Recombination between T-DNA insertions to cause chromosomal deletions in *Arabidopsis* is a rare phenomena

John F Seagrist [1] , **Shih-Heng Su** [2] , **Patrick J Krysan** Corresp. [1, 3]

[1] Department of Horticulture, University of Wisconsin-Madison, Madison, WI, United States

[2] Laboratory of Genetics, University of Wisconsin-Madison, Madison, WI, United States

[3] Genome Center of Wisconsin, University of Wisconsin-Madison, Madison, WI, United States

Corresponding Author: Patrick J Krysan
Email address: pjkrysan@wisc.edu

We previously described the identification of a chromosomal deletion in *Arabidopsis thaliana* that resulted in the elimination of genomic DNA between two T-DNA insertions located ca. 25 kilobases apart on chromosome IV. The mechanism responsible for this deletion appears to have been recombination between the closely spaced T-DNA elements located in trans in a parent plant. In our original study, we observed one such deletion event after screening ca. 2,000 seedlings using a polymerase chain reaction (PCR) assay. Because a method for precisely deleting a selected region of the Arabidopsis genome would have significant value as a research tool, we were interested in determining the frequency with which this type of T-DNA-directed deletion occurs. To do this we designed a genetic screen that would allow us to phenotypically screen for deletions caused by recombination between T-DNA inserts. This screen involved crossing T-DNA single-mutant lines in order to produce F1 plants in which the two T-DNA insertions flanked a *FUSCA* (*FUS*) locus present in the genome. Loss-of-function mutations of *FUS* genes cause a distinctive developmental phenotype that can be easily scored visually in young seedlings. We used T-DNA lines flanking *FUS2*, *FUS6*, *FUS7*, and *FUS11* for this study. Recombination between the T-DNAs in an F1 parent should result in deletion of the *FUS* gene located between the T-DNAs. Because the deletion would be heterozygous in the F2 generation, we screened the F3 progeny of pooled F2 individuals to search for the *fus* loss-of-function phenotype. Using this strategy we were able to evaluate a total of 28,314 meioses for evidence of deletions caused by recombination between the T-DNA inserts. No seedlings displaying the *fus* phenotype were recovered, suggesting that deletions caused by recombination between T-DNA inserts are relatively rare events and may not be a useful tools for genome engineering in *Arabidopsis*.

4 Recombination between T-DNA insertions to cause chromosomal deletions in *Arabidopsis*

5 is a rare phenomena

9 John Seagrist[1], Shih-Heng Su[2], and Patrick J Krysan[1,3]

13 [1]Department of Horticulture, University of Wisconsin-Madison, Madison, WI, USA

15 [2]Laboratory of Genetics, University of Wisconsin-Madison, Madison, WI, USA

17 [3]Genome Center of Wisconsin, University of Wisconsin-Madison, Madison, WI, USA

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22 Corresponding author: Patrick J Krysan

23   pjkrysan@wisc.edu

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31   ABSTRACT

32   We previously described the identification of a chromosomal deletion in *Arabidopsis*

33   *thaliana* that resulted in the elimination of genomic DNA between two T-DNA insertions

34   located ca. 25 kilobases apart on chromosome IV. The mechanism responsible for this

35   deletion appears to have been recombination between the closely spaced T-DNA elements

36   located in trans in a parent plant. In our original study, we observed one such deletion

37   event after screening ca. 2,000 seedlings using a polymerase chain reaction (PCR) assay.

