

1 Title

2 Tissue culture as a source of replicates in non-model plants: variation in cold
3 tolerance in *Arabidopsis lyrata* ssp. *petraea*

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19 Running title

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31

32 Abstract

33 Whilst genotype–environment interaction is increasingly receiving attention by
 34 ecologists and evolutionary biologists, such studies need genetically homogeneous
 35 replicates—a challenging hurdle in outcrossing plants. This could potentially be
 36 overcome by using tissue culture techniques. However, plants regenerated from tissue
 37 culture may show aberrant phenotypes and “somaclonal” variation. Here we examined
 38 the somaclonal variation due to tissue culturing using the response of the
 39 photosynthetic efficiency (chlorophyll fluorescence measurements for F_v/F_m , F_v'/F_m'
 40 and Φ_{PSII} , representing maximum efficiency of photosynthesis for dark- and light-
 41 adapted leaves, and the actual electron transport operating efficiency, respectively) to
 42 cold treatment, compared to variation among half-sibling seedlings from three
 43 different families of *Arabidopsis lyrata* ssp. *petraea*. Somaclonal variation was
 44 limited and we could successfully detect within-family variation in change in
 45 chlorophyll fluorescence by cold shock with the help of tissue-culture derived
 46 replicates. Icelandic and Norwegian families exhibited higher chlorophyll
 47 fluorescence, suggesting higher cold tolerance, than a Swedish family. Although the
 48 main effect of tissue culture on F_v/F_m , F_v'/F_m' and Φ_{PSII} was small, there were
 49 significant interactions between tissue culture and family, suggesting that the effect of
 50 tissue culture is genotype-specific. Tissue-cultured plantlets were less affected by cold
 51 treatment than seedlings, but to a different extent in each family. These interactive
 52 effects, however, were comparable to, or much smaller than the single effect of family.
 53 These results suggest that tissue culture is a useful method for obtaining genetically
 54 homogenous replicates for studying genotype–environment interaction related to
 55 adaptively relevant phenotypes, such as cold tolerance, in non-model outcrossing
 56 plants.

57 Introduction

58 Genotype–environment interaction on a phenotype or reaction norm may modulate
59 natural selection (Wright 1931; Sultan 1987). The genetic basis of genotype–
60 environment interaction is increasingly receiving attention (El-Soda *et al.* 2014; Yap
61 *et al.* 2011); however, such advances have been concentrated in inbreeding organisms
62 such as *Arabidopsis thaliana* (e.g. Bloomer *et al.* 2014; El-Soda *et al.* 2014; Sasaki *et*
63 *al.* 2015; Stratton 1998) and *Caenorhabditis elegans* (Gutteling *et al.* 2007), because
64 genetically isogenic individuals permit a given genotype to be exactly repeated in
65 multiple environments. Recently, the wild relatives of model organisms are
66 increasingly being exploited by evolutionary biologists to understand adaptation and
67 speciation (Clauss & Koch 2006; Mitchell-Olds 2001). However, one disadvantage of
68 non-model plants with outcrossing mating systems is that they cannot usually be
69 exploited to produce the genetically homogeneous or inbred recombinant lines that
70 enable researchers to study the reaction norms of a single genotype in multiple
71 environments (Dorn *et al.* 2000) or to map novel QTLs in previously-genotyped lines
72 (Alonso-Blanco *et al.* 2005). This disadvantage could be compensated for by using
73 cutting techniques to produce multiple clones from single genotypes (Sultan & Bazzaz
74 1993; Waitt & Levin 1993; Wu 1998). This method is only applicable to plants
75 capable of vegetative propagation, and it also needs relatively large plant bodies to
76 produce many replicate clones. Another technique applicable to a wider range of
77 plants with relatively small starting plant material is tissue culture (George &
78 Sherrington 1984). However, tissue culture has been exploited rarely for studies on
79 the genetic basis of genotype–environment interaction, and the few existing studies
80 (Glock 1989; Glock & Gregorius 1986) focused only on callus characteristics as
81 target phenotypes. One potential issue that should be carefully considered is that

