Suitability of two distinct approaches for the high-throughput study of the post-embryonic effects of embryo-lethal mutations in Arabidopsis

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Several hundred genes are required for embryonic and gametophytic development in the model plant Arabidopsis thaliana, as inferred from the lethality of their mutations. Despite many of these genes are expressed throughout the plant life cycle, the corresponding mutants arrest at early stages, preventing the study of their post-embryonic functions by conventional methods. Clonal analysis represents an effective solution to this problem by uncovering the effects of embryo-lethal mutations in sectors of mutant cells within an otherwise normal adult plant. In this pilot study, we have evaluated the suitability of two sector induction methods for the large-scale study of the post-embryonic effects of embryo-lethal (emb) mutations in Arabidopsis. In line with the interests of our laboratory, we selected 24 emb mutations that damage genes that are expressed in wild-type vegetative leaves but whose effects on leaf development remain unknown. For the induction of mutant sectors in adult plants, we followed one approach based on the X-ray irradiation of 'cell autonomy' (CAUT) lines, and another based on the site-specific excision of transgenes mediated by Cre recombinase. We conclude that both methods are time-consuming and difficult to scale up, being better suited for the study of emb mutations on a case-by-case basis.

Mutational approaches have greatly advanced our understanding of developmental processes in plants and animals. The isolation and characterization of viable mutants with defective growth and pattern formation has been crucial to identify both housekeeping and regulatory genes that are required for the organism to attain its normal size and shape. By focusing on viable mutations, however, these screenings are likely to have missed many genes that play important post-embryonic roles, because they are essential in early developmental stages and there are not viable alleles to study. This is particularly important in plants, whose development takes place mostly post-embryonically, after the basic body plan is laid out during the embryogenesis. Post-embryonic development includes the development of important plant organs, such as the leaves. Indeed, numerous viable mutants identified in such screenings turned out to be hypomorphic (partial loss-of-function) alleles of genes otherwise known only by their embryonic lethal effects. Some examples are the angulata1-1 (amu1-1), amu7-1, amu9-1 and scabral-1 (scal-1) mutants of Arabidopsis thaliana (hereafter, Arabidopsis), identified in a large-scale screen for viable mutants with abnormal leaf shape, size and pigmentation, which were later found to be hypomorphic alleles of the SECA2, EMBRYO DEFECTIVE 2737 (EMB2737), NON-INTRINSIC ABC PROTEIN 14 (NAPI4) and EMB3113 genes. Another example is the incurvata2-1 (icu2-1) mutant, identified in the same screen and found to be the first viable allele of the ICU2 gene, which encodes the catalytic subunit of DNA polymerase α. Because a significant fraction of the genes in the Arabidopsis genome is known to correspond to essential functions, and many such genes are expressed beyond the embryogenesis in wild-type plants, we hypothesized that many of them might also perform important roles in adult plants, after the embryogenesis has been completed.

Clonal analysis has been used to study embryo-lethal mutations by inducing genetic mosaics in many organisms, such as Drosophila melanogaster, maize and Arabidopsis. Clonal analysis experiments typically

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### Table 1. EMB genes, CAUT lines and pCB1 constructs used in this work.

