A transportome-scale amiRNA-based screen identifies redundant roles of Arabidopsis ABCB6 and ABCB20 in auxin transport

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Transport of signaling molecules is of major importance for regulating plant growth, development, and responses to the environment. A prime example is the spatial-distribution of auxin, which is regulated via transporters to govern developmental patterning. A critical limitation in our ability to identify transporters by forward genetic screens is their potential functional redundancy. Here, we overcome part of this functional redundancy via a transportome, multi-targeted forward-genetic screen using artificial-microRNAs (amiRNAs). We generate a library of 3000 plant lines expressing 1777 amiRNAs, designed to target closely homologous genes within subclades of transporter families and identify, genotype and quantitatively phenotype, 80 lines showing reproducible shoot growth phenotypes. Within this population, we discover and characterize a strong redundant role for the unstudied ABCB6 and ABCB20 genes in auxin transport and response. The unique multi-targeted lines generated in this study could serve as a genetic resource that is expected to reveal additional transporters.
Plants are complex organisms that have the ability to respond to environmental cues. These responses are mediated by the rapid transport of a wide variety of substrates from one part of the plant to another. In addition to the primary metabolites found in all plant species, there are estimated to be more than 200,000 secondary metabolites, and these organic molecules regulate all aspects of plant biology. In many cases, plants spatially balance metabolites and restrict them to specific tissues and cell-types to allow proper growth and response to biotic and abiotic stresses. The active movements of metabolites fluctuate due to changes in the environment and depending on the developmental stage. A variety of mechanisms are involved in long and short distance transfer of metabolites, and active transporters can regulate both. A few examples are the BOR and NIP boron transporters, the NPF nitrogen transporters, the GTR glucosinolate transporters, the SWEET sucrose transporters, and the AAP and LHT amino acid transporters. Active transport of organic small molecules is not restricted to cell-to-cell transport or vasculature loading and unloading but also takes place at the subcellular level to allow intracellular compartment allocation.

Plant growth and development are mediated to a large extent by a group of small and mobile signaling molecules named hormones. Plants regulate hormone response pathways at multiple levels including biosynthesis, metabolism, perception, and signaling. In addition, plants tightly control the spatial hormone distribution. This is illustrated most clearly in the case of auxin, where the combined activity of auxin influx and efflux carrier proteins generates auxin maxima and minima that inform developmental patterning. The regulation of the cellular localization of PIN-FORMED (PIN) efflux transporters determines the direction of auxin flow from one cell to another. In addition, PILS and ABC family proteins transport auxin. Over the past 2 decades, forward genetic screens have proven to be powerful approaches in the search for gene function in general and in the isolation of transporters; however, there are no observable phenotypes for many single-gene loss-of-function mutants. As of 2013, only 2400 Arabidopsis genes (~8%) were documented to have a loss-of-function mutant phenotype, and 401 Arabidopsis genes (1.5%) were found to exhibit a mutant phenotype only when disrupted in combination with a redundant paralog. Thus, as evidenced previously discernable phenotypes in plants mutated in single transporters may be masked by the low frequency of recombination between adjacent genes. Altogether, these data suggest that a large fraction of potential phenotypic plasticity is "hidden".

Here, we utilize a transportome-scale artificial microRNA (amiRNA) approach, to overcome the challenge of functional redundancy in plant transport processes. We generated a unique population of 3000 amiRNA lines wherein each amiRNA was designed to target closely homologous genes within sub-clades in transporter families in a variety of combinations. Our phenotypic screen revealed 95 reproducible shoot phenotypes lines. Among these lines, 80 presented phenotypes that had not been associated with the targeted genes before. Out of the 95 lines, 26 were shown to exhibit differential response to different plant hormone treatments, indicating an involvement in hormone regulation. We chose to characterize one of these lines (amiR1334) due to a striking auxin-related shoot phenotypes. We show that amiR1334 targets the previously unstudied ABCB6 and ABCB20 genes, and through expression analyses and transport assays verify that ABCB6 and ABCB20 are redundantly required for the basipetal movement of auxin in the shoot. By genotyping and phenotyping each of the 95 lines, we have created a genetic resource that will be useful in understanding plant metabolite transport.

Results

Transportome multi-targeted amiRNA-based phenotypic screen. To overcome the potential genetic redundancy among plant transporter families (transportome), we utilized the PHANTOM amiRNA library designed to target Arabidopsis gene families. Hauser et al. generated a genome-scale amiRNA library by synthesizing 22,000 amiRNA constructs that putatively silence 18,117 genes in different combinations with 96% of the amiRNAs co-silencing 2–5 closely related genes.