82 tissue-culture derived microshoots can express phenotypic, “somaclonal” variation
 83 (Larkin & Scowcroft 1981) or may sometimes show aberrant morphology and
 84 physiology *in vitro* (Joyce *et al.* 2003). This somaclonal variation resembles that
 85 induced by physical mutagens, with elevated levels of chromosome breakage and
 86 rearrangement, polyploidy, aneuploidy, transposon activation and point mutation (D’
 87 Amato & Bayliss 1985). Therefore, with a view to exploiting the techniques of tissue
 88 culturing more widely in studies of genotype–environment interaction in outcrossing
 89 plants, it is necessary to extend our knowledge on how propagation by tissue culture
 90 generates variation in phenotypes that are relevant to adaptation in natural
 91 environments, compared to other sources of genetically-related replicates such as
 92 outbred siblings.

93

94 Key plant properties that have attracted marked attention in the field of adaptation to
 95 various environments are stress tolerances (e.g. Hong & Vierling 2000; Kwon *et al.*
 96 2007; Lexer *et al.* 2003; Quesada *et al.* 2002; Steponkus *et al.* 1998; Zhang *et al.*
 97 2004; Zhen & Ungerer 2008). One trait that can be used to indicate tolerance against
 98 various physical stressors in plants is photosynthetic performance. Photosystem II
 99 (PSII) activity is sensitive to both biotic and physical environmental factors (Murchie
 100 & Lawson 2013). Chlorophyll fluorescence can be used to determine the maximum
 101 efficiency with which light absorbed by pigments of photosystem II (PSII) is used to
 102 drive photochemistry in dark- (F_v/F_m) or light- (F_v'/F_m') adapted material and the
 103 operating efficiency of PSII (Φ_{PSII}). It is a reliable indicator of photoinhibition and
 104 damage to the photosynthetic electron transport system (Maxwell & Johnson 2000;
 105 Quick & Stitt 1989). Changes in chlorophyll fluorescence have been successfully
 106 used in *Arabidopsis thaliana* to quantify tolerance to cold and freezing temperatures

107 (Ehlert & Hinch 2008; Heo *et al.* 2014; Mishra *et al.* 2014), drought (Bresson *et al.*
108 2015; McAusland *et al.* 2013; Woo *et al.* 2008), and salt and heavy-metal stress
109 (Yuan *et al.* 2013), as well as in various other plants for tolerance to cold and freezing
110 temperatures (Baldi *et al.* 2011; Khanal *et al.* 2015; Medeiros *et al.* 2012; Xie *et al.*
111 2015), drought (Jansen *et al.* 2009) and salt (Yuan *et al.* 2013). If variation in
112 chlorophyll fluorescence can be properly estimated using tissue-culture derived clones,
113 therefore, it would enhance studies in genotype–environment interaction for stress
114 tolerance in outcrossing plants.

115
116 To this end, we have studied change in chlorophyll fluorescence following cold shock
117 in a wild relative of a model plant species. *Arabidopsis lyrata* ssp. *petraea* is a close
118 relative of the model species *A. thaliana*, but with a different ecology, life history and
119 population genetics (Charlesworth *et al.* 2003; Davey *et al.* 2008; Davey *et al.* 2009;
120 Kuittinen *et al.* 2008; Kunin *et al.* 2009). Whilst *A. thaliana* is mainly selfing, with a
121 low level of genetic diversity within a population, *A. lyrata* ssp. *petraea* is outcrossing,
122 with a high level of genetic diversity even within a population (Clauss & Mitchell-
123 Olds 2006; Heide *et al.* 2006; Kunin *et al.* 2009; Schierup *et al.* 2008). Further
124 studies on genetic and phenotypic variation in spatially distinct individuals and in
125 closely-related plants will clarify whether or not locally advantageous alleles are fixed
126 and if local populations are in evolutionary equilibrium, and are thus important in our
127 understanding of the evolutionary responses to environmental change. Distinguishing
128 phenotypic variation among closely related individuals from measurement errors is
129 difficult; however, this becomes possible if we can quantify the error within the same
130 genotype using tissue-cultured clones.

