopolin (Sharan et al., 1998). The ectopic expression of the glycosyltransferase that is responsible for the final step in the conversion of scopoletin to scopolin has been shown to increase the levels of both compounds (Gachon et al., 2004; Matros and Mock, 2004). Antagonistic roles of the transcription factors MYB12 and MYB4 in scopolin production upon different types of stress have been described (Schenke et al., 2011). An indirect effect on scopolin accumulation was proposed for the transcription factors BRASSINOSTEROID ENHANCED EXPRESSION1 (BEE1) and G2-LIKE FLAVONOID REGULATOR (GFR), while suppressing transcription factors for anthocyanin biosynthesis (Petridis et al., 2016).

In order to identify hitherto unknown genes that modify scopolin accumulation we developed robust systems to elicit and detect scopolin production in the model plant *A. thaliana.* This allowed us to identify an RNA processing complex, named by analogy to its yeast homologue the TRanscription EXport (THO/TREX) complex (Piruat and Aguilera, 1998; Chavez et al., 2000; Sträßer et al., 2002), involved in scopolin accumulation. Our study indicates that mutation of *THO1* and other genes encoding components of the *A. thaliana* THO/TREX complex affect scopolin production upon exposure to abiotic stress. As mutations in *RNA-DEPENDENT RNA POLYMERASE 6* (*RDR6*), a well described key player in the RNA silencing (Dalmay et al., 2000; Mourrain et al., 2000), and mutations in other components of RNA silencing had similar effects on the levels of scopolin and co-affected metabolites, the involvement of RNA silencing pathway in the stress-related induction of scopolin accumulation is likely.

Results

Scopolin in plant roots can be detected by fluorescence microscopy.

A non-invasive method for the detection of scopolin was developed based on the strong fluorescence it displays in roots. Increasing the sucrose content of the growth medium increased the strength of root fluorescence, implying a rise in root scopolin content, which was confirmed by HPLC with fluorescence detection of the soluble root compounds. These analyses also showed that the fluorescence of scopolin exceeded the fluorescence of all other soluble compounds in roots by far. Two mutants defective for enzymes required for scopolin synthesis, caffeoyl-CoA-*o*-methyltransferase1 (mutant *ccoaomt1*) and feruloyl-CoA-6-hydroxylase1 (mutant *f6'h1*) (Kai et al., 2008), were checked for root fluorescence. The roots of the *ccoaomt1* mutant showed approximately half the fluorescence signal and the

scopolin content (as determined by HPLC) as the wild type, while in the *f6'h1* mutant, root fluorescence was very weak, and scopolin or scopoletin were virtually not detectable (Fig. S1). Inspection of micrographs of wild type and mutant roots confirmed that the fluorescent signal generated by scopolin was much stronger than the fluorescence of cell walls. Thus, scopolin and scopoletin were responsible for most of the roots' fluorescence. Scopolin fluorescence was mainly located in the endodermis and was occasionally visible in the cortex and pericycle, but it never occurred in the vascular tissue, rhizodermis or root hair cells.

THO1 is required for scopolin accumulation in roots induced by sucrose

A screen of a set of 8,000 activation-tagged *A. thaliana* lines (Weigel et al., 2000) for enhanced root fluorescence was performed, which eventually led to a focused analysis of a number of genes between loci At5g09710 and At5g09910. Among related T-DNA insertion lines analysed, substantial scopolin reduction was seen solely in line GK_052_D02 generated in accession Columbia (Col-0). This line, in which *At5g09860* is disrupted, produced significantly less scopolin in its roots compared to wild type when challenged by a high (3% w/v) sucrose regime and also under greenhouse conditions. The scopolin content in roots of the sucrose-induced GK_052_D02 plants was comparable to that of wild type under a low sucrose (0.5%) regime (Fig. 1A). The low-scopolin phenotype under high sucrose was confirmed for a second T-DNA insertion line with disrupted *At5g09860*, FLAG_191_B10, in Wassilewsija (Ws) background (Fig 1B). Gene *At5g09860* is annotated as *THO SUBCOMPLEX 1* (*THO1;* alias *HPR1*, *ATHPR1*, *ATTHO1* and *EMU;* Furumizu et al., 2010; Jauvion et al., 2010; Yelina et al., 2010 Pan et al., 2012; Xu et al. 2015; Tao et al. 2016). It shares homology with both yeast and animal *THO1* genes encoding components of the THO/TREX complex (Reed

and Cheng, 2005; Piruat and Aguilera, 1998; Furumizu et al., 2010; Jauvion et al., 2010; Yelina et al., 2010). Thus, line GK_052_D02 is referred to hereafter as *tho1* (Col-0) and line FLAG_191_B10 as *tho1* (Ws). In *A. thaliana*, *THO1/HPR1/EMU* gene function is linked to RNA processing and RNA silencing (Furumizu et al., 2010; Jauvion et al., 2010; Yelina et al., 2010). An involvement in stress defence and ethylene response has been reported for THO1 by Pan et al. (2012), Xu et al. (2015) and Tao et al. (2016). A small proportion of *A. thaliana tho1* plants display an altered pattern of differentiation (Furumizu et al., 2010). Considering that scopolin distribution was uneven in root tissues/cell types (Fig. S1), disturbed root tissues, rather than lack of functional THO1, might have been an alternative explanation for reduced scopolin content in *tho1* (Col-0) roots. There was, however, no evidence for abnormal development: *tho1* (Col-0) root zonation was indistinguishable from that of wild type, and the appearance of *tho1* (Col-0) rhizodermis, cortex, endodermis, pericycle and vascular tissue was perfectly normal. A comparison between wild type and *tho1* (Col-0) mutant roots showed that scopolin was present in precisely the same tissues – the only observable difference was quantitative (Fig. 1C). Under high sucrose regime, only one difference was noted: wild type primary roots became foreshortened and the number of secondary roots increased, while the *tho1* (Col-0) mutant formed longer, less branched roots (Fig. S2).

Scopolin accumulation in cold-induced leaves is reduced in tho1 mutants

A. thaliana leaves accumulate only trace amounts of scopolin under standard growth conditions (Kai et al., 2006). Nevertheless, the leaf scopolin content of wild type plants exposed to 10°C for one week was found to increase from less than three nmol per g fresh weight (FW) to up to 30 to 150 nmol per g FW. Leaves of both *tho1* mutants accumulated significantly less scopolin compared to respective wild type leaves when the plants were

subjected to low temperature stress. At control conditions, the low level of detectable scopolin in the *tho1* mutants did not show any significant differences from the respective wild type or was even slightly higher (Fig. 2A). Recently, line SAIL_1209_F10, a further independent T-DNA insertion line with disrupted *THO1* in Columbia background, became available. In line SAIL_1209_F10, scopolin accumulation is affected in a similar way as in the two mutants established previously (Fig. 2B). In accordance with the microscopic analyses of roots, less scopolin accumulated in mutant leaves under cold stress. No further phenotypical or developmental difference was observed between leaf tissues of mutants and wild type (Fig. 2C).