| Gene name | AGI code | Chromosome | Coordinates | Protein function/conserved domains | Predicted location | Mutant allele | CAUT line | pCB1 |
|-----------|----------|------------|-------------|------------------------------------|--------------------|---------------|-----------|-----|
| AT5G39750 | AT2G31430 | 2          | 13361506–13365200 | Subunit of SWI/SNF chromatin remodeling complex | Nucleus | emb1381-1 | --- | Yes |
| EMB1441   | AT3G49930 | 5          | 2308033–23081208 | Orthologue of metazoan Strawberry notch (Sno) that mediates stress-induced chromatin memory | Nucleus | emb1441-1 | L82 | Yes |
| EMB1513   | AT2G37920 | 2          | 15868580–15870071 | Copper ion transmembrane transporter | Plasma membrane | emb1513-1 | --- | Yes |
| EMB1586   | AT1G21770 | 1          | 4351064–4353685 | DEAD-box RNA helicase | Mitochondrion | emb1586-1 | --- | Yes |
| EMB1611   | AT2G34780 | 2          | 1466853–14673904 | Regulation of endoreduplication and maintenance of meristem cell fate | Plasma membrane | emb1611 | --- | Yes |
| EMB1637   | AT3G58780 | 3          | 2142849–21430200 | SUMO ligase | Nucleus | emb1637 | 25_12 | Yes |
| EMB1674   | AT1G58210 | 1          | 21553621–21558056 | Member of the NET superfamily that complexes membranes to the actin cytoskeleton | Plasma membrane | emb1674-1 | --- | Yes |
| EMB1688   | AT1G67440 | 1          | 25263804–25265719 | Minichromosome maintenance (MCM) family protein | Chloroplast | emb1688-1 | --- | Yes |
| EMB1691   | AT4G09980 | 4          | 6247735–6252288 | Required for N6-adenosine methylation of mRNA | Nucleus | emb1691-1 | L104 | Yes |
| EMB1706   | AT4G10760 | 4          | 6619817–6623351 | Required for N6-adenosine methylation of mRNA | Nucleus | emb1706-1 | L104 | Yes |
| EMB1745   | AT1G13120 | 1          | 4469181–4473213 | Nucleoporin GLE1-like protein | Nuclear envelope | emb1745 | --- | Yes |
| EMB1895   | AT4G20060 | 4          | 10854790–10859330 | Armadillo (ARM)-repeat superfamily protein involved in small nuclear RNAs (snRNA) maturation | Nucleus | emb1895-1 | --- | Yes |
| EMB1923   | AT4G28210 | 4          | 1399097–13992078 | Unknown function | Chloroplast | emb1923-1 | L4 | --- |
| EMB1990   | AT3G07430 | 3          | 2359193–2380198 | YGGT family protein involved in nucleoid distribution | Chloroplast | emb1990-1 | C413 | Yes |
| EMB2001   | AT2G22870 | 2          | 9739457–9741104 | P-loop containing nucleoside triphosphate hydrolases superfamily protein | Cytoplasm | emb2001-1 | 30B4 | Yes |
| EMB2036   | AT5G66055 | 5          | 26417156–2642964 | Ankyrin repeat protein | Chloroplast | emb2036-1 | --- | Yes |
| EMB2107   | AT5G99900 | 5          | 3089278–3092595 | Isoform of the 26S proteasome regulatory protein subunit RPNs | Nucleus, cytoplasm | emb2107 | --- | Yes |
| EMB2301   | AT2G46770 | 2          | 1922077–19292296 | Transcription factor | Nucleus | emb2301 | 7F | --- |
| EMB2410   | AT2G25660 | 2          | 1091620–1092790 | Unknown function | Chloroplast | emb2410-1 | 30B4 | --- |
| EMB2736   | AT3G15980 | 3          | 6961736–6965108 | Catalytic subunit of serine/threonine protein phosphatase 2A | Nucleus, plasma membrane, cytoplasm | emb2736 | --- | Yes |
| EMB3008   | AT5G39750 | 5          | 15096875–15097942 | MADS-box transcription factor | Nucleus | emb3008 | B111 | Yes |