38   Because a method for precisely deleting a selected region of the Arabidopsis genome would

39   have significant value as a research tool, we were interested in determining the frequency

40   with which this type of T-DNA-directed deletion occurs. To do this we designed a genetic

41   screen that would allow us to phenotypically screen for deletions caused by recombination

42   between T-DNA inserts. This screen involved crossing T-DNA single-mutant lines in order

43   to produce F1 plants in which the two T-DNA insertions flanked a *FUSCA* (*FUS*) locus

44   present in the genome. Loss-of-function mutations of *FUS* genes cause a distinctive

45   developmental phenotype that can be easily scored visually in young seedlings. We used T-

46   DNA lines flanking *FUS2*, *FUS6*, *FUS7*, and *FUS11* for this study. Recombination between the

47   T-DNAs in an F1 parent should result in deletion of the *FUS* gene located between the T-

48   DNAs. Because the deletion would be heterozygous in the F2 generation, we screened the

49   F3 progeny of pooled F2 individuals to search for the *fus* loss-of-function phenotype. Using

50   this strategy we were able to evaluate a total of 28,314 meioses for evidence of deletions

51   caused by recombination between the T-DNA inserts. No seedlings displaying the *fus*

52   phenotype were recovered, suggesting that deletions caused by recombination between T-

53   DNA inserts are relatively rare events and may not be a useful tools for genome

54   engineering in *Arabidopsis*.

55

56 INTRODUCTION

57 In order to understand the function of a genome, it is critical to have tools available for

58 modifying the DNA sequence of that genome. In the case of the model plant *Arabidopsis*

59 *thaliana*, one of the most widely used strategies for knocking out a gene of interest is

60 insertional mutagenesis based on the transferred DNA (T-DNA) of *Agrobacterium*

61 *tumefaciens* (Alonso et al. 2003; Krysan et al. 1999; O'Malley & Ecker 2010; Parinov &

62 Sundaresan 2000). A number of labs throughout the world have produced large

63 populations of *Arabidopsis* transgenic lines in which T-DNA elements are randomly

64 inserted throughout the genome (Alonso et al. 2003; Brunaud et al. 2002; Ito et al. 2002;

65 Rosso et al. 2003; Sessions et al. 2002; Woody et al. 2007). The exact positions of the T-

66 DNAs are determined by DNA sequencing (Alonso et al. 2003). Currently, there are over

67 400,000 T-DNA insertion lines cataloged in the Arabidopsis genome, with the

68 corresponding seed available through public stock centers. Although the T-DNA elements

69 are inserted "randomly", the existence of such a large catalog of lines means that most of

70 the genes in *Arabidopsis* have at least one T-DNA mutant allele available. These public T-

71 DNA collections are an extremely popular community resource for studying gene function

72 in *Arabidopsis*. Because approximately 17% of the genes in *Arabidopsis* are members of a

73 tandemly-duplicated gene family, however, it can be challenging to study gene function

74 using T-DNA knockouts when the gene of interest has a second copy nearby on the same

75 chromosome (Initiative 2000). If these duplicated genes display functional redundancy,

76 one must knockout both genes in order to reveal a mutant phenotype. Because of the

77 extremely tight genetic linkage between tandemly duplicated genes, it is not practical to

78 rely on meiotic recombination to produce a double-mutant for tandemly duplicated genes

79 using T-DNA insertion lines.

80   The *Arabidopsis* MAP kinase kinase kinase gene *MEKK1* is part of a tandemly

81 duplicated gene family in which *MEKK1*, *MEKK2*, and *MEKK3* are located within a ca. 25

82 kilobase (kb) region of chromosome IV. In a previous study we reported the identification

83 of a deletion mutant that appeared to be the result of recombination between a T-DNA

84 insertion in *MEKK1* and a second T-DNA located ca. 21 kb away in *MEKK3* (Su et al. 2013).

85 Because the two T-DNA insertions were produced using the same vector, meiotic

86 recombination directed by the homology within the T-DNA vector sequence seems to have

87 been responsible for producing the deletion of the intervening genomic DNA. This deletion
88 mutant proved to be valuable for dissecting the function of the *MEKK1/2/3* gene family (Su
89 et al. 2013).