Mutations in genes encoding subunits of the putative A. thaliana THO/TREX complex other than THO1 *partially reduce the scopolin content in roots under osmotic/high carbon stress and in leaves under cold stress*

The THO/TREX complex in *A. thaliana* is thought to comprise a dynamic set of subunits encoded by at least eight genes: *THO1*, *THO2*, *THO3*, *THO5A*, *THO5B*, *THO6*, *THO7A* and *THO7B* (Yelina et al., 2010). THO1, THO2, THO3 and THO6 are complex components that are distinct proteins encoded by unique genes in the *A. thaliana* reference genome, while THO5 and THO7 are encoded by duplicate genes, with THO5B being a truncated and considerably modified version of THO5A, and THO7A and THO7B differing from one another just with respect to a few amino acids. With these genes not being co-ordinately transcribed, Jauvion et al. (2010) have proposed that in *A. thaliana* the THO/TREX complex has a dynamic composition with different subunits possibly playing tissue-specific roles. Functionally, the *A. thaliana* THO/TREX complex is involved in the production of small interfering RNAs (siRNAs) (Yelina et al., 2010; Jauvion et al., 2010). Thus, T-DNA insertion lines in Columbia-0

background were obtained and confirmed, which in the following are referred to as *tho2*, *tho3*, *tho5a*, *tho5b*, *tho6*, *tho7a* and *tho7b* and scopolin contents in roots of plants grown in the presence of high (3% w/v) sucrose as well as leaves of cold-stressed plants were quantified (Fig. S3). A general trend towards reduction of scopolin content in comparison to wild type was seen for all further *tho* mutants tested in both treatments, but only for roots under high sugar treatment in *tho7b*, the reduction was significant. Nevertheless, fluorescence microscopy performed on leaves of cold-stressed *tho2* and *tho3* indicated the same general reduction of scopolin as observed for *tho1* (Fig. S4).

Mutations in AGO1, RDR6, and SGS3 known to be involved in RNA silencing pathways affect cold-induced scopolin accumulation in leaves

As THO1 and the THO/TREX complex were shown to contribute to RNA silencing pathways (Yelina et al., 2010; Jauvion et al., 2010), mutants of other components of the postulated pathways involving THO were also tested for decreased scopolin accumulation (Fig. 3). Mutant *rdr6-11* (Peragine et al., 2004) is defective in *RNA-DEPENDENT RNA POLYMERASE 6* (At3g49500***)***, a well-described key player for the production of tasiRNAs and other siRNAs. Consistently, scopolin was significantly reduced, in comparison to wild type, to levels below any of the tested *tho* mutant lines. That was also the case for mutants carrying *ago1-46*, a hypomorphic allele of *ARGONAUTE 1* (*At1g48410***,** phenotypes of *ago1* null alleles are too severe to perform experiments with the mutants (Smith et al., 2009)), and for *sgs3-12*, a defective allele of *SUPPRESSOR OF GENE SILENCING 3* (*At5g23570*) assumed to stabilize template RNA fragments before double-stranded (ds)RNA synthesis is performed by RDR6 (Yoshikawa et al., 2005).

RNA analyses revealed potential THO1-dependent transcripts

A micro-array-based transcript abundance analysis using RNA extracted from roots 18 h after the plants had been shifted from a low to a high sucrose regime (detailed results are provided in supplemental data set Data 1) and follow-up quantitative RT-PCR confirmed that transcripts from four loci, *At1g53490 (HEI10), At3g05770,* At5g14340

(*MYB40*) and At5g09870 (*CESA5,* downstream of *THO1*), show differential RNA levels in *tho1* (Col-0), *tho2*, and *tho3* in comparison to the wild type (Table S1). When related T-DNA insertion lines were analysed, however, no consistent effect of mutations in these loci on scopolin accumulation could be established (Figure S5, Figure S6).

High throughput RNA sequencing was performed on the aerial part of *tho1* (Col-0) and *tho1* (Ws) mutant plants in comparison to respective wild type plants 24 hours after a shift from 20°C to 10° C at 300 µmol m^{-2} s^{-1} light under a 12/12 hour day/night regime. Preparations of long RNA (>200nt) and small RNA (<200bp) fractions were analysed in five biological replications. The full data set can be found in the supplemental data sets Data 2 and Data 3. All three data sets are accessible via the eDAL repository of the IPK Gatersleben (https://doi.ipk-gatersleben.de/DOI/2ff827e5-8e06-42b0-950e-b5d3cc068721/0964bd45-7676-4b94-a988-c5827d43ffc8/2/1847940088; preliminary link for the reviewing process).

Reads from mutant loci *tho1* (Ws) and *tho1* (Col-0) containing T-DNA insertions were over-represented in comparison to reads from respective wild type plants (Table S2 and Table S3). Closer inspection, however, showed that in both mutants most reads were detected that were derived from regions close to the T-DNA integration sites close to the last exon and the untranslated 3´-region, respectively (Figure S7). Consistent with disrupted gene

function, however, reads from the 5' half of *THO1*, were partially reduced in *tho1* (Col-0) and severely reduced in *tho1* (Ws). Transcript levels of several genes were affected in both *tho1* mutants. The top-regulated are shown in Table S2 and Table S3.

The functional disruption of the THO/TREX complex leads to modulation of tasiRNAs and tasiRNA-precursors (Yelina et al., 2010; Francisco-Mangilet et al., 2015); this was also detectable in our material grown under cold stress conditions. There were, however, differences between the two *tho1* alleles analysed. Mutant *tho1* (Ws) showed an increased TAS1C precursor while this was not seen in *tho1* (Col-0) (Table S4). Rather, *tho1* (Col-0) accumulated more of the processed siRNAs siR850 (derived from TAS1C) and siR438 (derived from TAS1A), while this effect was not found in *tho1* (Ws) (Figure S8).

