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1 **A shortcut in forward genetics: concurrent discovery of mutant phenotype and causal mutation**
2 **in Arabidopsis M2 families via MAD-mapping**

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22 **CLASSIFICATION**

23 BIOLOGICAL SCIENCES (major) / Plant, Soil, and Microbial Sciences (minor)

24 **KEYWORDS**

25 EXORIBONUCLEASE 4 (XRN4) / ETHYLENE INSENSITIVE 5 (EIN5), DECAPPING 1 (DCP1),
26 diplontic selection, Transcription activator-like effector (TALE)

27 **AUTHOR CONTRIBUTIONS**

28 D.R.H., R.M., F.E.K and T.L. designed the research; D.R.H, R.M., and M.W. performed the research;
29 H.S. analysed the NGS data; and D.R.H., K.S., and T.L. wrote the paper.

30

31 **ABSTRACT**

32 Forward genetics is a powerful tool to establish phenotype-genotype correlations in virtually all areas
33 of plant biology and has been particularly successful in the model plant *Arabidopsis*. This approach
34 typically starts with a phenotype in an M2 mutant, followed by identifying a causal DNA change in F2
35 populations resulting from a cross between the mutant and a wildtype individual. Ultimately, two
36 additional generations are needed to pinpoint causal DNA changes upon mutant identification. We
37 postulated that genome-wide allele frequency distributions within the mutants of M2 families facilitate
38 discrimination of causal versus non-causal mutations, essentially eliminating the need for F2
39 populations. In a proof-of-principle experiment, we aimed to identify signalling components employed
40 by the executor-type resistance (*R*) protein, *Bs4C*, from pepper (*Capsicum pubescens*). In a native
41 setting, *Bs4C* is transcriptionally activated by and mediates recognition of the transcription activator-
42 like effector *AvrBs4* from the bacterial pathogen *Xanthomonas*. *Arabidopsis* containing an estradiol-
43 inducible *Bs4C* transgene was used in a conditionally lethal screen to identify second-site suppressor
44 mutations. Whole genome sequencing was used for M2 mutant allele-frequency distribution (MAD)
45 mapping in three independent M2 families. MAD-mapping uncovered that all three families harboured
46 mutations in *XRN4*, a novel component of executor R protein pathways. Our work demonstrates that
47 causal mutations observed in forward genetic screens can be identified immediately in M2 families
48 instead of derived F2 families. Notably, the timesaving concept of MAD mapping should be applicable
49 to most crop species and will advance the appeal of forward genetics beyond applications in
50 fundamental research.

51 **SIGNIFICANCE**

52 Forward genetics has uncovered numerous genes that govern plant immune reactions. This procedure
53 relies on mutant plants with modified immune reactions followed by identification of causal DNA
54 changes in derived F2 progeny. We developed a novel forward genetics concept where causal DNA
55 changes are identified in the initial M2 mutants, making time consuming establishment of F2 populations
56 obsolete. To confirm the feasibility of the concept, we mutagenized transgenic *Arabidopsis* seeds
57 containing the cell death executing resistance gene *Bs4C* from pepper. Whole-genome sequencing of
58 identified mutant families that lack a *Bs4C*-dependent cell death revealed the *XRN4* gene as a novel
59 component of *Bs4C*-dependent cell death. This confirms our hypothesis that causal mutations can be
60 identified directly within phenotypically selected mutant families.

61 MAIN TEXT

62 INTRODUCTION

63 To elucidate the molecular basis of biological phenomena, typically the function or activity of key
64 proteins are studied with the intent of uncovering physical or functional connections with other
65 components. Discovery and validation of the components that are involved in a biological process
66 typically involves a synergistic combination of genetic and biochemical approaches. Speed and
67 analytical power are the major parameters when selecting suitable experimental approaches to identify
68 novel elements of a biological process. Forward genetics has been a key discovery tool for biological
69 processes in *Arabidopsis thaliana* (*Arabidopsis* hereafter) and other plant species, since it requires no
70 prior knowledge of the molecular components that are involved in the process of interest, as it solely
71 relies on differential phenotypes (1). In forward genetics, mutagenesis is often used to induce loss-of-
72 function alleles that typically translate into a phenotypic change in M2 individuals that contain the
73 mutation in a homozygous configuration. Traditionally, causal mutations are located by linkage
74 mapping, usually carried out in F2 populations. Such F2 populations are established by the crossing of
75 M2 individuals to wildtype lines, followed by selfing of the F1. The advent of next generation sequencing
76 (NGS) technologies has drastically simplified this process. Using whole-genome sequencing of bulked
77 DNA of mutant recombinants enabled simultaneous mapping and identification of causal mutations in
78 segregating populations using a single sequencing experiment (2). The base pair resolution of whole-
79 genome sequencing technologies also allowed the use of isogenic crosses (i.e. crosses between the
80 mutant and non-mutagenized individual of the same strain) where random background mutations are
81 used as genetic markers instead of natural DNA polymorphisms between plant genotypes (3, 4). This
82 had the immediate advantage of bypassing practical challenges caused by phenotypic variation
83 between the parental lines of a regular cross that often complicate visual scoring of a specific mutant
84 phenotype in derived segregating populations.

85 Utilization of isogenic mapping populations also made way for the elimination of two additional
86 generations after the identification of the M2 mutant phenotypes to generate a mapping population.
87 Instead, selfing of heterozygous M2 mutants generates isogenic M3 mapping populations, thereby
88 minimizing the number of generations needed (5). The disadvantage of this method is that it requires
89 the generation of multiple offspring populations since the heterozygous M2 mutants that are needed to
90 establish M3 mapping populations cannot be phenotypically distinguished from wildtype individuals in
91 the M2 generation. As an alternative to genetic mapping, whole-genome sequencing of multiple allelic
92 mutants in one gene outlines a powerful way to identify causal genes without generating any
93 segregating populations (6). While two allelic mutants can already be sufficient for the identification of
94 a candidate gene, this approach is not free from crossing, as the allelism tests relies on pair-wise inter-
95 mutant crosses. Consequently, unless allelic mutants of one gene are known and available,
96 identification of causal DNA changes relies on segregating populations that need to be generated after
97 the identification of the mutant phenotype. This time-consuming and tedious task substantially reduces
98 the appeal of forward genetics. Due to this, only a small fraction of available M2 mutants are usually
99 used for follow up analysis. Therefore, a procedure that does not depend on laborious and time-
100 consuming crosses would enhance the attractiveness of forward genetics.

101 Plants have two interconnected layers of immunity that collectively provide protection against
102 parasites. Cell surface-localized pattern recognition receptors (PRRs) mediate recognition of conserved
103 pathogen-associated molecular patterns (PAMPs) such as bacterial flagellin (7). To overcome PAMP-
104 triggered immunity (PTI), pathogens have evolved virulence factors known as effectors that are typically
105 translocated into host cells to interfere with PTI and promote disease (8). In response, plants have
106 evolved resistance (*R*) genes that mediate recognition of microbial effectors. Typically, this effector-
107 triggered immunity (ETI) coincides with a plant cell death reaction (hypersensitive response). In most
108 cases, ETI is mediated by intracellular nucleotide-binding/leucine-rich-repeat proteins (NLRs), where
109 they sense activity and/or structural components of microbial effectors and in turn execute a defence
110 reaction (9-11).

111 Analysis of plant immune reactions triggered by transcription-activator-like effectors (TALEs)
112 from *Xanthomonas* uncovered a mechanistically novel plant *R* gene class where TALEs bind to
113 corresponding effector binding elements within *R* gene promoters and activate transcription of the
114 downstream encoded *R* protein (12, 13). In such TALE-activated *R* genes, the encoded *R* protein is not
115 involved in effector recognition, but only in the execution of the plant immune reaction. Accordingly,
116 these *R* proteins have been designated executors (13, 14).

117 As of yet, five plant *R* genes that are transcriptionally activated by and mediate recognition of
118 matching TALE proteins have been cloned. With the exception of rice Xa10 and Xa23, which share
119 50% identity, the predicted executor R proteins show no homology to each other. Recent studies of the
120 executor R protein Bs3 from pepper revealed that the Bs3-triggered immune reaction involves
121 accumulation of salicylic acid (SA), a plant defence hormone that is involved in NLR- and PRR-triggered
122 immune pathways (15). These findings possibly suggest that NLR-, PRR- and executor-type R proteins
123 use, at least in part, common signalling elements to trigger plant defence. However, at this point, little
124 is known about how executor R proteins trigger plant defence.

125 Bs4C is an executor-type R protein from pepper that was previously shown to mediate
126 recognition of the cognate TALE protein AvrBs4 (16). To identify components of the Bs4C-triggered cell
127 death reaction, we initiated a conditionally lethal forward genetic screen in Arabidopsis that identified
128 three abolishment of cell death by executor (*ace*) M2 mutant families. NGS-based M2 mutant allele-
129 frequency distribution (MAD) mapping was used instead of commonly used F2 mapping to identify
130 causal mutations and uncovered that all three *ace* mutant families carried mutations in the Arabidopsis
131 *XRN4/EIN5* gene.

