RESEARCH PAPER

A seed resource for screening functionally redundant genes and isolation of new mutants impaired in CO₂ and ABA responses

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Received 10 July 2018; Editorial decision 10 October 2018; Accepted 12 October 2018

Editor: Steve Penfield, John Innes Centre, UK

Abstract

The identification of homologous genes with functional overlap in forward genetic screens is severely limited. Here, we report the generation of over 14,000 artificial microRNA (amiRNA)-expressing plants that enable screens of the functionally redundant gene space in Arabidopsis. A protocol was developed for isolating robust and reproducible amiRNA mutants. Examples of validation approaches and essential controls are presented for two new amiRNA mutants that exhibit genetically redundant phenotypes and circumvent double mutant lethality. In a forward genetic screen for abscisic acid (ABA)-mediated inhibition of seed germination, amiRNAs that target combinations of known redundant ABA receptor and SnRK2 kinase genes were rapidly isolated, providing a strong proof of principle for this approach. A new ABA-insensitive amiRNA line that targets three avirulence-induced gene 2(-like) genes was isolated. A thermal imaging screen for plants with impaired stomatal opening in response to low CO₂ exposure led to the isolation of a new amiRNA targeting two essential proteasomal subunits, PAB1 and PAB2. The seed library of 11,000 T2 amiRNA lines (with 3000 lines in progress) generated here provides a new platform for forward genetic screens and has been made available to the Arabidopsis Biological Resource Center (ABRC). Optimized procedures for amiRNA screening and controls are described.

Keywords: Arabidopsis, artificial microRNA, forward genetic screen.

Introduction

The presence of large gene families in plants, including Arabidopsis (Arabidopsis Genome Initiative, 2000), leads to functional genetic redundancies or partial functional overlap among closely related genes. Functional overlap and partial or complete redundancy between different family members has been proposed to provide a buffer for loss or gain of function mutation events and mechanistic robustness of cellular networks (Wagner, 2005). This is considered to be a main reason for the lack of observable phenotypes in single-gene deletion mutants and increasing severity of phenotypes in higher
order mutants of homologous genes (Ma et al., 2009; Park et al., 2009). Identification and characterization of functionally overlapping genes in genetic screens is limited, as is evident from the relatively low number (591 of all Arabidopsis genes) of genes not associated with a single mutant phenotype (Lloyd and Meinke, 2012). Analysis of genome-wide gene family definitions showed that the Arabidopsis genome includes over 22,000 genes belonging to gene families (Hauser et al., 2013). Strategies and tools have been developed to enable screens of the functionally redundant gene space. Recently, an artificial microRNA (amiRNA)-based computational design approach was introduced (Hauser et al., 2013). AmiRNAs designed to specifically target diverse combinations of gene family members or combinations of subfamily members enable the screening of partial overlapping homologous gene functions at a genome-wide scale. The presented platform also provides an approach for the capture of homologous gene silencing phenotypes, for which higher order loss of function mutants would lead to lethality, as illustrated by a mutant identified here.

Here, we report the generation of over 11,000 T2 amiRNA lines and 3000 additional amiRNA lines by transformation of Arabidopsis Col-0 with a previously published amiRNA library (Hauser et al., 2013) and screening of T2 amiRNA lines for abscisic acid (ABA)-insensitive seed germination phenotypes or plants with low-CO$_2$-insensitive high-leaf-temperature phenotypes. Methods are described to identify robust amiRNA mutants and how to avoid pitfalls of this approach. The screen rapidly identified two amiRNAs that target three PYR/RCAR ABA receptor- (Ma et al., 2009; Park et al., 2009) or six SNF1-related kinase- (StaKK2; Mustilli et al., 2002; Yoshida et al., 2002; Fuji and Zhu, 2009) encoding genes known to be involved in ABA-mediated control of seed germination. One candidate line that shows an ABA-insensitive seed germination phenotype contains an amiRNA that targets three genes of unknown function, which are annotated as Avrincence-induced gene 2 (AI2G2A; AT3G28930), Avrincence-induced gene 2-like protein A (AI2G2A; AT5G39720), and Avrincence-induced gene 2-like protein B (AI2G2LB; AT5G39730). One amiRNA that causes a low-CO$_2$-insensitive high-leaf-temperature phenotype targets two genes encoding proteasomal α2-subunits, annotated as PAB1 (AT1G16470) and PAB2 (AT1G79210), for which double mutation causes lethality. New amiRNA lines that target the gene for proteasomal α7-subunit, annotated as PAG1 (AT2G27027), were constructed resulting in a similar stomatal phenotype. Together these observations indicate a rate-limiting role of the intact proteasome for stomatal opening responses.