The phased processing of tasiRNA is directed by microRNAs (Allen et al., 2005). Micro RNAs with a mature length of ~ 21nt are processed from micro RNA precursor molecules in a process involving DICER like enzymes. Francisco-Mangilet et al. (2015) showed that THO2 is necessary for the production of mature microRNAs. In accordance with this, in *tho1* (Ws) the precursor of miR164 was present at increased level (Table S5). This microRNA is connected to lateral root development and as well as leaf senescence. However, the amount of mature miRNAs was not reduced. On the contrary, both mutants accumulated more of the processed miRNA 398B*, tho1* (Ws) accumulated additionally the processed miRNA156, miRNA157, miRNA166 and miRNA390 (Table S6 and Table S7). Besides that, *tho1* (Ws) had increased levels of several other small RNAs, mostly originating from transposable element genes. Such effect of siRNA accumulation upon retrotransposition caused by stress has been described previously (Ito et al., 2011). Analysis of the small RNAs in *tho1* (Col-0) returned nearly no differences to wild type when applying the same strict filters (Table S8).

Non-targeted analysis of semi-polar secondary compounds of roots and leaves exposed to high-sucrose and cold stress, respectively

A non-targeted LC-MS based metabolite profiling analysis of the semi-polar metabolite content (covering mainly phenylpropanoids, glucosinolates and indoles) of the roots of wild type and *tho1* plants exposed to high sucrose revealed only minor differences (besides scopolin). Of 5000 extracted and aligned features, only two features with rather low intensities were found to be significantly different in both *tho1* mutants compared to the respective wild type: One with a retention time (RT) of 0.41 min and an *m/z* of 110.009 and another with a RT of 2.33 min and an *m/z* of 130.065. The most probable ion formula for the latter was C_9H_8N which is a typical diagnostic in-source fragment of indolic compounds (Figure S9).

The exposure of wild type plants to 10°C for one week induced an altered content in ~300 compounds in leaves; most prominent among these (apart from scopolin) were kaempferol and quercetin derivatives, sinapoyl glucose and a number of nitrogen- and sulphur-containing compounds (Petridis et al., 2016). A comparison between wild type and the two independent *tho1* mutants identified only four compounds (in addition to scopolin) of 4000 extracted and aligned features that were altered in *tho1* (Col-0) and *tho1* (Ws) in a similar fashion; all of them were sulphur-containing compounds with low signal intensities. The peak eluting at 5.21 min (*m/z* 205.080, estimated ion formula $C_{11}H_{12}N_2S$ + H) was increased in *tho1* (Col-0) and *tho1* (Ws). The same was true for the substance eluting at 4.43 min with the two fragments *m/z* 471.083 and *m/z* 440.042. As against that, the compounds eluting at 4.67 min (*m/z 3*79.095, estimated ion formula $C_{17}H_{19}N_2O_6S$) and 6.05 min ($C_{17}H_{19}N_2O_6S$, *m/z* 379.095) were decreased. These two substances were tentatively annotated as hydroxycamalexin hexoside #1 and #2, according to Pedras and Ahiahonu (2002), and

Böttcher et al. (2009). Substance *m/z* 205.080 accumulating in both *tho1* mutants accumulated also in *tho2* and *tho6*, and in *rdr6-11*. The compound eluting at 4.43min accumulated also in *rdr6-11*, but was decreased in *ago1-46*. The two substances with the mass *m/z* 379.095 were even stronger reduced in *ago1-46*, *rdr6-11* and *sgs3* (Figure S10). Both were not found in the 20°C control plants.

Transcript levels of phenylpropanoid pathway genes respond to high (3% w/v) sucrose stress

In order to test for potential targets of THO-dependent signalling, transcript levels of selected key genes of phenylpropanoid metabolism (Stracke et al., 2007) were determined by quantitative RT-PCR. Most of the genes assayed (*PAL1*, *F6'H1*, *CHS*, *FLS* and *F3'H*) had a higher transcript abundance in the root after a shift from a low (0.5% w/v) to a high (3% w/v) sucrose regime in the wild type (Figure 4). No *DFR* transcription was detectable. In *tho1* (Col-0) roots, both *F6'H1* and *F3'H* transcripts were less abundant than in wild type roots at one of the time points. In leaves, the shift from ambient temperature to 10°C initiated a raise of transcript abundance of *PAL*, *CHS*, *FLS*, *F3'H*, *F6'H1* and *DRF* in wild type within 6 h, which peaked after 12 to 24 h. In *tho1* (Col-0)*, PAL* transcripts were lower than in wild type, and transcripts of *F3'H* were higher (Figure S11).

Discussion

The screen performed in *A. thaliana* was successful in identifying *THO1* as a gene required for scopolin accumulation in response to stress imposed by either high osmotic/high carbon conditions or low temperature. The involvement of *THO1* was confirmed by the analysis of three independent *tho1* T-DNA insertion mutants. As in other contexts, *THO1* acts in the induction of scopolin accumulation most likely *via* its function as a subunit of the *A. thaliana*

THO/TREX complex involved in RNA silencing, along with AGO1 and SGS3 (binding single and double stranded RNAs, respectively) as well as RDR6, an RNA directed RNA polymerase acting downstream of the THO/TREX complex (Peragine et al., 2004; Howell et al., 2007; Jauvion et al., 2010; Yelina et al; 2010).

THO1 is necessary for stress-related scopolin accumulation

The roots of *tho1* mutants under sucrose stress accumulated only approximately 50% to 80% of the scopolin present in wild type roots under the same conditions. As there was no difference between *tho1* mutants and wild type in scopolin accumulation under non-stressed conditions, the effect of *tho1* in roots can be considered as dependent on stress. Similarly, under low temperature stress, leaves of three independent *tho1* mutants contained significantly less scopolin than their wild type counterparts. Pan et al. (2012) had shown that *THO1* is required for the signalling involved in mounting a hypersensitive response in the *edr1* mutant, a line that lacks a functional copy of a gene encoding a protein kinase which acts to suppress the transcription of a specific disease resistance gene. Thus, it is tempting to speculate that this requirement for *THO1* is connected to its role promoting scopolin accumulation in response to stress. In tobacco, scopolin is deposited around the margin of tissue undergoing necrosis, forming a characteristic, fluorescent ring (Dorey et al., 1997). Chong et al. (2002) have proposed a dual role for scopolin/scopoletin during the hypersensitive reaction of tobacco challenged with tobacco mosaic virus: firstly, it slows cell death by eliminating reactive oxygen species (ROS) in the surrounding cells and, secondly, it prevents the spread of the pathogen by forming an antibiotic barrier. A similar scenario has been suggested for the interaction of the bacterium *Pseudomonas syringae* with its host *A. thaliana* (Simon et al., 2010). Scopolin formation prompted by abiotic stress such as high

sucrose or cold may be triggered by an increased ROS content, and possibly also by an excess of carbon compounds which cannot be used for growth. Once accumulated, scopolin supports ROS scavenging as well as preformed pathogen defence. The latter may be advantageous during periods of low temperature, since it is known that some pathogens have the effect of reducing freezing tolerance (Lindow and Leveau, 2002). In conclusion, THO1 might form part of the signalling machinery leading to the accumulation of scopolin as a response to diverse stresses.