**Results and Discussion**

In an attempt to select an efficient strategy that is suitable for the systematic identification of essential genes that also function post-embryonically, we have carried out pilot experiments using two different approaches aimed at inducing somatic sectors that express the mutant phenotype, one based on the use of CAUT lines and another based on the use of the Cre-loxP site-specific recombination system. We focused on a subset of 24 EMBRYO DEFECTIVE (EMB) genes selected from the SeedGenes database (http://www.seedgenes.org/), which includes comprehensive information on the embryonic lethal genes of Arabidopsis. EMB genes were selected based on the availability of embryo-lethal mutant alleles and on their expression patterns beyond the embryogenesis (Table 1), particularly focusing on genes that are expressed in wild-type leaves and basal rosettes (i.e. during the vegetative phase) according to publicly available data from the electronic Fluorescent Pictograph (eFP) browser.
The genes selected encode proteins as diverse as transcription factors, proteasome subunits or epigenetic factors, which were considered good candidates to control leaf development at the transcriptional or post-transcriptional levels. We also selected some genes encoding proteins containing conserved domains whose functions remain unknown.

**Sector induction using CAUT lines and X-rays.** For the induction of marked somatic sectors in Arabidopsis, we initially took advantage of the availability of CAUT lines with insertions located on every chromosome arm. Thirteen different *EMB* genes (Table 1) were selected based on the availability of suitable CAUT lines carrying an insertion of the *CHLORATA-42* (*CH-42*) gene located between the *EMB* gene and the centromere of the corresponding chromosome. *CH-42* encodes the CHLI subunit of magnesium chelatase, which is required for chlorophyll biosynthesis. By choosing this configuration, we expect that all marked (yellow) sectors found after X-ray irradiation have also lost the wild-type allele of the *EMB* gene. To implement this strategy (Fig. 1), we systematically crossed heterozygous *EMB*/emb plants to the homozygous *ch-42*/*ch-42* mutant and isolated F2 plants displaying the recessive yellow phenotype caused by *ch-42*. The presence of aborted embryos or collapsed seeds in the siliques of these plants allowed us to select *ch-42/ch-42* plants segregating the corresponding embryo mutation in the F2 progeny (Fig. 2a,b). Plants with the *EMB*/emb; *ch-42/ch-42* genotype were subsequently crossed to appropriate CAUT lines. Ten different CAUT lines were used for this purpose (Table 1). Whenever possible, we selected CAUT lines carrying the *CH-42* insertion that maps closest to the *EMB* gene, because a higher frequency of chromosomal breaks is expected to occur as the distance between the insertion and the centromere increases. This crossing scheme allowed us to select phenotypically wild-type (green) plants that carry an insertion of the *CHLORATA-42* (*CH-42*) transgene and the wild-type copy of the *EMB* gene gives rise to a cell with pale-green genotype which might be accompanied by a mutant phenotype caused by the *emb* mutation.

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**Figure 1.** Detailed strategy to obtain hemizygous sectors for an embryo-lethal (*emb*) mutation by means of X-rays. Only the relevant genotype of each member from a pair of homolog chromosomes is indicated. The generation derived from a cross is indicated as F1, and the progeny of its self-fertilization is indicated as F2. The uppercase Greek letter delta (Δ) represents the loss of a chromosome fragment. In cells with the appropriate genotype, the loss of a chromosome fragment containing the *CHLORATA-42* (*CH-42*) transgene and the wild-type copy of the *EMB* gene gives rise to a cell with pale-green genotype which might be accompanied by a mutant phenotype caused by the *emb* mutation.
the *emb* mutations were also established from each cross as a control. We tested the Mendelian segregation of the yellow *ch-42* phenotype in these F3 families. Unexpectedly, we found a high number of plants exhibiting a yellow phenotype in seven (out of the thirteen) families segregating aborted seeds, suggesting that the CH-42 transgene fails to complement the *ch-42* allele (possibly due to silencing) or that it is located at a higher-than-expected chromosomal distance from the corresponding *EMB* gene.