Materials and methods

Plant material, growth conditions and transformation

Arabidopsis accession Columbia-0 was used as the background for all amiRNA transformations of the library. Surface-sterilized seeds (15 min 70% ethanol, 0.1% sodium dodecyl sulfate; three to four washes with ~100% ethanol; alternatively 10 min 50% bleach, 0.05% Tween-20; four to six washes with water; Lindsey et al., 2017) of Arabidopsis were cold-treated for 2–5 d at 4°C and germinated on half-strength Murashige and Skoog basal medium supplemented with Gamborg's vitamins (Sigma-Aldrich (Murashige and Skoog, 1962; Gamborg et al., 1968), 0.8% Phytoagar (Difco, Franklin Lakes, NJ, USA) and pH adjusted (pH 5.8; 2.6 mM MES titrated with potassium hydroxide). After 5–7 d, plants were transferred to plastic pots containing sterilized premixed soil (Sunshine Professional Blend LC1 RS; Sunshine; supplemented with an appropriate amount of insecticide (Marathon, Gnatrol) and propagated under the following conditions: long day (16 h light/8 h dark); 23–27°C; 20–70% humidity, 60–100 mmol m$^{-2}$ s$^{-1}$ light.

Plant transformation by floral dip was performed as described elsewhere (Clough and Bent, 1998) with the following modifications. Agrobacterium tumefaciens GV3101::pMP90 (Koncz and Schell, 1986) was grown under selection of all markers, i.e. genomic (rifampicin), Ti-plasmid (gentamicin), pSOUP (tetracycline) and T-DNA plasmid (spectinomycin). The infiltration medium for resuspension of the bacteria and floral dip contained 5% sucrose (w/v) and 0.02% (v/v) Shwet L-77 (Clough and Bent, 1998).

Large scale transformation with the amiRNA library pools (Hauser et al., 2013) was performed as described elsewhere (Cutler et al., 2000) with the following modifications. One microgram of DNA from each amiRNA sublibrary (Hauser et al., 2013) was electroporated into a total of 500 µl electrocompetent A. tumefaciens cells. The 20 bp and 21 bp amiRNA sublibrary variants for each pool were individually electroporated and combined at this stage. After 2 h at 30°C in non-selective Luria–Bertani–Miller medium (LB, Teknova), the cells were spread on 20 LB plates (1.5% agar; 150 mm diameter) containing all the appropriate antibiotics (rifampicin, gentamycin, tetracycline, spectinomycin) and grown for 3 d at 30°C. The bacteria were scraped from the plates, resuspended in 5 ml LB and concentrated by centrifugation for 20 min at 5855 g. Plants were transformed by spraying the flowers with this suspension of the bacteria in infiltration medium (adjusted to an optical density at 600 nm of 0.5) twice with 1 week between the treatments. T1 plants were selected on plates supplemented with 75 µM phosphotinric or directly on soil by spraying diluted herbicide (1000× dilution, Finalé®; Bayer, Research Triangle Park, NC, USA) four times with 2–7 d between the treatments. Herbicide-resistant plants were transferred to soil and grown to full maturity and T2 seeds collected from individual plants. When appropriate, media for growth of bacteria or plant selection contained the following concentrations of antibiotics (µg ml$^{-1}$): carbenicillin 100, gentamycin 25, kanamycin 30, rifampicin 50, spectinomycin 100, tetracycline 10, and phospotinric 15.

Screen for abscisic acid-insensitive cotyledon emergence phenotype

T2 plants were screened individually for insensitivity of seed germination to ABA in 96-well plates (100 µl 0.1% agarose supplemented with 2 µM (±)-ABA, Sigma-Aldrich). Approximately 10–20 seeds were used from each T2 plant. For the pooled screening, approximately 10–50 seeds of 90 individual T2 plants were mixed, surface sterilized and sprinkled onto agar plates (3 µM (±)-ABA; Sigma-Aldrich). As control for ABA insensitivity, abi4-1 (ABRC, CS8104) or abi5-1 (ABRC, CS8105) was used and Col-0 was used as a wild-type control. A putative ABA-insensitive phenotype was scored in a binary manner for similarity to the abi5-1 phenotype and difference from wild-type after 5–8 d using green cotyledons as indicator (Kuhn et al., 2006). For lines that showed a putative ABA insensitivity, the seed germination assay was repeated by propagating individual T2 seedlings to the next generation (T3) and using seeds of the T3 generation for ABA sensitivity assays. This time, seeds were placed on plates with and without ABA (2 µM (±)-ABA; Sigma-Aldrich) and images were taken daily for 7 d and emergence of radicles and cotyledons was counted manually using Fiji (Schindelin et al., 2012). For candidates of the individual screen the T2 seeds were used for the repetition of the germination assay.