A transporter-specific amiRNAs sublibrary, consisting of 1777 synthetic amiRNAs was transformed in bulk into wild-type (WT) Arabidopsis (Col-0). T1 seeds were selected for BASTA-resistance, and 3000 T1 plants were collected individually. T2 seeds of all 3000 lines were sown in soil and grown under normal conditions. A phenotypic screen was carried out from seedlings stage to flowering, with a focus on selecting phenotypes displaying altered shoot growth, leaf color, and morphology. We expected a dominant phenotype from the amiRNA, but in a few cases (3.6%) we detected clear recessive phenotypes that probably resulted from construct integration into a gene rather than from amiRNA activity. These lines were not included in further work. The screen identified 105 lines with interesting phenotypes. In order to validate the phenotypes, we selected homozygous T3 lines for the 105 lines and re-screened them. Of the 105 lines showing shoot growth or leaf morphology phenotype in the T2 generation, 95 had phenotypes that were reproducible in T3 lines. It is possible that the amiRNAs in the remaining lines were silenced over generations, which is a phenomenon that we often observe. We therefore continued to work only with the reproducible 95 amiRNA lines (Supplementary Table 1). The sequence of the amiRNA was successfully determined in 79 of these unique T3 lines, whereas we could not obtain sequence information of the amiRNA in 16 of the lines. The 79 amiRNAs collectively target 193 genes (Supplementary Table 1). Out of the estimated 1185 known Arabidopsis transporters, this number constitutes approximately 16% of the Arabidopsis transportome. We found 22 genes within 15 lines presenting previously reported phenotypes (Supplementary Figure 1), indicating on the screen strength. For example, we identified line 1162, targeting four PATL genes and line 686, targeting two PATL genes that show shoot growth defects (Supplementary Table 1). This result is in agreement with Tejos et al. who recently showed that while the single patl mutants do not present a phenotype, the quadruple patl2 patl4 patl5 patl6 present reduced PIN1 repolarization in response to auxin and disrupted plant development. We found 43 genes within 33 lines that presented previously reported phenotypes, which were different or partially different from the phenotypes we observed (Supplementary Figure 1). This could result from the co-silencing of additional genes in the family or by characterization of different developmental stages.

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and environmental conditions. Finally, we found 128 genes within 62 lines (66.3% of the identified genes) presenting previously unknown loss-of-function phenotypes (Supplementary Figure 1). Notably, the 193 target genes should be classified as putative targets as we have not validated off-targets effects for all lines. In addition, the results cannot exclude the possibility that several of the phenotypes are a result of down regulating only one of the target genes. In three different instances we found the same amiRNA transformed into multiple (2 or 3) independent lines showed similar phenotypes (Supplementary Table 1), suggesting that the screen is reliable but not saturated.

To further examine the reproducibility of the PHANTOM transportome amiRNA library results, we re-cloned and transformed plants with seven amiRNAs that showed strong growth phenotypes. Six out of the seven amiRNAs showed reproducible phenotypes that varied between independent transformation lines (Supplementary Figure 3). The observed variation between lines (52–87%) is equivalent to the report of amiRNA functionality and penetration by Schwab et al.40.

The 193 putative targets genes were classified into 11 groups according to the classification of the transporters database ARAMEMNON41 (Fig. 1a, Supplementary Table 1). In most groups, the percentage of potentially silenced genes within the family varied between 14 and 21% (Fig. 1b). The porins and p-type ATPase were at the extremes of percent at which phenotypes were observed, but this could not be statistically verified since the number of genes within these groups are relatively low. Interestingly, for the major facilitator superfamily (MFS) and the “other groups” (OG) less than 10% of genes in the family were co-silenced (Fig. 1b). In total, we identified 95 lines with observable phenotypes, among them 80 lines are association of gene function to phenotypes, exposing parts of the plant transportome phenotypic plasticity previously hidden by gene redundancy.

**Phenotypic and hormone response characterization.** Taking advantage of a high-throughput phenomics system, we evaluated photosynthesis, plant color shade, and shoot morphology parameters under normal growth conditions for the 95 homozygous amiRNA lines that showed reproducible growth phenotype. Data are summarized in Supplementary Table 1. Of the 95 lines, 65 showed significant plant height difference compared to WT. Six lines were taller than WT, whereas 59 lines were shorter (Fig. 1c). Color shade analysis showed several lines with differences in leaf color compared with WT plants. The color of amiRNA-32 leaves is visibly darker, whereas the color of amiRNA-426 leaves is visibly lighter (Fig. 1d, Supplementary Figure 1). In agreement, amiRNA-426 targets two genes, among them the ABC transporter AT3G17900, which shows resistance or hypersensitivity to different phytohormone treatments. In order to validate these hormone-related sub-screen results, we repeated all assays for all 32 lines. Twenty-six lines showed reproducible hypersensitivity or insensitivity to different phytohormone treatments; IAA: 7 lines; GA$_3$: 3 lines; ABA: 7 lines; t-Zeatin (CK): 6 lines; and JA: 7 lines (Fig. 2, Supplementary Figure 6). It should be noted that these results do not necessarily indicate that the amiRNA-targeted gene has a direct role in hormone transport. However, this sub-screen served as a starting point for identification of putative hormone transporter groups.

