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Kenta, T., Edwards, J.E., Butlin, R.K. et al. (2016) Tissue Culture as a Source of Replicates in Non-model Plants: Variation in Cold Response in Arabidopsis lyrata ssp. petraea. G3, 6 (12). pp. 3817-3823. ISSN: 2160-1836

https://doi.org/10.1534/g3.116.034314

Title

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

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Running title

Tissue culture and variation

Key words

Reaction norm, Stress tolerance, Genetic architecture, Genetic basis, Adaptive variation

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Abstract

Whilst genotype–environment interaction is increasingly receiving attention by ecologists and evolutionary biologists, such studies need genetically homogeneous replicates—a challenging hurdle in outcrossing plants. This could potentially be overcome by using tissue culture techniques. However, plants regenerated from tissue culture may show aberrant phenotypes and "somaclonal" variation. Here we examined the somaclonal variation due to tissue culturing using the response to cold treatment of the photosynthetic efficiency (chlorophyll fluorescence measurements for F_v/F_m, F_v'/F_m' and Φ_{PSII}, representing maximum efficiency of photosynthesis for dark- and light-adapted leaves, and the actual electron transport operating efficiency, respectively, which are reliable indicators of photoinhibition and damage to the photosynthetic electron transport system). We compared this to variation among half-sibling seedlings from three different families of *Arabidopsis lyrata* ssp. *petraea*. Somaclonal variation was limited and we could successfully detect within-family variation in change in chlorophyll fluorescence due to cold shock with the help of tissue-culture derived replicates. Icelandic and Norwegian families exhibited higher chlorophyll fluorescence, suggesting higher performance after cold shock, than a Swedish family. Although the main effect of tissue culture on F_v/F_m, F_v'/F_m' and Φ_{PSII} was small, there were significant interactions between tissue culture and family, suggesting that the effect of tissue culture is genotype-specific. Tissue-cultured plantlets were less affected by cold treatment than seedlings, but to a different extent in each family. These interactive effects, however, were comparable to, or much smaller than the single effect of family. These results suggest that tissue culture is a useful method for obtaining genetically homogenous replicates for studying

genotype–environment interaction related to adaptively-relevant phenotypes, such as cold response, in non-model outcrossing plants.

Introduction

Genotype–environment interaction effects on a phenotype, or variation in reaction norms, may modulate natural selection (Wright 1931; Sultan 1987). The genetic basis of genotype–environment interaction is increasingly receiving attention (El-Soda *et al.* 2014; Yap *et al.* 2011); however, such advances have been concentrated in inbreeding organisms such as *Arabidopsis thaliana* (e.g. Bloomer *et al.* 2014; El-Soda *et al.* 2014; Sasaki *et al.* 2015; Stratton 1998) and *Caenorhabditis elegans* (Gutteling *et al.* 2007), because genetically isogenic individuals derived by repeated inbreeding permit a given genotype to be exactly repeated in multiple environments. Recently, the wild relatives of model organisms have increasingly been exploited by evolutionary biologists to understand adaptation and speciation (Clauss & Koch 2006; Mitchell-Olds 2001). However, one disadvantage of non-model plants with outcrossing mating systems is that they cannot usually be exploited to produce the genetically homogeneous or inbred recombinant lines that enable researchers to study the reaction norms of single genotypes in multiple environments (Dorn *et al.* 2000) or to map novel QTLs in previously-genotyped lines (Alonso-Blanco *et al.* 2005). This disadvantage could be compensated for by using cutting techniques to produce multiple clones from single genotypes (Sultan & Bazzaz 1993; Waitt & Levin 1993; Wu 1998). This method is only applicable to plants capable of vegetative propagation, and it also needs relatively large plant bodies to produce many replicate clones. Another technique applicable to a wider range of plants with relatively small starting plant material is tissue culture (George & Sherrington 1984). However, tissue culture has been exploited rarely for studies on the genetic basis of genotype–environment