For candidates of the pooled screen ABA-insensitive seedlings were transferred to plates containing 75 µM phosphotinric, and after 7–10 d resistant seedlings were transferred to soil, grown up to full maturity, and the T3 seeds used for the validation of the ABA-insensitive germination phenotype.

Screen for CO$_2$-insensitive leaf temperature phenotype

Seeds of T2 plants were germinated in 96-spot pots (254 mm×508 mm; East Jordan Plastics, East Jordan, MI, USA) on soil with each pot
containing seeds from one plant. After 7 d, seedlings were sprayed with a 1000× dilution of Finale® (Bayer), and 2–3 d later pale seedlings were removed and only one healthy dark green seedling was left per pot. After 19 d under standard growth conditions, the plants were exposed to 150 ppm CO₂ for 2 h in a Percival growth chamber. A first set of thermal images was taken with a FLIR A320 thermal imaging camera (FLIR, Wilsonville, OR, USA). Subsequently the plants were exposed to ≥ 800 ppm CO₂ and after 2 h a second set of thermal images was taken.

Control plants included in the experiments were ht1-2 (Hashimoto et al., 2006), oit1-3 and wild-type Col-0. Thermal images were converted into Flexible Image Transport System format (fits) using the ExaminIR software (FLIR). For the screen using the 96-pot-flat format, the temperature of plant leaves and the surrounding soil were measured using Fiji (Schindelin et al., 2012). The soil temperature served as a location-specific reference to compensate for temperature variation depending on the position in the 96-pot flat. Either the temperature difference between plant leaves and surrounding soil or the average temperature of plant leaves was used as a quantitative measure. Plants with more than 1 °C difference from soil were considered as primary candidates and subject to further testing. The high-temperature leaf phenotype of ht1-2 was used as a reference for CO₂ insensitivity. To test the reproducibility of the CO₂ dependent leaf temperature phenotype of putative candidates, T2 plants were grown in triplicate and assayed again alongside with ht1-2 and wild-type control plants.

Identification of amiRNA sequences and testing reproducibility

Genomic DNA from candidates with a robust and reproducible phenotype was extracted and only one healthy dark green seedling was left per pot. After 19 d under standard growth conditions, the plants were exposed to 150 ppm CO₂ for 2 h in a Percival growth chamber. A first set of thermal images was taken with a FLIR A320 thermal imaging camera (FLIR, Wilsonville, OR, USA). Subsequently the plants were exposed to ≥ 800 ppm CO₂ and after 2 h a second set of thermal images was taken.

Control plants included in the experiments were ht1-2 (Hashimoto et al., 2006), oit1-3 and wild-type Col-0. Thermal images were converted into Flexible Image Transport System format (fits) using the ExaminIR software (FLIR). For the screen using the 96-pot-flat format, the temperature of plant leaves and the surrounding soil were measured using Fiji (Schindelin et al., 2012). The soil temperature served as a location-specific reference to compensate for temperature variation depending on the position in the 96-pot flat. Either the temperature difference between plant leaves and surrounding soil or the average temperature of plant leaves was used as a quantitative measure. Plants with more than 1 °C difference from soil were considered as primary candidates and subject to further testing. The high-temperature leaf phenotype of ht1-2 was used as a reference for CO₂ insensitivity. To test the reproducibility of the CO₂ dependent leaf temperature phenotype of putative candidates, T2 plants were grown in triplicate and assayed again alongside with ht1-2 and wild-type control plants.

Results and discussion

Generation of amiRNA library plants

We have previously described the generation of an amiRNA library consisting of 10 sublibraries that represent 22 000 individual amiRNA designs (Hauser et al., 2013). Deep sequencing of these 10 sublibraries showed that ≥95% of the designed amiRNAs were present in these sublibraries (Hauser et al., 2013). The amiRNA library was transformed first into Agrobacterium tumefaciens and then into Arabidopsis Col-0. Over a period of over 4 years, the amiRNA library consisting of 10 sublibraries was transformed and T1 seeds harvested. Using plate- or soil-based selection methods, herbicide-resistant T1 plants were grown and T2 seeds from over 14 000 individual plants were harvested (Table 1). The transformation rate varied over a range from 0.08% to 0.76% with an average of 0.25%. During the course of this research, approximately 3000 additional T2 lines (Zhang et al., 2018) were generated expressing amiRNAs that target homologous transporter-encoding gene family members. These 3000 lines will also be made available to the ABRC, such that in the end over 14 000 total T2 lines will be submitted for use by the community.

Screen for ABA-insensitive seed germination phenotype

In total over 2500 T2 amiRNA lines were screened individually and over 5000 T2 amiRNA lines were screened in pools for ABA-insensitive germination phenotypes (Fig. 1). In the primary screen using individual plants in a 96-well plate format, 59 putative candidates were identified. In the primary screen using pools of 90 plants with 25–80 seeds per line, approximately 340 putative candidates representing an unknown number of lines were identified (Fig. 1).