THO1 likely acts via its function as subunit of the A. thaliana *THO/TREX complex*

The *A. thaliana* THO/TREX complex has been reported to be a dynamic structure (Jauvion et al., 2010), which will not play the same role at all developmental stages or in every tissue or organ. THO1 is clearly required for stress-induced scopolin accumulation. When mutants affecting the other putative components of the complex were characterized, differences in tissue scopolin content could be established for a subset. Thus, the action of THO1 in the promotion of scopolin accumulation is most likely based on its function as subunit of the THO/TREX complex. However, the mutants defective in different THO/TREX components did not display precisely the same effect. The metabolite profiling indicated that the two most influential components over scopolin content were THO1 and THO2, whereas both Yelina et al. (2010) and Jauvion et al. (2010) have suggested that loss of THO3 has the strongest effect on tasiRNA accumulation, followed by THO1 and THO6. In the case of the potentially redundant AtTHO5A / AtTHO5B and AtTHO7A / AtTHO7B gene pairs, the lack of influence on detectable scopolin accumulation may be explained by the loss of one member being compensated by the continued presence of the other.

The THO/TREX Complex reduces tasiRNA production and modulates processing of other small RNAs

RNA silencing pathways that involve miRNAs as triggers and tasiRNAs as secondary signals broadly contribute to gene regulation in development and stress responses in plants (e.g. Poethig, 2009; Ding et al. 2013), with targets including, e.g., pentatricopeptide repeat proteins, auxin response TFs, and a squamosa-promoter binding protein (Peragine et al., 2004; Yoshikawa et al., 2005). Our results regarding changed abundance of tasiRNA precursors and microRNAs in *tho1* mutants support the contribution of THO/TREX complex to tasiRNA formation and accumulation of the respective precursors (Jauvion et al., 2010; Yelina et al., 2010). The tasiRNA silencing pathway is initiated by ARGONAUTE (AGO)-mediated cleavage of single-stranded transcripts from TAS loci under guidance of a miRNA. The THO/TREX complex is thought to participate in the trafficking of the single-stranded tasiRNA precursors to the catalytic centre of the AGO-miRNA complex. RNA-dependent RNA polymerase RDR6 is responsible for the conversion of TAS transcript cleavage products into a double-stranded form (Peragine et al., 2004; Yoshikawa et al. 2005), which is then processed into secondary tasiRNAs that serve as sequence-specific signals for the cleavage of target mRNAs or other means of down-regulation. Consequently, tasiRNAs are less abundant in *rdr6* mutants and regulation of target genes is impaired. The two-step design of the silencing process has the potential both to spread silencing *via* an amplification of the original signal and to move the signal away from its original source, thereby providing a mechanism for long distance signalling (Yelina et al., 2010). Metabolite profiling of leaves from cold-stressed *tho1, ago1, sgs3,* and *rdr6* plants revealed reduced scopolin contents for these mutants, with the difference in the *ago1, sgs3,* and *rdr6* mutant being more pronounced. The fact that these genes are required for the full scopolin stress reaction

suggests the involvement of an RNA silencing mechanism that might target a negative regulator of scopolin synthesis (Fig. 5). At control conditions, the initiating primary small RNA signal would be absent and the RNA silencing pathway inactive, allowing the expression of the negative regulator of scopolin synthesis and thus maintaining a low scopolin content. Upon stress, an initiating primary small RNA, as it was described for miRNA398 (Zhou et al., 2008), would be present, leading to inhibition of negative regulators of scopolin synthesis by silencing. Thus, scopolin will accumulate under stress in wild type plants. However, in absence of a functional THO/TREX complex or AGO1, SGS3 and RDR6, in the respective mutants, RNA silencing would be operating less efficiently, allowing the negative regulators of scopolin synthesis to be present under stress conditions, resulting in reduced scopolin levels.

Experimental procedures

Plant materials

Seeds of the *tho1* (Ws) mutant line FLAG_191_B10 (Dèrozier et al., 2011) were obtained from INRA Versailles, that of the *tho1* (Col-0) mutant line GABI-Kat GK_052_D02 from the GABI-Kat consortium, University of Bielefeld (Kleinboelting et al., 2012). All other seed stocks were obtained from the Nottingham Arabidopsis Stock Centre, University of Nottingham, UK (NASC) (Scholl et al., 2000): *ccoaomt1* (Insertion at *At4g34050*, SALK_151507C): NASC Stock No. N664340; *f6'h1* (Insertion at *At3g13610,* SALK_132418C): NASC Stock No. N658415; wild type Col-0: NASC Stock No. N1092; Activation tagging line collection: NASC Stock No. N21995 (Weigel et al., 2000); *tho1* (third allele, SAIL_1209_F10, Mc Elver et al., 2001): NASC Stock No. N844119; *tho2* (Insertion at *At1g24706,* SALK_051591): NASC Stock No. N55191; *tho3* (*At5g56130*, SALK_059869): NASC Stock No.

N559869; *tho5a* (*At5g42920*, SALK_100556): NASC Stock No. N60556; *tho5b* (*At1g45233*, SALK_122387C): NASC Stock No. (N656928); *tho6* (*At2g19430*, SALK_021789C): NASC Stock No. N658552; *tho7a* (*At5g16790,* SALK_078514C): NASC Stock No. N655638; *tho7b* (*At3g02950*, SALK_052697): NASC Stock No. N552697; *hei10* (At1g53490, SALK_014624): NASC Stock No. N514624; *At3g05770*, SALK_069840C: NASC Stock No. N669818; *myb40* (*At5g14340*, GABI-KAT GK_304_H04.09): NASC Stock No. N719473; *cesa5* (At5g09870, SALK_099008): NASC Stock No. N599008; *rdr6-11* (At3g49500, Peragine et al., 2004) NASC Stock No. N24285; *ago1-46* (AT1G48410, Smith et al., 2009): NASC Stock No. N67862; *sgs3-12* (AT5G23570, Peragine et al., 2004): NASC Stock No. N24290, *dcl4-2* (AT5G20320, GABI-KAT GK_160_G05): NASC Stock N9969; *ago6* (AT2G32940, SALK_021553C): NASC Stock No. N521553; wild type Wassilewskija: NASC Stock No. N915. All SALK lines were created by Alonso et al. (2003). Insertion mutant information for the SALK lines was obtained from the SIGnAL (Salk Institute Genomic Analysis Laboratory) website at http://signal.salk.edu. The presence of the expected T-DNA inserts was verified by PCRs based on primers defined in either the GABI-Kat instructions (http://www.gabi-kat.de/db/genotyping_details.php?lineid=052D02&genecode=At5g09860) or the SALK T-DNA Primer design tool (http://signal.salk.edu/tdnaprimers.2.html) on at least ten individual plants per line; the relevant primer sequences are listed in Table S9. The *rdr6-11* mutant was verified as described by Peragine et al. (2004). Experiments were performed on the off-spring of a homozygous single plant for each line.

