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How to avoid false negative results in transgenic selection

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Abstract Quality of *Arabidopsis thaliana* seeds deteriorates during prolonged storage, resulting in a low percentage of germinating seeds. The screening of older seed collections is therefore notoriously difficult. During screening of an older seed library in C24 background we observed both reduced germination frequency and a post-germination growth arrest that may represent a serious problem if unnoticed. The seedlings exhibiting post-germination growth arrest were detected both in wild-type and transgenic lines, and were not caused by silencing of the nptII gene. These seedlings resembled kanamycin-sensitive ones and thus interfered with the kanamycin selection. This severely impaired segregation experiments in transgenic lines. Although the post-germination growth arrest occurred as a probable consequence of prolonged or suboptimal seed storage, using sucrose containing growth medium could successfully prevent it. Addition of sucrose in solid medium is therefore highly recommended for germination of older seed batches and is vital for accurate kanamycin selection of *A. thaliana* seeds. Apart from sucrose we tested the influence of MES buffer and MS salts on post-germination growth arrest and efficiency of kanamycin selection in C24 and Landsberg *erecta* (L*er*) ecotypes.

Index terms: *Arabidopsis thaliana*, Sucrose, Post-germination growth arrest, Kanamycin selection

1. INTRODUCTION

The phytohormone abscisic acid (ABA) mediates many plant responses to environmental stress, particularly the ability to sense and respond to water status and to regulate seed dormancy. ABA is the hormone that maintains seed dormancy and causes developmental arrest of the embryo [1-4]. Studies done on ABA insensitive mutants, revealed that the inhibitory mechanisms, regulated by ABA, extend beyond seed dormancy and can arrest growth of seedlings as well. This other developmental checkpoint has been described as post-germination arrest of development [5-8]. ABI5 was identified as a key player in this process [7]. Post-germination growth arrest was previously observed either in some developmental mutants [9] or on medium containing ABA and increased concentrations of glucose [6, 7, 10].

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In *Arabidopsis thaliana* (L.) Heynh. as in many other species with non-persistent endosperm, nutrients stored in cotyledons are utilized to nourish the embryo before and shortly after germination [11-13]. Recent evidences suggest a new role for sugars as signaling molecules that interact with plant hormones and play important role during germination and vegetative plant growth [14, 15]. Both sugars and ABA can cause post-germination growth arrest [16].

Kanamycin selection is most frequently performed in germinating seeds of transgenic lines and is typically manifested as growth arrest followed by bleaching and death of the plant a few weeks after germination [17]. Any post-germination growth arrest triggered by other factors could therefore affect kanamycin selection and interfere with genetic segregation experiments. Germination of seeds depends on the genotype, age and environmental factors like for instance storage conditions (humidity, temperature etc). During prolonged storage or if not properly handled seeds might be damaged resulting in poor germination and growth. In several seed batches we observed post-germination growth arrest that could be completely abolished by externally supplied sucrose. In this paper we show the impact of sucrose, MES buffer and MS salts on post-germination growth arrest and efficiency of kanamycin selection in C24 and Landsberg *erecta* (L*er*) ecotypes.

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2. MATERIALS AND METHODS

Plant material

Plant transformation vector pMHAII contained the pnos-nptII plant selectable marker gene close to the left T-DNA border [18-20]. T-DNA lines were generated in the C24 ecotype of *A. thaliana* by root transformation. Approximately 50 seeds per 10 cm plate were germinated on MS medium [21] using either the MS medium basal salt mixture including vitamins with MES (2-N-morpholino/ethanesulfonic acid) (M0255) or without MES (M0222) from Duchefa. Selection medium contained 50 mg/l kanamycin (K4378, Sigma) while sucrose (S0809, Duchefa) was added to the germination medium in concentration 2% (w/v). Plants were grown at 23±3 °C and 70% humidity, in the Persival chamber under 40 W cool white fluorescent light (16/8 h light/dark). Seeds were harvested using Aracons (Arasystem) and dried in the desiccation chamber at the room temperature for few weeks. Alternative growth procedures: Surface sterilized seeds were sown on MS-2 plates (Murashige and Skoog 1962) and grown in the phytotron at 20–22◦C under LD conditions, i.e. 16 h light (100 μmol m^{-2} s^{-1}) and 8 h dark. Seeds were dried for two days at 42°C and stored at RT.