132 RESULTS

133 The pepper executor R protein Bs4C induces plant growth arrest in Arabidopsis

134 To identify genes that the pepper executor R protein Bs4C requires to trigger plant cell death, we
135 initiated a forward genetic screen in the model system Arabidopsis. To do so, we generated a T-DNA
136 encoding an epitope-tagged Bs4C derivative (*Bs4C-FLAG-GFP*) under the transcriptional control of an
137 estradiol-inducible promoter (Fig. 1A) (17). *Agrobacterium tumefaciens* mediated transient
138 transformation of *Nicotiana benthamiana* leaves (agroinfiltration) confirmed that the T-DNA construct
139 mediates cell death in the presence, but not in the absence, of the chemical inducer estradiol,
140 suggesting that the T-DNA construct would confer estradiol dependent *Bs4C* expression in transgenic
141 Arabidopsis plants (Fig. 1B). We then transformed the estradiol-inducible *Bs4C-FLAG-GFP* T-DNA
142 (*Estr:Bs4C-FLAG-GFP* hereafter) into the Arabidopsis ecotype Columbia (Col-0 hereafter). We
143 inspected seeds of numerous T2 lines to identify ones that showed a strong, estradiol dependent growth
144 inhibition phenotype. Segregation analysis of T2 seeds on kanamycin containing media identified lines
145 that presumably contain a single-copy transgene insertion. T2 lines with a single-copy transgene and
146 strong seedling growth inhibition phenotype were chosen to produce large quantities of T3 seeds for
147 ethyl methanesulfonate (EMS) mutagenesis. Before carrying out EMS mutagenesis, we confirmed
148 functionality of seedling growth inhibition in T3 seeds. To do so, we placed four-day old seedlings into
149 liquid media containing or lacking estradiol, and analysed seedling growth. We found that in the
150 presence, but not in absence of estradiol, the *Estr:Bs4C-FLAG-GFP* seedlings were severely stunted
151 in their growth (Fig. 1C). By contrast, a transgenic line containing a *GFP-GUS* reporter gene under
152 expressional control of the estradiol-inducible promoter (*Estr:GFP-GUS* hereafter) showed no signs of
153 estradiol-dependent growth inhibition. Hence, growth inhibition depends on presence of both the *Bs4C*
154 transgene and estradiol. Immunoblot analysis also showed that the *Estr:Bs4C-FLAG-GFP* transgenic
155 line contained an estradiol-dependent signal matching to the expected 50.6 kDa Bs4C-FLAG-GFP
156 fusion protein (Fig. 1D). Taken together, our data illustrate that the pepper executor R protein Bs4C
157 induces cell death when being expressed in the model plant Arabidopsis. Moreover, the established
158 transgenic Arabidopsis lines containing the *Bs4C* gene under control of an estradiol-inducible promoter
159 provide the basis for genetic dissection of Bs4C-dependent cell death in Arabidopsis.

160 A conditionally lethal screen identifies Arabidopsis mutants that do not execute a Bs4C 161 dependent cell death

162 To induce randomly distributed mutations across the Arabidopsis genome, approximately 10,000
163 *Estr:Bs4C-FLAG-GFP* T3 (M0) seeds were treated with EMS and planted into soil. Corresponding M1
164 plants were individually bagged, and derived M2 seeds were harvested, creating 4,000 M2 families.
165 About 100 seeds of each M2 family, equating to approximately 400,000 M2 seeds in total, were studied
166 as representatives for the entire M2 families. Seeds were allowed to grow on agar plates containing
167 estradiol in an effort to identify second-site suppressor mutants that inhibit Bs4C-dependent cell death.
168 After 14 days, most seedlings had stopped growing and neglected cotyledon emergence (Fig. 2A). M2
169 families containing putative suppressor mutations were easily detectable, as they were large in size
170 and developed roots and true leaves with a green colour similar to *Estr:GFP-GUS* (Fig. 2B). A total of

171 46 M2 families contained individual plants that grew like *Estr:GFP-GUS* plants on estradiol-containing
172 agar plates. A low percentage of survivors within most M2 families suggests recessive inheritance of
173 suppressor alleles. Survivors from each M2 family were transplanted from estradiol plates to soil for
174 further investigation. Here, we present the analysis of three representative *ace* mutant families.

175 ***ace* mutants show no systemic cell death despite having a functional *Bs4C* gene**

176 Two classes of mutations were expected to be identified from our forward genetic screen: those that
177 are within putative signalling and/or regulatory components that *Bs4C* requires to induce plant cell
178 death, and those that are within the transgene and affect expression and/or functionality of *Bs4C*. To
179 exclude plants that did not accumulate similar levels of *Bs4C* protein to that of the parental line, we
180 analysed *Bs4C* protein expression in *ace1*, *ace2*, and *ace3* mutants by immunoblot analysis. In all three
181 *ace* mutants, immunoblot analysis highlighted signals matching to the expected 50.6 kDa *Bs4C*-FLAG-
182 GFP fusion protein (Fig. 2D, Fig. S1). Moreover, we PCR-amplified and sequenced the *Bs4C* coding
183 sequence (CDS) in all three *ace* mutants' families and found that they all contained the wildtype *Bs4C*
184 CDS.

185 **Segregation in *ace* M2 seeds does not fit to the expected 1:7 ratio**

186 Arabidopsis M1 seeds contain two diploid cells that give rise to generative organs (inflorescence) of M1
187 plants that can be phenotypically studied in the M2 generation (1, 18). If EMS mutagenesis induces a
188 mutation in one of the two diploid M1 precursor cells, this translates into a 1:7 phenotypical segregation
189 of bulked M2 family seeds, assuming recessive inheritance. Thus 12.5% of the seeds of each *ace* M2
190 family are expected to survive on agar plates containing estradiol. We plated several hundred M2 seeds
191 for each of the three *ace* families on agar plates containing estradiol and observed survival rates of
192 6.1% (45/734), 2.0% (17/833), and 5.1% (57/1114) for *ace1*, *ace2*, and *ace3* mutant families,
193 respectively. Given the clear phenotype in all three *ace* mutant families and the large number of studied
194 M2 seeds, it seems unlikely that deviations of observed versus expected segregation ratios are due to
195 errors in phenotypical scoring. Thus, the observed distorted segregation is possibly the consequence
196 of diplontic selection, a process of competition between cells within a meristem that can result in
197 reduced proliferation of mutated cells (19).

198 **Segregation of EMS mutations in *ace* M2 families provides a basis to identify causal mutations**

199 Irrespective of the observed segregation data, such M2 plants that grow in the presence of estradiol
200 (survivors) should have the causal mutation exclusively in the homozygous configuration, assuming
201 recessive inheritance. By contrast, non-causal mutations are expected to segregate randomly in M2
202 survivors with exception of those that are linked to the causal mutations. NGS-mediated analysis of the
203 allele frequency for each EMS mutation within a pool of survivors from one M2 family can therefore
204 reveal mutations that are homozygous across all M2 survivors in one family and that are potentially
205 causal for the observed cell death suppression phenotype (Fig. 3). We termed this concept as M2
206 mutant allele-frequency distribution (MAD) mapping and studied the feasibility of MAD-mapping in three
207 representative *ace* mutant families.

208 **Three *ace* families have distinct mutations in the Arabidopsis *XRN4/EIN5* gene**

209 Whole-genome sequencing was performed on the parental line that was originally used for EMS
210 mutagenesis (*Estr:Bs4C-FLAG-GFP*) and three distinct DNA pools composed of survivors from *ace1*,
211 *ace2*, and *ace3* families, respectively. The DNA pools of *ace1*, *ace2*, and *ace3*, contained 38, 18, and
212 25 M2 survivors, respectively. Paired end sequencing was used with a minimum depth of 150X
213 coverage to determine EMS mutations and their allele frequencies in *ace1*, *ace2*, and *ace3* M2 families
214 (Fig. 4, Fig. S2-S4, Table S1). We detected 60, 150, and 487 EMS mutations specific for the *ace1*,
215 *ace2*, and *ace3* M2 families, respectively. Scanning the pooled genomes of M2 survivors for a selection-
216 induced increase in the frequency of mutant alleles, we identified genomic regions with increased (and
217 locally fixed) mutant allele frequencies on chromosome 1 for all three *ace* mutants (Fig 4). In order to
218 select candidate mutations in these genomic regions that possibly cause the observed cell death
219 suppression phenotype, we limited our search for causal mutations by considering only EMS mutations
220 with an allele frequency of 0.95 or higher. Moreover, our search was restricted to base pair changes
221 that are characteristic to EMS-induced mutations (C to T or G to A). We disregarded mutations that
222 occurred in either the non-coding regions (intronic or untranslated regions) or caused synonymous
223 mutations in the coding regions, thereby focusing on missense and nonsense mutations in coding
224 regions. This narrowed our search down to one candidate gene in *ace1* and *ace2* pools (*AT1G54490.1*),

225 and to three candidate genes in the *ace3* pool (*AT1G54490.1*, *AT1G52940.1*, and *AT1G55110.1*).
226 *AT1G54490.1*, which encodes the EXORIBONUCLEASE 4 (XRN4) / ETHYLENE INSENSITIVE 5
227 (EIN5) protein (Fig. 4) (20-22), was found to be mutated across all three *ace* families, indicating the
228 functional impact of mutations in this specific CDS on the common phenotype. As one would expect,
229 each M2 family had distinct mutations in the coding region of *AT1G54490.1* (Fig. 5, Fig. S5). The *ace1*
230 mutation lies within the sixth exon, and changes the wildtype aspartate to an asparagine (D to N). The
231 *ace2* mutation is in the eighth exon, and changes the wildtype tryptophan to a premature stop codon
232 (W to *). The *ace3* mutation is found in the third exon of *AT1G54490.1*, and alters the parental or
233 wildtype amino acid of an alanine to a valine (A to V) (Fig. 5).

234 Altogether, our data suggests that the identified *XRN4/EIN5* mutant alleles abolish Bs4C
235 dependent cell death in Arabidopsis. Moreover, our data demonstrates that MAD-mapping is a highly-
236 efficient approach for identification of causal mutations in M2 mutant families.

