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Madrid, Spain.A genetic screen for suppressors of a  
hypomorphic allele of *Arabidopsis*  
*ARGONAUTE1*

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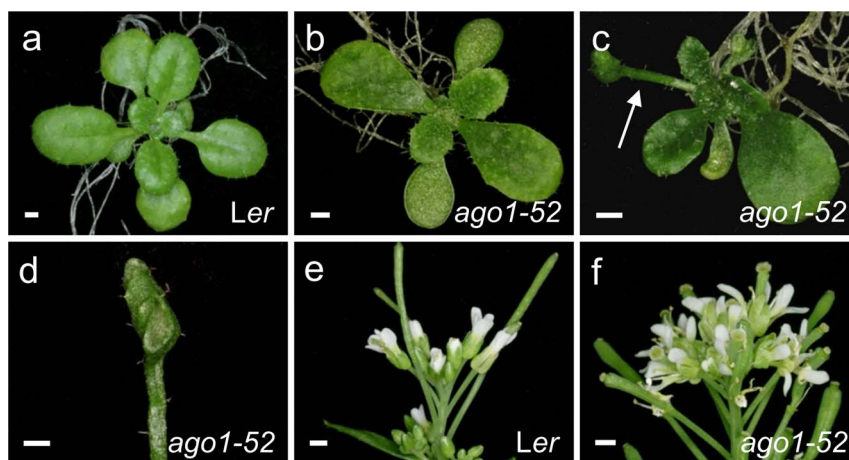
*ARGONAUTE1* (*AGO1*) encodes a key component of the complexes mediating microRNA (miRNA) function in *Arabidopsis*. To study the regulation, action and interactions of *AGO1*, we conducted a genetic screen to identify second-site mutations modifying the morphological phenotype of *ago1-52*, a partial loss-of-function allele of *AGO1*. Unlike null *ago1* mutations, the hypomorphic *ago1-52* allele does not cause lethality or sterility; however, *ago1-52* does produce a morphological phenotype clearly distinct from wild type. In our screen for modifiers of *ago1-52*, we identified suppressor mutations that partially restore wild-type morphology in the *ago1-52* background and we termed these *mas* (*m*orphology of *a*rgonaute1-52 *s*uppressed). We focused on 23 of these putative suppressors. Linkage analysis of the *mas* mutations together with sequencing of the *AGO1* gene in genomic DNA and cDNA from *ago1-52 mas* plants indicated that 22 of the *mas* lines contain extragenic suppressors, and one contains an intragenic suppressor that affects splicing of *ago1-52*. In the presence of the wild-type allele of *AGO1*, most of the *mas* mutations cause a mild or no mutant phenotype on their own, indicating that the *ago1-52* mutant may provide a sensitized background for examining the interactions of *AGO1*.

The existence of RNA molecules with repressor<sup>1</sup> or activator<sup>2</sup> functions was proposed in the first models of gene expression regulation. Despite these early predictions, however, experimental evidence of the existence of chromosomally encoded small regulatory RNAs was not published until the last two decades of the XX century in prokaryotes and eukaryotes, with the discovery of *MicF*<sup>3</sup> and *lin-4*<sup>4,5</sup>, respectively. *lin-4* belongs to a class of small RNAs that has received more attention in recent years, the microRNAs (miRNAs), single-stranded molecules of about 22 nt in length that hybridize by complementarity to their mRNA targets, and then induce the degradation and/or attenuation of the translation of these mRNA targets. These processes take place in the cytoplasm, in ribonucleoprotein complexes named RISC (RNA-Induced Silencing Complexes), whose catalytic component in all species studied so far is a protein of the ARGONAUTE (AGO) family<sup>6–8</sup>. Since the discovery of *lin-4*, hundreds of eukaryotic genes have been found to be negatively regulated by miRNAs in many organisms, including humans, and thousands have been predicted as miRNA targets using many different algorithms.

A collection of *Arabidopsis thaliana* (hereafter, *Arabidopsis*) mutants with morphologically abnormal leaves was obtained in the laboratory of J.L. Micol<sup>9,10</sup>, and 41 of these mutants were dubbed *incurvata* (*icu*), because of their upwardly curved leaves<sup>11–15</sup>. We positionally cloned the *icu3*, *icu8*, *icu9-1*, *icu9-2* and *icu15* mutations<sup>16,17</sup>, which were found to be novel loss-of-function alleles of the genes encoding HASTY (HST)<sup>18</sup>, HYPONASTIC LEAVES1 (HYL1)<sup>19</sup>, ARGONAUTE1 (AGO1)<sup>20</sup> and HUA ENHANCER1 (HEN1)<sup>21</sup>; these proteins are known components of the miRNA pathway<sup>22,23</sup>.

The above mentioned *icu* mutants were renamed as *hst-21*, *hyl1-12*, *ago1-51*, *ago1-52* and *hen1-13*, respectively, and intercrossed and crossed to *dcl1-9* (*dicer-like1-9*)<sup>24</sup>, a line carrying a mutant allele of *DCL1*, another gene encoding a component of the miRNA machinery. All of the double mutant combinations obtained in this way showed a strong, synergistic phenotype<sup>16</sup>. This observation led us to presume that loss-of-function alleles of *HST*, *HYL1*, *AGO1*, *HEN1* or *DCL1* would provide a sensitized genetic background, useful for a second-site mutagenesis aimed to identify novel genes directly or indirectly related to the miRNA pathway.

To study the regulation, action and interactions of *AGO1*, we decided to perform a second-site mutagenesis with the *ago1-52* mutant. The *ago1-52* hypomorphic allele causes a morphological phenotype easily distinguishable from wild type<sup>16</sup>, and only partially reduces viability and fertility, unlike null *ago1* alleles, which are completely sterile<sup>20</sup>. *ago1-52* carries a G→A transition mutation 12 bp upstream of the 3' end of its 21th intron<sup>17</sup>. The mutation creates a splicing acceptor signal that causes mis-splicing and gives rise to an mRNA 10 nt longer than



**Figure 1** | Some phenotypes of the *ago1-52* mutant. (a–c) Rosettes from (a) the wild type *Ler* and (b, c) *ago1-52*. An arrow in c highlights a trumpet-shaped, radialised leaf. (d) Detail of a radialised *ago1-52* leaf. (e, f) Terminal region of the inflorescences of (e) *Ler* and (f) *ago1-52*. Scale bars: 1 mm. Pictures were taken (a–d) 21 das and (e, f) 42 das.