GUS assay and Southern blot

GUS assay [22] was performed on non-fixed seedlings (line 601) using clearing technique [23]. Southern blot was prepared using 15 mg HindIII digested plant genomic DNA per lane.

Functional annotation of genes

Microarrays were performed as previously described [24]. Functional annotation of the microarray results was done using DAVID bioinformatic tool http://david.abcc.ncifcrf.gov/home.jsp [25, 26]. Expression results were calculated using GraphPad Prism software.

3. RESULTS

3.1. Post-germination growth arrest and kanamycin selection

In a typical selection experiment, wild type *A. thaliana* seedlings, germinate normally on kanamycin-containing plates but do not make true leaves and rosettes while the roots do not elongate. They bleach and gradually die after few weeks (Fig. 1A and B). Seedlings grown on non-selective plates remain green and develop normal rosettes, roots and reproductive organs (Fig. 1C).

We screened a collection of T-DNA mutants in C24 background [19, 20, 23, 24, 27] that was generated several years earlier [18]. It is known that the prolonged and suboptimal storage of seeds may lead to decline in germination efficiency. This was indeed observed (B. Stangeland, unpublished). Interestingly we also observed that a certain population of germinated seedlings suffered severe post-germination growth arrest (Fig. 1D). These seedlings were exclusively observed on the growth medium without sucrose and the phenomenon could be completely prevented by adding sucrose to the medium. The seedlings that showed post-germination growth arrests were observed on plates with and without kanamycin and both wild type and transgenic seeds could be affected.

The arrested seedlings did not make rosettes while their roots and the hypocotyls remained short and thick compared to the plants of the same age (Fig. 1D-E). Even though these seedlings stayed green few weeks after the kanamycin-sensitive seedlings turned white, the resemblance was strong especially in the early phase of the selection (Fig. 1E). Later on, the differences were more obvious because the arrested seedlings appeared "glassy" and stayed green for several weeks after the sensitive seedlings turned pale (Fig. 1E). Finally, arrested seedlings also turned pale and necrotic and died. In order to analyze this phenomenon we performed several tests.

3.2. Post-germination growth arrest is irreversible

To test if the observed post-germination growth arrest was reversible we transferred 3x50 arrested seedlings from the plates without sucrose to fresh MS plates containing 2% sucrose and incubated for up to four additional weeks (not shown). Affected seedlings were transferred immediately after phenotype became apparent. None of these were able to proceed with normal development on the medium with sucrose indicating some kind of long-lasting growth-arrest or permanent damage of the meristems. After prolonged incubation (>4 weeks) on the medium with sucrose, transferred seedlings, turned white and died.

The arrested seedlings were observed in several commercially available ecotypes (Lehle seeds). In L*er*, RLD, Col and Green wild arrested seedlings were rare while in the Niederzanz and C-24 ecotypes the frequency of occurrence was high (up to 14% of affected seedlings).

3.3. Influence of MES, MS and sucrose on germination, post-germination growth arrest and kanamycin selection

To test how different factors influence post-germination growth arrest we performed a series of experiments. Firstly, we tested how ingredients of growth medium influence germination, post-germination and kanamycin selection. Stages of kanamycin selection were here defined as following: (1) seedlings mildly affected (pale green color and eventually slightly smaller plants); (2) seedlings affected (pale green to yellowish color, eventually some accumulation of anthocyanins, smaller in size); (3) seedlings bleached (seedlings predominantly white, eventually accumulating anthocyanin, significant difference in size) and (4) seedlings necrotic (white to brown color, selection finished) (Fig. 1). Leaf size and anthocyanin accumulation were evaluated on a scale from 1 to 4 (where 4 corresponded to biggest leaves and highest accumulation of anthocyanins). We germinated seeds of C24 and L*er* ecotypes (~200/test) using five different media variations: **agar**; **MS** medium (full); -**MES MS** (MS medium without MES buffer); -**MES 1/2MS** (Medium without MES buffer and with ½ MS salts); and **½ (MES MS)** (MS medium

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with ½ MES buffer and ½ MS salts). The parameters like: color (green, yellow, white or red) and the approximate size of plants were scored. All experiments were performed on plates ± **sucrose** (2%) ±**kanamycin**. Results were scored after 7, 14, 21 and 28 days.