131

132 In this study, we measured the chlorophyll fluorescence parameters F_v/F_m , F_v'/F_m' and
 133 Φ_{PSII} before and after cold shock, as an index of cold tolerance, for seedlings from
 134 three families from geographically isolated populations of *A. lyrata* ssp. *petraea*, and
 135 tissue cultured plantlets derived from several genotypes (seeds) in each of those
 136 families (Table 1). In order to evaluate the usefulness of tissue culture for obtaining
 137 genetically homogenous replicates and to assess how much adaptively-relevant
 138 variation exists within the species, we tested whether (i) among-genotype phenotypic
 139 variation could be detected with the help of replication of tissue cultured plantlets, (ii)
 140 somaclonal variation would remain in the range of other components of variation such
 141 as within-family variation of seedlings, (iii) phenotypic variation in adaptively
 142 relevant traits would exist between families and (iv) tissue-culturing affected these
 143 measurements of chlorophyll fluorescence.

144 Material and Methods

145 *Plants*

146

147 Seeds of *Arabidopsis l. petraea* were collected from geographically separated
 148 populations in Ardal (Norway) (61°19'25"N, 7°50'00"E, alt. 63 m), Notsand
 149 (Sweden) (62°36'31"N, 18°03'37"E, alt. 3 m) and Sandfell (Iceland) (64°04'14"N,
 150 21°41'06"E, alt. 123 m). No specific permits were required for the seed collection for
 151 this study because these locations were not privately owned or protected in any way
 152 and because the species was not protected in these countries. The species is a
 153 perennial herb and keeps leaves throughout the year. We used a family of seeds that
 154 were at least half-siblings, from one mother plant in each population. We grew 28–40

155 seedlings per family and in each case derived 44–69 tissue-cultured plantlets from 2–3
156 seeds (1 genotype = cloned plantlets from one seed) of each family.

157

158 *Tissue culture*

159

160 Seeds were sterilised in 10% commercial bleach for 20 min, washed in sterile water
161 and stored at 4°C overnight. The seeds were then placed onto 50% strength Murashige
162 and Skoog (MS) medium (Melford Laboratories Ltd, Ipswich, UK), pH 5.7,
163 supplemented with 1 % sucrose, 5 mg/L silver thiosulphate and solidified with 1 %
164 plant agar (Melford Labs. Ltd). The agar plates were held vertically, allowing for
165 maximum recovery of root tissue. After 4 weeks the root systems were excised and
166 placed intact onto Callus Induction Medium (CIM) (Clarke *et al.*, 1992) solidified
167 with 0.55% plant agar. Plates were incubated at 23 °C for 3 days then the roots were
168 cut into 5 mm lengths and placed in bundles on fresh CIM plates that were further
169 incubated at 20°C for 2–3 days. The root sections from each plant were resuspended
170 in 10 ml molten Shoot Overlay Medium (SOM) (Clarke *et al.*, 1992) solidified with
171 0.8 % low gelling-temperature agarose and poured over a single 90 mm plate of Shoot
172 Induction Medium (SIM) (Clarke *et al.*, 1992) solidified with 0.55 % plant agar and
173 lacking antibiotics. The plates were incubated at 20 °C under a 16-hour day length.
174 Once shoots started to form from the calli they were transferred to 50 % strength MS
175 medium, pH5.7, supplemented with 1 % sucrose and solidified with 0.55% plant agar,
176 such that each plate contained 9 clones of the same genotype. A total of 4–9 plantlets
177 survived per plate. Each plate was treated as a block in the following experiment.

178

179 *Seedling growth*

180

181 Seeds were sown in Levington M3 compost within individual plug trays. Families
182 were randomised within each tray and trays were randomly repositioned every other
183 day. Plants were watered from the base of the pot as required with reverse-osmosis
184 (RO) purified water. No additional nutrients were added to the soil or water. Plants
185 were established to 6–8 leaf stage in controlled-environment growth cabinets
186 (Convion Controlled Environments Limited, Canada) set to a 12/12 hour day/night
187 cycle, 20/15 °C day/night, 70 % humidity; atmospheric CO₂ concentration was 400
188 ppm and photosynthetically-active radiation 250 μmol m⁻² s⁻¹. Chlorophyll
189 fluorescence measurements were taken just prior to and after a 24 hour cold treatment
190 in which plants were exposed to the same conditions as above, apart from the
191 temperature being decreased to 3 °C. 5–8 seedlings from the same family were treated
192 as a block in the following experiment.