Liquid (hydroponics) and solid (agar plates) plant culture

Seeds were surface sterilized by immersion for 2 min in 70% v/v ethanol, then in 5% w/v NaOCl / 4% v/v Tween 20 for 8 min and finally in three changes of sterile water. The seeds

were then suspended in 0.1% w/v sterile agarose, allowed to imbibe at 4°C for four days, and were reared hydroponically on ¼ MS including vitamins (Murashige & Skoog, 1962) for four weeks (Schlesier et al., 2003). 0.5% (w/v) or 3% (w/v) sucrose were added to media as required. Approximately ten seeds per container were cultivated at illumination with 100 µmol m^{-2} s^{-1}, with a 16/8 hour day/night period at 25/20°C. For experiments in which plants needed to be shifted from a low to a high sucrose regime, the plants were initially grown for four weeks on a medium containing 0.5% w/v sucrose, after which they were transferred to a medium containing 3% w/v sucrose. Roots were harvested at 0, 9, 18 and 36 h after the transfer. For the materials grown on agar (0.8% w/v), the medium was composed of half strength Murashige & Skoog (1962) including vitamins, and the plates were orientated vertically.

Root fluorescence screen of activation-tagged mutants

Plants were raised for three to four weeks on agar plates before taking root images with a gel documentation system (Diana II, raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany). The UV light required to excite scopolin fluorescence was obtained from full spectrum UV light by filtering with a 200 x 200 x 2 mm filter plate (transmittance 250-400 nm, optimum 340 nm) placed below the agar plate, while a filter to select emitted light (optimum transmission 460 nm) was placed in front of the camera lens. Resulting grey scale images were converted into a false colour image using Advanced Data Image Analyzer v2.3.007 software (Raytest Isotopenmessgeräte GmbH). The scopolin content of plants displaying enhanced root fluorescence was quantified *via* HPLC with fluorescence detection (FLD) at 336nm excitation/438nm emission wavelengths with the same LC separation conditions as described below.

Low temperature stress treatment

Seeds were sown into fine structured peat containing 140 mg L^{-1} N, 160 mg L^{-1} P, 180 mg L^{-1} K, 100 mg L^{-1} Mg, 120 mg L^{-1} S and trace elements, and exposed to a 9/15 hour day/night period at 21/18°C with 120-150 µmol m^{-2} s^{-1} light. After two weeks, the plants were transplanted into peat containing 280 mg L^{-1} N, 320 mg L^{-1} P, 360 mg L^{-1} K, mg L^{-1} Mg and 120 mg L^{-1} S. After another week, they were held for five days under a 12/12 hour day/night period at constant 20°C and 300 µmol m^{-2} s^{-1} light. The temperature was then adjusted to either a constant 10°C or to a 20°C/18°C regime. The sampling took place after 0 and 168 hours for metabolite analysis and at 0, 1, 6, 12, 24, and 36 hours for transcript analysis, respectively. Samples were frozen in liquid nitrogen and powdered in a ball mill.

Extraction of semipolar compounds

Root material harvested from hydroponically grown plants was chopped with a scalpel, blotted dry and snap-frozen. Soil-derived samples were rinsed three times, blotted dry and snap-frozen. Leaves were snap-frozen without any pre-treatment. Samples were kept at -80°C until processing. Large samples (>150 mg) were pre-homogenized before storage. For the extraction, 400 µL methanol per 100 mg was added to the frozen material and samples were homogenized with a tissue homogenizer. After centrifugation (28500 x *g*, 4°C, 10 min), the supernatant was transferred to a clean tube, while the pellet was re-extracted one time as above. The supernatants were combined. Prior to injection into an HPLC device (see below), aliquots were diluted to 80% with the aqueous solvent from the aqueous phase of the HPLC run and solids were sedimented by centrifugation for 5 min (Korn et al., 2008; modified).

LC-MS profiling

LC-MS was carried out as described previously (Petridis et al., 2016). In short, samples were separated on an Acquity UPLC® BEH Phenyl Column (130Å, 2.1 x 100 mm, 1.7 µm) with an Acquity UPLC® BEH Phenyl VanGuard precolumn (130Å, 2.1 x 5 mm, 1.7 µm, Waters, Eschborn, Germany). The column temperature was set to 35°C. The gradient was linear from 0 min, 100% solvent A (0.1% [v/v] formic acid in MS grade water), to 10 min, 40% B (0.1% [v/v] formic acid in acetonitrile) (Yonekura-Sakakibara et al., 2008). Solvent B was brought up to 100% after each run to clean the column, and the initial conditions were restored in a delay of 5 min. For MS detection, ESI was carried out in positive mode using a Bruker Daltonik GmbH (Bremen, Germany) Ultra High Resolution ToF mass spectrometer with settings optimized for small- to medium-sized molecules (mass range: 50–1,000 *m/z*; capillary voltage: 4 kV; nebulizer: 3 bar; dry gas: 8 L min^{-1}; dry temperature: 200°C; hexapole radio frequency (RF) voltage: 40 V peak-to-peak (Vpp); funnel 1 RF: 300 Vpp; funnel 2 RF: 300 Vpp; prepulse storage time: 5 ms; transfer time: 50 ms; low mass: 40 *m/z*; collision cell RF: 500 Vpp; collision energy: 10 eV). Data analysis was carried out using Compass DataAnalysis v4.1 and Compass QuantAnalysis v2.1 software (Bruker Daltonik). Mass-retention time pairs ("Buckets") were generated by the DataAnalysis "Find Molecular Features" function (settings: intensity threshold 1,000, signal-to-noise threshold 3, minimum compound length 7 spectra, smoothing width 3, no limitation to CHNO), then filtered by a Profile Analysis Software routine (Bruker Daltonik) to exclude compounds not present in all replicates of at least one treatment. A principle component analysis was applied after Pareto scaling; the confidence level for the critical distances of the PCA model was 95%. Differences in compound concentrations were tested for significance using the Student's *t*-test with $p < 0.05$. Molecular sum formulae were generated from the

DataAnalysis Smart Formula facility. Compounds were annotated using the generated molecular sum formula for a data base search (KEGG, ChemSpider, ChEbI, Metlin), and the in-source fragmentation patterns were used to identify sugar moieties. A compound was deemed to have been fully identified when its retention time and fragmentation pattern matched those of a known standard. Unknown compounds were quantified by their peak areas from extracted ion chromatograms of the respective *m/z* ratios.