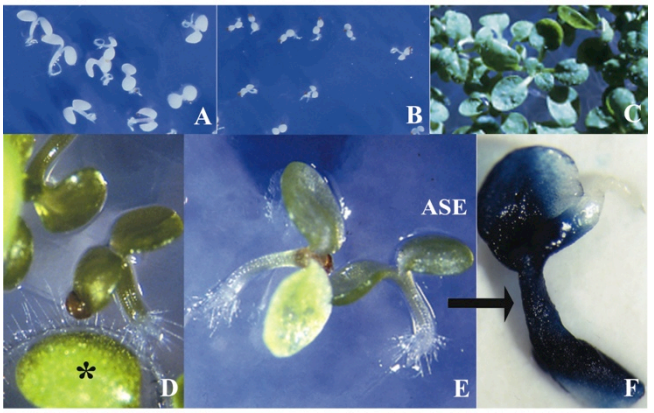

Figure 1. Kanamycin selection in C24 ecotype of *A. thaliana*. **A**: Kanamycin selection of wild type *A. thalaiana* seedlings on MS medium containing 2% sucrose; **B**: Kanamycin selection of wild type seedlings on MS medium without sucrose; **C**: Wild type seedlings of the same age as A and B grown on the MS medium without kanamycin; **D**: growth-arrested seedling (right) and the leaf of the wild type seedling from the same plate (*); **E**: Kanamycin-sensitive seedling before the end of the selection and ahead of complete bleaching (left) and the growth-arrested seedling (ASE) of the same age (right);

Minimal medium (agar+water): After two weeks on agar kanamycin-sensitive seedlings showed yellowish cotyledons of the size comparable to the plants on non-selective medium. The roots were bushy and much shorter than the control plants on non-selective agar plates (not shown). In both ecotypes the selection was finished after about three weeks (Fig. 3)

Influence of MES: MES deprivation led to faster selection especially in C24 (Fig. 3A-D) but did not prevent the post-germination growth arrest. Lack of MES also accelerated bleaching of the green leaves but did not influence the accumulation and bleaching of red pigments anthocyanins (Fig. 3E). MES deprivation had a negative effect on leaf size in absence of sucrose (Fig. 3F).

Influence of MS salts: Presence of MS salts had a positive effect on the leaf size (Fig. 3F). The concentration of MS salts did not have any influence on the post-germination growth arrest. Using medium of half a strength had a positive effect on the speed of selection as well (except for L*er* on sucrose). In presence of 1xMS salts we noticed less anthocyanins when compared to 0.5xMS salts but only in co-presence of sucrose and kanamycin (Fig. 3E).

Influence of sucrose: The main effect of sucrose (2%) was that it could completely abolish post-germination growth arrest. The effect of sucrose on the kanamycin selection was also in: 1) increasing the leaf size (Fig. 3F), 2) prolongation of the selection (Fig. 3B and D) and 3) as a major cause of anthocyanin accumulation in leaves (Fig. 3E). There was no apparent extensive accumulation of anthocyanins on the control non-selective plates with sucrose. Neither was it observed on the selective (+ kanamycin) plates without sucrose (not shown).

The kanamycin selection on plates without MES and without sucrose (Fig. 3A and C) was on average more than a week faster than on the other selective plates (Fig. 3B and D). Medium of half a strength [1/2 (MES; MS)] was superior over the full medium (MS) because the selection was faster especially if sucrose was added to the medium (Fig. 3). Bleaching of green leaves increased without MES. The accumulation of anthocyanins was caused by the co-presence of sucrose and kanamycin and was also influenced by MS salts (Fig. 3E). The speed and the efficiency of the selection on agar plates were the same in both ecotypes. In all other cases the selection was on average one week faster in L*er* ecotype (Fig. 3A and B). Our experience is that Col ecotype (not shown) usually behaves more like L*er* than C24 in the kanamycin selection experiments. Quite good selection in L*er* was achieved already 7-10 days after germination on the medium without MES and without sucrose (Fig. 3A). In this experimental setup we did not observe presence of arrested seedlings in wt plants of L*er* ecotype.