193

194 *Chlorophyll fluorescence*

195

196 Pre-cold and post-cold treatment measurements of chlorophyll fluorescence were
197 obtained using a chlorophyll fluorescence imager using Fluorimager software
198 (Technologica Ltd., Colchester, UK). Each block of plants was dark adapted for at
199 least 15 minutes before the maximum efficiency of photosystem II (F_v/F_m) was
200 measured to a blue light pulse at 3000 μmol m⁻² s⁻¹ for 200 ms. Following this pulse,
201 the plants were exposed to an actinic light of 150 μmol m⁻² s⁻¹ for six minutes,
202 followed by pulses of 3000 μmol m⁻² s⁻¹ for 200 ms to obtain measures of maximum
203 efficiency of photosystem II (F_v'/F_m') of light-adapted plant material and the
204 operating efficiency of photosystem II (Φ_{PSII}) in light-adapted plant material. Mean

205 values of F_v/F_m , F_v'/F_m' and Φ_{PSII} for each plant were taken from the image of each
206 whole plant.

207 All these phenotypic data are available in Dryad Digital Repository:

208 <http://dx.doi.org/10.5061/dryad.xxxxx>.

209

210 *Statistical analyses*

211

212 To examine the relative importance of among-family and among-genotype variation
213 in cold tolerance, we used nested ANOVA to partition the total variance in the
214 difference in each chlorophyll fluorescence measurement (F_v/F_m , F_v'/F_m' or Φ_{PSII})
215 induced by cold shock:

216

$$217 \quad VD \sim VF / VG / VB$$

218

219 where VD was the total variance in difference in each type of chlorophyll
220 fluorescence for a plant individual between two measurements (i.e. value after cold
221 shock minus that before cold shock), VF was the component of among-family
222 variance, VG was the component of among-genotype variance nested in VF and VB
223 was the component of among-block variance nested in VG. We did this analysis
224 separately for the tissue-cultured plants and seedlings, in order to evaluate variation in
225 each natural and tissue-cultured condition. The VG term was not applied to the
226 analysis for seedlings. We also conducted variance component analysis using the
227 varcomp function in the ape library and the lme function using R 2.8.0(R
228 Development Core Team 2008).

229

230 We tested whether variance in the change of F_v/F_m , F_v'/F_m' or Φ_{PSII} due to cold
 231 shock among tissue-culture derived plantlets within each genotype was different from
 232 that in seedlings of half-siblings of the same family using Bartlett tests. Because the
 233 number of blocks differed between seedlings and tissue-cultured plantlets (Table 1),
 234 we checked first whether the difference in the number of blocks affected the variance,
 235 by re-sampling all possible combinations of 4 blocks from the 10 blocks of half-
 236 siblings in Ardal and Notsand. Reducing block number changed the original variance
 237 for 10 blocks only $< \pm 3\%$ without systematic bias.

238

239 Finally, we evaluated the effect of several factors on each type of chlorophyll
 240 fluorescence measurement before and after cold treatment. We constructed the
 241 following linear mixed-effect model, in which plant individual was treated as a
 242 random effect:

243

$$244 \quad CF = I|B/P + C + T + F + C \times T + T \times F + C \times F + C \times T \times F$$

245

246 where CF was a single measurement of either F_v/F_m , F_v'/F_m' or Φ_{PSII} and I|B/P was
 247 the intercept with random effects of block, and individual plant nested in each block,
 248 C was a binary variable of cold shock (1 for shocked and 0 for not), T was a binary
 249 variable of tissue culture (1 for tissue cultured and 0 for not) and F was a categorical
 250 variable of family (3 families), followed by the interaction terms among those
 251 variables. The effect of each term was estimated by the lme function using the
 252 statistical software R 2.8.0 (R Development Core Team 2008). Akaike's Information
 253 Criterion (AIC) was compared between the full model and a model lacking each term

254 in a stepwise manner and the best model with the lowest AIC was selected, followed
255 by testing the significance of each selected parameter using the Wald test.