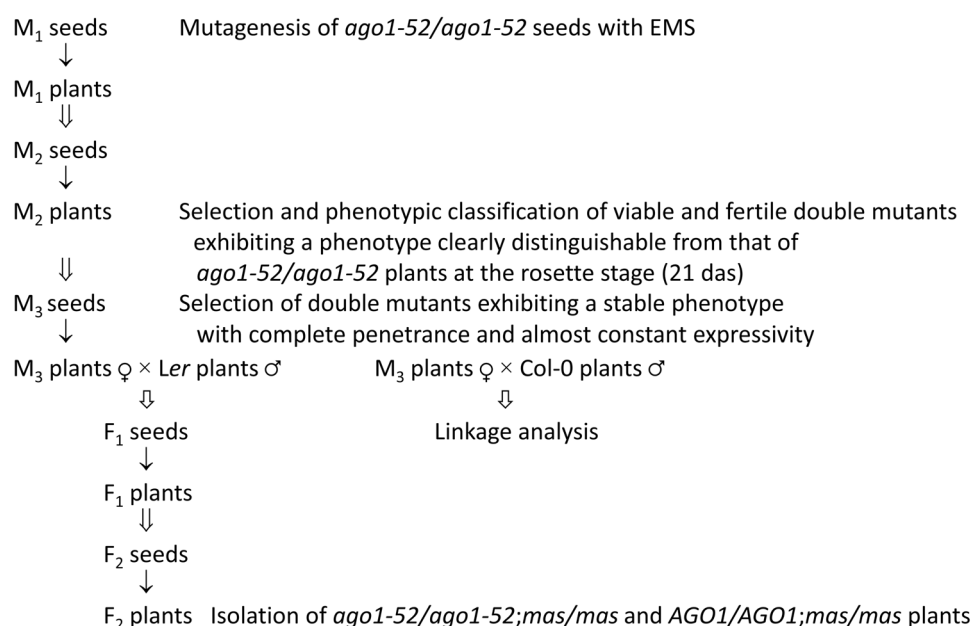
that of the wild type; the *ago1-52* mutant mRNA is translated into a mutant protein with 15 aa different than those of the wild type at the carboxyl terminus, and with 55 fewer residues than the wild type (Figure S1). For the mutagen, we chose ethyl methanesulfonate (EMS) for its strong mutagenicity and its capacity to generate hypomorphic alleles, which might provide a broader range of suppressor mutations than null alleles<sup>25–27</sup>.

## Results and Discussion

**Morphological phenotype of the *ago1-52* mutant.** The *ago1-52* recessive mutation causes a pleiotropic phenotype with complete penetrance and variable expressivity (Figure 1). At 21 days after stratification (das) the *ago1-52* plants have an average of  $4.07 \pm 0.25$  vegetative leaves, in contrast to the *Ler* plants, which have an average of  $8.33 \pm 0.84$  leaves ( $n=30$ ). As in other *ago1* mutants, leaves of *ago1-52* show no clear boundary between petiole and lamina<sup>17</sup>. Juvenile *ago1-52* leaves (the first three) exhibit abaxial trichomes, which are seen in the wild type only in adult leaves (fourth and following). Leaf adaxialisation is apparent in some

*ago1-52* plants, which exhibit different extents of radialisation of the two first leaves<sup>17</sup>. The *ago1-52* plants do not exceed 20 cm in height ( $13.79 \pm 1.82$  cm in *ago1-52* versus  $25.5 \pm 1.73$  cm in *Ler*;  $n=20$ ) and possess a short, compact inflorescence. The *ago1-52* plants have shorter siliques than the wild type ( $5.77 \pm 1.52$  mm in *ago1-52* versus  $10.10 \pm 1.54$  mm in *Ler*;  $n=30$ ), and have fewer seeds than wild type ( $10.79 \pm 8.49$  in *ago1-52* versus  $44.14 \pm 16.92$  in *Ler*;  $n=30$ ).

**Screen design and mutagenesis.** To isolate modifiers of the morphological phenotype of the *ago1-52* mutant, we mutagenised homozygous *ago1-52* plants with EMS. The procedure followed to isolate and characterise new mutants (Figure 2) was similar to that described by Berná *et al.*<sup>9</sup>. Approximately 67,500 seeds of *ago1-52* were sent to Lehle Seeds ([www.arabidopsis.com](http://www.arabidopsis.com)) to be mutagenized. Mutagenized seeds ( $M_1$  generation) were sown on soil and grown in a growth chamber to obtain  $M_2$  seeds, which were harvested in bulk to form 15 parental groups; these groups were then sent to our laboratory for screening.



**Figure 2** | Flowchart describing the screening strategy for the isolation and genetic analysis of suppressors of *ago1-52*. The ↓ and ⇓ symbols indicate growth and selfing, respectively. The ⇓ symbol indicates outcrossing or backcrossing.

Table 1 | Modifiers of the mutant phenotype of *ago1-52* identified in the  $M_2$  population screened

Parental group <sup>a</sup>	Number of $M_2$ seeds screened	Early lethality	Albino seedlings	Putative double mutant plants			
				Fertile	Sterile	Lethal	Total
1	3,891	1,215	92	113	148	102	363
2	2,373	1,035	56	26	68	222	316
3	4,212	1,651	29	47	89	422	558
4	6,002	2,422	11	28	75	709	812
5	4,984	1,893	12	11	41	478	530
6	6,310	2,302	24	32	51	675	758
7	4,594	1,851	7	20	22	401	443
8	4,444	1,752	18	25	27	357	409
Total	36,810	14,121	249	302	521	3,366	4,189

Values indicate the number of seeds, seedlings or plants of each type. <sup>a</sup>The number of  $M_1$  plants in each parental group was 1,283.