These candidates were subjected to further analysis in a secondary screen (Fig. 2). The cotyledon emergence phenotype of 24 T3 seedlings from a total of 76 retested plants showed a more reduced ABA sensitivity that was clearly different from wild-type and less severe than the abi4-1 and abi5-1 controls (Fig. 2A). From the 59 putative candidates identified using the individual screening approach, the amiRNA line p8l1257 showed a reproducible partial insensitivity to ABA in the T3 generation (Fig. 3). Only the amiRNA in candidates with the most robust phenotypes was determined by sequencing. Two of the amiRNA-targeted gene sets identified in 24 seedlings with reproducible phenotypes are known core components of the ABA signal transduction cascade (Fig. 2; Table 2). These include amiRNA lines that target the three ABA receptors PYR1 (RCAR11), PYL4 (RCAR10), and PYL6 (RCAR9) (Fig. 2B, C; Table 2).
Furthermore, amiRNA-expressing plants that target six members of the SnRK2 protein kinase family (Mustilli et al., 2002; Yoshida et al., 2002; Fujii and Zhu, 2009) were isolated in this screen, including the three SnRK2 protein kinases, SnRK2.2, SnRK2.3, and SnRK2.6 (OST1), that are known to be required for abscisic acid signaling (Fig. 2B, C; Table 2; Mustilli et al., 2002; Yoshida et al., 2002; Fujii and Zhu, 2009).

Notably, Fig. 2D shows a strong variation in the cotyledon emergence phenotype among plants expressing the same amiRNA that targets six SnRK2 kinase transcripts. This variation might be responsible for the high number of variable candidates that did not show a robust phenotype following the primary screen. Additional amiRNA lines were isolated as putative mutants and the amiRNA sequence was determined (Supplementary Table S4). Although some of the predicted targets might be expected to affect abscisic acid responses, rescreening of these putative mutants did not show consistently robust reproducible phenotypes. Thus, amiRNAs appear to produce phenotypes that may be variable even within the same line. These findings

### Table 1. Overview of the 10 amiRNA libraries as described by Hauser et al. (2013), the number of amiRNAs designed for each library, and the number of individual T2 amiRNA transformants that were generated here

| Pool name               | Pool description                                                                 | AmiRNA designs | T2 lines |
|-------------------------|----------------------------------------------------------------------------------|----------------|----------|
| Kinase receptor (PKR)   | Protein kinases, protein phosphatases, receptors and their ligands                | 1860           | 817      |
| Binding (BNO)           | Proteins binding small molecules                                                 | 1968           | 1771     |
| Protein (CSI)           | Proteins that form or interact with protein complexes including stabilization of those | 2313           | 842      |
| RNA DNA (TFB)           | Transcription factors and other RNA and DNA binding proteins                     | 2964           | 831      |
| Metabolism (TEC)        | Metabolic and other enzymes catalysing transfer reactions (EC: 2)                | 1521           | 1548     |
| Diverse enzymes (PEC)   | Catalytically active proteins, mainly enzymes                                    | 1881           | 1113     |
| Non-classified (UNC)    | Genes for which the function is not known or cannot be inferred                  | 4082           | 1387     |
| Diverse mofun (DMF)     | Protein with diverse functional annotation not found in the other categories      | 1505           | 1152     |
| Hydrolase (HEC)         | Hydrolytic enzymes (EC class 3), excluding protein phosphatases                  | 2129           | 971      |
| Transporter (TRP)       | Proteins that transport molecules across membranes                               | 1777           | 3844     |
| Total                   |                                                                                   | 14276          |          |

Note that for the generation of each pool the 20 bp and 21 bp libraries were combined (see Hauser et al., 2013 for details). All T2 lines have been submitted to the Arabidopsis Biological Resource Center (ABRC).
led us to develop a protocol in which: (i) only putative mutants that showed a consistent phenotype when screening seeds from the next generation of plants were selected, and (ii) Only lines that showed similar phenotypes upon re-transformation with new amiRNAs that are predicted to target the same genes were selected. Furthermore, based on the variation observed here in the secondary screen it is advisable to investigate over 10 independent transformed lines (Schwab et al., 2006; Hauser et al., 2013) in the future to determine which amiRNAs produce phenotypes that can be carried forward. The isolation of amiRNA lines targeting functionally overlapping PYR/RCAR ABA receptor and SnRK2 protein kinase genes, which could not be isolated in traditional forward genetic mutant screens, provides a proof of principle that functionally redundant genes can be isolated in forward genetic screening using this new amiRNA resource. The inclusion of control lines and the validation steps described above should enable screening for diverse phenotypes using the lines generated here that are being provided to ABRC.