90   Due to the prevalence of tandemly-duplicated gene families in Arabidopsis, we were
91 interested in determining if deletions caused by recombination between T-DNA inserts
92 might be useful tools for performing genome engineering in Arabidopsis. In our study of
93 the *MEKK1/2/3* tandem gene family, we identified the deletion mutant using a PCR-based
94 screen of ca. 2,000 seedlings. Since we only found one individual carrying the deletion, it
95 was not possible to accurately estimate the frequency with which this type of T-DNA
96 directed deletion might occur. In order for deletions caused by recombination between T-
97 DNA insertions to be useful genome engineering tools, the frequency with which it occurs
98 needs to be within a practical range to make screening for deletions cost- and time-
99 effective. We therefore designed a genetic screen that enabled us to phenotypically screen
100 for deletions caused by recombination between T-DNA inserts at a number of locations in
101 the *Arabidopsis* genome. Despite screening 28,314 meioses for evidence of T-DNA-directed
102 deletions, none were found. These results suggest that deletions caused by recombination
103 between T-DNA inserts may be relatively rare events and that their utility for genome
104 engineering may be limited.

105

106 MATERIALS AND METHODS
107 **Plant Material and Growth Conditions**
108 *Arabidopsis* lines carrying T-DNA insertions flanking *FUS2* (At4g10180), FUS6
109 (At3g61140), *Fus7* (At4g14110), and *Fus11* (At5g14250) were obtained from the
110 Arabidopsis Biological Resource Center at The Ohio State University (abrc.osu.edu). *FUS2*:
111 SALK_046021 and SALK_004560. *FUS6*: WiscDsLox453-456F5, WiscDsLox402F03,
112 SALK_203944, and SALK_062171. *FUS7*: SALK_073580 and SALK_150313. *FUS11*:
113 SALK_150318 and SALK_150319. Seedlings were germinated on 1% agar (w/v) plates
114 containing 0.5× Murashige and Skoog (MS) basal salt mixture under continuous light at
115 20°-23°C. Seedlings were transferred to a 2:1 mixture (vol:vol) of potting mix:pearlite 5 to
116 7 days after germination and grown under constant light at 20°-23°C.

117

118 **Genotyping**

119 PCR-based genotyping was used to determine the presence of the desired T-DNA insertion

120 elements. Genomic DNA was extracted from 5mm square pieces of leaf tissue as previously

121 described (Kasajima et al. 2004). PCR was performed using the gene-specific PCR primers

122 indicated in Table 1 in conjunction with the T-DNA left border primer p745: 5'-

123 AACGTCCGCAATGTGTTATTAAGTTGTC-3'. The thermal cycling conditions used were as

124 follows: 96°C for 2 minutes, followed by 40 cycles of 94°C for 10 seconds, 58°C for 30

125 seconds, and 72°C for 30 seconds. PCR products were visualized by agarose gel

126 electrophoresis followed by ethidium bromide staining.

127

128 **Phenotypic Screening**

129 F1 plants were produced by crossing two single-mutant T-DNA lines. PCR genotyping was

130 used to confirm that the F1 plants carried the desired T-DNA insertions. F2 seed was then

131 collected from individual F1 plants. F2 seedlings were germinated on petri dishes

132 containing agar growth media and transplanted to potting mix at a density of 9 seedlings

133 per 3.5 inch square pot. The F2 plants were allowed to self-pollinate, and the resulting

134 seed was collected in bulk from each pot of 9 plants. Ca. 1,200 F3 seeds from each pool of

135 nine F2 plants were placed onto water-saturated 125 mm diameter circular pieces of

136 Whatman filter paper (Whatman cat. #1004-125) inside 140mm diameter plastic petri

137 dishes. The plates were then incubated in the dark at 4°C for 2 to 3 days to stratify the

138 seeds. The plates were then placed under light at 20°-23°C for four hours to stimulate

139 germination, and then transferred to black box with no light at 20°-23°C for four days to

140 allow for etiolated growth. Plates were then visually inspected to search for seedlings

141 displaying the distinctive *fus* phenotype.