**Estimation of the efficiency of the mutagenesis.** One of the methods used in Arabidopsis to estimate the efficiency of mutagenesis is based on determining the frequency of initial cells with mutations ( $P$ ) and the mean number of mutations per initial cell ( $M$ )<sup>28</sup>. The values of  $P$  and  $M$  are calculated from the frequency of  $M_1$  plants that show siliques with some sort of mutation ( $m_a$ ) or from the frequency of siliques with mutations ( $m_b$ ), which, in turn, are determined according to the appearance of easily-observable mutant phenotypes in the silique, phenotypes such as embryo lethality or albinism, which occur very rarely in wild-type populations.  $P$  and  $M$  are then calculated as follows<sup>28,29</sup>:

$$P = 1 - \sqrt[n]{1 - m_a} = m_b \quad (1)$$

$$M = -\ln(1 - m_a)n^{-1} = -\ln(1 - m_b) = -\ln(1 - P) \quad (2)$$

where  $n$  is the mean number of initial cells, whose most widely accepted value is 2–3. About 50  $M_1$  plants are usually examined, and 5–6 siliques in each plant, to count the presence of albino embryos. Any mutagenesis with EMS that leads to a  $P$  value of above 0.3 can be considered effective. Indeed, using the equation in which the  $P$  value was defined, it can be seen that, assuming  $n = 2$ ,  $P$  will = 0.3 when half the  $M_1$  plants show at least one mutation that causes embryo albinism ( $m_a = 0.5$ ).

Ninety siliques from 18  $M_1$  plants were examined, and albino embryos were found in all of them. As a consequence, the proportion of siliques revealing the existence of a mutation causing albinism was  $m_b = 90/90 = 1$ , which implies  $P = 1$ . From the above, we can deduce that the frequency of  $M_1$  plants with siliques showing some mutation was  $m_a = 1$ . In other words, all the  $M_1$  plants were carriers of at least one mutation causing embryo albinism. This value is surprisingly high, since in a similar mutagenesis carried out by Lehle Seeds for J.L. Micol on a wild type *Ler* genetic background, the value of  $P$  was 0.54<sup>30</sup>. Perhaps in the sensitized *ago1-52* genetic background many phenotypes, including albinism, arise that are much less visible in a wild-type background.

Of the  $M_2$  seeds studied, 38% showed no or abortive germination. The causal mutations for such lethal phenotypes in mature embryos (seeds) or seedlings seem to occur in all the  $M_1$  parental lines. This conclusion is based on the assumption that almost all these mutations were recessive, as is common for hypomorphic or null mutations, and that each of them was homozygous in one of the three  $M_2$  seeds representing by each average  $M_1$  parental.

The number of albino seedlings also indicates the presence of mutations in the genomes of the plants screened. We found one case of albinism per every 91  $M_2$  plants that did not show early lethality, which indicates that the mutagenesis was very effective. We use the term albinism here to refer to the absence of a green colour in the cotyledons of the seedlings, some of which were completely white and others yellowish.

**Characterisation of the mutants.** *Phenotypic classification of putative double mutants.* The screen for modifiers of the phenotype of *ago1-52* was carried out in two steps, using eight and seven parental groups, respectively. We first screened 36,810  $M_2$  seeds, the progeny of 10,264  $M_1$  plants (parental groups P1–P8; Table 1) sowing seeds in plates in a controlled number with *Ler* and *ago1-52* seeds as controls (see Methods). Whereas the *ago1-52* and *Ler* seeds germinated at a rate of 93.0% and 97.2%, respectively, and developed into viable plants, we observed no or abortive germination in 38.0% (14,121) of the  $M_2$  seeds sown. Such a high percentage of early lethality shows the sensitivity of the *ago1-52* genetic background to EMS. Another indicator of the efficiency of the mutagenesis was the large number (249) of seedlings with albino cotyledons and no or only rudimentary leaves; all these seedlings died before 21 das (Table 1).

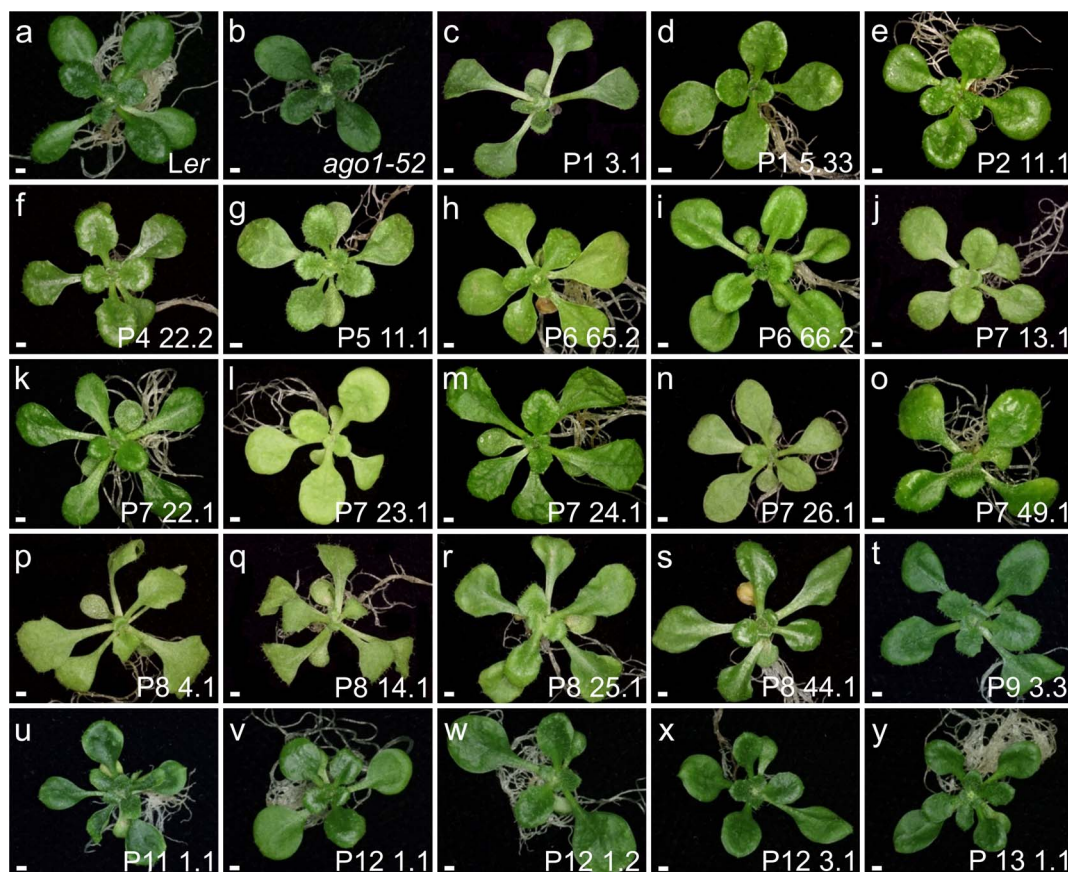
About 11% of the  $M_2$  seeds sown produced plants that survived more than 21 das and were considered double mutants because they showed unexpected phenotypes or an increased or reduced mutant phenotype compared to that of *ago1-52* plants. Among the 4,189 double mutants isolated, 3,366 exhibited lethality after 21 das and died before completing their life cycle, 521 completed their life cycle but did not produce seeds, and only 302 produced  $M_3$  seeds (Table 1).