AmiRNA lines targeting three avirulence-induced genes show partial insensitivity to ABA inhibition of seed germination but not to ABA-induced stomatal closure

The amiRNA in line p811257 isolated in the present screen targets a new set of three genes (Fig. 3A, 3B). Previous research...
Hauser et al. annotated these genes based on their mRNA upregulation in a transcriptomic study after infection with *Pseudomonas syringae pv maculicola* carrying avrRpt2 (avrRpt2-induced gene, AIG2) (Reuber and Ausubel, 1996). However, these genes have not been previously described to be involved in ABA-mediated control of seed germination or other phenotypic responses in plants.

The line p8l1257 was named *amiRNA-AIG* here and was further tested by analysing seed germination with additional T2 generation seeds from the original p8l1257 stock (Fig. 3). Germination properties were compared with a control amiRNA line targeting the human myosin 2 (*amiRNA-HsMYO*), which has no targeted genes in Arabidopsis (Hauser et al., 2013). After 12 d on plates containing 2 µM ABA, the

![Fig. 3. Avirulence-induced genes (AIG2s) targeted by an amiRNA cause a reduced ABA sensitivity in cotyledon emergence assays. (A) Seedlings of the control amiRNA line, which has no target gene in Arabidopsis (*amiRNA-HsMYO* control), and an amiRNA line that targets three AIG2 genes (*amiRNA-AIG*) germinated in the presence of 0 or 2 µM ABA. Photographs were taken after 12 d of exposure. (B) Five new amiRNAs were designed to target the three AIG2(-like) genes. Three of these amiRNAs, amiRNA1, 2 and 3, target a single gene each. AmiRNA4 targets two tandem-repeat AIG2(-like) genes and amiRNA5 targets all three genes at non-identical nucleotides compared with the original amiRNA isolated in the primary screen (*amiRNA-AIG*, see Supplementary Table S2 for amiRNA sequences). (C) The new T2 amiRNA lines were tested in cotyledon emergence assays. Seedlings were germinated in the presence of 0 or 0.5 µM ABA. Photographs were taken after 4 d of incubation.](#)

| No. of sequenced T3 plants | AmiRNA sequence | Target gene | Gene name |
|---------------------------|----------------|-------------|-----------|
| 18                        | TGGATATGCTCCAACOGGCAT | AT1G10940 | SNRK2.4   |
|                            |                | AT1G60940 | SNRK2.10  |
|                            |                | AT2G23030 | SNRK2.9   |
|                            |                | AT3G50500 | SNRK2.2   |
|                            |                | AT4G33950 | SNRK2.6 (OST1)|
|                            |                | AT5G66880 | SNRK2.3   |
| 5                         | TATCAACGACGTAAGACTCGT | AT2G38310 | PYL4 (RCAR10) |
|                            |                | AT2G40330 | PYL6 (RCAR9)|
|                            |                | AT4G17870 | PYR1 (RCAR11) |
| 1                         | TTAATACATGGATGCACACGT | AT3G28930 | AIG2     |
|                            |                | AT5G39720 | AIG2LA   |
|                            |                | AT5G39730 | AIG2LB   |
amiRNA-AIG line showed cotyledon greening in contrast to the control amiRNA-HsMYO line (Fig. 3A). The effect of the amiRNA-AIG on the expression of a known ABA-induced gene, RAB18, was analysed by qRT-PCR (see Supplementary Fig. S1). The ABA-mediated induction of RAB18 expression was substantially reduced in the amiRNA-AIG line indicating a role of the targeted avirulence-induced genes (AIG2s) in ABA signal transduction.

Since two out of the three genes are tandemly repeated, generation of double mutants using T-DNA insertion knockouts would be limited. Therefore, five new amiRNA lines were generated that target subsets of genes targeted by the original amiRNA-AIG to verify the relevance of the predicted AIG2 target genes. AmiRNA1, 2 and 3 targeted each a single AIG2 (Fig. 3B; Supplementary Table S3). AmiRNA4 targeted two tandem-repeat AIG2 genes and amiRNA5 targeted all three AIG genes targeted in the original amiRNA-AIG line, but with a different amiRNA sequence (Fig. 3B; Supplementary Table S3 for amiRNA sequences). When the T2 seeds expressing these five new amiRNAs were tested in a seed germination assay with 0.5 µM ABA, only the amiRNA4 and amiRNA5-expressing lines showed less sensitivity to ABA compared with the control amiRNA-HsMYO line in cotyledon greening (Fig. 3C). The expression of all three putative target genes (AT5G39720, AT5G39730, AT3G28930) was analysed using qRT-PCR in the originally isolated amiRNA-AIG line and in all the amiRNA4 lines 1–5 (see Supplementary Fig. S2). The amiRNA efficiency of transcriptional inhibition varies between the lines, target transcript(s) and amiRNA sequence. Note that microRNA silencing in plants occurs via two mechanisms, (i) the degradation of transcripts and (ii) inhibition of translation (Brodersen et al., 2008). Thus, quantification of targeted transcripts may not fully show the degree of silencing of target genes. Combined, these data provide evidence that the original amiRNA-AIG phenotype is attributable to silencing of more than one AIG2 gene, suggesting overlapping homologous gene functions.