In phenotypically wild-type *ch-42*/*ch-42*; *EMB* *CH-42*/*emb* plants, X-rays can cause chromosomal breaks between the centromere and the T-DNA insertion, and are expected to generate hemizygous yellow sectors when the acentric fragment carrying the extra copy of CH-42 and the *EMB* wild-type allele is lost. A drawback of irradiating F3 families, which comprise seeds with a mixture of genotypes, is that recombination events between the loci of the T-DNA insertions and the linked *EMB* genes might lead to yellow sectors that still keep a functional copy of the *EMB* gene. Any developmental or other visible phenotypes occurring specifically in the yellow sectors can be attributed to the post-embryonic effects of the corresponding *emb* mutation only if they are not observed in the irradiated control families. Because the cells in the L1 layer are colorless and those in the L3 contribute comparatively little to most organs, the *ch-42* yellow phenotype is best scored in the cells of the L2 layer, making this marker most useful for the study of genes that function in this layer.

Two different X-ray dosages were used to induce sectors. On the one hand, water-imbibed seeds were subjected to a dosage of 1000 rad (10 Gy) based on previous reports from the Arabidopsis and maize literature. On the other, dry seeds received a dosage of 16000 rad (160 Gy), as previously described. The irradiation of dry seeds allowed us to stagger the sowing of the irradiated families. Plants were periodically examined under the stereomicroscope to identify yellow sectors. The temperature sensitivity of the *ch-42* mutation, which determined a paler pigmentation at 26 °C than at 20 °C, made the yellow sectors easier to spot and helped us to select plants with the correct genotype. Sectors occurred at a very low frequency in the families irradiated.

**Figure 2.** Selection of *EMB/emb* lines and effects of temperature on *ch-42* plants. (a,b) F2 mature seeds derived from a cross involving *EMB/emb;CH-42/CH-42* and *EMB/EMB;ch-42/ch-42* plants. (a) Absence of abortive seeds indicates that the F2 line does not carry the *emb* mutation, and (b) presence of abortive seeds indicates that the F2 line carries the *emb* mutation. (c,d) Plants from different genotypes growing at (c) 20 °C, and (d) 26 °C. Scale bars represent (a,b) 1 mm, and (c,d) 1 cm.
at 1000 rad (Fig. 3a–c). In these families, we only found 6 sectors, one half of which appeared in control families lacking an emb mutation (Fig. 3c). Three of these sectors, including two in the control families, were completely albino, rather than yellow, suggesting that rearrangements caused by X-rays lead to visible phenotypes even when emb mutations are not involved. By contrast, we found sectors in every family in about 1% of the plants irradiated at 16000 rad (Fig. 3d–f), a frequency that is roughly similar to the frequency reported by Furner et al.\textsuperscript{14}. In the six families that exhibited a clear distortion of the Mendelian segregation of the yellow phenotype caused by ch-42, we found somatic sectors in both types of irradiated families (segregating and not segregating the emb mutation; Fig. 3f), making it difficult to draw conclusions on the post-embryonic roles of the corresponding genes.

Incidentally, this approach occasionally allowed us to find escapers for some emb mutations, i.e. plants that completed the embryogenesis and reached the seedling stage or beyond, potentially providing information on the post-embryonic function of the genes. Escapers were found for mutant alleles of three EMB genes (Fig. 4), in all cases at a very low frequency in the F\textsubscript{2} generation (0.72\% for emb1135, 1.92\% for emb1706-1, and 0.48\% for emb2410-1). The majority of escapers were pale green, as expected from our crossing scheme, and exhibited

Figure 3. Sectors identified after X-rays irradiation. Plants from irradiated families segregating (a,b) emb1441, (d) emb2001, and (e) emb1706 mutations. (c,f) Plants from irradiated families that are not segregating emb mutations. Plants were irradiated at dosages of (a–c) 1000 and (d–f) 16000 rad. Plants were collected (a,c–f) 14 and (b) 40 days after stratification. Scale bars represent (a,c–f) 1 mm and (b) 1 cm.
additional developmental phenotypes. Although we did not genotype the T-DNA insertions in the escapers, the observed phenotypes were absent from the control families (which lacked collapsed seeds), suggesting that they were specifically caused by the loss of a given EMB gene. The emb1135 escapers were small, with fused cotyledons, wrinkled surface and irregular margins (Fig. 4a). The emb2410 escapers expanded their cotyledons and then died (Fig. 4e,f). The emb1706 escapers formed small rosettes, which included leaves with long petioles and adaxially curved leaf laminae (Fig. 4b). When transferred to soil, the emb1706 escapers produced numerous secondary shoots (Fig. 4c) with abnormally patterned flowers (Fig. 4d).