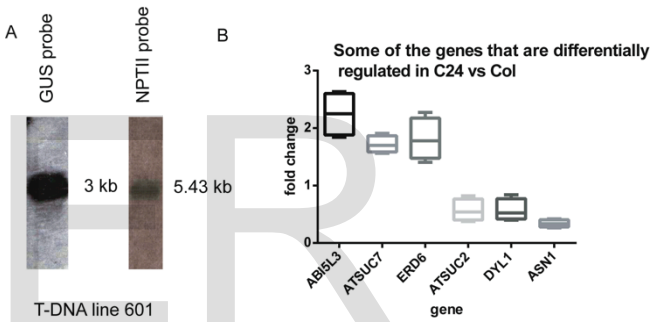

Figure 2 Molecular analysis of the transgenic line 601 and the expression analysis in C24 seedlings. **A:** Southern blots show presence of a single T-DNA insert. DNA was cut with HindIII enzyme. Probes corresponding to the GUS gene (left) and nptII gene (right) were hybridized to the genomic DNA (for the whole blot see Supplementary file 1). **B:** Expression of some ABA and sucrose signaling related genes in C24 seedlings compared to Col seedlings of the same age.

Influence of environmental factors: The Numerous *A. thaliana* growth protocols suggest drying and storage of seed at room temperature ([28], ABRC, http://abrc.osu.edu, Nottingham Arabidopsis Stock Centre NASC, http://arabidopsis.info etc.). These steps are usually not considered as critical. We observed that proper seed drying (with optional incubation at 42°C) had a beneficial effect on post-germination growth arrest (B. Stangeland, unpublished).

Several environmental factors like quality of light (Phytotrone vs. Persival incubator) and temperature in the growth chamber were tested but did not seem to make a significant difference (not shown). However differences between ecotypes and seed batches were significant indicating that seed handling and storage are the most probable causes of the growth arrest.

3.4. Post-germination growth arrest can severely impair kanamycin selection and genetic segregation experiments

To test if the post-germination growth arrest could impair genetic segregation experiments we chose several transgenic

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lines from the previously described T-DNA collection in C24 background [19, 20, 27]. Each transgenic line contained a single T-DNA insert as previously determined by segregation analysis (not shown) and Southern blot (Fig. 2A and Supplementary file 1). Transgenic marker line 601 exhibited strong GUS staining in seedlings (Fig. 1F, Supplementary file 2) and a single T-DNA copy was confirmed by Southern blots (Fig. 2A). The line 601

while in the homozygous lines all seedlings were expected to be resistant to kanamycin (lower panel, Table 1). We tested the observed Ratio O^R where the arrested (ARR) seedling were included in Km-resistant (KmR) population and ratio O^S where ARR were counted as Km-sensitive (KmS) seedlings. Observed (Ratio O^R and O^S) and expected ratios (Ratio E) were then compared to each other and the null hypothesis that these ratios were significantly different (sign. diff) was tested using Chi square. In the experiment with the hemizygous lines, the ratios E (E=3:1) and O^R were not different and the null hypothesis could be discarded (Table 1). When the arrested seedlings were counted as Km-sensitive (Ratio O^S) the incorrect answer was obtained for half of the plates (the null hypothesis could not be rejected). These results indicated that there is a 50% chance to make a mistake when interpreting the results of a genetic (selection) experiment because of the post-germination arrest phenotype. In the experiment with the homozygous plants the probability for misinterpreting segregation results was even higher. We chose ratio E to be either 3:1 or 3:2. The null hypothesis was that Ratio E (only one of the two suggested ratios E was tested) was significantly different from Ratios O^R and O^S. We tested if we can accept the null hypothesis for E vs. O^R and discard it for E vs. O^S. When the arrested seedlings were counted as KmS (Ratio O^S) the incorrect answer was obtained in more than 75% of the cases. In practice it would mean that the homozygous plants would be mistaken for hemizygous (ratio 3:1) or for plants containing multiple T-DNA insertions (for example 3:2).

Genetic segregation experiments on plates with sucrose and kanamycin showed the expected segregation ratio and

However, if the sucrose from some reason can not be included in the growth medium special attention should be paid to seed handling procedures. Seed drying at room temperature is widely recommended by seed banks and other sources [17, 28]. Our results indicate that seed drying at RT might not always be enough and additional incubation at 42°C and cautious handling of seeds are recommended as these could eliminate some of the problems with germination and the post-germination growth-arrest.