The original amiRNA-AIG line was also investigated to determine if it affects ABA-induced stomatal closure using an intact leaf gas exchange analysis approach. When ABA was applied to the transpiration stream of intact leaves at a final concentration of 2 µM, both the control amiRNA-HsMYO line and the amiRNA-AIG line showed an ABA-induced decrease in stomatal conductance to H2O (g, Fig. 4A). Normalization of the stomatal conductance data showed no dramatic difference in ABA-induced stomatal closure between amiRNA-HsMYO and amiRNA-AIG (Fig. 4B). Together, the present data show that the isolated amiRNA-AIG line is less sensitive to ABA inhibition of seed germination.

The AIG2 genes are functionally annotated as putative γ-glutamyl cyclotransferases (GGCTs, EC:4.3.2.9) based on their similarity to the human orthologue (HsGGCT; O75223). AIG2LA and AIG2LB share only 16% and 17% identity, respectively, to the human orthologue. GGCTs have been described to cleave γ-glutamyl-amino acid dipeptides to release the free amino acid and 5-oxoproline (Oakley et al., 2008). Further research will be required to determine the mechanism by which AIG2s affect ABA inhibition of seed germination.

Screen for CO2-insensitive leaf temperature phenotype

In total, over 2500 T2 amiRNA lines were screened individually for an altered leaf temperature response to a low CO2 concentration (150 ppm) by infra-red thermal imaging (Fig. 5). Leaf temperature depends on various parameters including radiation absorption, air temperature, and humidity (Merlot et al., 2002). Low ambient CO2 concentration leads to stomatal opening in Arabidopsis, causing an increased transpiration rate and thus a decrease in leaf temperature compared with the surrounding air. Mutants impaired in CO2-induced stomatal opening appear warmer compared with wild-type plants. In the screen, we used the soil temperature as reference to compensate for the local temperature differences due to various factors including humidity of the soil. Wild-type plants and plants of the HIGH LEAF TEMPERATURE1-2 (ht1-2) mutant (Hashimoto et al., 2006) were included in all trays as controls. Based on visual inspection of the thermal images, plants with relatively higher leaf temperature under low [CO2] compared with the other plants in the same image were selected and the difference between the average leaf temperature and the surrounding soil was determined. The difference between leaf temperature and soil temperature was determined as reference

![Fig. 4.](image-url) The isolated amiRNA line targeting three AIG2 genes (amiRNA-AIG) responds to ABA in whole leaf gas exchange analyses. Time-resolved stomatal conductance to H2O (g) in response to application of 2 µM ABA to the transpiration stream (red arrows) is shown in the amiRNA control line (amiRNA-HsMYO) and in the amiRNA line targeting three AIG2 genes (amiRNA-AIG). Stomatal conductance was analysed using whole leaf gas exchange. (A) Stomatal conductance in mol m−2 s−1. (B) Data from (A) were normalized to stomatal conductance at the beginning of the experiment. Data are the mean of n=3 leaves per genotype ±SEM.
for overall temperature and to compensate for local temperature differences. A set of 106 plants with more than one degree difference between the leaf temperature and the surrounding soil was defined as initial putative candidates for further testing (see Methods for details). For the rescreening of putative mutants, we set a high threshold for temperature differences in the selection of mutants compared with the wild-type strain of 1 °C. The constitutive CO₂ response mutant ht1-2, when exposed to low [CO₂], shows a delta temperature above 1 °C between leaf and soil. Rescreening of these candidates in the T2 generation revealed an amiRNA line (p9l22) with a robust and reproducible impaired response to low CO₂ (Fig. 5B).

After exposure to low [CO₂], the leaf temperature of the p9l22 line was compared with wild-type (Col-0) and with the constitutive high-CO₂-response mutant ht1-2 (Fig. 6A; Hashimoto et al., 2006). The leaves of the p9l22 line had a higher temperature than wild-type leaves and a similar temperature to ht1-2 leaves (Fig. 6A). Stomatal index (SI) and density (SD) were calculated for wild-type, the control line, the shift from ambient (400 ppm) to low (150 ppm) [CO₂] led to an increase in stomatal conductance (g) with a reduced rate of g, increase when compared with the control line. The same was observed when blue light (at 10 μmol m⁻² s⁻¹) was superimposed on the red light background (Fig. 6C). Thus, the amiRNA line p9l22 causes reduced responses to low CO₂ concentration, red light, and blue light.