142

143

144 RESULTS

145 **Design of the Genetic Screen**

146 We use the term "deletion caused by T-DNA recombination" to refer to the situation where

147 meiotic crossing over between T-DNA insertions located nearby to each other leads to

148 deletion of the intervening genomic DNA (Fig. 1). In our previous study describing the

149   identification of a deletion caused by T-DNA recombination in the *MEKK1/2/3* gene family

150   we used a PCR-based method to identify a single deletion mutant after screening ca. 2,000

151   seedlings (Su et al. 2013). In the present study, our objective was to screen a larger

152   population of individuals in order to better ascertain the frequency with which deletions

153   caused by T-DNA recombination occur. In order to do this in a cost-effective manner, we

154   chose to develop a genetic screen that would let us visually screen for evidence of deletions

155   caused by T-DNA recombination. The rationale for the screen was to choose loci in the

156   *Arabidopsis* genome for which null mutants produced a striking visual phenotype that

157   could be easily scored at the early seedling stage. We chose to use *FUSCA* (*FUS*) genes for

158   this purpose (Misera et al. 1994). The *FUS* genes are negative regulators of

159   photomorphogenesis such that null mutants cause dark-grown seedlings to display

160   characteristics normally seen in light-grown seedlings. When Arabidopsis seeds are

161   germinated and grown in the dark for four days, the hypocotyls elongate, the apical hook

162   remains closed, and the seedlings are pale white in appearance. By contrast, *fus* mutant

163   seedlings grown in the dark have short hypocotyls, the apical hook is open, and they are

164   dark purple due to anthocyanin accumulation (Misera et al. 1994). A *fus* mutant can

165   therefore be easily distinguished on a plate of thousands of wild-type dark grown seedlings

166   as a short purple seedling with open cotyledons against a background of long, skinny, white

167   seedlings. This made the *fus* mutant phenotype a good candidate for our genetic screen.

168   There are at least fourteen *FUS* loci (Misera et al. 1994) in the genome, and these genes

169   have also been identified as *CONSTITUTIVE PHOTOMORPHOGENIC* (*COP*) and *DE-*

170   *ETIOALTED* (*DET*) (Huang et al. 2014).

171   To identify specific *FUS* loci for our screen, we searched the available T-DNA mutant

172   collections for *FUS* genes that had T-DNA insertions in close proximity upstream and

173   downstream of the FUS gene. Suitable T-DNA lines were found for *FUS2*, *FUS6*, *FUS7*, and

174   *FUS11* (Fig. 2). In the case of *FUS6*, we chose two different sets of T-DNA lines flanking

175   *FUS6*, one set from the SALK T-DNA line collection (Alonso et al. 2003) and the other from

176   the WiscDsLox collection (Woody et al. 2007). The T-DNA lines for *FUS2*, *FUS7*, and *FUS11*

177   were from the SALK collection.

178   In our previous study involving deletion of the *MEKK1/2/3* locus, we determined that

179   T-DNA left border sequences were present on both sides of the T-DNA inserts for *MEKK1*

180   and *MEKK3*. This observation suggested that the at least two copies of the T-DNA

181   monomer were inserted at each locus in an inverted repeat orientation. Since inverted

182   repeats have the potential to form hairpin structures that may have the ability to stimulate

183   recombination, we were interested in determining if some of the T-DNA inserts used in the

184   present study had left border sequences on both sides of the insert. PCR reactions using

185   left-border specific primers and gene specific primers were used to map the left-border

186   sequences present at each locus. As shown in Figure 2, at least one of the T-DNA inserts in

187   each pair had left border sequence on both sides of the insert. In the case of *FUS7* and

188   *FUS11*, both the upstream and downstream T-DNA inserts had left border sequences on

189   both sides.

190   For a given *FUS* gene, the upstream and downstream T-DNA lines were crossed

191   together to produce F1 plants carrying two T-DNA insertions: one upstream of the *FUS*

192   gene and one downstream (Fig. 1). If recombination occurs between those two T-DNA

193   insertions during meiosis, one would expect one of the meiotic products to carry a deletion

194   of the *FUS* gene. Unless both the pollen and egg carried deletion events, one would expect

195   T-DNA directed deletions produced by an F1 plant to be heterozygous in the F2 progeny

196   (Fig. 1). Since the *fus* phenotype is a recessive condition, it is necessary to perform F3