$M_2$  double mutants of the most represented class showed a strong synergistic phenotype, in some cases reminiscent of those of the double mutant combinations of mutations in two miRNA machinery genes, and almost all showed a lethal phenotype. We also defined six phenotypic classes based on morphological traits, all of which included viable double mutants (Table 2 and Figure S2). Although some showed more than one of the traits considered characteristic of each class, we provisionally assigned each mutant to only one class. Plants showing leaf variegation or generalized chlorosis were assigned to the “De-pigmented plants” class. The “Severe Ago1-52 phenotype” and “Weak Ago1-52 phenotype” classes included plants

Table 2 | Assignment of  $M_2$  putative double mutants to phenotypic classes

Phenotypic classes	Viability and fertility			
	Fertile	Sterile	Lethal	Total
Synergistic phenotypes	0	3	3,130	3,133
De-pigmented plants	34	70	68	172
Reticulate leaves	48	19	7	74
Compact rosette	25	104	37	166
Severe Ago1-52 phenotype	42	67	23	132
Weak Ago1-52 phenotype	93	91	29	213
Other phenotypes	60	167	72	299
Total	302	521	3,366	4,189

Values indicate the number of plants of each type.



**Figure 3** | Vegetative phenotype of the suppressor lines identified in this work. Rosettes of  $M_3$  plants are shown from 23 of the double mutants isolated, in which the morphological phenotype of *ago1-52* is partially suppressed by a second-site mutation. Pictures were taken 21 das. Scale bar: 1 mm.

in which the phenotype of *ago1-52* was accentuated or alleviated, respectively. The “Reticulate leaves” class included plants with leaves in which the veins stood out because of their colour compared with a pale lamina. Plants with small vegetative leaves or short petioles were included in the “Compact rosette” class. The “Other phenotypes” class included a number of mutants with phenotypes that could not be categorized into the other classes.

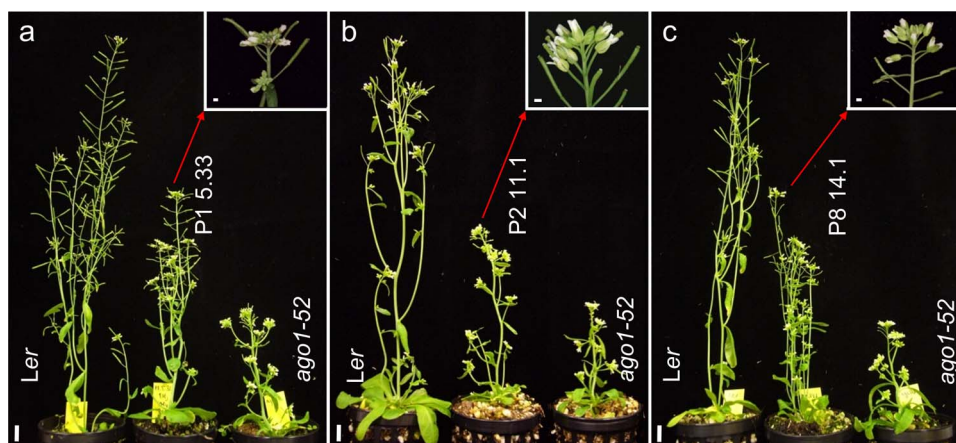
**Analysis of the transmission of the suppression of the phenotype of *ago1-52*.** To study the inheritance of the suppressed phenotype, we examined the progeny of the isolated  $M_2$  plants. We sowed several tens of  $M_3$  seeds derived from each  $M_2$  plant self-pollination. Transmission of the mutant phenotype was unequivocally Mendelian in only 92 of the 302  $M_2$  putative mutants that were fertile. We observed variable expressivity in 104  $M_3$  families, a phenotype weaker than that of their  $M_2$  parentals in 21  $M_3$  families, and in 85 families the  $M_2$  phenotype did not reappear in the  $M_3$  generation.

Of the 92  $M_2$  putative double mutants that showed complete penetrance and almost invariable expressivity in the  $M_3$  progeny, we focused on studying 17 lines of the 21 that we initially assigned to the class we denominate “Weak Ago1-52 phenotype” (Figure 3c–s). All these  $M_3$  lines exhibited suppression of the *ago1-52* phenotypes during their vegetative and reproductive development. Leaves of the  $M_3$  plants were less spatulated than those of *ago1-52*, with a well-defined boundary between petiole and lamina. No radialized leaves were seen in these suppressor lines, which exhibited in addition more vegetative leaves than *ago1-52*. Suppression was also shown by these plants after bolting: they exhibited increased stature and number of stems compared to *ago1-52*, from which they also differ in having a less compact inflorescence (Figure 4). The number of seeds in the siliques of these suppressor lines was also higher than that of *ago1-52* plants.

We hypothesized that partial suppression of the phenotype of the *ago1-52* mutation shown by these lines may have been caused by loss of function in genes whose products have an antagonistic effect on *AGO1*, or by gain of function alleles of genes that act together with *AGO1* in the silencing of miRNA targets. We did not study the lines of the remaining phenotypic classes, most of which were poorly viable and semi-fertile or sterile. We also did not study lines with phenotypes that seemed merely additive to that of *ago1-52*, since the corresponding double mutants were considered likely carriers of novel alleles of genes involved in processes unrelated to *AGO1*<sup>31</sup>.

We also screened an additional set of about 20,000  $M_2$  seeds later, belonging to the P9–P15 parental groups. In this case we sowed the seeds at high density (500 seeds for plate) in top agar and directly looked for plants with a weak Ago1-52 phenotype. We found six additional suppressor lines in this way (Figure 3t–y), which were studied together with the 17 lines already chosen in our first screen.