LC-MS data sets were deposited at the European Informatics Institute's (EMBL-EBI) MetaboLights data base (Haug et al., 2013), ID: MTBLS389. (Currently accessible for reviewing purposes only at http://www.ebi.ac.uk/metabolights/reviewer3b16278b-f47a-4f5b-a04e-5f957fce1650. The public link will be http://www.ebi.ac.uk/metabolights/MTBLS389).

Confocal laser scanning microscopy (CLSM)

After degassing, the sample was placed on a slide in water under a coverslip. Fluorescence was recorded no later than 30 min after sample preparation. The settings for CLSM (model LSM 510 Meta, Carl Zeiss MicroImaging GmbH, Jena, Germany) were: 364 nm laserline, 40% power, objective 40x/1.2 NA, zoom 1.4, 512 x 512 pixels, pixel dwell time 12.8 µs, averaging 2x, pinhole 600 µm, gain 684, offset 10. A lambda stack for 364 nm excitation / 376 – 708 nm emission was recorded. All pixels with the scopolin-like emission spectrum were extracted. For leaf samples, an intensity-absolute frequency histogram was used to determine the maximum intensity at 451-461 nm over a 25829.08 μm^2 area.

Micro-array analysis

Wild type Col-0 and *tho1* (Col-0) GK_052_D02 plants were grown for four weeks in hydroponic culture (¼ Murashige and Skoog including vitamins, 0.5% [w/v] sucrose). Roots were harvested 18 hours after replacing the medium with ¼ Murashige and Skoog including vitamins, 3% w/v sucrose in three pools (1 pool consisting of roots from five containers), obtaining three biological replicates of 50 plants per line. RNA was extracted using an RNA extraction kit with on-column DNAse digest (RNeasy Plant Kit, Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Affymetrix 3' expression profiling and the subsequent bioinformatic analysis were performed by ATLAS Biolabs GmbH (Berlin, Germany). Six micro-arrays (GeneChip Arabidopsis ATH1 Genome Arrays, Affymetrix, Santa Clara, CA, USA) were hybridized (three for wild type Col-0 and three for *tho1* (Col-0) GK_052_D02), and signals were normalized over all six arrays. Mean signal strengths for each probe set were compared between the two groups using Student's *t*-test. A threshold of $p<0.05$ and an at least two fold up- or down-regulation was considered the minimum to call a significant effect. Probe sets not represented in at least two out of three samples of one group were rejected. The TAIR database (Rhee et al., 2003) provided the annotation of significantly regulated genes.

RNA abundance analysis by high throughput RNA sequencing

Plants were reared and exposed to low temperature stress as described above. Samples for RNA preparation were harvested 24 hours after application of cold stress and 4 hours after beginning of the light phase. 5 times 3 trays with 9 plants were pooled to gain 5 biological replicates for *tho1* (Col-0) GK_052_D02, t*ho1* (Ws) FLAG_191_B10, and respective wild type Col-0 and Ws (20 samples in total). 150 mg powdered leaf material was used to prepare a

small RNA fraction and a long (=small-RNA-depleted) RNA fraction using the Ambion® mirVana™miRNA isolation kit (Thermo Scientific, Waltham, MA, USA): Following the manufacturer's protocol, after "Organic extraction" (E), "Enrichment procedure for SmallRNAs" (FII) was applied, followed by the recovery of the RNA fraction depleted of small RNAs (FI 3-5). This delivered two RNA fractions per sample, one containing all RNAs < 200 nts and one containing the long RNAs >200 nts. The RNA preparations were quality-checked using NanoDrop and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA; small-RNA-depleted fraction only) delivering small-RNA concentrations between 100 and 200 ng per µl, long-RNA concentrations between 600 und 1400 ng per µl, a A260/A280 ranging between 1.91 and 2.01 (small RNAs) or 2.15-2.17 (depleted fraction), and RNA integrity numbers (RIN) between 8.5 and 9.

For small RNAs, sequencing was performed with RNA Illumina next generation sequencing technology Hiseq2000 (Illumina, San Diego, Ca, USA) as described in Meyer et al. (2015). Adapter trimming was performed via CLC Genomics Workbench 7.0 (Qiagen/CLC bio, Aarhus, Denmark). The default workflow for transcriptome analysis for small RNAs was used to quantify processed micro RNAs: Sample files were extracted and counted after size selection (from 15 to 55 nts). 100% identical sequences of the same size constituted one "feature". The trimmed reads were mapped on the *A. thaliana* reference genome extracted from TAIR10 (Lamesch et al., 2012) with no mismatch allowed and strand specific annotation. The single feature read counts were normalized to reads per million. Differences of mean values were calculated with Baggerly's t-test (Baggerly et al., 2003). As the default workflow does not perform a summation of all reads mapping to one locus, the detection of significantly differential loci was performed using the open source software R (R Core Team, 2013, version 3.0.0.) Reads matching the same annotated locus tag were

merged and their normalized counts were added up. A locus was considered "differential" if it showed at least two-fold up- or down-regulation of the normalized counts. To check for normality, Shapiro-Wilk test, implemented in stats::shapiro.test, was performed per gene and probe. Variance homogenity was tested gene-wise using F-test, implemented in stats::var.test. Student's *t*-test was performed genewise to prove significance of the differential loci (Bonferroni corrected p-value threshold < 0.05).

Hiseq2000 was also used for high throughput RNA sequencing analysis of the long RNAs. The Illumina TruSeq RNA Sample Preparation Kit was used to prepare the sequencing library. After adapter trimming, the reads were mapped on the *A. thaliana* reference genome extracted from TAIR10 (Lamesch et al., 2012) with CLC Main Workbench (CLCbio) using standard parameters. Mapping options were set with 2 mismatch cost, 3 insertion cost, 3 deletion cost and 0.8 length and similarity fraction. The expression value was given as total read count and normalized afterwards. For statistical analysis within the software, unpaired two-group comparison was chosen. Comparison of digital gene expression (DGE) was performed on normalized data (rpkm) delivering p-values and fold changes from an EDGE test with tag-wise dispersions (Robinson et al., 2010) with additional Bonferroni correction.