The amiRNA in the p9l22 line was sequenced and is predicted to target two homologous proteasomal subunit genes (PAB1, AT1G16470; and PAB2, AT1G79210). PAB1 and PAB2 are the sole genes that encode the 20S proteasome α2 subunit (Baumeister et al., 1998). First, we attempted to isolate a double mutant (pab1 pab2) using T-DNA insertion lines (SALK_099950 and SALK_144987; Alonso et al., 2003). After genotyping over 100 plants in the F2 generation, no homozygous double mutant was recovered. We concluded that the double mutant is very likely lethal.

Alternatively, a new amiRNA sequence targeting solely the PAB1 gene was cloned and transformed into the pab2-1 single mutant (SALK_144987). This new amiRNA line, pab2-1mut pa1amIRNA, was investigated in stomatal conductance analyses of [CO₂] responses (Fig. 7). Leaves were first exposed to high (900 ppm) [CO₂] for 1 h and steady-state g was recorded. Shifts to low (150 ppm) [CO₂] led to an increase in g in both the pab2-1mut pa1amIRNA line and the control amiRNA-HsMYO line (Fig. 7A). The normalized stomatal conductance data show that the pab2-1 amiRNA-PAB1 line responds to low [CO₂] with a reduced magnitude compared with the control line (Fig. 7B).

Initial experiments were pursued to determine if modifications in the α-ring of the 20S proteasome might be linked to the above phenotypes, or whether this mutation is specific to only α subunit mutations of the proteasome. The α-ring of the 20S proteasome is composed of seven α-subunits (Kurepa and Smalle, 2008). The p9l22 amiRNA targets the only two genes that encode the α2 subunit of the proteasome (Fig. 7, inset highlighted in red). To determine whether other α-subunits also affect the response to low [CO₂], a second amiRNA line was generated, which targets the PAG1 gene (α7 subunit, inset in Fig. 7 highlighted in green),
named amiRNA-PAG1. The α7 subunit is encoded by a single gene in Arabidopsis (Kurepa and Smalle, 2008). When an amiRNA-PAG1 line was tested in gs responses to [CO2] shifts, it showed a lower rate of stomatal opening when compared with the control amiRNA-HsMYO line (Fig. 6, C, D; one-way ANOVA P > 0.05). The expression levels of PAB1, PAB2, and PAG1 were analysed in the p9l22 amiRNA line and also in pab2-1mut pab1amiRNA and amiRNA-PAG1 lines using qRT-PCR (see Supplementary Fig. S4). With the exception of the severely reduced PAB2 expression in the pab2-1mut pab1amiRNA line when compared with control lines, no clear evidence for knock down at the transcriptional level could be detected in the amiRNA lines, which may point to amiRNA-mediated inhibition of translation (Brodersen et al., 2008).

The present findings show that the p9l22 amiRNA line is also partially impaired in red light–induced stomatal opening. Red light mediates stomatal opening in part via activation of photosynthesis and the resulting drop in internal concentration of CO2 (Ci) (Roelfsema et al., 2002; Matrosova et al., 2015).
In addition, the p9l22 line is also partially impaired in blue light-induced stomatal opening. This suggests that a general regulator of stomatal opening is impaired in this line. As the proteasome mediates the degradation of proteins and reduced functions of α-ring subunits are predicted to increase protein levels, it is tempting to speculate that the phenotype observed might be correlated with an increased abundance of a negative regulator of stomatal opening. Further research will be required to test this or other hypotheses. In other studies, the 26S α2 subunit, when overexpressed, enhanced thermostolerance and adaptation in rice and Arabidopsis, suggesting that proteasomal subunits can have rate-limiting roles in regulating plant physiological responses (Li et al., 2015).

Summary and future use

A library of over 11 000 plus 3000 additional T2 generation amiRNA lines was created as a new resource to screen the redundant gene space in Arabidopsis. These amiRNA-expressing lines are being provided as individual lines to the Arabidopsis Biological Resource Center (ABRC). Given the observations and findings in the present study, lines will be available for high-throughput screening in pools of 90 lines per pool with approximately 25–50 seeds per individual amiRNA line in each pool. In each pool, the pooled seeds for screening will originate from one of the 10 sublibraries that target gene family members with defined functional annotations (Table 1; Hauser et al., 2013). This approach will increase the probability of identifying interesting putative mutants in future screens despite the biological variability in amiRNA silencing lines found here (Fig. 2D).