To exclude the possibility of contamination with wild-type seeds, we sequenced the *AGO1* gene in all the suppressor lines, confirming the presence of the original, homozygous *ago1-52* mutation in all of the lines when genomic DNA was used as template. We also confirmed the effect of *ago1-52* on splicing when cDNA was used as template. The genomic and cDNA sequences revealed that one of the lines, P9 3.3, carried an intragenic suppressor mutation (which we termed *ago1-52S*), since it carries a G to A transition adjacent to the *ago1-52* mutation (Figure 5a). Sequencing of *AGO1* cDNA from P9 3.3 showed that the splicing acceptor (AG) site of the 21st intron had been shifted 1 nt downstream, causing the inclusion in the mutated mRNA of 9 nt that are intronic in the wild type, instead of 10 nt as occurs in *ago1-52* mRNA; this restored the wild-type reading frame, although inserting three additional codons that were not present in the wild-type mRNA (Figure 5b, c).



**Figure 4** | Examples of other phenotypes of the suppressor lines identified in this work. Plant height of the (a) *ago1-52 mas1-1* (P1 5.33), (b) *ago1-52 mas2-1* (P2 11.1) and (c) *ago1-52 mas3-1* (P8 14.1) double mutants was intermediate between those of the wild type *Ler* and the *ago1-52* single mutant. As shown in the insets, the structure of the terminal region of the inflorescence in the double mutants was also intermediate between those of *Ler* (Figure 1e) and *ago1-52* (Figure 1f). Pictures were taken (a) 54 das, (b) 43 das and (c) 48 das. Scale bars: (a–c) 1 cm and (insets) 1 mm.

We named the genes whose mutations suppressed the morphological phenotype of *ago1-52* as *MORPHOLOGY OF argonaute1-52 SUPPRESSED (MAS)*. We backcrossed the *ago1-52 mas* suppressor lines to *Ler* twice. We transferred 1–5  $F_1$  plants derived from each backcross into pots to complete their life cycle and collected the  $F_2$  seeds resulting from self-pollination. Between 150 and 450 seeds of several  $F_2$  families from each line were sown and the morphological phenotypes of the corresponding plants were examined to identify *ago1-52/ago1-52;mas/mas* and *AGO1/AGO1;mas/mas* plants.

We assumed that, as is usual for EMS-induced mutations, the suppressor mutations would be recessive, in most if not all cases. Hence, we expected to find four phenotypic classes in the  $F_2$  progeny: (1) the wild type (*AGO1-;MAS/-*) class, (2) that of the mutation to be identified (*AGO1-;mas/mas*), (3) *Ago1-52* (*ago1-52/ago1-52;MAS/-*) and (4) the double mutant (*ago1-52/ago1-52;mas/mas*) class. As seen in Table S2, some of the *mas* mutations did not show any visible morphological phenotype in the *AGO1-;mas/mas* genotype (P1 5.33, P2 11.1, P4 22.2, P5 11.1 and P8 14.1) whereas others do (P7 13.1, P7 23.1, P7 26.1 and P8 25.1).

We also note that in 6 of the 9  $F_2$  derived from the first backcrosses to *Ler* we found mutant phenotypes additional to those initially seen in the  $M_2$  (Table S2). This suggests the presence of mutations in different genes in the  $M_2$  individual initially selected as a double mutant, and reinforces the importance of backcrossing to *Ler* several times to reduce the number of mutations not relevant to the phenotype. In addition, the proportions of the phenotypes that we observed in the  $F_2$  did not fit any known Mendelian segregation. No or abortive seed germination contributed to this, as well as lethality and plants showing phenotypes that had not been seen in the  $M_2$  or  $M_3$ .

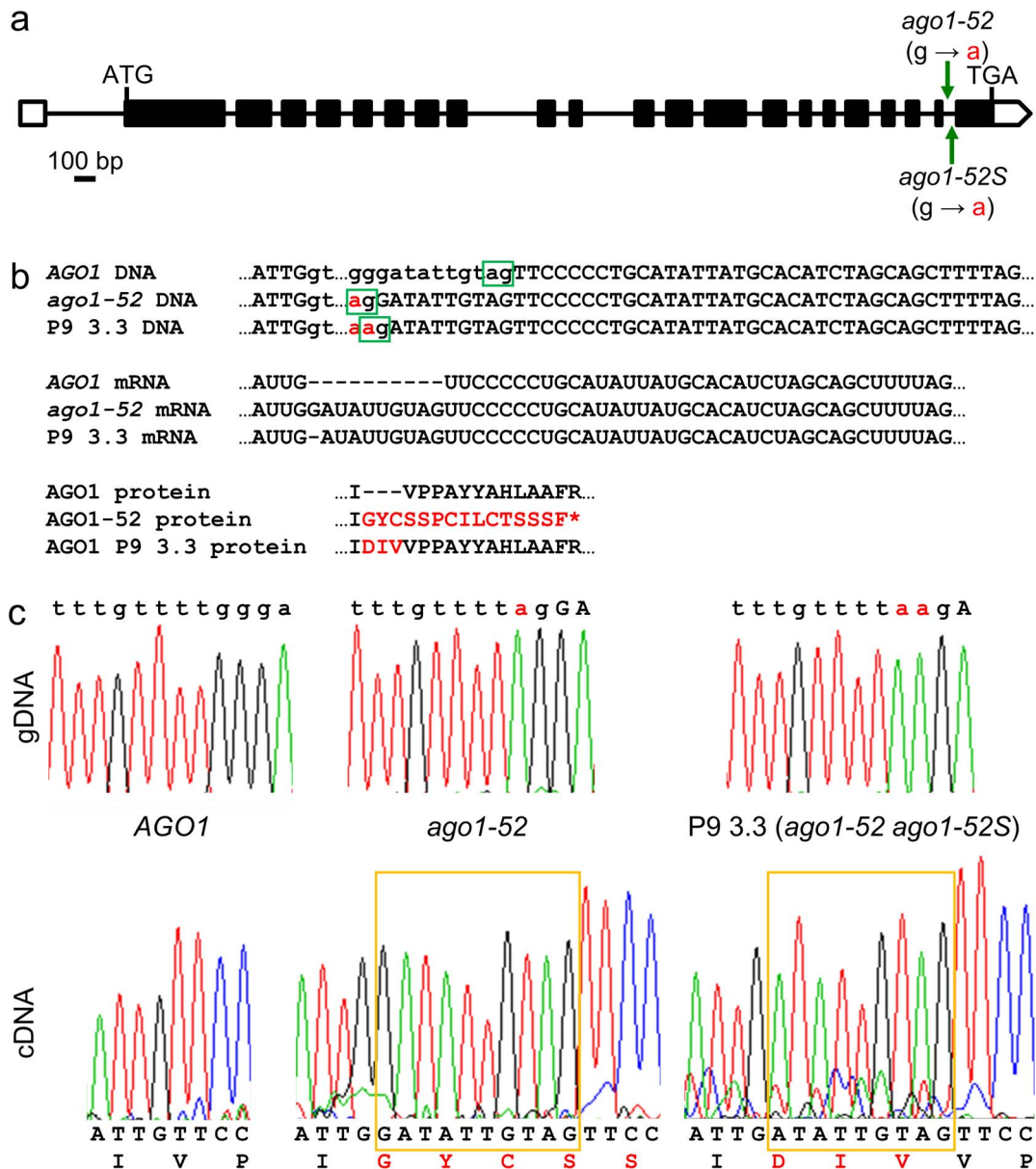
In all the lines studied except two (P5 11.1 and P7 26.1), the double mutant class is more numerous than that of the single mutants. Given that the mutations under study normalise the body architecture of the *ago1-52* mutant, it is reasonable to suppose that they also increase its fertility. As previously shown, the number of seeds per silique of the *ago1-52* mutant is 25% that of the wild type, which explains why the *ago1-52/ago1-52;mas/mas* plants are more numerous than *ago1-52/ago1-52;MAS/-* plants. The absence of the *AGO1-;mas/mas* class in some of the lines suggests that plants of this genotype are included in the phenotypically wild-type class. As a consequence, the populations of the first three lines in Table S2 should fit a 12:3:1 segregation (*AGO1-;-/-* : *ago1-52/ago1-52;MAS/-* : *ago1-52/ago1-52;mas/mas*), but they do not because of the much better viability of the suppressed plants compared with the plants showing the full *ago1-52* mutant phenotype.