81 interaction, and the few existing studies (Glock 1989; Glock & Gregorius 1986)
82 focused only on callus characteristics as target phenotypes. One potential issue that
83 should be carefully considered is that tissue-culture derived microshoots can express
84 phenotypic, "somaclonal" variation (Larkin & Scowcroft 1981) or may sometimes
85 show aberrant morphology and physiology *in vitro* (Joyce *et al.* 2003). This
86 somaclonal variation resembles that induced by physical mutagens, with elevated
87 levels of chromosome breakage and rearrangement, polyploidy, aneuploidy,
88 transposon activation and point mutation (D' Amato & Bayliss 1985). Therefore, with
89 a view to exploiting the techniques of tissue culturing more widely in studies of
90 genotype–environment interaction in outcrossing plants, it is necessary to extend our
91 knowledge on how propagation by tissue culture generates variation in phenotypes
92 that are relevant to adaptation in natural environments, compared to other sources of
93 genetically-related replicates such as outbred siblings.
94
95 Key plant properties that have attracted marked attention in the field of adaptation to
96 various environments are stress tolerances (e.g. Hong & Vierling 2000; Kwon *et al.*
97 2007; Lexer *et al.* 2003; Quesada *et al.* 2002; Steponkus *et al.* 1998; Zhang *et al.*
98 2004; Zhen & Ungerer 2008). One trait that can be used to indicate tolerance against
99 various physical stressors in plants is photosynthetic performance. Photosystem II
100 (PSII) activity is sensitive to both biotic and abiotic factors (Murchie & Lawson 2013).
101 Chlorophyll fluorescence can be used to determine the maximum efficiency with
102 which light absorbed by pigments of photosystem II (PSII) is used to drive
103 photochemistry in dark- (F_v/F_m) or light- (F_v'/F_m') adapted material and the operating
104 efficiency of PSII (Φ_{PSII}). It is a reliable indicator of photoinhibition and damage to
105 the photosynthetic electron transport system (Maxwell & Johnson 2000; Quick & Stitt

106 1989). Changes in chlorophyll fluorescence have been used in *Arabidopsis thaliana*
107 to quantify tolerance to cold and freezing temperatures. Ehlert and Hincha (2008)
108 showed that chlorophyll fluorescence imaging detected difference in freezing
109 tolerance between two *A. thaliana* lineages both before and after cold acclimation.
110 Mishra *et al*. (2014) applied chlorophyll fluorescence imaging for nine *A. thaliana*
111 lineages under cold and freezing temperature and suggested that freezing tolerance of
112 lineages could be screened by chlorophyll fluorescence under cold (4 °C) condition
113 without exposing plants to sub-zero temperature. Chlorophyll fluorescence have also
114 been used to study tolerance to drought (Bresson *et al.* 2015; McAusland *et al.* 2013;
115 Woo *et al.* 2008), and salt and heavy-metal stress (Yuan *et al.* 2013) in *A. thaliana*, as
116 well as in various other plants for tolerance or response to cold and freezing
117 temperatures (Baldi *et al.* 2011; Heo *et al.* 2014; Khanal *et al.* 2015; Medeiros *et al.*
118 2012; Xie *et al.* 2015), drought (Jansen *et al.* 2009) and salt (Yuan *et al.* 2013). If
119 variation in chlorophyll fluorescence can be properly estimated using tissue-culture
120 derived clones, therefore, this method would enhance studies in genotype–
121 environment interaction for stress tolerance in outcrossing plants.
122
123 To this end, we have studied change in chlorophyll fluorescence following cold shock
124 in a wild relative of a model plant species. *Arabidopsis lyrata* ssp. *petraea* is a close
125 relative of the model species *A. thaliana*, but with a different ecology, life history and
126 population genetics (Charlesworth *et al.* 2003; Davey *et al.* 2008; Davey *et al.* 2009;
127 Kuittinen *et al.* 2008; Kunin *et al.* 2009). Whilst *A. thaliana* is mainly selfing, with a
128 low level of genetic diversity within a population, *A. lyrata* ssp. *petraea* is outcrossing,
129 with a high level of genetic diversity even within a population (Clauss & Mitchell-
130 Olds 2006; Heidel *et al.* 2006; Kunin *et al.* 2009; Schierup *et al.* 2008). Further

studies on genetic and phenotypic variation in spatially distinct individuals and in closely-related plants will clarify whether or not locally advantageous alleles are fixed and if local populations are in evolutionary equilibrium, and are thus important in our understanding of the evolutionary responses to environmental change. Distinguishing phenotypic variation among closely related individuals from measurement errors is difficult; however, this becomes possible if we can quantify the error within the same genotype using tissue-cultured clones.