237 DISCUSSION

238 We demonstrated that the estradiol-inducible expression of the pepper executor-type R protein Bs4C
239 triggers systemic cell death in transgenic Arabidopsis plants. This conditionally lethal phenotype was
240 used in a forward genetic screen to identify three distinct Arabidopsis *ace* mutants that do not execute
241 Bs4C-dependent plant cell death. We determined the frequency of EMS-mutations in three distinct *ace*
242 M2 families, a process that we designated as MAD-mapping. This identified mutations for all three M2
243 families within *AT1G54490.1*, which encodes the exoribonuclease XRN4 (22).

244 As of yet, *XRN4* is the first known genetic component required for cell death triggered by
245 executor-type R proteins. XRN4 is the plant cytoplasmic homolog of yeast and metazoan XRN1, and
246 catalyses degradation of uncapped mRNAs from the 5' end (23, 24). In a simplistic model, XRN4 could
247 degrade a transcript encoding a negative regulator of the Bs4C-dependent cell death. Absence of
248 functional XRN4 in *ace* mutant plants would presumably cause increased expression of the putative
249 negative regulator and inhibit Bs4C-triggered cell death, being consistent with the observed mutant
250 phenotype.

251 Recent studies uncovered that PAMP-induced activation of PRRs results in phosphorylation of
252 the DECAPPING 1 (DCP1) protein that in turn, interacts with and activates XRN4 (25). It is assumed
253 that activated XRN4 degrades transcripts encoding positive and negative regulators of PRR-triggered
254 immune reactions. It is therefore conceivable that XRN4 could be a shared regulator of PRR- and
255 executor R protein-triggered immune pathways. Future studies will have to clarify the exact role of
256 XRN4 in Bs4C-dependent cell death reactions, and whether or not XRN4 is also involved in other plant
257 defence pathways.

258 Forward genetic screens and subsequent isolation of causative mutations by positional cloning
259 is an essential gene discovery tool for elucidation of any kind of biological process in plants (26). The
260 advent of next-generation sequencing technology introduced several innovations into the process of
261 mutation identification, including simultaneous mapping and identification of causal mutations as well
262 as the utilization of isogenic mapping populations (3, 4). Unless allelic groups are available, mutation
263 identification still relies on the time-consuming process of generating numerous segregating
264 populations. Therefore, the workload and time that is needed to establish segregating populations
265 remains a major limitation in forward genetics. We postulated and experimentally validated that the
266 segregation of causal mutations in M2 families, which is regularly used for the initial identification of
267 mutant phenotypes, can already be used to identify causal mutations, ultimately removing the need for
268 tedious generation of segregating populations. Therefore, upon mutagenesis of seeds, only two
269 generations are needed to identify causal mutations. Given the generation time of approximately 8
270 weeks in Arabidopsis, it essentially takes less than one year to identify causal mutations via MAD-
271 mapping. While we demonstrated the feasibility of MAD-mapping in the model plant Arabidopsis, the
272 concept could be applicable to any plant and even non-plant species.

273 We combined MAD-mapping with a conditionally lethal screen (Fig. 3); and a benefit of this
274 combination is that it can be carried out at the seedling stage. Accordingly, large numbers of mutants
275 can be studied in a short time and the need for space remains quite limited. Plant defence reactions
276 typically rely on the execution of cell death reactions, and as a result, several conditionally lethal screens
277 have been conducted in the past to study plant R proteins and to identify signalling components of R
278 pathways (27-29). These screens depend on inducible promoters that typically contain constitutively
279 expressed elements. For example, the estradiol-inducible system that we used contains the

280 constitutively expressed synthetic transcription factor XVE that is activated by estradiol (Fig. 1). It has
281 been noted in the past, that estradiol-inducible transgenes lose inducibility throughout generations (30).
282 This phenomenon typically starts in the T4 and T5 generations and is likely the consequence of
283 transgene silencing. We identified causal mutations in the M2 generation, which corresponds to the T5
284 generation (Fig. 3). In previous studies, we used the estradiol-inducible system in a conventional
285 forward genetic screen and established conventional F2 mapping populations, which corresponds to
286 the T7 generation, to identify causal mutations for given M2 survivors. However, we did not observe the
287 expected segregation of cell death in F2 individuals and ultimately could not identify causal mutations
288 by this approach, possibly caused by transgene silencing in the F2/T7 mapping generation. In MAD-
289 mapping, phenotypic identification and isolation are both carried out in the M2 generation, essentially
290 overcoming the problem of gene silencing that possibly occurs in mapping populations derived from a
291 single transgenic M2 plant.

292 While the principle of MAD-mapping is broadly applicable, it cannot be carried out on bulked
293 M2 populations since it is based on the analysis of individual M2 families. Accordingly, after EMS
294 mutagenesis, each M1 plant must be harvested individually to generate a collection of M2 families.
295 Similarly, each M2 family must be studied individually for phenotypic changes. Although MAD-mapping
296 is generally time-saving, it is more laborious in the harvesting and screening phase than conventional
297 screens that are typically based on bulked M2 seeds. On the upside, however, screening of separate
298 M2 families offers the possibility for recovering mutations that are infertile when homozygous via the
299 heterozygous siblings of the mutant plants. Moreover, this strategy guarantees the independence of
300 mutants isolated from distinct M2 families. In the long run, while the analysis of M2 families is more
301 laborious than analysis of bulked M2 seeds, the benefits of MAD mapping vastly overcome the short-
302 term extra work that is required.

303 Overall, we envision that the ease and speed of MAD-mapping will substantially increase the
304 attraction of forward genetic approaches and it stands to reason that MAD-mapping will make a major
305 contribution towards the elucidation of biological pathways in the near future.

306 MATERIALS AND METHODS

307 Plant material and growth conditions

308 *Arabidopsis thaliana* plant material used in this study: Col-0, *Estr:Bs4C-FLAG-GFP*, *Estr:GFP-GUS*,
309 *ace1 family*, *ace2 family*, *ace 3 family*. For the seedling growth assay, seeds were sterilized using 80%
310 ethanol and 0.05% Triton X-100 solution, and left to stratify in the darkness at 4 °C for two days on ½
311 MS plates (0.43% (w/v) MS Salts (Gibco), 1% (w/v) Sucrose, 0.05% MES, pH 5.8) containing 200 µg/mL
312 Cefotaxim. Seeds were put to long day (16hr light/8hr dark) at 20 °C in light and 18 °C in dark for four
313 days. On the fourth day, seedlings were transplanted to 48-well plate, each well containing either 20
314 µM estradiol, or mock treatment (1% (v/v) DMSO), and left for 10 more days. On the 14th day, seedling
315 growth was analysed. For seedling immunoblot detection, four 14 day old seedlings of each genotype
316 were placed in either 20 µM estradiol or 1% DMSO, vacuum infiltrated, and left at room temperature for
317 24 hours. Samples were then flash frozen and used for immunodetection, as described below. For EMS
318 mutagenesis, approximately 200 mg of *Estr:Bs4C-FLAG-GFP* *Arabidopsis* seeds were allowed to swell
319 in water for 3 days. Afterwards, these seeds were incubated in 50 mL of 0.3% EMS solution for 6 hours,
320 shaking. The seeds were then transferred to a Nalgene Filter Unit and washed six times with water.
321 The seeds were then resuspended in 0.1% phytoagar and sowed on soil. After 10 days, the seedlings
322 were transplanted into individual pots in an outdoor greenhouse (16hr light/8hr dark, temperature
323 minimum 18° C, no humidity control). These individual plants now generated the M1 population. After
324 6 weeks, seeds from each individual M1 plant were harvested, creating 4000 individual M2 families.
325 For the screening of *ace* families, 100 seeds of each of the 4000 M2 families were placed in a 96 well
326 plate, and were gas sterilized (80 mL NaClO and 3 mL 32% HCl solution) overnight. The following day,
327 200 µL of 1% phytoagar was placed in each well, sealed, and left to stratify at 4 °C in the dark for 2
328 days. The seeds were then plated on ½ MS plates (described previously) containing 20 µM estradiol,
329 and put to long day. Plates were left for 14 days, suppressing families were selected, and survivors
330 were transplanted to soil. Five week old plants were used for immunodetection by taking three 4 mm
331 punches and vacuum infiltrating them with 20 µM estradiol, and letting them sit for 24 hours before flash
332 freezing and going forward with immunodetection (as described below). To sequence the transgene,
333 gDNA was collected from leaf tissue, PCR amplified, and sent for Sanger sequencing.

334 **Plasmid construction**

335 For *Estr:Bs4C-FLAG-GFP* and *Estr:GFP-GUS* T-DNA constructs coding sequences of *Bs4C*, *3xFlag*,
336 *GFP* and *uidA* were PCR amplified and cloned via GoldenGate cloning into pENTR CACC-AAGG.
337 Resulting pENTR-Bs4C-FLAG-GFP and pENTR-GFP-GUS were used in LR reaction together with
338 pER10-GW generating pER10-Bs4C-FLAG-GFP and pER10-GFP-GUS.

339 **Transgenic lines**

340 *Estr:Bs4C-FLAG-GFP* and *Estr:GFP-GUS* were generated using *Agrobacterium* GV3101 containing
341 pER10-Bs4C-FLAG-GFP or pER10-GFP-GUS in a floral dip method. Transgenic *A. thaliana* were
342 selected with Kanamycin on ½ MS plates.