**ABC6 and ABC20 redundantly regulate shoot growth.** amiR1334 (amiRNA-1334 line) presented a striking developmental phenotype of shoot growth inhibition and small leaves (Fig. 3a, Supplementary Figure 7A). As the plants progressed through developmental stages, the amiR1334 phenotype was enhanced showing short inflorescence stems and skewed small leaves (Fig. 3a, Supplementary Figure 7A, Supplementary Figure 8D, F), similar to known defects in auxin responses and distribution43,44. Interestingly, amiR1334 targets two closely related genes from the ABCB family; ABCB6 and ABCB20 (AT2G39480 and AT3G55320) (Fig. 3b). These genes are of particular interest since several members of the ABCB family, including ABCB11, ABCB19, ABCB21, and ABCB4 have been shown to mediate auxin transport through the plasma membrane45–53. The functions of the closely related ABCB6 and ABCB20 have not been previously studied nor linked to auxin transport. The amiR1334 target sequence has two nucleotide mismatches with ABCB6 and ABCB20, and five mismatches with ABCB1 (AT2G36910), including a mismatch in the 3’ region of the seed sequence, and no additional targets are detected in silico (Supplementary Figure 7b). Therefore, we speculated that the phenotype of amiR1334 is predominately caused by the down-regulation of ABCB6 and ABCB20.

In order to determine if the amiR1334’s target genes (ABCB6 and ABCB20) are involved in auxin response, we generated homozygous T-DNA-insertion lines for ABCB6 and ABCB20 (Supplementary Figure 8a) and performed a hypocotyl elongation assay on the amiR1334 line, the single T-DNA mutant lines and WT (Fig. 3c). Plants were transferred to Murashige-Skoog plates with 5 µM IAA, 3 µM NAA, or 100 nM of the auxin derivative 2,4-dichlorophenoxyacetic acid (2,4-D). Hypocotyls of amiR1334 seedlings showed a normal response to IAA, but a reduced response to the synthetic auxin NAA and 2,4-D treatment (Fig. 3d). A similar trend was observed in the single T-DNA-insertion mutants of ABCB6 and ABCB20 (Fig. 3d). In addition, the amiR1334 line showed shoot growth hypersensitivity to the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) (enhanced shoot growth inhibition) (Supplementary Figure 8b,c). The partial insensitivity of amiR1334 to synthetic auxin and the hypersensitivity to NPA suggested that ABCB6 and ABCB20 may be involved in auxin response in the shoot.

In order to genetically verify the cause of the amiR1334 phenotype and assess the specific contribution of ABCB6 and
ABC20 genes to the phenotype, we generated single and double mutant T-DNA abc6-1 abc20-1 lines (Fig. 3c). Whereas the single abc6-1 (GABI_5401D12) and abc20-1 (GABI_520G10) mutants did not show any significant growth phenotype, the abc6-1 abc20-1 double mutant exhibited a strong developmental phenotype that mimicked that of the amiR1334 line with dwarf stature and small skewed leaves (Fig. 3e, f, Supplementary Figure 8D,F). We further obtained and genotyped an additional, independent abc20 allele (GABI_387F09) termed abc20-2 (Fig. 3c, Supplementary Figure 9A). The abc6-1 abc20-2 double mutant showed identical phenotypes to abc6-1 abc20-1 double mutant and amiR1334 line (Fig. 3g, Supplementary Figure 8D, F, Supplementary Figure 9B-E). Nanostring experiments showed that transcript levels of ABC6 and ABC20 are significantly
down regulated in both alleles of abcb6 abcb20 double mutant and amiR1334 line (Fig. 3h, Supplementary Figure 9F, Supplementary Figure 13B). The transcript levels of the related auxin transporters ABCB1 and ABCB19 did not change in these lines (Supplementary Figure 10a,b). This result suggests that the phenotype of the amiR1334 line is due to down-regulation of ABCB6 and ABCB20 transcripts and that ABCB6 and ABCB20 share overlapping activities.

We further tested the expression level of all ABCBs in amiR1334 plants and abcb6-1 abcb20-1 compared to WT. While ABCB6 and ABCB20 were significantly down regulated in both amiR1334 and abcb6-1 abcb20-1 compared to WT, four additional ABCBs (ABCBS 5,7,16,17) were slightly down regulated (non-significant) and two ABCBs (ABCBS 18 and 22) were slightly up-regulated (non-significant) (Supplementary Figure 11).