256 Results

257 *Variance components in cold-response of F_v/F_m , F_v'/F_m' and Φ_{PSII}*

258

259 In the seedlings, the changes in F_v/F_m , F_v'/F_m' or Φ_{PSII} by cold treatment varied
260 significantly among families, explaining 4.9–9.1 % of the total variance (Table 2). For
261 the tissue-cultured plantlets, the change in those indices by cold treatment did not vary
262 significantly among families, but did vary significantly among genotypes within
263 family, this component explaining 8.5–31.5 % of the total variance. The within-block
264 variance component for tissue-cultured plantlets was 61.7–81.8 % and tended to be
265 smaller than this component for seedlings (89.1–92.2 %).

266

267 *Evaluation of somaclonal variation in comparison to within-family variation*

268

269 Variances in the change of F_v/F_m , F_v'/F_m' or Φ_{PSII} among clones within genotype
270 were clearly smaller than those among half-siblings of the same family in the Sandfell
271 family. Most genotypes had significantly smaller variances in F_v/F_m , F_v'/F_m' and Φ_{PSII}
272 than half-sibs as shown by the Bartlett test (Fig. 1). Similar patterns were observed in
273 Notsand and Ardal. No studied genotype had larger variance among clones than the
274 variance among half-siblings in any family.

275

276 *Effects of cold shock, tissue culturing and family on F_v/F_m , F_v'/F_m' and Φ_{PSII}*

277

278 All single effects of cold shock, tissue culture and family and all possible interaction
 279 combinations among them affected F_v/F_m and F_v'/F_m' , and all such effects except the
 280 3-way interaction between cold shock, tissue culture and family affected Φ_{PSII} ,
 281 according to the best model (Table 3) based on Akaike's Information Criterion (AIC).
 282 Cold shock and family were the strongest single effects. The interaction between these
 283 two factors was also found to change all three measurements of chlorophyll
 284 fluorescence, indicating that the effect of cold shock depended on family. The effect
 285 of tissue culture was relatively small and not significant for any of the chlorophyll
 286 fluorescence measures. We found substantial interactions between tissue culture and
 287 family and interactions among cold shock, tissue culture and family, indicating that
 288 the effect of tissue culture depended on family.

289

290 Discussion

291

292 *Among-genotype variance*

293

294 We were able to test for among-genotype variance using replicates generated by tissue
 295 culture within genotypes and we detected such variance in F_v/F_m , F_v'/F_m' and Φ_{PSII}
 296 measurements (Table 2). On the other hand, we showed significant but low
 297 somaclonal variation. The within-block variance component for tissue-cultured
 298 plantlets was relatively small (Table 2). The Bartlett tests showed that somaclonal
 299 variation was smaller than, or at least remained within the range of, the within-family
 300 variance, which is the smallest naturally observed component of variation in the
 301 hierarchy of genetic structure (Fig. 1). In *A. thaliana*, studies of natural variation have
 302 focused mainly on between-population variation (e.g. (Shindo *et al.* 2007). In contrast,

303 *A. lyrata* has substantial within-population variation, for example in the composition
 304 of glucosinolates (Clauss *et al.* 2006) or self-incompatibility genes (Schierup *et al.*
 305 2008). In this paper, we showed that there is within-family as well as among-family,
 306 and thus among-population, genetic variation in *A. lyrata* ssp. *petraea*. Within-family
 307 genetic variance was relatively large in Sandfell (Iceland). The observed within-
 308 family genetic variances in adaptively relevant traits highlight the wide potential for
 309 evolutionary adaptation of the species and further validates the usefulness of relatives
 310 of model organisms in evolutionary biology (Clauss & Koch 2006; Mitchell-Olds
 311 2001).

312

313 *Among-family variance*

314

315 There was significant or marginally significant among-family variance in the change
 316 of F_v/F_m , F_v'/F_m' and Φ_{PSII} values by cold treatment for seedlings (Table 2). In *A.*
 317 *thaliana*, the change in chlorophyll fluorescence from before to after cold shock
 318 correlates with tolerance to sub-zero temperatures measured by electrolyte leakage or
 319 survival and, therefore, this is regarded as an indicator of cold tolerance (Ehlert &
 320 Hinch 2008; Heo *et al.* 2014; Khanal *et al.* 2015). Therefore, our result also
 321 represents evidence for among-family (thus possibly among-population) variance in
 322 cold tolerance. Linear mixed models (Table 3) showed that F_v'/F_m' after cold-shock
 323 was higher in family Ardal and Sandfell, and F_v/F_m and Φ_{PSII} after cold-shock was
 324 higher in family Sandfell, compared to Notsand (Sweden). These results are consistent
 325 with families Aradal (Norway) and Sundfell (Iceland) being derived from relatively
 326 high latitude and high altitude and so having high cold tolerance. This among-family
 327 effect was weaker for tissue-cultured plantlets (Table 3). This may be due to the small

number of genotypes for each family in our nested experimental design, or, could be explained by the main part of the among-family variance detected for seedlings being due to among-genotype variance within families.