343 **Genomic DNA extraction**

344 Approximately 100-150 mg of leaf material was collected. 600 µL of CTAB buffer (100 mM Tris-HCl pH
345 8.0, 20 mM EDTA, 1.4 M NaCl, 2% (w/v) cetyltrimethyl ammonium bromide) was added, and
346 homogenized using a vortex. Samples were incubated at 65 °C for 30 minutes. Heated samples were
347 spun down at room temperature. 500 µL of the supernatant was transferred to a new tube. 2.5 µL of
348 RNase A (10 mg/mL, ThermoFisher) was added, and gently vortexed, and incubated at 37 °C for 30
349 min. 500 µL of chloroform was added, and mixed. Samples were spun down at room temperature, and
350 450 µL of the aqueous phase was added to a new tube. 450 µL of 100% isopropanol was added, and
351 gently mixed. The tubes were then spun down until a pellet formed, and the supernatant was discarded.
352 500 µL of 70% ethanol was added, mixed, spun down at room temperature, and then the supernatant
353 was discarded. This was repeated twice. The pellet was then dried at 35 °C. The dried pellet was
354 dissolved in 35 µL of 10 mM Tris-HCl pH 8.0, and quantified using a Qubit (ThermoFisher). These
355 samples were then sent for NGS.

356 **Next generation sequencing and mapping populations**

357 Raw reads of each sample were aligned to Col-0 reference genome (The Arabidopsis Genome Initiative
358 2000; www.arabidopsis.org) using *GenomeMapper* (31), after which short-read alignments were
359 corrected for read-pair information and consensus bases were called with *shore* (31). After removing
360 common SNPs between each mutant and the parental line, the causal mutation of each mutant was
361 predicted by analysing allele frequencies with SHOREmap v3.0 (2, 32).

362 **Immunoblotting**

363 Samples were flash frozen and then ground to a fine powder. 50 µL of SDS loading buffer (50 mM Tris-
364 HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) was added, and boiled at
365 95 C for 10 min. Samples were loaded onto a SDS-polyacrylamide gel (4% stacking, 10% resolving),
366 and then transferred to a PVDF-membrane (BioRad). Samples were blocked in 5% milk/1X TBST (50
367 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20), and anti-bodies were then applied. Anti-FLAG primary
368 antibody (F1804, Sigma-Aldrich) raised in mouse, at 1:5000 dilution, was used, shaking overnight. The
369 next day, membranes were washed with 1X TBST (50 mM Tris base, 150 mM NaCl, 0.05% (v/v) Tween-
370 20), and the anti-mouse-HRP secondary antibody (A9044, Sigma-Aldrich) was used at a 1:2500
371 dilution, and incubated for 2 hours. Anti-GFP-HRP conjugated primary antibody (SC-9996, SantaCruz)
372 at 1:2500 dilution was used, and incubated for 2 hours. The blot was washed 3 times with 1X TBST,
373 and once with 1X TBS (1X TBST, but no Tween-20 added). The Clarity ECL Substrate (BioRad) and
374 the Amersham™ Imager 600 (GE Life Sciences) machine were used for imaging. All membranes were
375 stained with Ponceau.

376 ACKNOWLEDGEMENTS

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384 plants, as well the ZMBP gardeners for taking care of these plants.

385 CONFLICTS OF INTEREST

386 The authors have no conflicts of interest to report.

387 FIGURE LEGENDS

388 **Figure 1. Bs4C induces growth arrest in Arabidopsis.**

389 **A** | A T-DNA construct for estradiol-inducible expression of the pepper executor protein Bs4C. Driven
390 by the constitutive G10-90 promoter, the *XVE* gene translates into a chimeric transcriptional activator
391 that contains an estrogen receptor domain. When estrogen (E) is present (here in the form of β -
392 estradiol), it binds to the XVE protein, mediates XVE homodimerization and enables XVE to bind to the
393 LexA operator (OlexA). This induces transcription of the downstream gene encoding a Bs4C-FLAG-
394 GFP protein. The Bs4C fusion protein requires the putative signaling elements A, B, C to trigger plant
395 cell death. Methylation of the G10-90 promoter (M) can cause transcriptional silencing of the G10-90
396 promoter and results in a non-inducible promoter.

397 **B** | A *Bs4C* transgene triggers estradiol-dependent cell death in *Nicotiana benthamiana* leaves. The
398 depicted T-DNA constructs were delivered into *N. benthamiana* leaves via *Agrobacterium* mediated
399 transient transformation. Leaf areas into which the inducer estradiol was infiltrated are highlighted with
400 a red line.

401 **C** | An inducible *Bs4C* transgene triggers systemic cell death in Arabidopsis. Four day old seedlings of
402 indicated genotypes were placed in liquid media either containing estradiol or a lacking estradiol (Mock).
403 Ten days later, the seedlings show cell death in presence of estradiol and the *Bs4C* transgene.

404 **D** | Immunoblot analysis using anti-FLAG antibody of tissue from two week old Arabidopsis seedlings
405 of depicted genotypes (Col-0, *Estr:Bs4C-FLAG-GFP*, *Estr:GFP-GUS*). Plants were incubated for 24
406 hours in liquid media either containing estradiol or lacking estradiol (mock). Ponceau stained membrane
407 serves as a loading control.

408 **Figure 2. ace screen identifies suppressors of Bs4C-dependent cell death in Arabidopsis.**

409 **A** | Identification of the *ace3* M2 family. Seeds of twelve distinct M2 families are placed in rows on
410 estradiol-containing agar. Boxes framed by dashed lines indicate the region that is covered by seeds
411 of one M2 family. One M2 family (*ace3*; bottom right) contains individual M2 plants that grow despite
412 the presence of the inducer chemical.

413 **B** | Estradiol triggers a Bs4C-dependent cell death reaction. Seeds containing either an estradiol-
414 inducible Bs4C (*Estr:Bs4C-FLAG-GFP*) or a *GFP-GUS* reporter gene (*Estr:GFP-GUS*) were placed on
415 an estradiol-containing agar plate.

416 **C** | Bs4C protein is expressed in different *ace* mutants. Immunoblot analysis of five week old
417 Arabidopsis leaves treated with estradiol for 24 hours. Bs4C was detected using an anti-FLAG antibody.
418 Ponceau stain provides an info on total protein content in the samples.

419 **Figure 3. MAD-mapping excludes crosses to expedite isolation of causative mutation.**

420 MAD-mapping identifies causal mutations in the M2 generation. The parental transgenic line (P/T3)
421 contains an inducible transgene (not indicated) that triggers systemic cell death (black skull) upon
422 application of estradiol. Seeds of the parental line were mutagenized by EMS treatment (red arrow)
423 producing M1/T4 plants with EMS-induced mutations (ovals). The causal mutation (red oval), that
424 inhibits activity of the inducible transgene is heterozygous in the M1. EMS mutations of a given M1
425 segregate in M2/T5 descendants. M2 plants that are homozygous for the causal mutation will survive
426 in presence of the inducer chemical. Survivors of a given M2 family are used to generate DNA pools in
427 which the frequency of EMS-induced mutations is determined by next generation sequencing (NGS).
428 The causal mutation will be homozygous in all survivors and thus will be present at a frequency of 1
429 (100%) in the pool DNA.

430 **Figure 4. M2 allele frequency-distribution (MAD) mapping identifies mutations in *AT1G54490.1***
431 **that suppress *Bs4C*-dependent cell death.**

432 The frequency of EMS-induced mutations (red dots) on chromosome 1 is displayed for *ace1*, *ace2* and
433 *ace3* DNA pools. Boxes provide information on mutations that occur at frequencies of 0.95 or higher.
434 Italic font indicates the chromosomal location of the mutation with the frequency provided in square
435 brackets. Boldface font provides the gene designation. If the mutation is within a gene, the third row
436 reveals the consequences of this mutation. Underlining indicates that the given mutation has likely
437 functional consequences.

438 **Figure 5. *ace* families harbor distinct mutations in *AT1G54490.1*.**

439 **A** | Location of mutations in *ace1*, *ace2*, and *ace3* mutants. Black boxes represent *AT1G54490.1* exons.
440 The location of each causal mutations in *ace3*, *ace1*, and *ace2*, is indicated. Black bar indicates length
441 of 500 bp.

442 **B** | Mutations in *ace* mutants and its consequences at the protein level. Underlined letters indicate the
443 affected codons with the encoded amino acid shown below. Letters in black bold display the base pair
444 or amino acid found in the parental line. Red bold font indicates EMS-induced mutations with the
445 encoded amino indicated below. Numbers above mutated base pairs indicate positions of the mutations
446 within the transcript sequence. An asterisk (*) indicates a translational stop codon.

447 **SUPPLEMENTAL FIGURES**

448 **Figure S1. | Transgenic lines containing inducible transgenes express transgene-encoded**
449 **proteins in an estradiol-dependent fashion.** Immunoblot analysis using anti-FLAG and anti-GFP
450 antibody of tissue from two week old Arabidopsis seedlings of depicted genotypes (*Col-0*, *Estr:Bs4C-*
451 *FLAG-GFP*, *Estr:GFP-GUS*). Plants were incubated for 24 hours in liquid media either containing
452 estradiol or lacking estradiol (mock). Ponceau stained membrane serves as a loading control. Molecular
453 mass markers are indicated by triangles.

454 **Figure S2. | M2 allele frequency-distribution in the *ace1* mutant family.** The frequency and position
455 of EMS-induced mutations (red dots) on chromosome 1-5 are displayed.

456 **Figure S3. | M2 allele frequency-distribution in the *ace2* mutant family.** The frequency and position
457 of EMS-induced mutations (red dots) on chromosome 1-5 are displayed.

458 **Figure S4. | M2 allele frequency-distribution in the *ace3* mutant family.** The frequency and position
459 of EMS-induced mutations (red dots) on chromosome 1-5 are displayed.