Quantitative RT-PCR

RNA was extracted from 100 mg frozen material from three biological replicates using the TRIzol® reagent (Life Technologies GmbH, Darmstadt, Germany) and treated with DNAse (Thermo Scientific). 1 µg aliquots of total RNA provided the template for oligo-dT primed cDNA synthesis, carried out using Maxima Reverse Transcriptase (Thermo Scientific). Gene-specific primers for the subsequent PCR were designed using Quantprime software (Arvidsson et al., 2008) and are listed in Table S10. Four of the reference genes suggested by

Czechowski et al. (2005) were tested for transcription stability on 12 samples (AT2G28390, "SAND family protein"; AT5G08290, "mitosis protein YLS8"; AT5G15710, "F-box protein"; AT5G55840, "PPR gene") and two of them were selected for normalization, namely "SAND family protein" and "mitosis protein YLS8". Primers were diluted to 2 pmol μl^{-1} (for *F3'H*, *ATPSK2*, *At1g43590* to 20 pmol μl^{-1}), cDNA to 5ng μl^{-1}. Each 6 µL PCR set up comprised 1 µL cDNA, 2 µL primer mix and 3 µL SYBR Green Mix (SSoAdvanced, BioRad Laboratories GmbH, München, Germany). The cDNA was first denatured at 95°C for 2 min and then given 40 cycles of 95°C for 10 s, 58°C for 30 s, using a CFX96 icycler (BioRad). Each sample was represented by three technical replicates. Quantification cycle (Cq) values were determined in regression mode. Relative transcript abundances were based on the global mean of transcript abundance in all samples, as calculated using qBase software (Biogazelle, Zulte, Belgium).

Statistical analyses

SigmaPlot software (Systat Software Inc., San Jose, CA, USA) was used to compare mean values and to detect significant differences between two or more groups. Numerical data were tested for normality (Shapiro-Wilk test) and equality of variances. Data which passed both tests were subjected to a Student's *t*-test against the control or, where multiple comparisons were required, to an ANOVA with a Holm-Sidak *post-hoc* test. For non-normally distributed and for non-numerical data, the Mann-Whitney U test or, for multiple comparisons, a Kruskal-Wallis One Way Analysis of Variance on Ranks with Dunn's correction were performed.

Acknowledgements

Funding of research by grants to H-P Mock from the EU (ATHENA; KBBE-2009-13) and the BMBF (FKZ 031A108B) are gratefully acknowledged. A Petridis was supported by a post-doctoral fellowship from the Leibniz Association and the German Academic Exchange Service (DAAD). We thank the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed *Arabidopsis* T-DNA insertion mutants. Funding for the SIGnAL indexed insertion mutant collection was provided by the National Science Foundation. We also thank the Nottingham Arabidopsis Stock Centre, the GABI-KAT consortium and INRA Versailles for providing further seed stocks. Furthermore we are grateful for the assistance of Katja Witzel (Leibniz Institute of Vegetable and Ornamental Crops, Großbeeren, Germany) with the transcript analysis. We would also like to thank Steffen Neumann (Leibniz Institute for Plant Biochemistry, Halle, Germany) for the upload of LC-MS data into the MetaboLights database. The authors declare no conflict of interest.

Abbreviations:

ABA: abscisic acid; AGO: ARGONAUTE; CCoA*o*MT1: Caffeoyl-CoA *ortho* methyltransferase1; CHS: chalcone synthase; CLSM: confocal laser scanning microscopy; Col-0: Columbia 0; DCL: Dicer nuclease; DFR: dihydroflavonol 4-reductase; *EDR1: ENHANCED DISEASE RESISTANCE1; EMU: ERECTA mRNA UNDER-EXPRESSED;* ESI: Electron spray ionization; F3'H: flavonoid 3'-hydroxylase; F6'H1: feruloyl-CoA 6-hydroxylase1; FLS: flavonol synthase; FW: fresh weight; *HEI10: ENHANCER OF CELL INVASION NO. 10; HPR1: HYPERRECOMBINATION1;* HR: hypersensitive reaction; LC-MS: Liquid Chromatography – Mass Spectrometry; *m/z*: mass-to-charge ratio; MYB: myeloblastosis family of transcription factors; PAL: phenylalanine ammonia-lyase; RDR: RNA-dependent RNA polymerase; siRNA: small interfering RNA; SGS:

SUPPRESSOR OF GENE SILENCING; tasiRNA: trans-acting small interfering RNA; T-DNA: transfer DNA; TF: transcription factor; THO: suppressors of the transcriptional defects of *hpr1*Δ by overexpression; TREX: transcription and (mRNA) export; Ws: Wassilewskija; WT: wild type

Short Supporting Information Legends

Fig. S1. Fluorescence of the *A. thaliana* root induced by scopolin and/or scopoletin.

Fig. S2. Root growth of the *tho1* mutant and wild type Col-0 in response to available sucrose.

Fig. S3. Scopolin accumulation in roots and leaves of mutants of individual members of the proposed THO complex of *A. thaliana* in comparison to wild type plants

Fig. S4. The distribution of scopolin in the leaf of wild type and *tho* mutant plants exposed to 10°C for one week.

Fig. S5. Scopolin content of roots from mutants of genes with different transcript abundance in roots of *tho1* (Col-0).

Fig. S6. Scopolin content of leaves from mutants of genes with different transcript abundance in roots of *tho1* (Col-0) after one week at 10°C.

Fig. S7. RNA sequencing reads mapping to *THO1* (At5g09860) cDNA.

Fig. S8. Relative transcript abundance of known siRNAs according to Allen et al. (2005).

Fig. S9. Relative contents of the substances significantly altered in *tho1* roots in comparison to wild type in a number of mutant lines tested.

Fig. S10. Relative contents of the substances significantly altered in *tho1* leaves in comparison to wild type in a number of mutant lines tested.

Fig. S11. Relative transcript levels of phenylpropanoid key genes and THO1 in leaves after placement in 10°C and 100µmol m-2 s-1.

Table S1. Transcript abundance in *tho* roots assayed using qRT-PCR.

Table S2. RNA sequencing results of *A. thaliana* leaves, RNAs > 200nt, comparison wildtype Wassilewskija (Ws) vs. *tho1* (Ws).

Table S3. RNA sequencing results of *A. thaliana* leaves, RNAs > 200nt, comparison wildtype Columbia 0 (Col) vs. *tho1* (Col-0).

Table S4. RNA sequencing results for TAS RNA precursors, measured in *A. thaliana* leaves, RNA fraction > 200nt, comparison wildtype vs. *tho1.*

Table S5. RNA sequencing results of *A. thaliana* leaves, RNAs < 200nt, comparison wildtype Wassilewskija (Ws) vs. *tho1* (Ws).

Table S6. RNA sequencing results of *A. thaliana* leaves, RNAs < 200nt, detected 20-21nt processed microRNAs, comparison wildtype Wassilewskija (Ws) vs. *tho1*(Ws). Changes in ~21 nt cDNA sequences of processed micro RNAs.