Auxin regulates many aspects of plant growth and development including phyllotaxis. Interestingly, the abcb6-1 abcb20-1 double mutant showed strong phyllotaxis defects as siliques initiated in a non-spiral phyllotactic arrangement with an average angle of 150° compared to 134° in the WT (Fig. 3i, j). The defects in phyllotaxis were observed both in angle and in spacing between lateral organ initiation sites (Supplementary Figure 12a). However, the defects on angle organ initiation is also attributed by the twisting of the stem in abcb6-1 abcb20-1 double mutant.

**Fig. 2** Transporter-targeted amiRNA lines show significant developmental responses to plant hormones. Gray lines connect transporters groups, amiRNA lines, and their putative gene targets. Transporter groups are indicated by the inner gray circles. amiRNA lines showing significant developmental responses to particular hormones are indicated by enlarged color-coded diamonds (middle circle). Putative amiRNA target genes are indicated in enlarged gray squares (outer circle). amiRNA lines with insignificant developmental responses to hormones are indicated by the small gray diamonds (middle circle) with putative amiRNA target genes indicated by small gray squares (outer circle). amiRNA designated 973* indicates for three independent amiRNA lines (973, 2182, 2410) with the same target sequence in Supplementary Table 1. Abbreviations: ABC ATP-binding cassette transporter, DMT drug/metabolite transporter, MFS major facilitator superfamily, OG other groups, APC amino acid/polyamine/organo-cation, OCCG other cation carrier groups (i.e., CPA, CaCA, CDF, CaCA2), MC mitochondrial carrier, Others (additional transporters that do not belong to families mentioned above), NS non-sequenced.
In addition, the siliques of amiR1334 plants were slightly twisted and the siliques of abcb6–1 abcb20–1 double mutant plants were highly twisted (Fig. 3k), possibly due to uneven growth of the epidermis and inner mesophyll layers as reported for abcb1 abcb19 and twd1 mutants55. The amiR1334 and abcb6 abcb20 double mutant but not the single mutants showed weak but significant root growth phenotype (Supplementary Figure 8e). In summary, these findings strongly suggest that ABCB6 and ABCB20 redundantly function to regulate shoot growth.

**ABCB6 and ABCB20 function redundantly in auxin export.** In order to determine if ABCB6 and ABCB20 facilitate auxin export,
transport, as suggested by loss-of-function phenotypes and activities of the characterized family members, we carried out auxin transport and response assays in whole plants and protoplasts. First, we utilized the auxin reporter pDR5::GUS and evaluated the response to auxin following IAA treatment on the apical side of the inflorescence stem (Fig. 4a, b). Strong GUS staining was detected after 5.5 h at the basal side in WT background, but in contrast, no signal was detected from pDR5::GUS in the amiR1334 background (Fig. 4b, Supplementary Figure 13a), suggesting that basipetal auxin transport was disrupted in this line. Next, we analyzed polar auxin transport in stems by applying radiolabeled IAA to the apical or basal side of the inflorescence stem and detected its accumulation on the opposing side after 4 h. This showed a statistically significant reduction (~50%) in polar auxin transport in the inflorescence stems of the double mutant abcb6-1 abcb20-1 compared to that in WT (Fig. 4c), in agreement with a significant ABCB6 and ABCB20 transcriptional down-regulation in stem segments compared to WT (Supplementary Figure 13a). This indicates that ABCB6 and ABCB20 are essential for basipetal polar auxin transport. We furthermore tested export of radiolabeled IAA in a cellular system using isolated Arabidopsis leaf mesophyll protoplasts. In this system, abcb6-1 and abcb20-1 single mutants showed a 54% and 70% reduction in export activity, respectively, compared to WT, while the abcb6-1 abcb20-1 double mutant and the amiR1334 auxin export was lower by about 73% and 66% compared to that in WT protoplasts (Fig. 4d). We further tested the export of benzoic acid (BA), which is often used as a diffusion control in auxin research and is known to be transported by ABCB1456. Transport of both substrates was not significantly different in abcb6-1 and abcb20-1 mutant protoplasts indicating the specificity of ABCB6 and ABCB20-mediated auxin transport (Supplementary Figure 14a,b). Altogether, these results suggest that ABCB6 and ABCB20 specifically promote auxin export and act redundantly to regulate shoot growth.

In order to better understand how ABCB6 and ABCB20 participate in the cellular regulation of auxin transport activity we tested their subcellular protein localization. We were unable to participate in the cellular regulation of auxin transport activity we used promoter fragments to drive GUS-GFP reporters. Transgenic plants expressing pABCB6::GUS-GFP showed a GUS signal in true leaves and the differentiated root vasculature tissues (Fig. 4g). Consistent results of ABCB6 expression in root vasculature were obtained by imaging pABCB6::YFP plants (Fig. 4h). pABCB20::GUS-GFP showed a similar GUS signal in leaves (Fig. 4i). In addition, auxin treatment did not affect the expression levels of ABCB6 and ABCB20 (Supplementary Figure 15). Altogether, these results indicate that ABCB6 is plasma membrane localized and that, together with ABCB20, is required for auxin transport to regulate shoot growth and development.