197   progeny testing to identify the presence of a T-DNA directed deletion carried by an F2

198   plant. To streamline this process, we grew 9 F2 plants together in a single pot and

199   collected their F3 progeny in bulk. Ca. 1,200 F3 seeds from each pot were then germinated

200   in the dark on moist filter paper and visually scored after four days to screen for the *fus*

201   phenotype. This represents an average of 133 progeny for each F2 parent. If any of the F2

202   parents were carrying a heterozygous deletion of the *FUS* gene, then we would expect 25%

203   of those progeny, or ca. 33 seedlings, to display the *fus* phenotype. In dark growth

204   conditions, *fus* seedlings are short and purple with open cotyledons, which would stand in

205   stark contrast to the long, white, etiolated form of wild-type seedlings.

206

207   **Results of Genetic Screen**

208   F3 progeny collected from a total of 14,157 F2 parent plants were screened for the *fus*

209   phenotype as described above in order to find seedlings potentially carrying deletions

210   caused by T-DNA recombination of the *FUS* gene (Table 2). Since the recombination event

211   that would lead to a deletion could occur during meiosis in either the male of female

212   gametophyte, the population of 14,157 F2 plants that we screened represented 28, 314

213   meioses. The distribution of these F2 plants between the different *FUS* loci used in the

214   study are shown in Table 2. No *fus* mutant seedlings were observed in any of the F3

215   progeny tested.

216

217   DISCUSSION

218   The objective of this study was to determine the rate with which recombination occurs

219   between T-DNA inserts located nearby to each other in the *Arabidopsis* genome. One of the

220   consequences of this type of recombination is the deletion of chromosomal DNA located

221   between the two T-DNA inserts. We use the term "deletions caused by T-DNA

222   recombination" to describe this phenomena. This type of deletion is caused by

223   recombination between two established T-DNA inserts that were generated via

224   independent transformation events in separate plants. Recently, it has been shown that

225   during the initial T-DNA integration process that heterogeneous T-DNA integration can

226   cause chromosomal rearrangements, including deletions, as part of the double-stranded

227   break repair mechanism driving T-DNA insertion (Hu et al. 2017). This type of T-DNA

228   associated deletion is mechanistically distinct from the phenomena that we were targeting

229   with our study.

230   In our previous work we observed one instance of a deletion caused by T-DNA

231   recombination in a population of ca. 2,000 seedlings (Su et al. 2013). Because of the

232   extensive collections of *Arabidopsis* T-DNA insertion lines available through public stock

233   centers, we were intrigued by the possibility that deletions caused by T-DNA

234   recombination could provide an effective strategy for genome engineering. If the rate of

235   deletion caused by T-DNA recombination was high enough, one could imagine obtaining a

236   pair of T-DNA lines flanking a genomic region of interest, producing an F1 parent carrying

237   those two T-DNA insertions, and then screening for deletions in the F2 progeny. Since the

238   genetic screen described in this study did not reveal any evidence for deletions caused by

239   T-DNA recombination from a population of 14,157 F2 parent plants, it appears that this

240   type of deletion may be a relatively rare event.