Exposure to exogenous ABA during germination arrests development reversibly, enabling seedlings to withstand early water stress without loss of viability. Similar to seedlings exposed to high concentrations of glucose, during germination, seedlings exposed to ABA, show arrested-growth [6, 7, 10]. The post-germination arrest that we observed phenocopied ABA exposure thus indicating involvement of ABA signaling in this process. However, unlike post-germination arrest caused by

did not show visible phenotypes. We tested seeds from four hemizygous and nine homozygous transgenic lines on selection plates without sucrose. The numbers of Km-sensitive (Km^S), Km-resistant (Km^R) and arrested seedlings (ARR) were counted three weeks after germination (Table 1). The expected segregation ration (Ratio E) was 3:1 for the hemizygous plants (upper panel, Table 1) confirmed that the nptII gene was functional and that there was no silencing (not shown). GUS assay was performed on growth-arrested seedlings (line 601) two weeks after germination. This test revealed strong blue staining thus confirming absence of gene silencing for this locus (Fig. 1F). Our tests thus confirmed that the two loci: nptII and GUS were functional and not silenced.

4. DISCUSSION

Post-germination growth arrest is observed on medium containing either ABA or increased concentrations of glucose [6, 7, 10]. We show that a post-germination growth arrest can occur also in wild type *A. thaliana* seedlings grown under normal growth conditions such as on solid MS-agar medium without sucrose. The trigger of this phenomenon is probably embryo damage. We think that the embryos could be impaired in storage or mobilization of the nutrients leading to the subsequent meristem damage. Our results indicate that the post-germination growth arrest was probably caused by prolonged storage or suboptimal handling of seeds although additional involvement of environmental factors can not be totally excluded.

If the sucrose (2%) was added to the medium the post-germination growth arrest could be successfully prevented. Our results thus indicate that sucrose should be included whenever possible and especially when screening long-term stored seeds of C24 ecotype. Although sucrose is routinely used, its importance in preventing post-germination growth arrest has never been shown before. Actually, many protocols recommend to avoid sucrose in order to prevent infections [17, 28].

externally added ABA we observed irreversible growth arrest indicating that processes other than ABA signaling may also be involved. Moreover the frequency of the arrested seedlings was very low in most of the tested ecotypes except for C24 indicating that genetic factors may also play a role. The *A. thaliana* accession C24 is a vernalization-responsive and a moderately late flowering ecotype [24]. Several genes involved in ABA signaling, sugar sensing and sugar transport are differentially expressed in C24 ecotype (Fig. 2B). Crucial role of ABI5 in post-germination growth arrest has been previously demonstrated [7]. In C24 background ABI5like protein 3 (AtDPBF4, At2g41070) is naturally up-regulated (Fig. 2B). This transcription factor is highly functional in C24 [29] and might activate downstream genes thus mimicking a phenotype similar to hypersensitivity to ABA. This is indeed what we observed in our seedlings: growth arrest resembling action of ABA or hypersensitivity to ABA.