Discussion

As sessile organisms, plants are particularly susceptible to the ever-changing environment and other external conditions. In order to compete genetically under such conditions, plants have developed robust genetic functional redundancy at the expense of gene specialization. Functional redundancy can be achieved by two mechanisms: First, multiple parallel metabolic pathways that eventually lead to the same outcome can be driven by different genes. Second, multiple homologous genes, resulting from gene duplication, can perform the same or overlapping functions. Retaining duplicated genes can be beneficial as these duplicates buffer fundamental processes from the detrimental effects of random mutations. Plant genomes are highly redundant as over 75% of Arabidopsis genes belong to families with at least two members. As a result, most single null mutants do not present an evident phenotype as the overlapping function of one (or more) paralogs might mask phenotypic effects. This fact limits the utility of classic forward genetic studies, which use random mutagenized lines created by chemical or radiation treatments, to identify the players responsible for different processes in plant development and response to the environment. In order to reveal the functions of redundant genes, Hauser et al. developed and produced libraries of amiRNAs targeting different gene families. By utilizing Hauser’s amiRNA plasmid library targeting genes of the transportome, we identified 80 amiRNA lines presenting novel phenotypes. In this screen, the sequence of 79 amiRNA collectively targeted 193 genes which constitutes approximately 16% of the known Arabidopsis transporters (estimated 1185 transporters in Arabidopsis). Classified into 11 groups according to the classification on ARAMEM-NOX (Fig. 1a), the percentage of potentially silenced genes within the family varied between estimated 14 and 21% in most groups (Fig. 1b). This suggests that genetic redundancy plays an important role across the transportome. The porins and p-type ATPase were at the extremes of percent at which phenotypes were observed, but this could not be statistically verified since the number of genes within these groups are relatively low. Interestingly, for the MFS and the OG less than 10% of genes in the family were co-silenced (Fig. 1b). One explanation for this difference is that these families have relatively low redundancy, in other words high specification. Another explanation is that these
families are too redundant to be exposed in our experimental design.

The evolutionary conserved ABC family is one of the largest transporter families in plants, and plant ABCs have been shown to transport diverse hormones. For example, ABCB isoforms have been demonstrated to transport auxin, while some ABCG isoforms transport cytokinin and ABA as well as other compounds (reviewed in ref. 59). The redundant auxin transport activity of ABCB1 and ABCB19 and redundant arsenic tolerance of ABCC1 and ABCC2 in Arabidopsis are an example of phenotypic robustness of this family50,60. Therefore, the ABCs are an extreme example of gene expansion that provides redundantly robust responses and diverse specialization in plant hormone transport.

Unlike single T-DNA mutants of ABCB6 and ABCB20, the abcb6 abcb20 double mutant showed a strong developmental phenotype revealing a very small stature, curly, skewed leaves, and shorter inflorescence stems, which resembled that of amiR1334 plants suggestive of defects in auxin transport.
result strongly indicates that the homologous genes, ABCB6 and ABCB20, are functionally redundant and have a crucial role in shoot growth.

Functional redundancy of ABCB6 and ABCB20 in auxin transport was further supported by quantification of developmental phenotypes of amiR1334 and of the single T-DNA mutants on various natural and synthetic auxins as well as on auxin transport inhibitors. The shoot surface area of amiR1334 and the single T-DNA mutants grown on the non-competitive auxin efflux inhibitor, NPA, was significantly smaller than with mock treatment indicating hypersensitivity to NPA (Supplementary Figure 8b,c). Since NPA inhibits ABCB protein function in auxin transport\(^{38}\), the combination of down-regulating ABCB6 and ABCB20 activity together with NPA treatment (which has multiple ABCB targets\(^{61}\)), resulted in a synergistic effect and a hypersensitivity of amiR1334. In another classical auxin-related assay, hypocotyl elongation of amiR1334 and the single abcb6 and abcb20 mutants did respond to IAA but not to the synthetic auxin transport inhibitors. The shoot surface area of mutants did respond to IAA but not to the synthetic auxins, 2,4-D and 1-NAA (Fig. 3d), both shown to either not act as an ABCB substrate or to bypass carrier-mediated import by simple diffusion\(^{40}\). In most of the phenotypic and transport assays, the impact in abcb6 abcb20 is similar to amiR1334 (Fig. 3, Supplementary Figure 7-9), suggesting that the phenotype generated by amiR1334 is indeed caused by silencing of its putative targets ABCB6 and ABCB20. Although the results show some specificity towards auxin transport activity (ABCB6 and ABCB20 did not affect the transport of BA and malic acid), it is possible that these ABCBs may have additional substrates; dual or multiple substrate specificities are not uncommon among ABCB transporting proteins\(^{32}\). It is unclear at this point whether ABCB6 and ABCB20 have further redundant activities with other known ABCB auxin exporters, such as ABCB1 and ABCB19.