Figure 4. Putative escapers for (a) emb1135, (b–d) emb1706, and (e,f) emb2410 mutations. Plants were collected (a,e,f) 21, (b) 40 and (c,d) 50 days after stratification. Scale bars represent (a,b,d–f) 1 mm, and (c) 1 cm.
Sector induction using Cre recombinase. We also tested a strategy based on the site-specific excision of transgenes driven by a heat-inducible Cre recombinase (Fig. 5). To this end, we prepared two Gateway-compatible versions of the pCB1 vector (see Material and Methods), which is intended for the induction of clonal sectors by means of the Cre-mediated excision of a cassette containing a wild-type copy of the gene of interest (Fig. 6). We used the Gateway cloning technology to systematically create 20 entry clones, each containing a different genomic region able to complement the embryonic lethality of a selected $emb$ mutation (Table 1). These entry clones were transferred to the Gateway-compatible version of pCB1 using LR reactions in order to obtain constructs for plant transformation. Because the Gateway cassette is flanked by two $loxP$ sites, the genomic inserts of these constructs can be excised by expressing Cre to produce GFP-marked, $emb$ mutant sectors.

In order to obtain transgenic lines for 20 non-allelic $emb$ mutations (Fig. 7), we first transformed homozygous $HS_{pro}$:Cre plants with the pCB1-Gateway constructs, each carrying a wild-type copy of a different $EMB$ gene. The resulting $T_1$ transformants are expected to carry insertions of two T-DNAs, one from the pCB1-Gateway vector and another to allow the inducible expression of Cre driven by a heat shock promoter. These transgenic plants were subsequently crossed to $EMB$:emb heterozygotes to isolate plants carrying the $emb$ mutation and both constructs. The $F_2$ progenies of these crosses were genotyped by PCR to verify the presence of both constructs before sector induction. Ideally, the induction of informative sectors should be performed on plants homozygous for the $emb$ mutation and hemizygous for the pCB1-Gateway construct, which would require additional generations and a complex crossing scheme before the plants can be heat-shocked. For this reason, we induced the sectors directly in the $F_2$ plants, some of which must have the desired genotype, although at the expense of screening a larger plant population. Plates containing 6-days-after-sowing $F_2$ seedlings were sealed with Parafilm.

Figure 5. Transgene-mediated approach to generate hemizygous marked sectors for embryo-lethal mutations. (a) Cell with the appropriate genotype for induction of fluorescent sectors by heat shock. This cell is homozygous for the embryo-lethal mutation ($emb$/$emb$) and carries two different constructs, one of them providing a wild-type copy of an $EMB$ gene that allows its normal development, and the other with a heat-shock promoter driving the inducible expression of Cre recombinase. (b) A heat pulse causes the activation of Cre and a concomitant loss of the wild-type copy of the $EMB$ gene through the excision of the Gateway cassette mediated by the action of Cre recombinase on the $loxP$ sites. The subsequent action of GAL4 on the UAS drives the expression of GFP and marks the cell, which is fluorescent and might exhibit any mutant phenotype associated with the loss of function of the $EMB$ gene in adult tissues.
Figure 6. Maps of pCB1 and pCB1-Gateway vectors. (a) The pCB1 binary vector, and (b) the modified pCB1-Gateway vector. LB: T-DNA left border; T: transcriptional terminator; BAR: bialaphos resistance gene; pNOS: nopaline synthase promoter; 35 S: constitutive promoter; loxP: Cre recombination site; tpCRT1: resistance gene; GAL4VP16: transcriptional activator; UAS: upstream activating sequence; GFPem: endoplasmic reticulum-localized green fluorescent protein; RB: T-DNA right border.