Effects of tissue culturing

We detected genotype-specific effects of tissue culture on F_v/F_m , F_v'/F_m' and Φ_{PSII} (Table 3). This is consistent with a previous report of a genotype-specific effect on callus characteristics (Glock 1989; Glock & Gregorius 1986). The three measured parameters of chlorophyll fluorescence (F_v/F_m , F_v'/F_m' and Φ_{PSII}) all decreased after the cold treatment (Table 3), indicating a decrease in photosystem II activity, as reported in previous studies (Finazzi *et al.* 2006). A positive effect of interaction between tissue culture and cold shock for Φ_{PSII} suggests that tissue-cultured plants were less affected by cold shock than seedlings, and an interaction between tissue culture, cold shock and family suggests that the extent to which tissue-cultured plants were less affected by cold shock differed among families. Any differences among families in traits related to responses to the tissue-culture environment, including root-cutting, callus formation and growth on medium, might explain these observed interactions between tissue culture and family. This finding is consistent with the report that somaclonal variation is genotype-dependent and influenced by both the explant source and the tissue-culture protocol (George & Sherrington 1984), and a recent study that found that the effect of tissue culture on somatic mutations depended on genotype (Zhang *et al.* 2010). The effects of tissue culture–genotype interaction, however, were comparable to, or much smaller than the single effect of family (Table 3), indicating that such interactions would not mask the single effect of genotype. The

353 interaction between tissue culture and family was much smaller in Φ_{PSII} (the range
354 between maximum and minimum estimates was $0.043 - (-0.005) = 0.048$, Table 2)
355 than in F_v/F_m ($0.082 - 0 = 0.082$) and F_v'/F_m' ($0.181 - 0 = 0.181$). The interaction
356 between cold shock, tissue culture and family was detected only in F_v/F_m and F_v'/F_m' .
357 Also, the relative impact of among-genotype variance was smaller for Φ_{PSII} (8.5% of
358 the total variance, Table 2) than F_v/F_m (31.5 %) and F_v'/F_m' (10.9 %). These results
359 imply that, although the maximum efficiencies of photosynthesis for dark- (F_v/F_m)
360 and light-adapted leaves (F_v'/F_m') were affected by tissue culturing in genotype-
361 specific ways, the actual electron transport operating efficiency (Φ_{PSII}) was less
362 affected by tissue culture.

363

364 *Conclusion*

365 Overall, we successfully detected among-genotype variance, with low somaclonal
366 variation, indicating that the advantage of tissue culturing in generating genetically
367 isogenic replicates exceeded its disadvantage in amplifying somaclonal variation in
368 our study system. We detected interaction effects of tissue culture with genotype for
369 an adaptively relevant trait, cold tolerance; however, such variation would not mask
370 the single effect of genotype. Therefore, although one should carefully consider
371 effects of tissue culturing when interpreting any results relying on the technique,
372 tissue culturing is a useful method for obtaining genetically homogenous replicates in
373 this, and probably other non-model organisms. It can provide critical additional
374 power when studying phenotypes such as cold tolerance related to adaptation in
375 natural environments, the variation in the phenotypes among families or populations,
376 the reaction norms of a genotype or the QTLs accounting for phenotypes.