We chose for subsequent study the P1 5.33, P2 11.1 and P8 14.1 lines for several reasons. Lines P1 5.33 and P2 11.1 were the first to be

isolated in our screen and, therefore, the first ones to be studied. Line P8 14.1 was chosen because it showed a more pronounced suppression of the phenotype of *ago1-52* during the reproductive phase. In the  $F_2$  of the backcrosses involving P1 5.33, P2 11.1 and P8 14.1 we only found three phenotypic classes: entirely wild type phenotype, *Ago1-52* and double mutants, which indicates that the suppressor mutations did not cause a mutant phenotype on their own, either as homozygotes or heterozygotes in the presence of the wild type allele of *AGO1*. We did not perform complementation analyses by intercrossing the suppressor lines. Rather, we first used linkage analysis to determine their genetic map positions (see examples in Table S3), and then crossed for allelism tests only the lines carrying mutations with neighbouring map positions. Linkage analyses demonstrated in all cases that the suppressor mutations are extragenic suppressors that map to chromosomes 2, 3, 4 or 5, or on chromosomes 1 but far from *AGO1*; the only exception was the above-mentioned *ago1-52S* intragenic suppressor mutation. The genetic and molecular characterization of each of these suppressors will be described elsewhere.

**Concluding remarks.** EMS has been widely used to induce mutations in forward and second-site genetic screens with many experimental organisms, but also to increase crop diversity. Further, EMS has been used in saturation mutagenesis in several TILLING (Targeting Induced Local Lesions in Genomes)<sup>32,33</sup> projects with different animal and plant species, including *Arabidopsis*, because it causes randomly distributed point mutations and only rarely produces DNA rearrangements that could result in lethality<sup>26</sup>. Mutagenesis in *Arabidopsis* predominantly relies on EMS and its non-lethal dose treatment has been well established. Therefore, it was striking that our *ago1-52* mutagenesis led to a very high percentage of lethality. EMS mainly induces alkylation of G residues, which then pair with T instead of with C; if these G:T mismatches are not repaired, they produce G/C to A/T transitions.

Universal repair mechanisms, such as the mismatch repair pathway that recognizes G:T mismatches, as well as repair of alkylated nucleotides by DNA glycosylases, act in *Arabidopsis*<sup>34–36</sup>. The apparently high sensitivity to EMS that we observed in the *ago1-52* background could be the result of defects in these DNA repair mechanisms. If this were true, *ago1-52* plants should accumulate mutations at a higher density than wild-type plants or other non-sensitive backgrounds under similar non-lethal EMS doses. Another interpretation of our results could be that since *AGO1* affects many pivotal processes, these processes are all de-regulated in the *ago1-52* mutant and second-site mutations in genes involved in other pivotal processes could result in lethality. Comparative analysis of whole-genome sequences of  $M_2$  plants obtained from either *ago1-*



**Figure 5** | The P9 3.3 line carries an intragenic suppressor mutation of *ago1-52*. (a) Structure of the *AGO1* gene with indication of the nature of the *ago1-52* and *ago1-52S* mutations (green arrows and red letters). Positions of the start (ATG) and stop (TGA) codons are also indicated. Exons are shown as boxes, and introns as lines between boxes. Open boxes represent untranslated exon sequences. (b) Effects of the *ago1-52* and *ago1-52S* mutations on splicing and mRNA translation. Intron sequences are shown in small case, and exon sequences in capital letters. The preferred splicing acceptor sites are boxed in green. Amino acids different from those of the wild type are shown in red. The asterisk indicates a premature stop codon. (c) Sequencing electropherograms obtained from *Ler* (*AGO1*), *ago1-52* and P9 3.3 genomic DNA (gDNA; top) and cDNA (bottom). Nucleotide sequences that are intronic in the *AGO1* wild type allele but exonic in the *ago1-52* mutant and the *ago1-52 ago1-52S* double mutant are boxed in orange.

52 or wild-type plants both treated with EMS could settle this question.