Table S7. RNA sequencing results of *A. thaliana* leaves, RNAs < 200nt, comparison wildtype Columbia-0 (Col) vs. *tho1*(Col). Changes in ~21 nt cDNA sequences of processed micro RNAs.

Table S8. RNA sequencing results of *A. thaliana* leaves, RNAs < 200nt, comparison wildtype Columbia 0 (Col) vs. *tho1* (Col-0).

Table S9. Primer sequences used for the validation of mutants.

Table S10. Primer sequences used for qRT-PCR based transcription analyses.

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Figure legends

Figure 1. Scopolin content and morphology of roots of *tho1* mutant lines in comparison to the respective wild type accessions. (A) HPLC-based analysis of root extracts from *tho1* (Col-0) in comparison to Col-0 wild type grown in low (0.5% [w/v]) and high (3% [w/v]) sucrose as well as in soil culture. Column heights indicate arithmetical means, error bars standard deviations [n=10 pools of 9 plants (sterile culture) or 20 individual plants (soil culture)]. *** indicates significantly different from wild type with $p<0.001$, ** significantly different from wild type with $p<0.01$, according to Student's *t*-test (sterile culture) or Mann-Whitney *U*-test (soil culture). (B) The scopolin content from plants grown for 24 days in sterile hydroponic culture of a second, independent T-DNA *tho1* (Ws) insertion mutant line in comparison to Ws wild type (n=30 pools of 9 plants). Box plots indicate medians and the 25% and 75% percentiles ("boxes"), the 5% and 95% percentiles ("whiskers") and outliers (circles); * indicates significantly different with $p<0.05$ according to Mann-Whitney *U*-test. (C) Root hair zone in *tho1* (Col-0) and wild type Col-0 plants exposed to 3% w/v sucrose in hydroponic culture. (a) Bright field image (va: vascular tissue, pe: pericycle, en: endodermis, co: cortex, rh: rhizodermis), (b) overlay of bright field and fluorescence images, and (c) fluorescence image; In *tho1* (Col-0) roots, the full set of cell layers is present, the tissues appear normal and scopolin is accumulated in the same tissues as in wild type roots, but to a lesser degree. Scale bars = 50µm.

Figure 2. Scopolin content of leaves of *tho1* mutants and respective wild type accession plants. (A) Mutants *tho1* (Col-0) and *tho1* (Ws) as well as Col-0 and Ws wild type. Left: Grown 4 weeks at 20°C. Right: Grown 4 weeks at 20°C plus 1 week at 10°C. Column heights indicate arithmetical means, error bars standard deviations (n=15 pools of 9 plants). ***, **, * indicate significantly different with $p<0.001$, <0.01 and <0.05, respectively, according to

Mann-Whitney *U*-test (left, 20°C) or Student's *t*-test (right, 10°C). (B) A second, independent T-DNA insertion *tho1* mutant in Col-0 background (SAIL_1209_F10) grown 4 weeks at 20°C and 1 week at 10°C (n=6 plants). Box plots indicate medians and the 25% and 75% percentiles ("boxes"), the 5% and 95% percentiles ("whiskers") and outliers (circles); ** indicates significantly different with p<0.01 according to Mann-Whitney *U*-test. (C) Scopolin distribution in leaves from plants grown 4 weeks at 20°C and 1 week at 10°C in wild type and the *tho1* (Col-0). Blue = scopolin fluorescence. Red = chlorophyll fluorescence. From top to bottom: Upper epidermis. Palisade parenchyma. Spongy parenchyma. Lower epidermis (Confocal laser scanning microscopy). Scale bars = 20 µm.

Figure 3. Scopolin content in further mutants affecting RNA silencing. LC-MS analysis of the leaf scopolin content of: (A) Mutant *rdr6-11* (*RNA-DEPENDENT POLYMERASE 6*) and wild type Col-0 grown for 4 weeks at 20°C (left) and 4 weeks at 20°C plus 1 week at 10°C (right) (n=15 pools of 9 plants). *** indicate significantly different with p<0.001 according to Mann-Whitney u-test. (B) Hypomorphic mutant *ago1-46* (*ARGONAUTE1*) and mutant *ago6* (*ARGONAUTE6*) compared to wild type Col-0 (n=10 plants). *** indicate significantly different with p<0.001 according to One-Way-ANOVA with Holm-Sidak correction. (C) Mutation *sgs3* (*SUPPRESSOR OF GENE SILENCING3*) and mutation *dcl4-2* (*DICER-LIKE4*) compared to wild type Col-0 (n=20 plants). *** indicates significantly different with p<0.001 according to Kruskal-Wallis One Way Analysis of Variance on Ranks with Dunn's correction. Box plots indicate medians and the 25^{th} and 75^{th} percentiles ("boxes"), the 5^{th} and 95^{th} percentiles ("whiskers") and outliers (circles).

Figure 4. Transcript levels of phenylpropanoid metabolism associated genes in the roots of Col-0 wild type and *tho1* (Col-0) plants at different time points after shifting from 0.5% to

3% [w/v] sucrose. Black columns represent Col-0 wild type, white columns mutant *tho1* (Col-0), column heights represent arithmetical means, and error bars standard deviations. ***, **, * indicate significantly different with $p<0.001$, <0.01 and <0.05, respectively, according to Student's *t*-test. The accompanying flow chart shows the key steps of phenylpropanoid metabolism simplified from Stracke et al. (2007). PAL1, phenylalanine ammonium lyase1; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; F3'H, flavonoid 3'-hydroxylase; DFR, dihydroflavonol 4-reductase; F6'H1 feruloyl-CoA 6' hydroxylase1; UGTs UDP-glycosyltransferases.

Figure 5. Explanatory model for the involvement of the THO complex in the control of scopolin accumulation based on RNA silencing according to Yelina et al. (2010) and Melnyk et al. (2011). DICER proteins mediate generation of primary short (s)RNAs such as miRNAs or siRNAs (phase 1), which are bound to ARGONAUTE proteins and direct cleavage of single-stranded target RNAs. SGS3 protein is thought to stabilize resulting cleavage fragments, which serve as templates for RNA-DEPENDENT RNA POLYMERASE6 synthesis of double-stranded (ds)RNA. DsRNAs are again processed by DICER proteins into secondary siRNAs, which in turn are loaded to ARGONAUTE proteins and act as signals for further gene silencing (phase 2). The THO/TREX complex might enhance RNA silencing by supporting the transport of sRNA precursors from their site of synthesis to their site of processing. The relevant target of RNA silencing in the context of scopolin accumulation might be a repressor of scopolin synthesis. Thus, with RNA silencing being defective in *tho* and *ago1*, *sgs3* and *rdr6* mutants, the repressor of scopolin synthesis would be expressed at higher levels and scopolin accumulation reduced.