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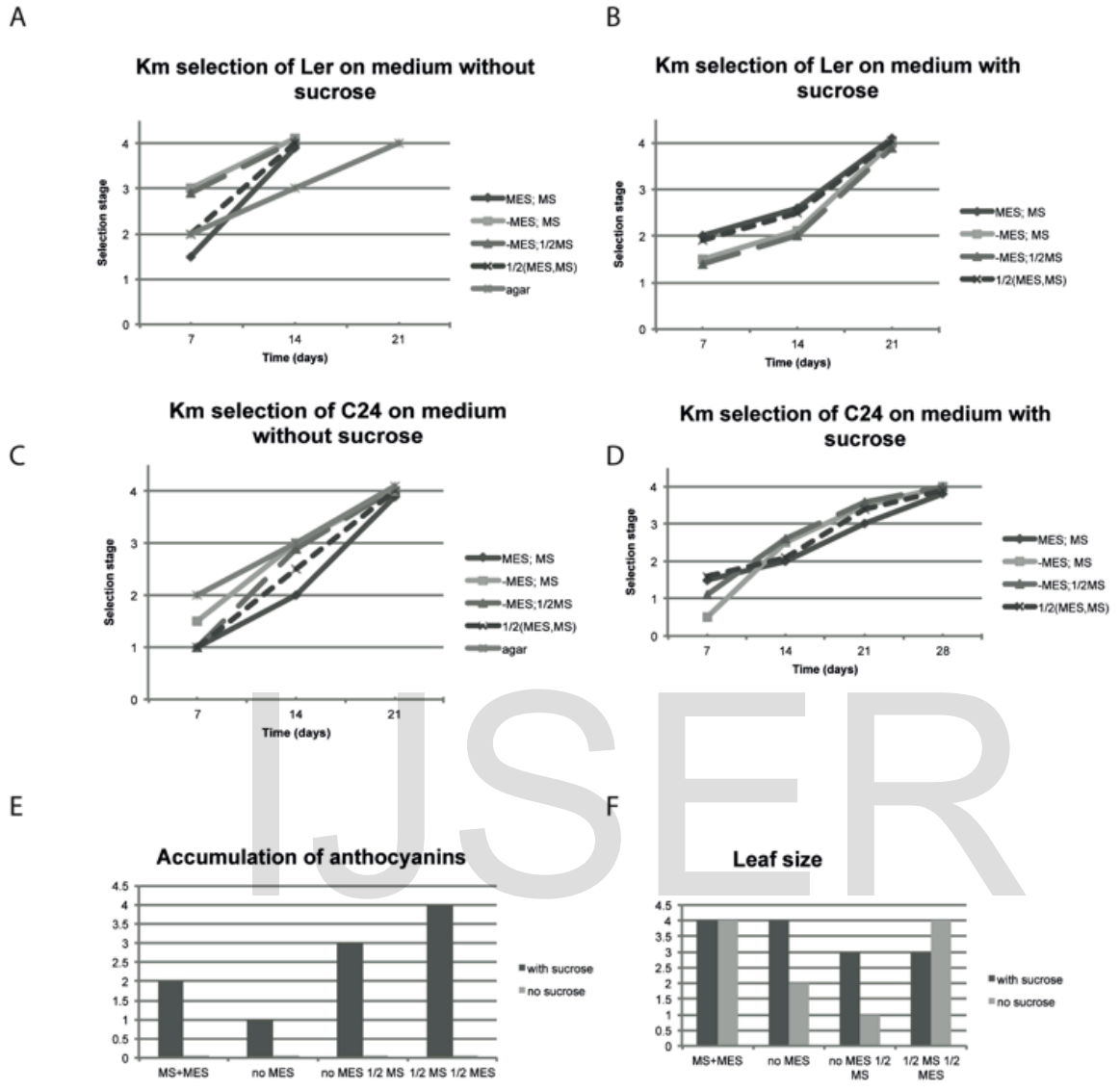

Figure 3. Effect of growth medium on kanamycin selection. A-D: Speed of the selection. Wild type plants of ecotypes L*er* (**A-B**) and C24 (**C-D**) were grown on medium without (**A** and **C**) and with (**B** and **D**) sucrose. **E**: Accumulation of anthocyanins in C24 with and without sucrose (14 days after the selection start). Results for L*er* were very similar. **F:** Leaf size in C24 (day 14). Results for L*er* were very similar).

Successful transgenic research depends on good germination and reliable antibiotic selection. Ideally, the antibiotic present in the medium should be the only growth-limiting factor. The germination medium recipe is very simple and usually contains only 1 or 0.5x MS salts, agar and water. MES and sucrose are optional. Sucrose is frequently omitted because of microbial contamination [17, 28].

In our tests sucrose in the medium led to the prolongation of kanamycin selection in both tested ecotypes (C24 and L*er*). The fastest selection was observed on plates without both sucrose and MES buffer (especially in L*er* ecotype). The other impractical consequence (besides microbial infections) of externally added sucrose was the accumulation of anthocyanins. In kanamycin selection context, this means browning rather than bleaching of the leaves. In this case anthocyanin accumulation is induced in synergy between kanamycin and sucrose and is also influenced by MS salts. MS salts and MES buffer are not considered critical for kanamycin selection. While MS and sucrose influenced size of the plants MES depletion was efficient in accelerating bleaching of the green leaves. However, lack of MES in the medium could not eliminate accumulation of anthocyanins. The fastest kanamycin selection was observed on plates that did not contain sucrose and MES. Our results show that without actually altering the concentration of kanamycin the selection in *A. thaliana* might take all from slightly more than a week to four weeks depending solely on germination

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medium and the ecotype. The necessity of externally added sucrose becomes more apparent in C24 ecotype. Quick and reliable kanamycin selection of L*er* plants can be safely performed in MS medium without sucrose and without MES. The selection of C24 seeds on very commonly used ½ strength medium without sucrose takes one week longer than L*er*. With additional 2% sucrose the selection of C24 would thus take approximately four weeks or roughly two weeks more than for L*er*.