Figure 7. Detailed strategy to obtain GFP-marked sectors which are hemizygous for an embryo-lethal (emb) mutation by means of a heat-shock. Only the relevant genotype of each member from a pair of homolog chromosomes is indicated. The generation derived from a cross is indicated as F1, and the progeny of its self-fertilization is indicated as F2. In cells with the appropriate genotype, the activation of Cre recombinase causes the excision of the wild-type copy of the EMB gene and gives rise to a cell marked with GFP that exhibits an additional mutant phenotype caused by the emb mutation.
and heat-shocked for 30 min at 37 °C in a water bath. We reproducibly found leaf sectors for four different emb mutations: emb1408-1, emb1586-1, emb1637-1 and emb2001-1 (Fig. 8). However, sectors similar to those for emb1637-1 and emb2001-1 occurred in the control lines (i.e. lines carrying the pCB1-Gateway and HSp65::Cre constructs but lacking an emb mutation, which we selected in parallel based on the absence of segregating collapsed seeds; Fig. 8g,h), showing that sectors with a mutant phenotype can arise from Cre-induced chromosomal rearrangements even in the absence of an embryonic-lethal mutation. In addition to these, we found some heat-shocked families segregating plants with impaired growth and a chlorotic phenotype (Fig. 8e). These plants exhibited intense and generalized GFP fluorescence (Fig. 8f), questioning whether the observed phenotypes were indeed caused by the loss of a specific EMB gene or if they were instead due to deleterious, non-specific consequences of elevated Cre expression in the affected tissues.

Concluding Remarks
In this report, we have tested two different strategies for the induction of somatic sectors in adult plants. The first approach, based on the use of CAUT lines, did not scale up well for high-throughput studies. In addition to being labour-intensive and time-consuming, this strategy required a complex crossing scheme with several generations before plant materials were ready for irradiation. According to Furner et al., the timing required for preparing a single line is about 40 weeks. This approach is further complicated when the emb mutations reside in the same chromosome arm as the ch-42 marker (on chromosome 4) or when they map very close to a centromere. The latter problem might make it difficult to identify an appropriate CAUT line for a given emb mutation, and a short distance between the CH-42 transgene and the centromere is expected to result in a low frequency of sectors. Furthermore, scoring the boundaries of yellow sectors is a problematic task, particularly when the sectors are small or hard to distinguish from other pale-green necrotic sectors that occur non-specifically (i.e. which might be present in control families) as a secondary effect of the X-ray treatment.

Implementing the second strategy, based on the use of the site-specific Cre recombinase and transgenes, was more straightforward. To establish an efficient cloning pipeline, we first prepared a Gateway destination vector based on the pCB1 vector, which has previously been used effectively to characterize the effects of individual embryonic-lethal mutations in somatic sectors in Arabidopsis thaliana. We found that a skilled operator can efficiently streamline the making of entry clones containing large genomic inserts by using high-fidelity DNA polymerases (e.g. Phusion High-Fidelity DNA Polymerase) and primers containing attB1 and attB2 sites for subsequent recombination into the Gateway-compatible version of pCB1. However, scaling up this approach was also time-consuming because, similar to the approach based on CAUT lines, it required crossing, genotyping and propagating the plants for several generations before obtaining families with an adequate genotype for sector induction. Fine-tuning the X-ray dosages or the duration of the heat-shock treatment should help to minimize the secondary effects of both treatments while optimizing the frequency of somatic sectors specifically being due to loss of EMB functions.