460 **Figure S5. | Mutations in *ace* families are independent from one another.** Underlined letters
461 indicate the 3 letter codon corresponding to the amino acid directly below. Letters in black bold display
462 reference base pair or amino acid found in the parental line, and letters in red bold designate altered
463 base pair or amino acid found in indicated mutant family.

464

465 **SUPPLEMENTAL TABLES**

466 Table S1 – EMS-induced mutations with increased allele frequency in Arabidopsis *ace* mutants.

Table S1. Allele frequencies of EMS-induced SNPs in three *ace* families.

Family	Position	Ref./Alt.	Cover.	Allele freq.	Gene ID	Feature	CDS position	Effect	Change
<i>ace1</i>	18,641,436	G/A	81	0.77	—	intergenic			
	20,352,190	G/A	98	0.97	<u>AT1G54490.1</u>	CDS	706	Nonsyn	D/N
<i>ace2</i>	18,117,826	G/A	61	0.85	AT1G48970.1	5' UTR			
	19,031,508	G/A	61	0.87	—	intergenic			
	20,105,279	G/A	59	0.97	AT1G53850.1	intronic			
	20,105,279	G/A	59	0.97	AT1G53850.2	intronic			
	20,352,595	G/A	44	0.98	<u>AT1G54490.1</u>	CDS	911	Nonsyn	W/*
	20,765,374	G/A	59	1	AT1G55580.1	CDS	1269	Syn	Q/Q
	21,402,987	G/A	47	0.96	—	intergenic			
<i>ace3</i>	18,062,237	C/T	101	0.9	AT1G48840.1	intronic			
	19,722,708	C/T	129	0.98	AT1G52940.1	CDS	478	Nonsyn	H/Y
	20,351,467	C/T	146	0.99	<u>AT1G54490.1</u>	CDS	329	Nonsyn	A/V
	20,386,527	C/T	102	0.99	—	intergenic			
	20,560,738	C/T	139	0.95	AT1G55110.1	CDS	1036	Nonsyn	E/K
	20,613,630	C/T	98	0.88	—	intergenic			
	20,616,883	C/T	96	0.89	—	intergenic			
	20,830,617	C/T	123	0.95	AT1G55720.1	5' UTR			
	21,512,799	C/T	130	0.93	AT1G58100.1	CDS	1087	Nonsyn	G/S
	21,512,799	C/T	130	0.93	AT1G58100.2	CDS	1015	Nonsyn	G/S

SNPs identified on Arabidopsis chromosome 1 in *ace1*, *ace2* and *ace3* M2 families, stating the position, the reference base (Ref.), identified altered base (Alt.), the coverage (Cover), allele frequency (Allele freq.), the annotated Gene ID, the feature within the gene, the coding sequence position (CDS position), the effect of the mutation, and the resulting amino acid change (Change). SNPs that were below an allele frequency of 0.77 are not listed. Underlined font indicates the gene that was identified in all three *ace* families.

467

468

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Figure 1

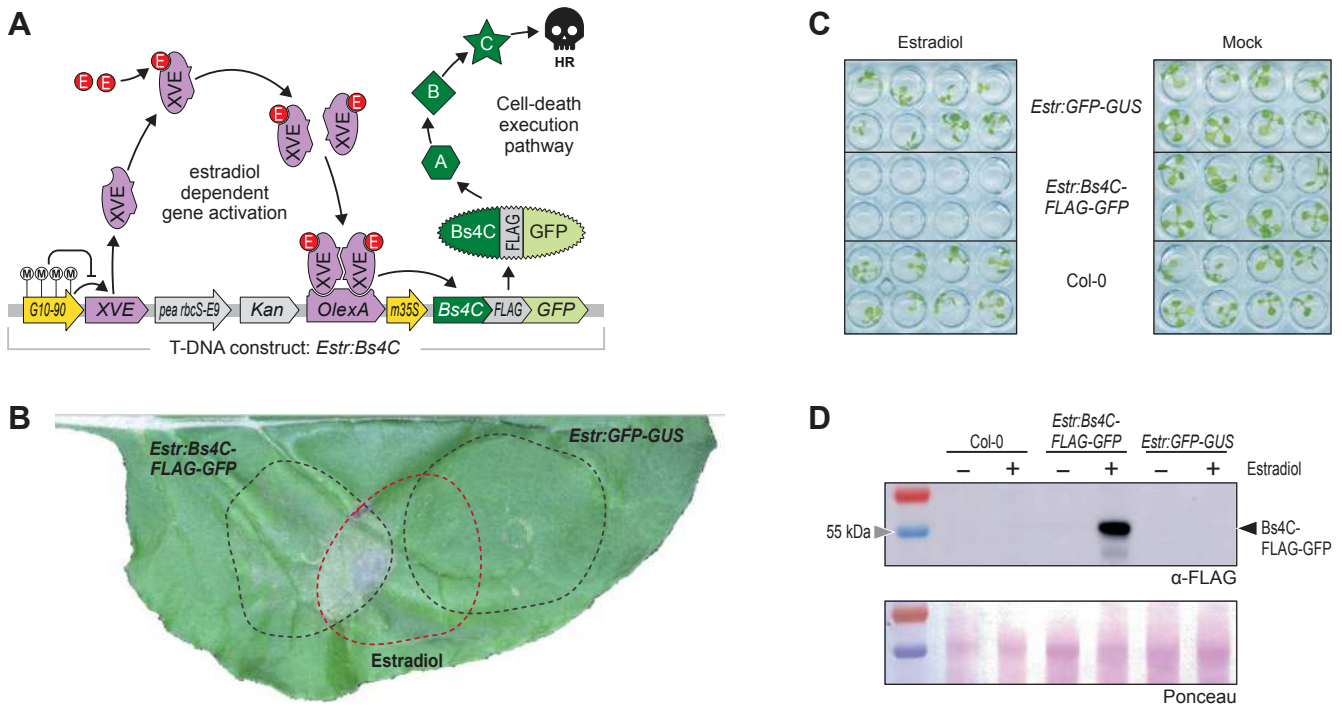


Figure 1. Bs4C induces growth arrest in Arabidopsis.

A | A T-DNA construct for estradiol-inducible expression of the pepper executor protein Bs4C. Driven by the constitutive G10-90 promoter, the XVE gene translates into a chimeric transcriptional activator that contains an estrogen receptor domain. When estrogen (E) is present (here in the form of β -estradiol), it binds to the XVE protein, mediates XVE homodimerization and enables XVE to bind to the LexA operator (OlexA). This induces transcription of the downstream gene encoding a Bs4C-FLAG-GFP protein. The Bs4C fusion protein requires the putative signaling elements A, B, C to trigger plant cell death. Methylation of the G10-90 promoter (M) can cause transcriptional silencing of the G10-90 promoter and results in a non-inducible promoter.

B | A *Bs4C* transgene triggers estradiol-dependent cell death in *Nicotiana benthamiana* leaves. The depicted T-DNA constructs were delivered into *N. benthamiana* leaves via Agrobacterium mediated transient transformation. Leaf areas into which the inducer estradiol was infiltrated are highlighted with a red line.

C | An inducible *Bs4C* transgene triggers systemic cell death in Arabidopsis. Four day old seedlings of indicated genotypes were placed in liquid media either containing estradiol or a lacking estradiol (Mock). Ten days later, the seedlings show cell death in presence of estradiol and the *Bs4C* transgene.

D | Immunoblot analysis using anti-FLAG antibody of tissue from two week old Arabidopsis seedlings of depicted genotypes (Col-0, *Estr:Bs4C-FLAG-GFP*, *Estr:GFP-GUS*). Plants were incubated for 24 hours in liquid media either containing estradiol or lacking estradiol (mock). Ponceau stained membrane serves as a loading control.

Figure 2

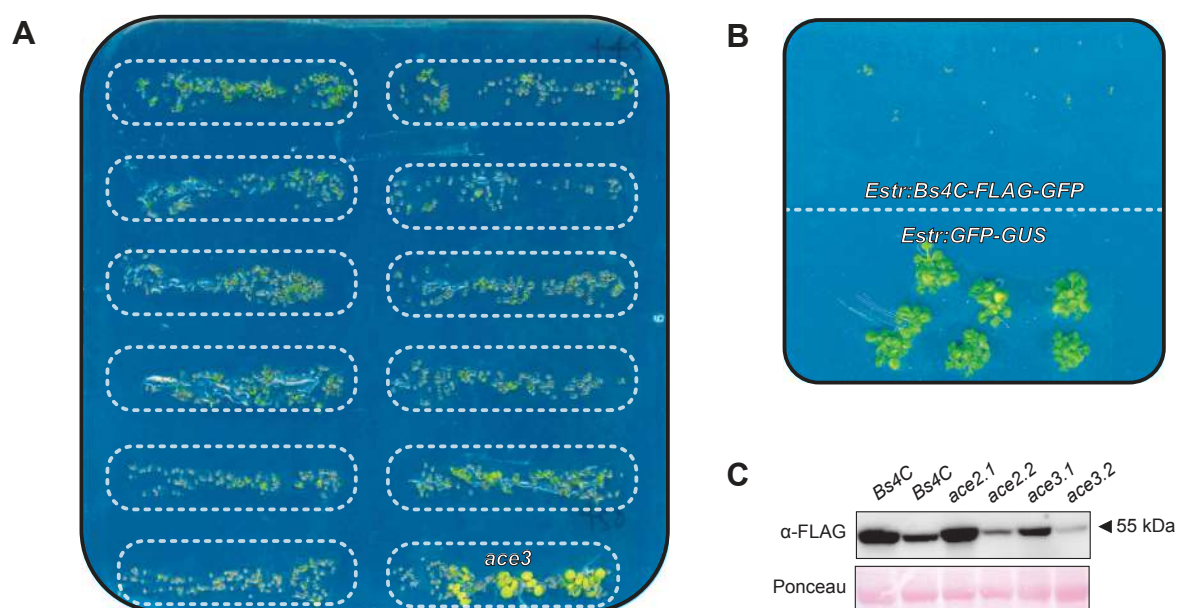


Figure 2. *ace* screen identifies suppressors of Bs4C-dependent cell death in Arabidopsis.