Table 1

hemizygous															
					ARR are KmR					ARR are KmS					
No.	KmR	ARR.	KmS	RATIO E	RATIO OR	Chi	p	Sign. Diff.	RESULT	RATIO OS	Chi	p	Sign. Diff.	Tested ratio	RESULT
1	27	8	11	3:1	35:11	0.029	0.8648	NOT	CORRECT	27:19	6.522	0.0107	YES	3:1	WRONG
2	37	5	15	3:1	42:15	0.053	0.8185	NOT	CORRECT	37:20	3.094	0.0786	NOT QUITE	3:1	CORRECT
3	19	11	10	3:1	30:10	0.000	1.000	NOT	CORRECT	19:21	16.133	<0.0001	EXTREMELY	3:1	WRONG
4	36	0	13	3:1	36:13	0.061	0.8046	NOT	CORRECT	36:13	0.061	0.8046	NOT	3:1	CORRECT
total	119	24	49												

homozygous															
					ARR are KmR					ARR are KmS					
No.	KmR	ARR.	KmS	RATIO E	RATIO OR	Chi	p	Sign. Diff.	RESULT	RATIO OS	Chi	p	Sign. Diff.	Tested ratio	RESULT
1	20	9	0	all R	29:0	19.333	<0.0001	EXTREMELY	CORRECT	20:9	0.971	0.3244	NOT	3:2	WRONG
2	26	14	1	all R	40:1	24.102	<0.0001	EXTREMELY	CORRECT	26:15	0.199	0.6554	NOT	3:2	WRONG
3	20	4	0	all R	24:0	8.000	0.0047	VERY	CORRECT	20:4	0.889	0.3458	NOT	3:1	WRONG
4	29	4	0	all R	33:0	11.000	0.0009	EXTREMELY	CORRECT	29:4	2.919	0.0875	NOT QUITE	3:1	WRONG
5	29	6	0	all R	35:0	11.667	0.0006	EXTREMELY	CORRECT	29:6	1.152	0.2831	NOT	3:1	WRONG
6	25	1	0	all R	26:0	8.667	0.0032	VERY	CORRECT	25:1	6.205	0.0127	YES	3:1	CORRECT
7	48	3	0	all R	51:0	17.000	<0.0001	EXTREMELY	CORRECT	48:3	9.941	0.0016	VERY	3:1	CORRECT
8	31	9	0	all R	40:0	13.333	0.0003	EXTREMELY	CORRECT	31:9	0.133	0.7150	NOT	3:1	WRONG
9	23	18	0	all R	41:0	13.667	0.0002	EXTREMELY	CORRECT	23:18	0.260	0.6100	NOT	3:2	WRONG
total	127	31	1												

Results of the kanamycin selection performed on several T-DNA lines. Seedling categories are indicated as wild type (wt), growth-arrested (ARR), kanamycin-resistant seedlings (Km^R) and kanamycin-sensitive seedlings (Km^S). Each transgenic line contained a single T-DNA insert as previously determined by segregation analysis (not shown) and Southern blot (Supplementary file 1).

NptII or kanamycin selection, typically performed in presence of kanamycin, is a positive selection, meaning that only the resistant plants survive. Although a whole of non-transgenic seedlings can be easily eliminated the disadvantage is that the non-surviving population might include not only the kanamycin-sensitive plants but also those that, from various reasons, may show arrested vegetative growth. Using transgenic lines (each containing a single T-DNA insert) we showed that the deviation from correct segregation ratios was significant when the arrested seedlings were counted as kanamycin-sensitive. Instead of one T-DNA copy the genetic analysis indicated presence of several.

Conclusion: Our study shows that cautious seed handling and presence of sucrose in the medium are important for proper germination and vegetative growth of seedlings and successful kanamycin selection especially in older seed batches and C24 ecotype. We hope that these results may come in handy when doing high-throughput screenings and other experiments where drug selection is important.

Supplementary file 1. Southern blots of transgenic T-DNA lines performed with nptII and GUS probes. Southern blots show that each transgenic line contained a single T-DNA insert. DNA was cut with HindIII enzyme. Probes corresponding to the GUS gene (right) and nptII gene (left) were hybridized to the genomic DNA.