377

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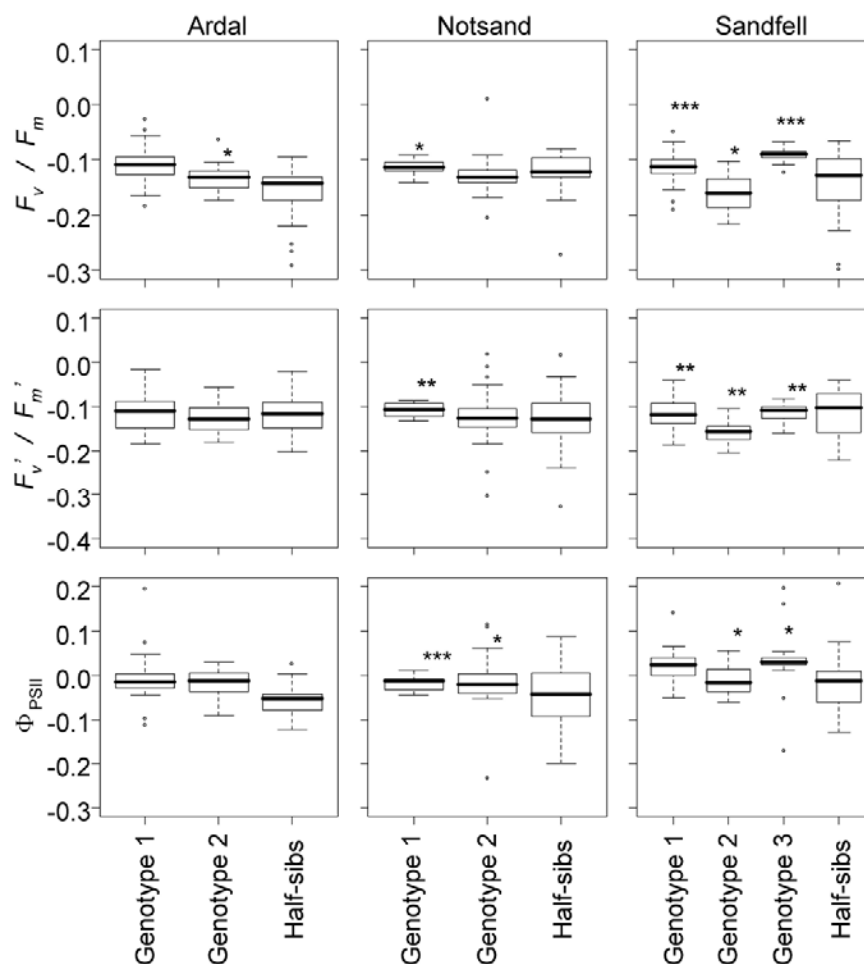
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543

544

Figure



546

547 Figure. 1. Change in chlorophyll fluorescence (F_v/F_m , F_v'/F_m' and Φ_{PSII}) in seedlings
548 or plantlets originating from Norway (Ardal), Sweden (Notsand) and Iceland
549 (Sandfell) after cold-treatment (*values after shock – those before shock*). *, ** and
550 *** = $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, (Bartlett test) indicate a
551 significantly lower variance of the genotype than among half-siblings in the same
552 family. Three F_v/F_m values (0.340, 0.375, 0.592) and an F_v'/F_m' value (0.354) in

553 Sandfell half-siblings were out of the vertical ranges shown but were included in the
554 statistical tests.
555

556 Tables

557 Table 1. Numbers of plants and blocks in each family (Ardal, Notsand and Sandfell).

558 Plants were either seedlings in a half-sibling family or tissue-cultured clonal plantlets

559 from genotypes derived from a seed from each family.

	Genotype 1	Genotype 2	Genotype 3	Half sibs
Ardal				
Number of plants	33	36	-	40
Number of blocks	4	4	-	10
Plants / block (min - max)	6 - 9	9 - 9	-	4 - 4
Notsand				
Number of plants	13	31	-	40
Number of blocks	2	4	-	10
Plants / block (min - max)	5 - 8	4 - 9	-	4 - 4
Sandfell				
Number of plants	45	28	23	28
Number of blocks	5	4	3	4
Plants / block (min - max)	9 - 9	5 - 9	5 - 9	5 - 8

560

561

Table 2. Analysis of variance for change in F_v/F_m , F_v'/F_m' and Φ_{PSII} by cold treatment for non-tissue cultured seedlings and tissue-cultured plantlets. Family and Block refer to variation among families and among blocks within families, respectively