The amiRNA screen offers many advantages over classical forward genetic screens. Unlike chemical mutagenesis, often utilized in classical forward genetic screens, amiRNAs are gene-specific and a single amiRNA can be designed to target multiple, related genes. The ease of identification of silenced genes in the amiRNA lines is another huge advantage. In addition, there are multiple examples of gene duplication that result in tandem duplicates in the genome; often these genes are highly redundant. Since these genes are strongly linked, it is very complicated to create double and triple mutant T-DNA-insertion lines. The amiRNA approach overcomes this limitation of targeting multiple linked genes while enabling forward genetic screens.

The amiRNA library screen is a new tool that needs to be further studied, however, we do not know, for example, the off-target rate\(^{40}\) for the different amiRNAs investigated here, which will lead to noise in the screen. Off-target effects have been extensively explored in the CRISPR system for genome editing\(^{42}\) and should be evaluated in amiRNA systems as well. In addition, the fact that amiRNAs can target multiple homologous genes at once is a two-sided coin. The amiRNA library used in the present screen was designed such that 96% of the amiRNAs are predicted to target 2–5 genes\(^{38}\). This makes it an outstanding tool to reveal functions of unknown redundant genes. amiRNAs produce knock-down mutants, and are predicted to minimize generation of lethal knock-out mutations\(^{38,40}\). Nevertheless, lethality cannot be completely excluded. This could be partially overcome by expressing amiRNAs under an inducible promoter. In addition, from our observations, activities of a few amiRNA lines weakened over generations. This might be a result of silencing of amiRNA expression. To overcome this difficulty for a specific line one can reproduce the phenotype with additional methods that result in stable mutations such as T-DNA and CRISPR. To overcome the silencing issue at the screening level the amiRNA could be expressed under an inducible promoter.

In some rare cases, the amiRNA insert produces a loss-of-function mutant where the phenotype results from interference with the coding sequence in the genome thus knocking out a gene rather than down regulating expression of other genes through an RNA interference mechanism. We observed 3.6% recessive mutations in our screen. These are easily identifiable, since the mutation, in most cases, is recessive and the phenotype is present in only 25% of T2 plants (in contrast to the dominant amiRNA effect which appears in 75% of T2 plants). By re-cloning and transforming several amiRNAs into plants we showed that the transportome amiRNA screen results are highly reproducible (6 out of 7 lines). However, among these lines, we observed significant variation between independent transformation lines, as 52–87% of the lines showed the expected phenotype. While such variation was previously reported for amiRNA activity in plant\(^{40}\) one might take this into account in future work and should examine multiple independent transformation lines (around 50 lines).

Finally, we found that the most challenging step in the screen was designing the screen in a way that allows the isolation of lines directly involved in the desired process. For example, it is likely that only a few of the 26 lines identified here that show significant hormone responses are indeed the direct down regulation of hormone transporters. Image-based screens using specific fluorescent markers or selection-based screens might be a powerful tool to combine with the amiRNA multi-targeted approach.

In summary, we showed that ABCB6 and ABCB20 redundantly function to regulate shoot growth and likely function as auxin transporters involved in basipetal auxin transport. We expect that the resources generated here linking uncharacterized genes with novel quantitative phenotypes, arising from multi-targeted gene silencing, will be utilized by the community to reveal new mechanisms in hormone signaling and small molecule transport.
Methods
Plant material and growth conditions. All Arabidopsis thaliana lines used in this work are in Columbia background (Col-0 ecotype from Salk Institute). For assays on plates, sterilized seeds were plated on 16 × 16 cm square petri dishes with growth media containing 0.5x Murashige-Skoog (MS) medium (pH 5.7, 1% sucrose, 0.8% plant agar). The seeds were stratified for 2–3 days at 4°C then transferred to growth chambers (Percival, CU41L5) at 21°C, 100 µE·m⁻²·s⁻¹ light intensity under long-day conditions (16 h light/8 h dark). For seed production, transformation, cross-feeding, and soil pot assays, seeds were sown onto wet soil, and plants were grown in growth rooms under long day conditions (16 h light/8 h dark) at 21°C. Sequence data for Arabidopsis genes used in this study can be found in the Arabidopsis Genome Initiative under the following accession numbers: ABCB6 (AT1G39480) and ABCB20 (AT3G55320).

amiRNA transportome library generation. A plasmid library expressing a pool of amiRNAs was designed and synthesized as described in Hauser et al.88. Agrobacterium (GV3101) was transformed with Plasmid pSOUP. The amiRNA library, constructed in pGREEN, was then transformed into the Agrobacterium and grown on antibiotic selections (Gentamycin, Rifampicin, Spectinomycin, and Tetra-cycline). The Agrobacterium plasmid library was transformed in bulk into six trays of Arabidopsis Col-0 plants. Seeds were collected in bulk and sown on 120 trays with soil and sprayed with BASTA for selection at the age of 2 weeks. About 3000 resistant transgenic plants were propagated, and T2 seeds were collected from individual lines. Phenotypic screens were done on the T2 generation. Lines showing a reproducible phenotype were selected, and phenotypes were characterized in T3 homozygous plants.