Supplementary file 2. GUS staining in transgenic line 601. GUS staining of two plants of the same age. A: Seedling showing arrested growth. B: Normal seedling.

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POTENTIAL CONFLICT OF INTERESTS

None.

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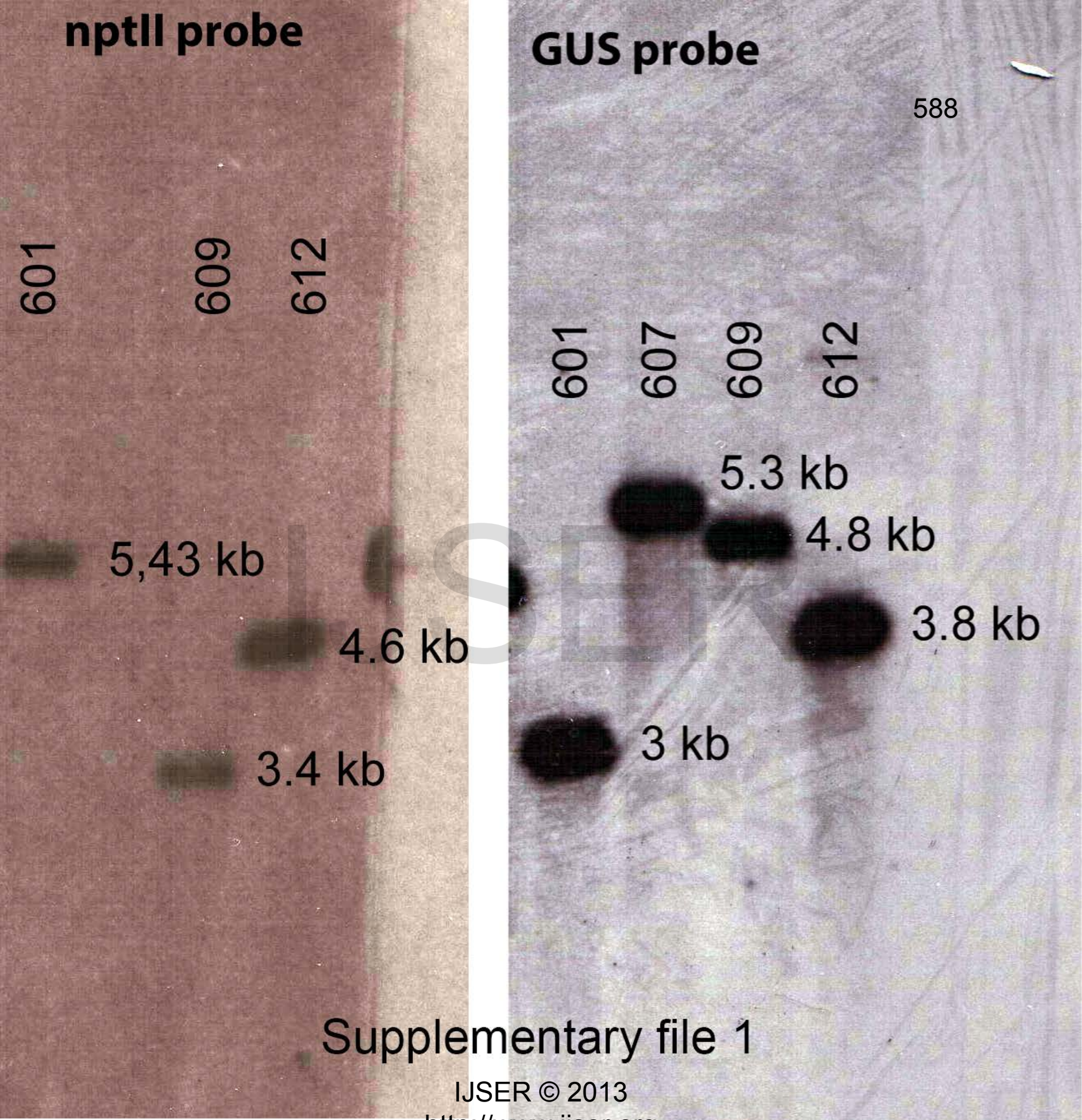

Supplementary file 1

Supplementary file 2