	Seedlings						Tissue cultures					
	Df	Sum Sq	Mean Sq	F	P	Variance component (%)	Df	Sum Sq	Mean Sq	F	P	Variance component (%)
<i>F_v/F_m</i>												
Family	2	0.027	0.013	2.84	0.081	4.9	2	0.002	0.001	0.06	0.946	0.0
Genotype							4	0.080	0.020	11.52	0.000	31.5
Block	21	0.098	0.005	1.11	0.351	6.1	19	0.033	0.002	1.91	0.016	6.8
Within Block	84	0.353	0.004			89.1	183	0.167	0.001			61.7
<i>F_v'/F_m'</i>												
				10.0								
Family	2	0.081	0.041	1	0.001	7.8		0.005	0.002	0.24	0.798	0.0
Genotype							4	0.041	0.010	3.27	0.034	10.9
Block	21	0.085	0.004	0.33	0.997	0.0	19	0.059	0.003	2.54	0.001	14.1
Within Block	84	1.048	0.012			92.2	183	0.225	0.001			74.9
<i>Φ_{PSII}</i>												
Family	2	0.026	0.013	8.44	0.002	9.1	2	0.044	0.022	2.81	0.173	7.7
Genotype							4	0.031	0.008	3.37	0.030	8.5
Block	21	0.032	0.002	0.45	0.978	0.0	19	0.044	0.002	1.23	0.241	2.0
Within Block	84	0.282	0.003			90.9	183	0.349	0.002			81.8

Table 3. The best linear mixed models for F_v/F_m , F_v'/F_m' and Φ_{PSII} , based on AIC.
Intercepts represent the mixture of background conditions, i.e. not cold shocked, not tissue cultured, and family Notsand. Fam A and Fam S refer to families Ardal and Sandfell, respectively.

	Estimates	SE	DF	<i>t</i>	<i>P</i>
<i>F_v/F_m</i>					
Intercept	0.787	0.011	311	71.3	<0.001
Cold shock	-0.122	0.008	311	-15.9	<0.001
Tissue culture	-0.017	0.015	302	-1.1	0.252
Fam A	-0.026	0.015	302	-1.7	0.093
Fam S	-0.091	0.017	302	-5.4	<0.001
Cold shock x Tissue culture	-0.007	0.011	311	-0.7	0.506
Cold shock x Fam A	-0.035	0.011	311	-3.2	0.002
Cold shock x Fam S	-0.007	0.012	311	-0.5	0.584
Tissue culture x Fam A	0.029	0.020	302	1.5	0.147
Tissue culture x Fam S	0.082	0.021	302	3.9	<0.001
Cold shock x Tissue culture x Fam A	0.043	0.014	311	3.0	0.003
Cold shock x Tissue culture x Fam S	0.015	0.015	311	1.0	0.327
<i>F_v'/F_m'</i>					
Intercept	0.695	0.014	311	50.9	<0.001
Cold shock	-0.131	0.011	311	-12.1	<0.001
Tissue culture	-0.019	0.019	302	-1.0	0.304
Fam A	-0.050	0.019	302	-2.6	0.009
Fam S	-0.167	0.021	302	-7.9	<0.001
Cold shock x Tissue culture	0.011	0.015	311	0.8	0.446
Cold shock x Fam A	0.015	0.015	311	0.9	0.345
Cold shock x Fam S	0.068	0.017	311	4.0	<0.001
Tissue culture x Fam A	0.070	0.025	302	2.8	0.006
Tissue culture x Fam S	0.181	0.026	302	6.9	<0.001
Cold shock x Tissue culture x Fam A	-0.013	0.020	311	-0.7	0.514
Cold shock x Tissue culture x Fam S	-0.077	0.021	311	-3.7	<0.001
Φ_{PSII}					
Intercept	0.403	0.012	313	34.2	<0.001
Cold shock	-0.047	0.006	313	-7.7	<0.001
Tissue culture	-0.027	0.016	302	-1.7	0.090
Fam A	-0.029	0.016	302	-1.8	0.081
Fam S	-0.086	0.018	302	-4.7	<0.001
Cold shock x Tissue culture	0.034	0.006	313	5.8	<0.001
Cold shock x Fam A	-0.004	0.007	313	-0.5	0.610
Cold shock x Fam S	0.028	0.007	313	3.9	<0.001
Tissue culture x Fam A	-0.005	0.021	302	-0.2	0.822
Tissue culture x Fam S	0.043	0.022	302	2.0	0.051

573