Second-site mutagenesis screens are common strategies for finding functionally related genes. The pleiotropic phenotype of the *ago1* mutants results from the alteration of many biological processes, which involve many genes that are directly or indirectly regulated by the miRNA pathway. It may, therefore, seem surprising that we obtained *ago1-52 mas* double mutants with morphological phenotypes very close to that of the wild type. Suppressor mutations usually fall into two classes: informational suppressors and functional suppressors. Informational suppressors act through a generic mechanism, such as the transcription machinery, RNA processing and translation, and they can also suppress a premature stop codon, modify aberrant splicing of mRNA or control mRNA translation or protein degradation. For example, in *Caenorhabditis elegans*, three

kinds of informational suppression have been described so far: nonsense suppression, suppression by modified splicing and suppression by loss of nonsense-mediated decay. By contrast, functional suppressors act through mechanisms directly related to the process of interest<sup>37,38</sup>. Since informational suppressors generally are allele-specific but not gene-specific, we will test allele- and gene-specificity in all our *mas* mutations.

## Methods

**Plant material and growth conditions.** *Arabidopsis thaliana* (L) Heynh. Landsberg *erecta* (*Ler*) and Columbia-0 (Col-0) wild-type accessions were obtained from the Nottingham Arabidopsis Stock Center (NAS; Nottingham, UK) and then propagated at our laboratory for further analysis. Seed sterilization and sowing, plant culture and crosses were performed as previously described<sup>37,39</sup>. In brief, seeds were sown on plates containing MS agar medium (half-strength Murashige and Skoog salts, 0.7% plant agar [Duchefa], pH 5.7, and 1% sucrose) and stratified (4°C in the



dark) for 48 h and then transferred to either Conviron TC16 or TC30 growth chambers set to our standard conditions (continuous light at approximately 75  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ,  $20 \pm 1^\circ\text{C}$ , 60–70% relative humidity). When required, plants were transferred into pots containing a 2:2:1 mixture of perlite:vermiculite:sphagnum moss and grown in walk-in growth chambers set to our standard conditions.

**Mutagenesis and mutant isolation.**  $\approx 67,500$  *ago1-52* seeds (1 g) were sent to a commercial supplier, Lehle Seeds (<http://www.arabidopsis.com>), where they were mutagenised by immersion in a solution of 0.23% (v/v) EMS for 12 hours at  $25^\circ\text{C}$ , stratified for 7 days, and then sown in pots. The  $M_2$  progeny obtained from selfed  $M_1$  plants was sent to us as 15 envelopes, each of which contained the pooled  $M_2$  seed progeny of a parental group: 1,283  $M_1$  plants developed from seeds exposed to EMS. Seeds were sown in 15 cm-diameter Petri dishes containing solid MS medium. Each dish was sown with 94  $M_2$  seeds, together with 8 *Ler* and 8 *ago1-52* seeds, which served as controls. Selection of putative double mutants was carried out 21 days after stratification (das) by eliminating all the  $M_2$  plants not showing clear morphological differences from *ago1-52*. All plants distinguishable from *ago1-52* were presumed to be double mutants, and they were transplanted into pots and allowed to complete their life cycles. Putative double mutants were given protocol numbers, as PN X.Y: PN indicates the corresponding parental group, X refers to the number of the plate where the mutant was isolated and Y is an ordinal assigned to each of the mutants found in a given plate.

**Linkage analysis, RNA isolation, and genomic DNA and cDNA sequencing.** Low-resolution mapping of the suppressor mutations was performed by linkage analysis as described in Ponce *et al.*<sup>40,41</sup>. Genomic DNA was isolated as previously described<sup>42</sup>. Total RNA from 20–30 mg of rosette leaves, collected 21 das, was isolated using TRI Reagent (Sigma), and first-strand cDNA synthesis was performed as described in Jover-Gil *et al.*<sup>17</sup>. Genomic DNA and cDNA PCR amplification and sequencing were performed as previously described<sup>14,42</sup>. Primers for PCR amplifications and sequencing were *ago1-52-27-F* (5'-TTACCAGTCTCTTTGGGATGAG-3') and *ago1-52-27-R* (5'-GCAGTAGAACATGACACGCTTC-3'). The chromatograms shown in Figure 5 were obtained with Chromas Lite 2.1.1. (<http://technelysium.com.au/>).