A | Identification of the *ace3* M2 family. Seeds of twelve distinct M2 families are placed in rows on estradiol-containing agar. Boxes framed by dashed lines indicate the region that is covered by seeds of one M2 family. One M2 family (*ace3*; bottom right) contains individual M2 plants that grow despite the presence of the inducer chemical.

B | Estradiol triggers a Bs4C-dependent cell death reaction. Seeds containing either an estradiol-inducible Bs4C (*Estr:Bs4C-FLAG-GFP*) or a *GFP-GUS* reporter gene (*Estr:GFP-GUS*) were placed on an estradiol-containing agar plate.

C | Bs4C protein is expressed in different *ace* mutants. Immunoblot analysis of five week old Arabidopsis leaves treated with estradiol for 24 hours. Bs4C was detected using an anti-FLAG antibody. Ponceau stain provides an info on total protein content in the samples.

Figure 3

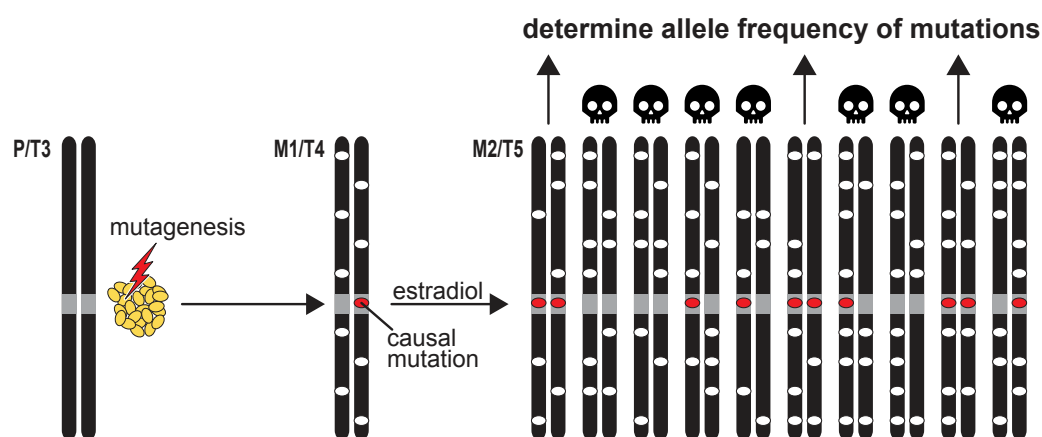


Figure 3. MAD-mapping excludes crosses to expedite isolation of causative mutation.

MAD-mapping identifies causal mutations in the M2 generation. The parental transgenic line (P/T3) contains an inducible transgene (not indicated) that triggers systemic cell death (black skull) upon application of estradiol. Seeds of the parental line were mutagenized by EMS treatment (red arrow) producing M1/T4 plants with EMS-induced mutations (ovals). The causal mutation (red oval), that inhibits activity of the inducible transgene is heterozygous in the M1. EMS mutations of a given M1 segregate in M2/T5 descendants. M2 plants that are homozygous for the causal mutation will survive in presence of the inducer chemical. Survivors of a given M2 family are used to generate DNA pools in which the frequency of EMS-induced mutations is determined by next generation sequencing (NGS). The causal mutation will be homozygous in all survivors and thus will be present at a frequency of 1 (100%) in the pool DNA.

Figure 4

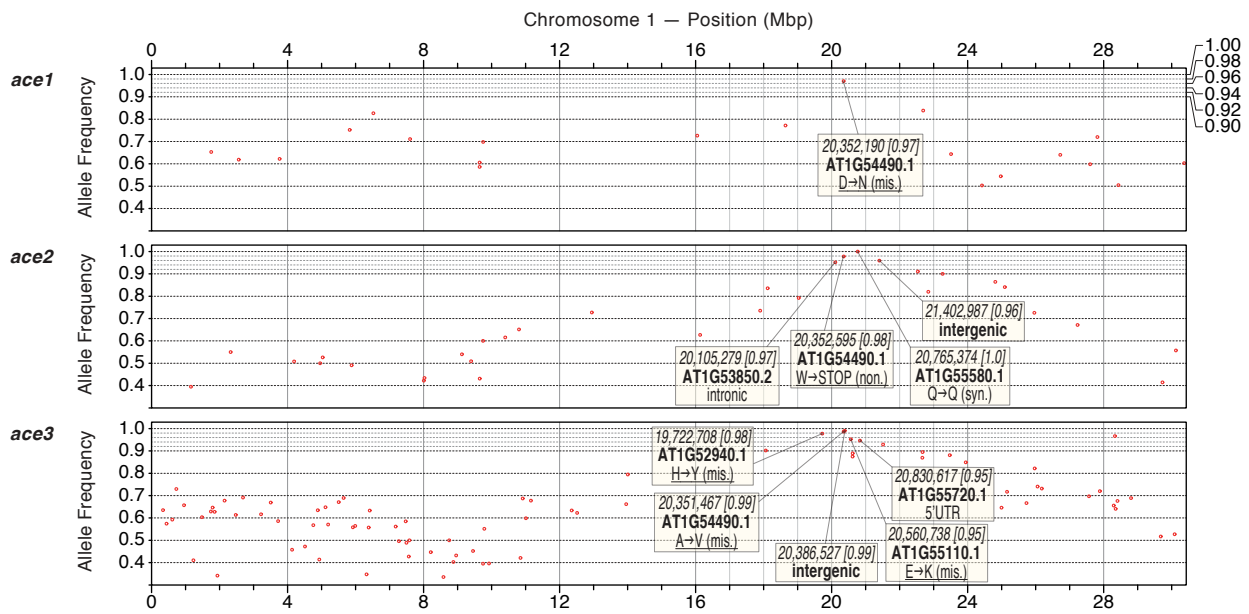


Figure 4. M2 allele frequency-distribution (MAD) mapping identifies mutations in *AT1G54490.1* that suppress *Bs4C*-dependent cell death.

The frequency of EMS-induced mutations (red dots) on chromosome 1 is displayed for *ace1*, *ace2* and *ace3* DNA pools. Boxes provide information on mutations that occur at frequencies of 0.95 or higher. Italic font indicates the chromosomal location of the mutation with the frequency provided in square brackets. Boldface font provides the gene designation. If the mutation is within a gene, the third row reveals the consequences of this mutation. Underlining indicates that the given mutation has likely functional consequences.

Figure 5

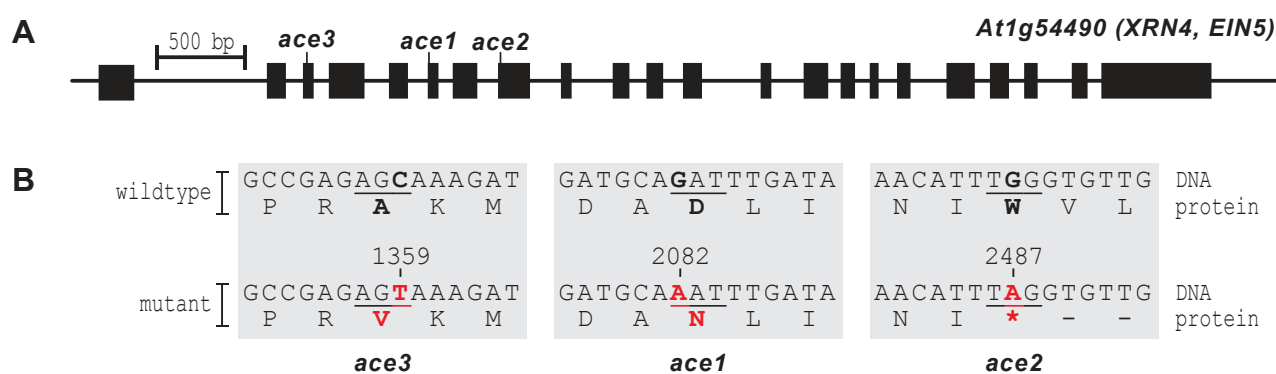


Figure 5. *ace* families harbor distinct mutations in *AT1G54490.1*.

A | Location of mutations in *ace1*, *ace2*, and *ace3* mutants. Black boxes represent *AT1G54490.1* exons. The location of each causal mutations in *ace3*, *ace1*, and *ace2*, is indicated. Black bar indicates length of 500 bp.

B | Mutations in *ace* mutants and its consequences at the protein level. Underlined letters indicate the affected codons with the encoded amino acid shown below. Letters in black bold display the base pair or amino acid found in the parental line. Red bold font indicates EMS-induced mutations with the encoded amino indicated below. Numbers above mutated base pairs indicate positions of the mutations within the transcript sequence. An asterisk (*) indicates a translational stop codon.