Agrobacterium transformation. Electro-competent Agrobacterium tumefaciens strain GV3101 was incubated on ice with 100ng plasmid for 5 min, then electroporated in a MicroPulser (Bio-Rad Laboratories) (2.2 kV, 5.9 ms). Bacteria were transferred immediately to 1-ml liquid LB tubes and shaken for 2 h at 28°C. T1 colony was chosen and verified by sequencing.

Genotyping. amiRNA lines were genotyped by PCR using primers listed in Supplementary Table 2. The sequence obtained was aligned with the miRNA319 backbone and the 21 amiRNA base pairs were used to identify putative gene targets using the PHANTOM database.38 T-DNA lines for the single mutants, listed in Supplementary Table 3, were ordered from Gabi Kat (https://www.gabi-kat.de/) and The Arabidopsis Information Resource (http://www.arabidopsis.org). Primers for the T-DNA genotyping were designed using the T-DNA Primer Design Tool powered by Genome Express Browser Server (GEBD) (http://signal.salk.edu/tdnaprimer2.2.html). Homozygous mutants were selected by PCR performed with primers listed in Supplementary Table 4.

Cloning. ABCB6 CDS was cloned from Col-0. CDS was amplified in PCR using primers listed in Supplementary Table 5. ABCB6 and ABCB20 promoters (2323 bp upstream to AGC start codon of ABCB6 and 638 bp for ABCB20) were cloned using primers listed in Supplementary Table 5. The T-DNA sequences are deposited in GenBank (accession number KX2400).

Phenomics. Morphological and photosynthesis parameters were analyzed with the PlantScreen™ Phenotyping System, Plants Systems Instruments (PSI), Czech Republic. Plants were sowed in PSI standard pots and imaged at day 25.

Hypocotyl elongation assay. Seeds were sown on MS plates and stratified at 4°C for 2–3 days. Seedlings were grown vertically in long day conditions (16 h light) for 4 days then transferred to treatment plates (MS with chemicals) and grown vertically for another 3 days before seedlings were imaged using a Zeiss Steco 2000-C stereo microscope and measured using the ImageJ software (http://rsweb.nih.gov/ij/index.html).

Shoot characterization. Shoot size, biomass, height, and angles between silencers, and silencing time measurements, plants were sown on soil, one plant per pot, and measurements were taken at time points indicated in figure legends with at least 12 plants for each genotype. Phyllotactic patterns were analyzed on fully grown inflorescence stems of 7-week-old plants as described in Peaucelle et al.85. Divergence angles and internode length were measured simultaneously.

Hormone treatment assays. All the lines were sown on 1/2 MS plates and incubated in a Percival growth chamber for 4 days after the cold stratification (4°C for 2 days). Seedlings were transferred to 1/2 MS plates supplemented with hormone: 1 µM trans-ABA, 1 µM CK (t-Zein, trans-isomer), 5 µM GA3, 250 nM IAA (root assays), 5 µM IAA (hypocotyl assays), or 50 µM JA. After 3-day treatment, plates were scanned and root length was measured using a Fiji system89.

Construction of phylogenetic tree and network construction. A phylogenetic tree of ABCB family members based on protein sequence was constructed using sequences alignment by Clustal W (software http://www.clustal.org/clustal2). One neighbor-joining phylogenetic tree was constructed using MEGA7.0 (Molecular Evolutionary Genetics Analysis) software, with 1000 of bootstrap replications. The network graph (Fig. 2) was visualized using Cytoscape (http://www.cytoscape.org/).

Statistical analysis. Two-tailed Student’s t-test was performed whenever two groups were compared. Statistical significance was determined at P = 0.01 unless otherwise indicated. All pairs, Tukey HSD in JMP software (JMP Statistical Discovery) was used for Nonparametric statistical analysis.

Fluorescent imaging. Seedlings were imaged on a laser scanning confocal microscope (Zeiss LSM 780) with argon laser and GaAsP detector. For protoplast imaging, protoplasts were stained in 100 µM FM-64 for 1 min and washed by W5 solution and imaged within 80 min. For root imaging, seedlings were stained in 10 mg/L propidium iodide for 1 min, rinsed, and mounted in water.