- Jacob, F. & Monod, J. Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* **3**, 318–356 (1961).
- Britten, R. J. & Davidson, E. H. Gene regulation for higher cells: a theory. *Science* **165**, 349–357 (1969).
- Esterling, L. & Delilhas, N. The regulatory RNA gene *micF* is present in several species of gram-negative bacteria and is phylogenetically conserved. *Mol. Microbiol.* **12**, 639–646 (1994).
- Lee, R. C., Feinbaum, R. L. & Ambros, V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* **75**, 843–854 (1993).
- Wightman, B., Ha, I. & Ruvkun, G. Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell* **75**, 855–862 (1993).
- Höck, J. & Meister, G. The Argonaute protein family. *Genome Biol.* **9**, 210 (2008).
- Meister, G. Argonaute proteins: functional insights and emerging roles. *Nat. Rev. Genet.* **14**, 447–459 (2013).
- Peters, L. & Meister, G. Argonaute proteins: mediators of RNA silencing. *Mol. Cell* **26**, 611–623 (2007).
- Berná, G., Robles, P. & Micol, J. L. A mutational analysis of leaf morphogenesis in *Arabidopsis thaliana*. *Genetics* **152**, 729–742 (1999).
- Serrano-Cartagena, J., Robles, P., Ponce, M. R. & Micol, J. L. Genetic analysis of leaf form mutants from the *Arabidopsis* Information Service collection. *Mol. Gen. Genet.* **261**, 725–739 (1999).
- Serrano-Cartagena, J. *et al.* Genetic analysis of *incurvata* mutants reveals three independent genetic operations at work in *Arabidopsis* leaf morphogenesis. *Genetics* **156**, 1363–1377 (2000).
- Ochando, I. *et al.* Mutations in the microRNA complementarity site of the *INCURVATA4* gene perturb meristem function and adaxialize lateral organs in *Arabidopsis*. *Plant Physiol.* **141**, 607–619 (2006).
- Pérez-Pérez, J. M. *et al.* A role for *AUXIN RESISTANT3* in the coordination of leaf growth. *Plant Cell Physiol.* **51**, 1661–1673 (2010).
- Barrero, J. M., González-Bayón, R., del Pozo, J. C., Ponce, M. R. & Micol, J. L. *INCURVATA2* encodes the catalytic subunit of DNA polymerase alpha and interacts with genes involved in chromatin-mediated cellular memory in *Arabidopsis thaliana*. *Plant Cell* **19**, 2822–2838 (2007).
- Esteve-Bruna, D., Pérez-Pérez, J. M., Ponce, M. R. & Micol, J. L. *incurvata13*, a novel allele of *AUXIN RESISTANT6*, reveals a specific role for auxin and the SCF complex in *Arabidopsis* embryogenesis, vascular specification, and leaf flatness. *Plant Physiol.* **161**, 1303–1320 (2013).
- Jover-Gil, S., Candela, H. & Ponce, M. R. Plant microRNAs and development. *Int. J. Dev. Biol.* **49**, 733–744 (2005).
- Jover-Gil, S. *et al.* The microRNA pathway genes *AGO1*, *HEN1* and *HYL1* participate in leaf proximal-distal, venation and stomatal patterning in *Arabidopsis*. *Plant Cell Physiol.* **53**, 1322–1333 (2012).
- Telfer, A. & Poethig, R. S. *HASTY*: a gene that regulates the timing of shoot maturation in *Arabidopsis thaliana*. *Development* **125**, 1889–1898 (1998).
- Lu, C. & Fedoroff, N. A mutation in the *Arabidopsis HYL1* gene encoding a dsRNA binding protein affects responses to abscisic acid, auxin, and cytokinin. *Plant Cell* **12**, 2351–2366 (2000).
- Bohmer, K. *et al.* *AGO1* defines a novel locus of *Arabidopsis* controlling leaf development. *EMBO J.* **17**, 170–180 (1998).
- Chen, X., Liu, J., Cheng, Y. & Jia, D. *HEN1* functions pleiotropically in *Arabidopsis* development and acts in C function in the flower. *Development* **129**, 1085–1094 (2002).
- Park, W., Li, J., Song, R., Messing, J. & Chen, X. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Current Biol.* **12**, 1484–1495 (2002).
- Han, M. H., Goud, S., Song, L. & Fedoroff, N. The *Arabidopsis* double-stranded RNA-binding protein HYL1 plays a role in microRNA-mediated gene regulation. *Proc. Natl. Acad. Sci. USA* **101**, 1093–1098 (2004).
- Jacobsen, S. E., Running, M. P. & Meyerowitz, E. M. Disruption of an RNA helicase/RNase III gene in *Arabidopsis* causes unregulated cell division in floral meristems. *Development* **126**, 5231–5243 (1999).
- Weigel, D. & Glazebrook, J. EMS mutagenesis of *Arabidopsis* seed. *Cold Spring Harb. Protoc.* **2006** (2006).
- Kim, Y., Schumaker, K. S. & Zhu, J. K. EMS mutagenesis of *Arabidopsis*. *Methods Mol. Biol.* **323**, 101–103 (2006).
- Qu, L. J. & Qin, G. Generation and identification of *Arabidopsis* EMS mutants. *Methods Mol. Biol.* **1062**, 225–239 (2014).
- Mednik, I. G. On methods evaluating the frequencies of induced mutations in *Arabidopsis* based on embryo-test data. *Arabidopsis Inf. Serv.* **26**, 67–72 (1988).
- Ivanov, V. I. Estimation of induced mutation rate in *Arabidopsis*. *Arabidopsis Inf. Serv.* **9**, 31–32 (1973).
- Quesada, V., Ponce, M. R. & Micol, J. L. Genetic analysis of salt-tolerant mutants in *Arabidopsis thaliana*. *Genetics* **154**, 421–436 (2000).
- Pérez-Pérez, J. M., Candela, H. & Micol, J. L. Understanding synergy in genetic interactions. *Trends Genet.* **25**, 368–376 (2009).
- McCallum, C. M., Comai, L., Greene, E. A. & Henikoff, S. Targeted screening for induced mutations. *Nat. Biotechnol.* **18**, 455–457 (2000).
- McCallum, C. M., Comai, L., Greene, E. A. & Henikoff, S. Targeting induced local lesions IN genomes (TILLING) for plant functional genomics. *Plant Physiol.* **123**, 439–442 (2000).
- Culligan, K. M. & Hays, J. B. *Arabidopsis* MutS homologs-AtMSH2, AtMSH3, AtMSH6, and a novel AtMSH7-form three distinct protein heterodimers with different specificities for mismatched DNA. *Plant Cell* **12**, 991–1002 (2000).
- Dany, A. L. & Tissier, A. A functional *OGG1* homologue from *Arabidopsis thaliana*. *Mol. Genet. Genom.* **265**, 293–301 (2001).
- García-Ortiz, M. V., Ariza, R. R. & Roldán-Arjona, T. An *OGG1* orthologue encoding a functional 8-oxoguanine DNA glycosylase/lyase in *Arabidopsis thaliana*. *Plant Mol. Biol.* **47**, 795–804 (2001).
- Hodgkin, J. in *WormBook* (2005), pp. 1–13.
- Fay, D. & Johnson, W. in *WormBook* (2006), pp. 1–4.
- Ponce, M. R., Quesada, V. & Micol, J. L. Rapid discrimination of sequences flanking and within T-DNA insertions in the *Arabidopsis* genome. *Plant J.* **14**, 497–501 (1998).
- Ponce, M. R., Robles, P. & Micol, J. L. High-throughput genetic mapping in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **261**, 408–415 (1999).
- Ponce, M. R., Robles, P., Lozano, F. M., Brotons, M. A. & Micol, J. L. Low-resolution mapping of untagged mutations. *Methods Mol. Biol.* **323**, 105–113 (2006).
- Pérez-Pérez, J. M., Ponce, M. R. & Micol, J. L. The *ULTRACURVATA2* gene of *Arabidopsis* encodes an FK506-binding protein involved in auxin and brassinosteroid signaling. *Plant Physiol.* **134**, 101–117 (2004).

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## Author contributions

M.R.P. conceived and designed the research. R.M.-P. and V.A. performed the research. M.R.P. and R.M.-P. wrote the article.

## Additional information

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