Additional information might be obtained from the characterization of hypomorphic (non-null) alleles of EMB genes, which might be difficult to isolate, or from the complementation of null alleles with transgenes carrying a copy of the corresponding wild-type EMB gene driven by an embryo-specific promoter, two approaches that have been successfully applied to the study of individual genes. As an example, weak mutations in EMB2107 and EMB1611 have recently been found to cause post-embryonic phenotypes in leaves. Such alleles would be ideal controls in future clonal analysis experiments with a larger number of plants or aimed at defining an optimal set of experimental conditions.
Methods

Plant materials, growth conditions and crosses. Seeds of the *Arabidopsis thaliana* L. Heynh. wild-type accessions Landsberg erecta (Ler) and Columbia-0 (Col-0), as well as heterozygous EMB/emb lines and CAUT lines (Table 1) were obtained from the Nottingham Arabidopsis Stock Centre (NASC; http://arabidopsis.info/). Transgenic seeds carrying the Hspro:CRE construct were kindly supplied by Dr. Guy Wachsmann. Seed sterilization, sowing, plant culture and crosses were performed as previously described. Briefly, seeds were sown on plates containing Murashige and Skoog (MS) agar medium (half-strength MS salts, 0.7% plant agar (Duchefa), pH 5.7, and 1% sucrose), stratified at 4 °C in the dark for 24 h and then transferred to TC16 or TC30 growth chambers (Conviron) set to our standard conditions (continuous light at approximately 75 μmol·m⁻²·s⁻¹, 20 °C, 60-70% relative humidity). When required, plants were transferred to pots containing a 2:2:1 mixture of perlite:vermiculite:sphagnum moss and grown in walk-in

| Gene name | Amplified region (bp) | Primers |
|-----------|-----------------------|---------|
| ATSW13A   | 4001                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB1381   | 5694                  | F: ggggacaagttgtgacaaaaaag-aggctTTGACCGTAATACACATCC<br>R: ggggaccactttgcacaaaaaag-aggctTAAAAGAGAGGATTCCAC |
| EMB1408   | 5191                  | F: ggggacaagttgtgacaaaaaag-aggctCTCATCTGCTTGCACCA<br>R: ggggaccactttgcacaaaaaag-aggctTAAAAGAGAGGATTCCAC |
| EMB1441   | 8456                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB1513   | 4593                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB1586   | 3901                  | F: ggggacaagttgtgacaaaaaag-aggctTTGACCGTAATACACATCC<br>R: ggggaccactttgcacaaaaaag-aggctTAAAAGAGAGGATTCCAC |
| EMB1611   | 7464                  | F: ggggacaagttgtgacaaaaaag-aggctCTCATCTGCTTGCACCA<br>R: ggggaccactttgcacaaaaaag-aggctTAAAAGAGAGGATTCCAC |
| EMB1637   | 4001                  | F: ggggacaagttgtgacaaaaaag-aggctCTCATCTGCTTGCACCA<br>R: ggggaccactttgcacaaaaaag-aggctTAAAAGAGAGGATTCCAC |
| EMB1674   | 6435                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB1688   | 3384                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB1691   | 7693                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB1706   | 8220                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB1745   | 6539                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB1895   | 8109                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB1990   | 2533                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB2001   | 3822                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB2036   | 3501                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB2107   | 4995                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB2736   | 5372                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| EMB3008   | 5953                  | F: ggggacaagttgtgacaaaaaag-aggctACTTTTGCGGTGCACGGA<br>R: ggggaccactttgcacaaaaaag-aggctCTCATCTGCTTGCACCA |
| GAL4      | 489                   | F: TCAAGTCGCTCCCAAAGAGAGC<br>R: TGAAGTTGCTCCATAACGAG |
| CRE       | 1031                  | F: CACCATGCCAAATTACGAGTTAC<br>R: CTAATGGCCATCCATCCAGCAG |

Table 2. Primers used in this work. F: forward primer. R: reverse primer. attB1 and attB2 sites are represented in lower case.
growth chambers set to our standard conditions. For selection of transgenic plants, T1 seeds were sown in flat pots containing perlite and river sand and were sub-irrigated with ATM supplemented with 15 mg/l glufosinate ammonium (Finale).