241   The most straightforward interpretation of our failure to observe *fus* mutant
242   seedlings in our genetic screen is that deletions caused by T-DNA recombination did not
243   occur in the F1 plants used in our study. It is also possible, however, that deletions may
244   have been generated, but were subsequently selected against due to lethality or reduced
245   fitness of the specific deletion allele that was formed. In most of the *FUS* loci used in our
246   study, the upstream and downstream T-DNA inserts are located in neighboring genes. It is
247   possible that deleting those neighboring genes in addition to the FUS gene could have a
248   detrimental effect on the plant. It should be noted that each of the T-DNA lines used for this
249   study was viable when the T-DNA insert was homozygous, indicating that the neighboring
250   genes are not essential genes and that deleting them on their own should not cause
251   lethality. It nevertheless remains possible that genetic interactions between the loci or
252   other functional elements contained within the intervals between the flanking T-DNAs
253   could lead to unexpected lethality of the predicted deletions.
254
255   CONSLUSIONS
256   Our study has suggested that the deletion caused by T-DNA recombination
257   phenomena is unlikely to be a useful strategy for genome engineering in *Arabidopsis*. Given
258   recent advances in the application of CRISPR-Cas9 methods in plants, a more effective
259   approach may be to direct double-strand breaks using Cas9 targeted to sites flanking the
260   region of interest (Yin et al. 2017). This type of approach has been used in rice to delete
261   regions up to 245 kb in size (Zhou et al. 2014) and *Arabidopsis* to delete regions up to 120
262   kb (Ordon et al. 2017). Despite the lack of an immediate practical application, our previous
263   observation of recombination between established T-DNA insertions highlights the
264   dynamic nature of genome structure. As our ability to modify genomes advances, there
265   remains a need to more fully understand all of the mechanisms and processes that shape
266   genome structure and behavior.
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269
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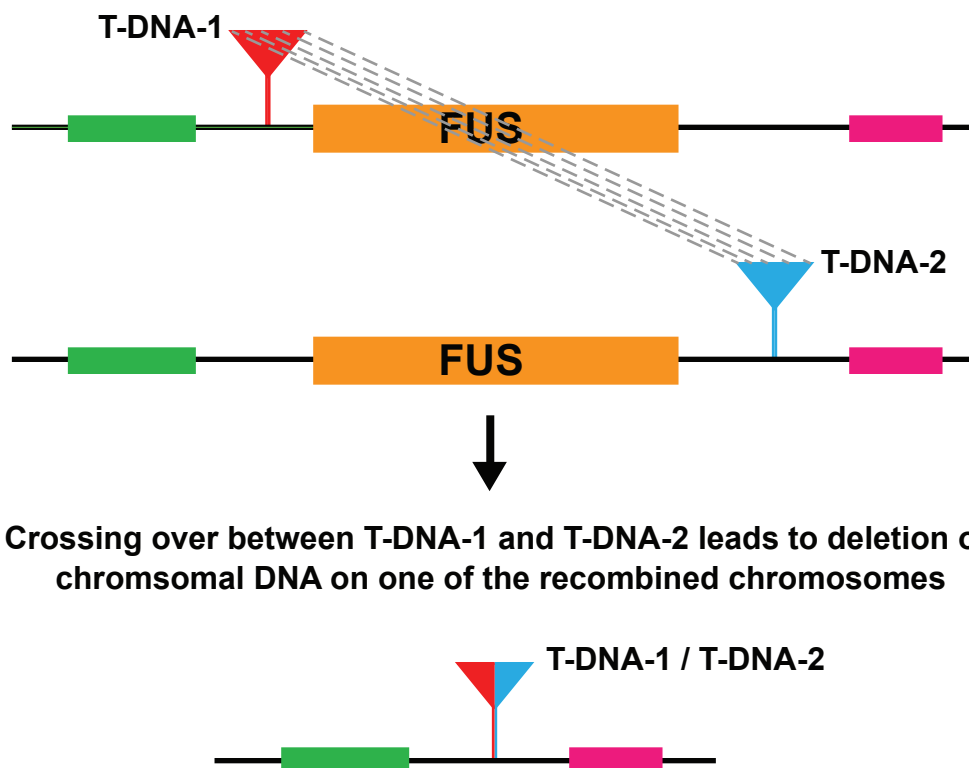
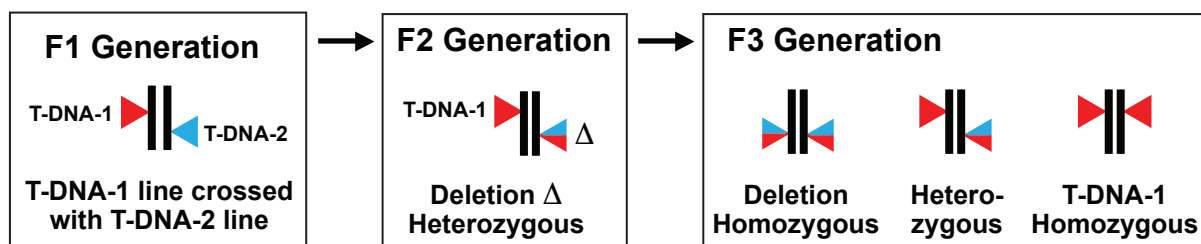
Schematic of T-DNA directed deletion and genetic screen.