Scanning electron microscopy. Scanning electron microscopy was conducted as described89, with the following exception: samples were fixed in 2.5% glutaraldehyde in PBS. After washing, samples were dehydrated by successive ethanol treatments. Following critical point drying (Balzer’s critical point dryer), the samples were mounted on aluminum stubs and sputter-coated (SC7620, Quorum) with gold. Images were captured using a Jeol JCM-6000 scanning electron microscope.

Histochemical GUS-staining. The GUS assay was performed as previously described86 with minor modifications. Plant tissues were incubated for approximately 16 h at 37°C in 100 mM sodium phosphate buffer (pH 7.0) containing 0.1% Triton X-100, 1 mM 5-bromo-4-chloro-3-indolyl-phosphate (BrdU) and 4-methylumbelliferyl-D-glucuronide salt (X-gluc, Sigma-Aldrich), 2 mM potassium ferricyanide, and 2 mM potassium ferrocyanide. Tissues were immersed with 70% ethanol until transparent. GUS-stained tissues were examined and imaged using Zeiss Steemi 2000-C stereomicroscope. Images were captured using ZEN software (Zeiss).

Auxin transport assays. Polar auxin transport assays in Arabidopsis inflorescence stems were measured using [14C]indole acetic acid ([14C]IAA) using a modification of a procedure described in Okada et al.87. When Arabidopsis inflorescence stems were 14–15 cm long, 2.5-cm long segments from the apical tip (not including floral parts) were harvested. The apical/basal side of each segment was then submerged in 30 µl of 0.25 MS solution (0.5% sucrose, pH 5.7) containing 1.8 µM [14C]IAA and 250 nM IAA and incubated at room temperature for 8 h. Based on the orientation of the inflorescence segment in the tube, apoplastic (polar auxin transport) or basipetal (control) auxin transport was measured. After incubation, the segment was removed and the last 5 mm of the non-submerged end was excised and placed into 3 ml scintillation liquid (Opti-Fluor). The samples were allowed to sit for at least 24 h in darkness before being counted in a liquid scintillation counter. The amount of [14C]IAA transported to the end of the segment was reported as CPM. Relative export of radioactivity from protoplasts was calculated as described90, with the following exception: samples were fixed in 2.5% glutaraldehyde in PBS. After washing, samples were dehydrated by successive ethanol treatments. Following critical point drying (Balzer’s critical point dryer), the samples were mounted on aluminum stubs and sputter-coated (SC7620, Quorum) with gold. Images were captured using a Jeol JCM-6000 scanning electron microscope.

Polar auxin transport assays based on pDR5:GUS expression were performed in inflorescence stems of Arabidopsis plants expressing amiR1334 in the pDR5:GUS background. The assay was performed on the F1 generation of the amiR1334 × pDR5:GUS cross and WT crossed with pDR5:GUS plants as a control. When Arabidopsis inflorescence stems were 16–15 cm long, 2.5 cm long segments from the apical tip (not including floral parts) were harvested. The apical side of each segment was then submerged in 30 µl of 0.25 MS solution (0.5% sucrose, pH 5.7) containing 5 µM IAA and incubated at room temperature for the duration of 5.5h. After incubation, an overnight GUS staining was carried.
Nanostring and quantitative RT-PCR. Total RNA was isolated from the indicated plant materials using NCode Plant Mini Kit (QIAGEN 74,904). For NanoString, 66 NanoString probes were synthesized by NanoString Technologies Inc., Seattle, USA. The custom-designed probes included 100-bp region targeting the mRNA, with sequence-specific, fluorescent-barcoded probes for each target. Probes and 100 ng total RNA were hybridized overnight at 65 °C according to the manufacturer’s protocol. A NanoString nCounter Digital Analyzer (NanoString Technologies, Seattle, USA) was used to count the digital barcodes representing the number of transcripts. The resulting data were normalized again with the geometric mean of the housekeeping genes (PP2A, CBP20, and ACT2). Nanostring probes are listed in Supplementary Table 6.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Generated plasmids and Arabidopsis lines are available from the corresponding author upon request.

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**Author contributions**

Y.Z. and E.S. conceived and designed the study and wrote the manuscript. Y.Z. performed the research, V.N. carried out parts of the experiments presented in Figs. 3 and 4. O.P. carried out the amiRNA library transformation and the Nanostring experiments. M. O. assisted in ABCB6 cloning and imaging. N.W. performed hormone transport assays. I. T. assisted with library transformation and data analysis. M.D.D. and P.H. carried out auxin transport assays and the ABCB6 subcellular localization. O.R. performed the SEM experiments. F.H., H.F., J.I.S., H.H.N.-E., and M.G. designed and supervised the work and edited the manuscript. F.H. and J.I.S. also provided amiRNA library information prior to publication. All authors discussed the results and commented on the manuscript.

**Additional information**

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