Irradiation and sector screening. Irradiation of Arabidopsis seeds was performed using a Philips MG102 X-ray cabinet. Seeds were irradiated at doses of 10 Gy for sterilized seeds and 160 Gy for dry seeds, as previously described[6,14]. At least two control wild-type lines and two heterozygous EMB/emb lines of each of the 13 genotypes were irradiated. After irradiation, seeds were sown in Petri dishes and the resulting plants were checked periodically, looking for mutant sectors. Pictures of the different sectors were taken, and the leaves that contained them were collected and stored. Plants containing sectors were moved to soil pots in order to verify if they spread to other plant organs like secondary shoots, cauline leaves or flowers, as previously described[18].

Modification of pCB1 vector. We modified the pCB1 vector[7] for use with the Gateway cloning technology (Fig. 6). For this, pCB1 was linearized with NotI, and the resulting cohesive ends were filled in with Klenow to generate blunt ends. A PCR product corresponding to a Gateway cassette (Frame A) was amplified with Phusion DNA polymerase (Finnzymes) and ligated to pCB1 using T4 ligase (Fermentas). The ligation products were transformed into the Escherichia coli DB3.1 strain, and colonies resistant to both kanamycin and chloramphenicol were selected. This modified plasmid was called pCB1-Gateway. After purifying the plasmids that carried the insert of interest, its orientation was checked with a Smal and Sall double digestion. We obtained two different versions of the pCB1-Gateway vector, with the Gateway cassette oriented in both possible directions, (+) and (−).

Generation of pCB1-Gateway constructs. In order to introduce a wild-type copy of the EMB genes of interest into the pCB1-Gateway empty vector, we amplified genomic regions containing each EMB gene spanning from the end of the previous gene coding region to the beginning of the following gene coding region, to make sure that the regulatory sequences were also included. We designed primer pairs containing attB1 and attB2 sites (Table 2), in order to amplify the regions that contain each EMB gene of interest from its corresponding bacterial clone. These regions were PCR amplified using the Phusion polymerase (Finnzymes). The amplification products were purified and used in different BP reactions (Invitrogen), in which the pGEM-T Easy 221 plasmid was used as entry vector. Chemocompetent DH5α Escherichia coli cells were transformed by heat shock with the products of BP reactions. Colonies carrying the pGEM-T Easy 221 plasmid were selected in Petri dishes with LB medium supplemented with ampicillin. Insert presence was checked by rapid size screen with lysis buffer22, digestion with the restriction enzyme NotI and PCR with plasmid and insert primers (Table 2). Positive colonies were used to perform LR reactions (Invitrogen) with the appropriate pCB1-Gateway destination vector. Each LR reaction was performed twice, using the pCB1-Gateway plasmids with the Gateway cassette in both orientations. Chemocompetent DH5α Escherichia coli cells were transformed by heat shock with the LR products and colonies carrying the pCB1-Gateway vector were selected in LB medium supplemented with kanamycin. The presence of each insert was checked by double digestion with Xbal and Sall restriction enzymes. Positive clones were mobilized into Agrobacterium tumefaciens C58C1 pSOUP cells by electroporation. Every pCB1-Gateway construct was transferred to plants carrying the HSpro::CRE construct by the floral dip method[21].

Heat shock sector induction. Plants carrying HSpro::CRE and pCB1-Gateway constructs combined with emb mutations were sowed in Petri dishes. After growing for 6 days, plates were sealed with Parafilm and submerged in water at 37 °C during 4 hours. They were put inside the plant growth chamber again and, after 5-6 days, the different lines were observed using fluorescence microscopy, in order to detect sectors with GFP signal.

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Additional Information
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