(A) Recombination between the T-DNA insertions results in deletion of the fus gene. (B) A T-DNA directed deletion that occurs during meiosis in an F1 parent will not produce a visible fus phenotype until the F3 generation

A.

Crossing over between T-DNA-1 and T-DNA-2 leads to deletion of chromsomal DNA on one of the recombined chromosomes

B.

Maps of *FUS* loci used in this genetic screen.

Positions of the T-DNA inserts used for each FUS locus are indicated with blue triangles. The presence of the T-DNA Left Border sequence is indicated by "LB". "LB" on both sides of the T-DNA suggests that there are at least two copies of the T-DNA element inserted in an inverted repeat orientation. All maps are drawn to the same scale as indicated by the scale bar.

SALK_046021
LB

AT4G10170
AT4G10180
Fus2
SALK_004560
LB **LB**

AT4G10190

WiscDsLox 453-456F5
LB

AT3G61120
AT3G61130
AT3G61140
Fus6
WIscDsLox 402F03
LB **LB**

AT3G61150

SALK_203944
LB **LB**

AT3G61120
AT3G61130
AT3G61140
Fus6
SALK_062171
LB

AT3G61150

SALK_073580
LB **LB**
AT4G14105

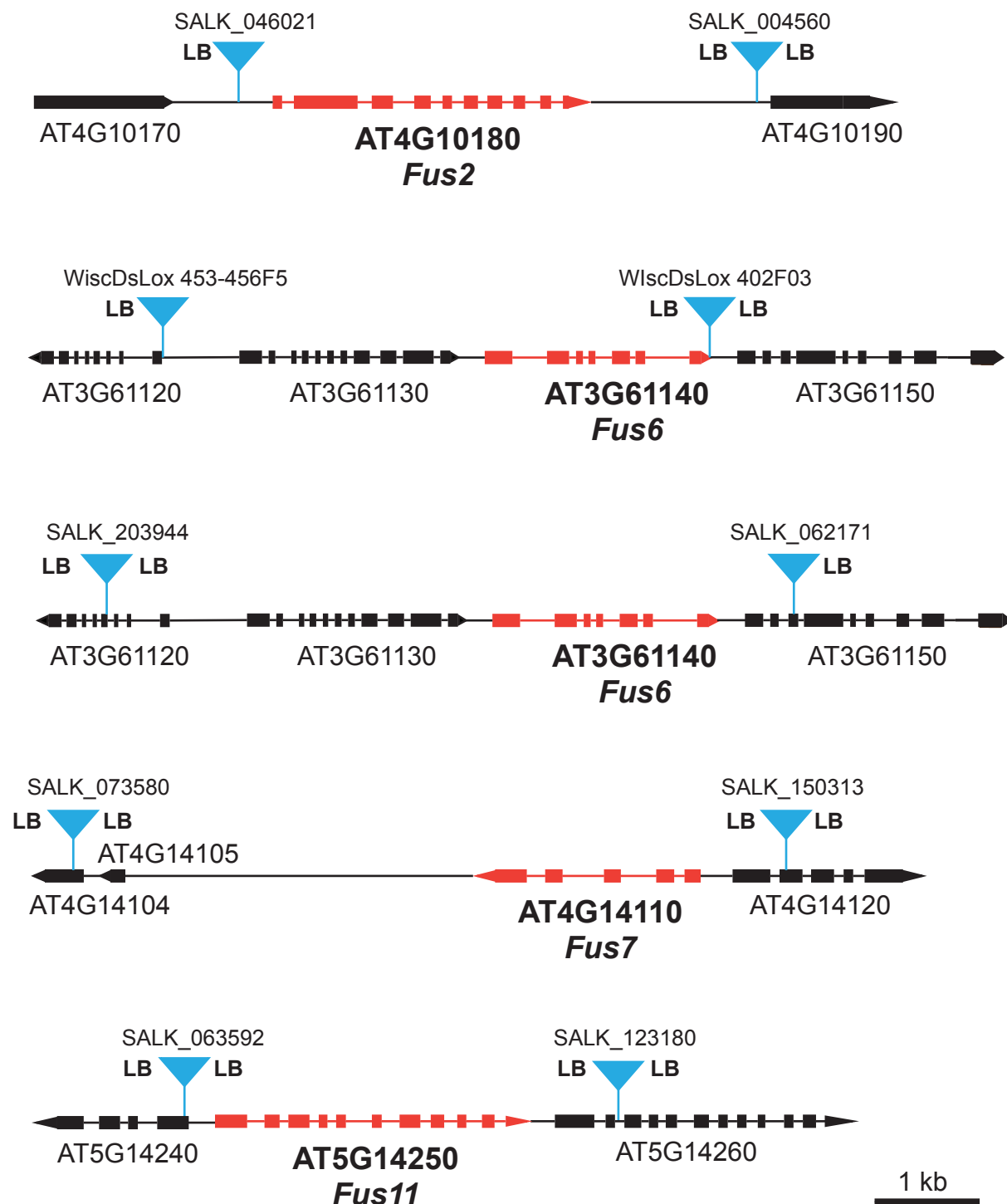
AT4G14104
AT4G14110
Fus7
SALK_150313
LB **LB**