Figure S1

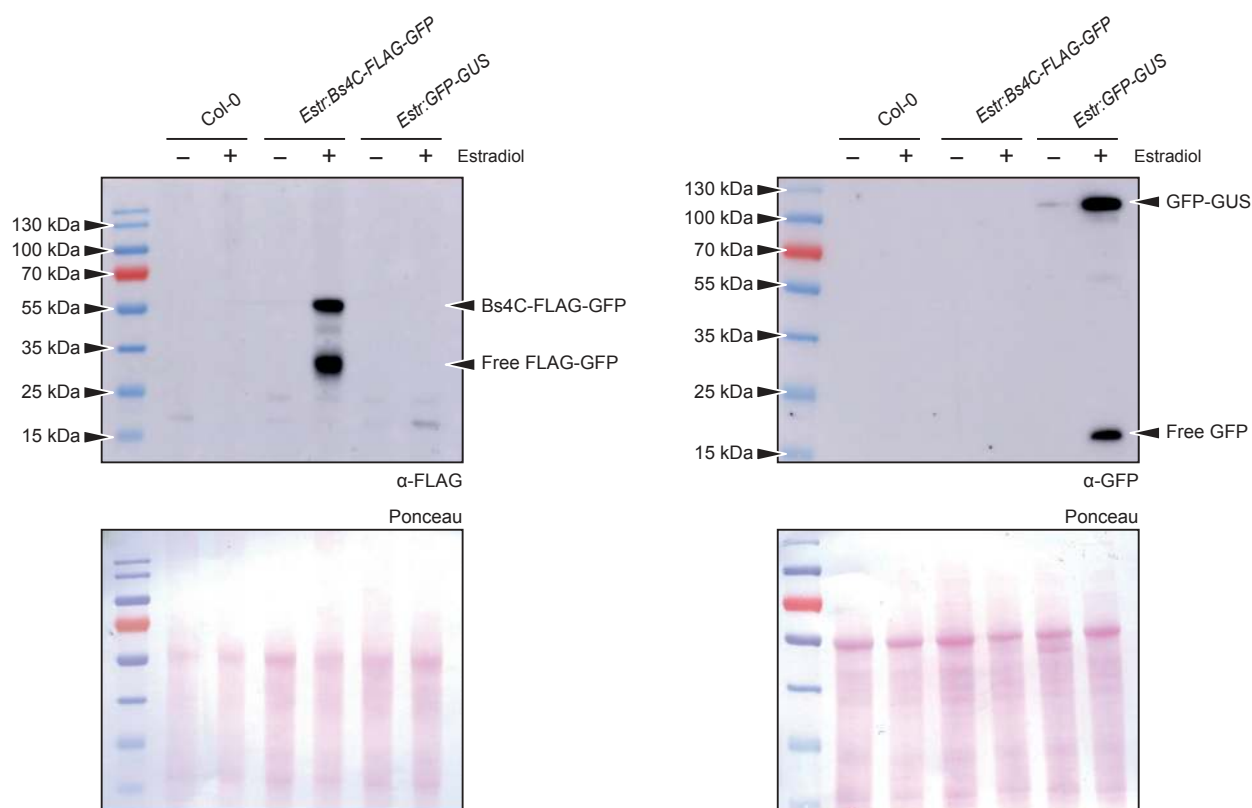


Figure S1. | Transgenic lines containing inducible transgenes express transgene-encoded proteins in an estradiol-dependent fashion. Immunoblot analysis using anti-FLAG and anti-GFP antibody of tissue from two week old Arabidopsis seedlings of depicted genotypes (*Col-0*, *Estr:Bs4C-FLAG-GFP*, *Estr:GFP-GUS*). Plants were incubated for 24 hours in liquid media either containing estradiol or lacking estradiol (mock). Ponceau stained membrane serves as a loading control. Molecular mass markers are indicated by triangles.

Figure S2

ace1

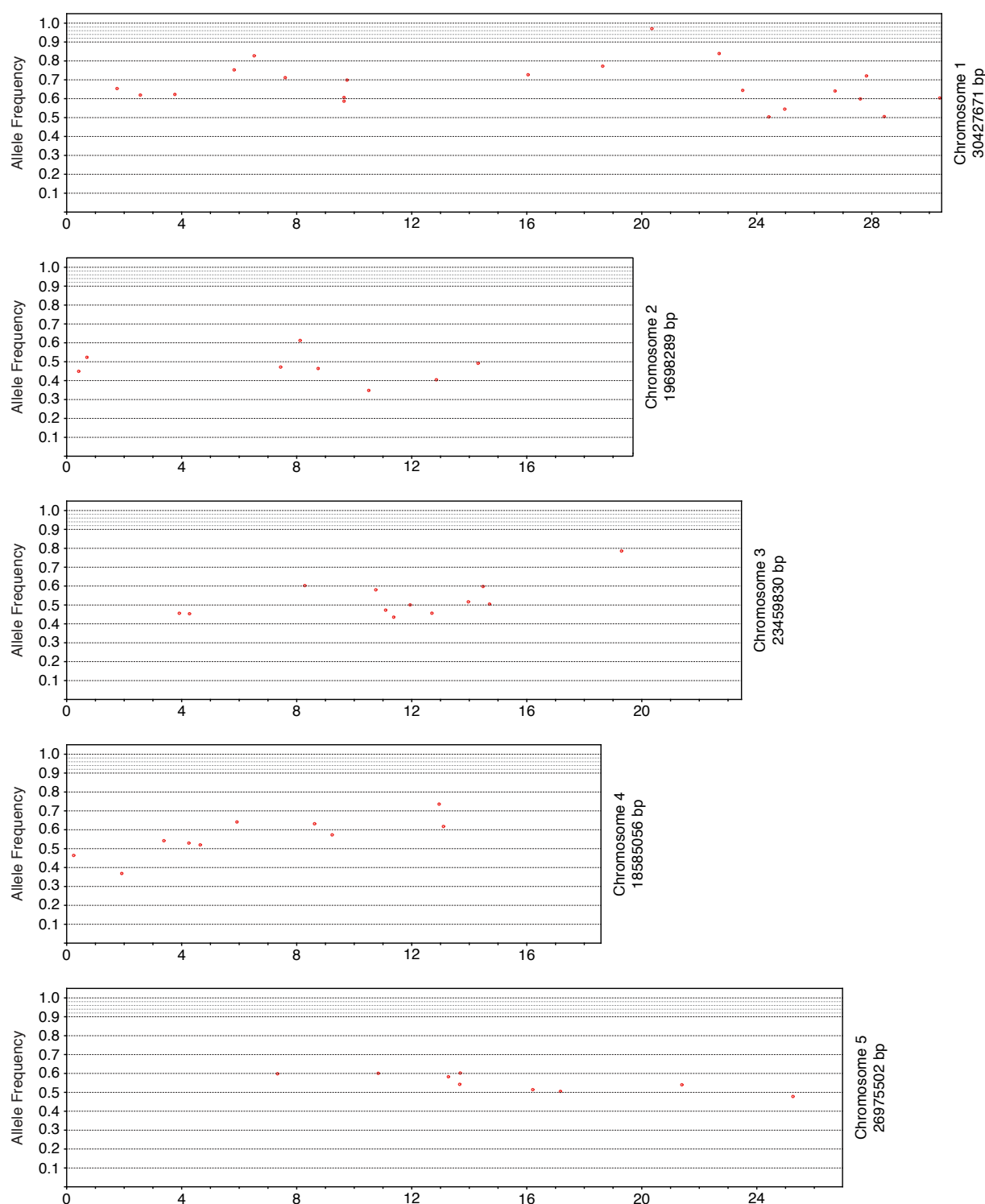


Figure S2. | M2 allele frequency-distribution in the *ace1* mutant family. The frequency and position of EMS-induced mutations (red dots) on chromosome 1-5 are displayed.