The screen for ABA-insensitive seed germination phenotypes identified two amiRNAs targeting PYR/RCAR ABA receptor genes and SnRK2 genes, which are both known groups of redundant key genes and proteins required for ABA signal transduction (Ma et al., 2009; Park et al., 2009). Isolating amiRNA lines in these known components serves as proof of principle for our approach. Moreover, screening this amiRNA population enables the identification of mutants that require co-silencing of homologous gene family members, which are less likely to be found in forward genetic screens of ethyl methanesulfonate or T-DNA mutagenized seed populations. Overall the presented amiRNA screen shows that amiRNA lines are prone to showing a high rate of variable candidates with weak or non-robust phenotypes. Nevertheless, as shown here new mutants can be isolated. Furthermore, during the course of this research, this amiRNA library resource has been used to isolate long-sought functionally redundant auxin transporter genes (e.g. ABCB6, ABCB20; Zhang et al., 2018). Approaches to circumvent the inherent limitations of forward genetic screening with amiRNAs were developed in the present study. As a first step, it is recommended to rescreen the next generation to identify robust and reproducible phenotypes in individually isolated putative mutant lines. As a second step, the amiRNA sequence of confirmed mutant lines needs to be determined (see Methods). AmiRNA sequences linked to the observed phenotypes are retransformed, and testing over 10 independent lines for the phenotype is recommended. Alternatively, amiRNA on one line without break that target a subset of the initially predicted targets can be used to narrow down the causative genes (e.g. Fig. 3). For cases where only two to three genes are targeted, T-DNA lines or CRISPR/Cas9 mutants can be used to narrow down the genes relevant for the phenotype.

Over 95% of the amiRNAs in this library were designed to target only two to five genes (Hauser et al., 2013), meaning that identification of causal genes is facilitated. Using the above approach, we report on two newly identified mutants: (i) amiRNA lines targeting three genes encoding avirulence induced gene (2-like) proteins show an ABA-insensitive seed germination phenotype; and (ii) amiRNA lines targeting two proteasomal subunits show insensitivity to low-CO2-induced stomatal opening. Further analyses of the two targeted genes in this amiRNA line suggest that stronger T-DNA alleles result in lethality. This indicates the usefulness of the generated amiRNA lines for forward genetic isolation of higher order mutants that would be lethal upon knock-out. Our data suggest that the wild-type expression levels of two α-subunits of the 20S proteasome, α2 and α7, are required for fully functional stomatal opening mediated by physiological stimulation. This indicates that the proteasomal subunits are likely controlling an unknown general negative regulator of stomatal opening. The amiRNA seed resource generated here provides a new and potent tool to identify redundant genes and also lethality causing higher order mutants in many biological processes in Arabidopsis.

In conclusion, the amiRNA library resource is well suited for screening of phenotypes that can be easily verified in subsequent generations. This population may be best suited for screens that permit high throughput or medium throughput screening for phenotypes with a large dynamic range. Many such powerful screens have been performed in classical Arabidopsis mutant screens that were previously not designed to identify functionally redundant genes.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. The induction of RAB18 gene expression by ABA is lower in the amiRNA-AIG lines.

Fig. S2. The expression of AIG2 genes in amiRNA-AIGs lines.

Fig. S3. The p9l22 amiRNA line has normal stomatal indices and density.

Fig. S4. The expression of PAB1, PAB2, and PAG1 genes in amiRNA lines.

Table 1. List of relevant primers used in this study.

Table 2. List of relevant plasmids used in this study.

Table 3. List of new amiRNAs designed and cloned in this study.

Table 4. AmiRNA sequences and predicted target genes found in candidate plants.

Acknowledgements

Seeds for 11 000 T2 amiRNA lines described here have been made available to, and 3000 are being prepared for, the Arabidopsis Biological Resource Center.
Resource Center (ABRC) for screening by the research community (order numbers: CS99427, CS99428, CS99429, CS99430, CS99431, CS99432, CS99433, CS99434, CS99435, CS99436). We thank Dr. Jianyan Huang, Kellie Tao Kim, Wilma Lee, Sandra Vogel, Marianne Kreusch, and Elly Poretsky for help in the transformation of the miRNA library into Arabidopsis, and generation of the lines and support during the various stages of the screen for novel phenotypes. This research was funded by grants from the National Science Foundation (MCB161236) and the National Institutes of Health (GM06396-ES010337) to JJS and was in part supported by grants from the Israel Science Foundation (50110003977; 1832/14) and a European Research Council Starting Grant (1001006663; 757683 – RobustHormoneTrans) to ES. PHOC was supported by a Ciencias Sem Fronteiras/CNPq fellowship (203406/2014-1). DG was supported by an EMBO long-term postdoctoral fellowship (50110003043; ALTF334-2018). DG was supported by a China Scholarship Council (501100004543; 201706580016) Scholarship.

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