AT4G14120

SALK_063592
LB **LB**

AT5G14240
AT5G14250
Fus11
SALK_123180
LB **LB**

AT5G14260

1 kb

PCR Primers

1 **Table 1. PCR primers**

Gene	T-DNA	Primer F	Primer R	T-DNA PCR[1]
FUS2	SK_046021	CCGATCAACAAAATGGGAAGT	TCACATTTTGGATCGGATTTT	F+p745
	SK_004560	TCCATTAACTCCAGATTTGTGAA	GTAATCGGACCAAAGTTACGG	F+p754 or R+p745
FUS6	SK_203944	TGCACCAAAAAGCAGAAGATT	TGTCCTTAAGATCCATGAAACC	F+p754 or R+p745
	SK_062171	TGTAAATTTGGCAGCAAACAA	TCATAGCTTCGCGTACAGACA	R+p745
FUS6	WiscDsLox-453-456F5	TACGGCTACCCATGATAAACC	TTGCATGGTAATTGAAATGAGAA	F+p745
	WiscDsLox-402F03	TGCATTTCTTTGAAGTTGTTCTC	TTTGTTGAGCGTTTCATTTGTT	F+p754 or R+p745
FUS7	SK_073580	GAATAGCCACAAGGAGGAACC	TTGGAGCATTTATGAGATCGAG	F+p754 or R+p745
	SK_150313	TCTCAGACGCGTATTCTCTCC	TTTCCGGATGACGAATCTGTA	F+p754 or R+p745
FUS11	SK_063592	ATGGCAACAGATCCGAGAAA	CGGTTTAGCCAGACCAATTT	F+p754 or R+p745
	SK_123180	GCGTCTCTCTTCCAATTCGT	CCCGTAATCCCACCCTTATC	F+p754 or R+p745

[1]"T-DNA PCR indicates" which gene-specific primers can be used to detect the T-DNA insertion when used in combination with the T-DNA left border primer p745.

Genetic Screen Results

Table 2. Genetic Screen Results

Gene	T-DNA 1	T-DNA 2	# of F2 Parents	# of Deletions
FUS2	SK_046021	SK_004560	2,916	0
FUS6	SK_203944	SK_062171	2,592	0
FUS6	WiscDsLox 453-456F5	WiscDsLox 402F03	3,312	0
FUS7	SK_073580	SK_150313	2,583	0
FUS11	SK_063592	SK_123180	2,754	0
Total			14,157	0