Figure S3

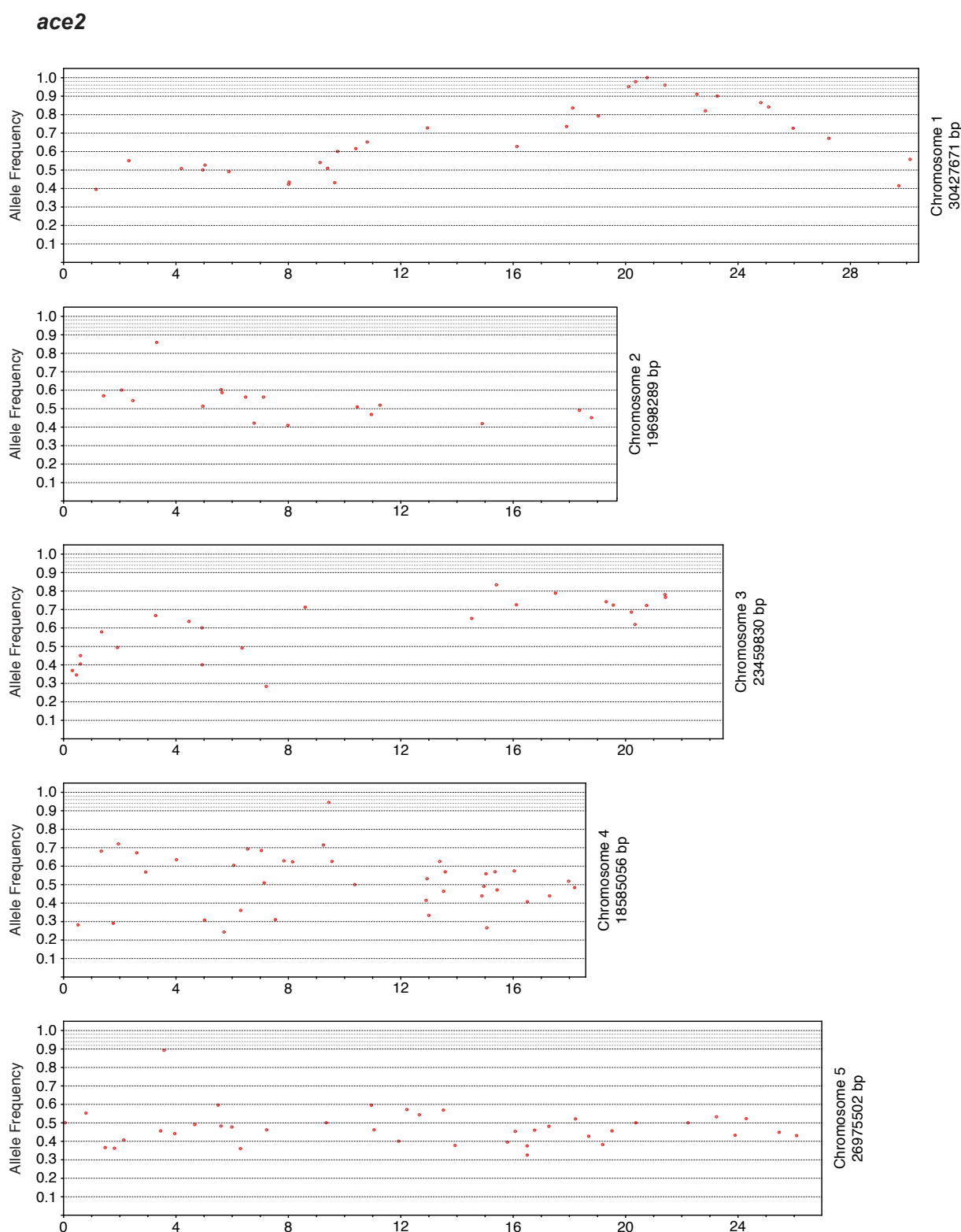


Figure S3. | M2 allele frequency-distribution in the *ace2* mutant family. The frequency and position of EMS-induced mutations (red dots) on chromosome 1-5 are displayed.

Figure S4

ace3

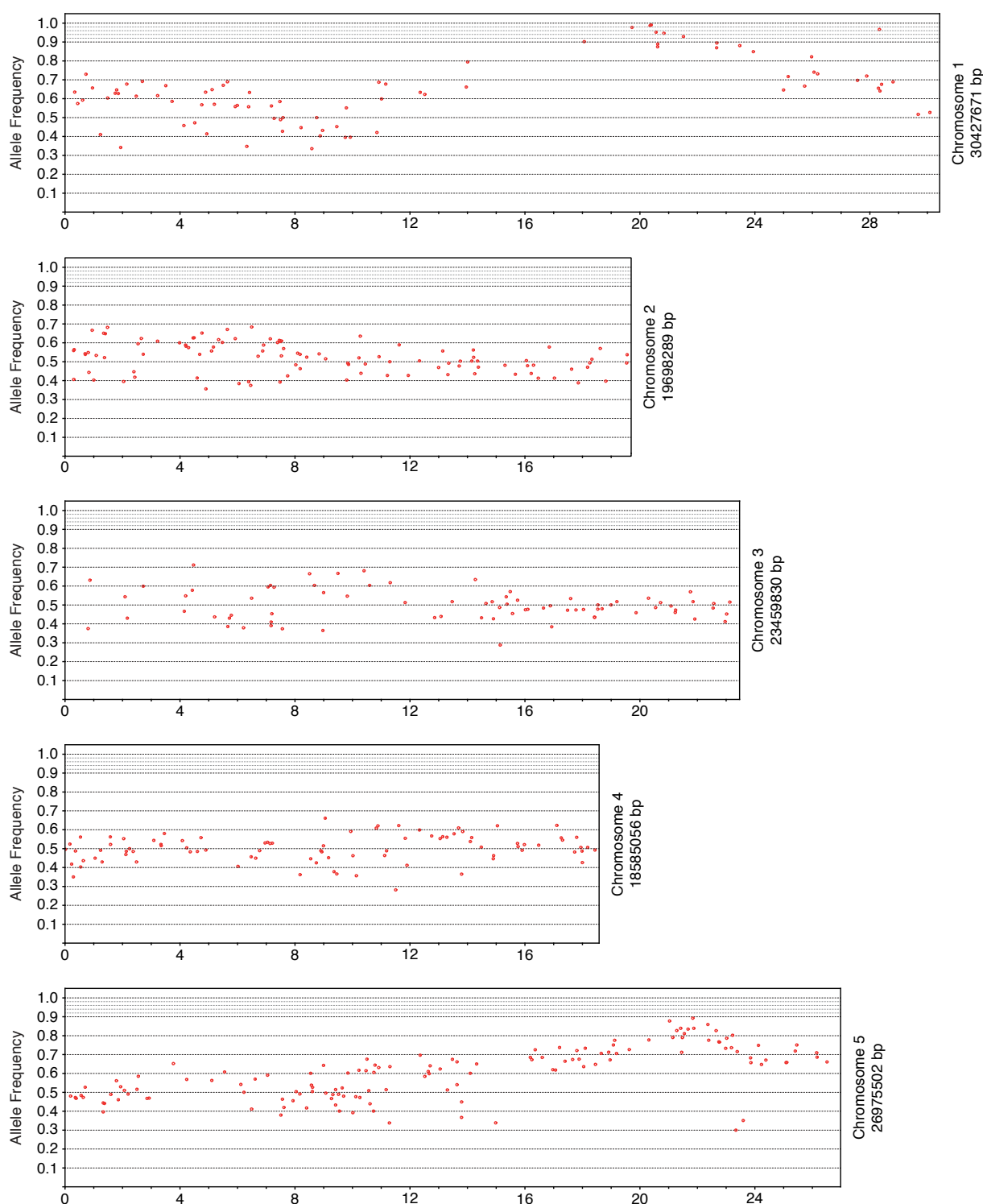
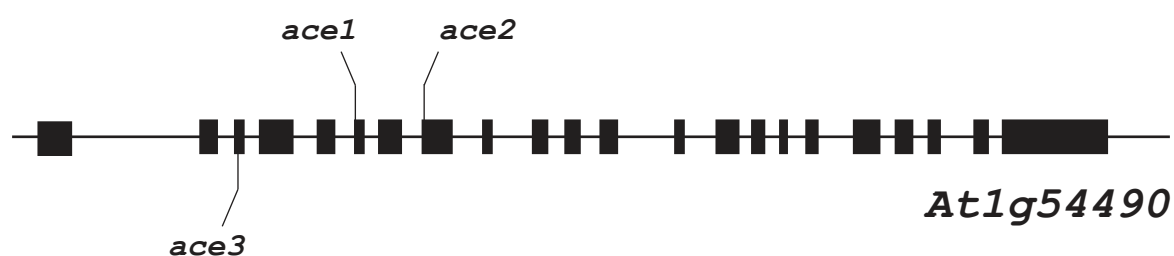


Figure S4. | M2 allele frequency-distribution in the *ace3* mutant family. The frequency and position of EMS-induced mutations (red dots) on chromosome 1-5 are displayed.

Figure S5



WT	AGATGGAGTTGCGCCGAGAG <u>C</u> AAAAGATGAATCAGCAGCGTTCTCGACGTTTC	360
	D G V A P R A K M N Q Q R S R R F	
ace3	AGATGGAGTTGCGCCGAGAG <u>T</u> AAAAGATGAATCAGCAGCGTTCTCGACGTTTC	360
	D G V A P R V K M N Q Q R S R R F	
ace2	AGATGGAGTTGCGCCGAGAG <u>C</u> AAAAGATGAATCAGCAGCGTTCTCGACGTTTC	360
	D G V A P R A K M N Q Q R S R R F	
ace1	AGATGGAGTTGCGCCGAGAG <u>C</u> AAAAGATGAATCAGCAGCGTTCTCGACGTTTC	360
	D G V A P R A K M N Q Q R S R R F	
WT	atattattatgcagGATGCAG <u>ATT</u> TGATAATGCTCTCCTTAGCTACACAT	735
	Intronic----- D A D L I M L S L A T H	
ace1	atattattatgcagGATGCAG <u>ATT</u> TGATAATGCTCTCCTTAGCTACACAT	735
	Intronic----- D A N L I M L S L A T H	
ace2	atattattatgcagGATGCAG <u>ATT</u> TGATAATGCTCTCCTTAGCTACACAT	735
	Intronic----- D A D L I M L S L A T H	
ace3	atattattatgcagGATGCAG <u>ATT</u> TGATAATGCTCTCCTTAGCTACACAT	735
	Intronic----- D A D L I M L S L A T H	
WT	AAATATCAGTTCCTGAACATTT <u>G</u> GGTGTTCGAGAATATCTGCAATATGAA	939
	K Y Q F L N I W V L R E Y L Q Y E	
ace2	AAATATCAGTTCCTGAACATTT <u>A</u> GGTGTTCGAGAATATCTGCAATATGAA	939
	K Y Q F L N I * - - - - -	
ace1	AAATATCAGTTCCTGAACATTT <u>G</u> GGTGTTCGAGAATATCTGCAATATGAA	939
	K Y Q F L N I W V L R E Y L Q Y E	
ace3	AAATATCAGTTCCTGAACATTT <u>G</u> GGTGTTCGAGAATATCTGCAATATGAA	939
	K Y Q F L N I W V L R E Y L Q Y E	

Figure S5. | Mutations in ace families are independent from one another. Underlined letters indicate the 3 letter codon corresponding to the amino acid directly below. Letters in black bold display reference base pair or amino acid found in the parental line, and letters in red bold designate altered base pair or amino acid found in indicated mutant family.