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

Material and Methods

Plants

Seeds of *Arabidopsis l. petraea* were collected from geographically separated populations in Ardal (Norway) (61°19′25″N, 7°50′00″E, alt. 63 m), Notsand

(Sweden) (62°36′31″N, 18°03′37″E, alt. 3 m) and Sandfell (Iceland) (64°04′14″N, 21°41′06″E, alt. 123 m). No specific permits were required for the seed collection for this study because these locations were not privately owned or protected in any way and because the species was not protected in these countries. The species is a perennial herb and keeps leaves throughout the year. We used a family of seeds that were at least half-siblings, from one mother plant in each population. We grew 28–40 seedlings per family and in each case derived 44–69 tissue-cultured plantlets from 2–3 seeds (1 genotype = cloned plantlets from one seed) of each family.

Tissue culture

Seeds were sterilised in 10% commercial bleach for 20 min, washed in sterile water and stored at 4˚C overnight. The seeds were then placed onto 50% strength Murashige and Skoog (MS) medium (Melford Laboratories Ltd, Ipswich, UK), pH 5.7, supplemented with 1 % sucrose, 5 mg/L silver thiosulphate and solidified with 1 % plant agar (Melford Labs. Ltd). The agar plates were held vertically, allowing for maximum recovery of root tissue. After 4 weeks the root systems were excised and placed intact onto Callus Induction Medium (CIM) (Clarke *et al*., 1992) solidified with 0.55% plant agar. Plates were incubated at 23 ˚C for 3 days then the roots were cut into 5 mm lengths and placed in bundles on fresh CIM plates that were further incubated at 20˚C for 2–3 days. The root sections from each plant were re-suspended in 10 ml molten Shoot Overlay Medium (SOM) (Clarke *et al*., 1992) solidified with 0.8 % low gelling-temperature agarose and poured over a single 90 mm plate of Shoot Induction Medium (SIM) (Clarke *et al*., 1992) solidified with 0.55 % plant agar and lacking antibiotics. The plates were incubated at 20 ˚C under a 16-hour day length.

Once shoots started to form from the calli they were transferred to 50 % strength MS medium, pH5.7, supplemented with 1 % sucrose and solidified with 0.55% plant agar, such that each plate contained 9 clones of the same genotype. A total of 4–9 plantlets survived per plate. Each plate was treated as a block in the following experiment.

Seedling growth

Seeds were sown in Levington M3 compost within individual plug trays. Families were randomised within each tray and trays were randomly repositioned every other day. Plants were watered from the base of the pot as required with reverse-osmosis (RO) purified water. No additional nutrients were added to the soil or water. Plants were established to 6–8 leaf stage in controlled-environment growth cabinets (Conviron Controlled Environments Limited, Canada) set to a 12/12 hour day/night cycle, 20/15 °C day/night, 70 % humidity; atmospheric CO_2 concentration was 400 ppm and photosynthetically-active radiation 250 µmol m^{-2} s^{-1}. Chlorophyll fluorescence measurements were taken just prior to and after a 24 hour cold treatment in which plants were exposed to the same conditions as above, apart from the temperature being decreased to 3 ºC. 4–8 seedlings from the same family that were tested together were treated as a block in the following experiment.

Chlorophyll fluorescence

Pre-cold and post-cold treatment measurements of chlorophyll fluorescence were obtained using a chlorophyll fluorescence imager using Fluorimager software (Technologica Ltd., Colchester, UK). Each block of plants was dark adapted for at

least 15 minutes before the maximum efficiency of photosystem II (F_v/F_m) was measured to a blue light pulse at 3000 µmol m^{-2} s^{-1} for 200 ms. Following this pulse, the plants were exposed to an actinic light of 150 µmol m^{-2} s^{-1} for six minutes, followed by pulses of 3000 µmol m^{-2} s^{-1} for 200 ms to obtain measures of maximum efficiency of photosystem II (F_v'/F_m') of light-adapted plant material and the operating efficiency of photosystem II (Φ_{PSII}) in light-adapted plant material. Mean values of F_v/F_m, F_v'/F_m' and Φ_{PSII} for each plant were taken from the image of each whole plant.

All phenotypic data are available in Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.xxxxx.

Statistical analyses

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

P ~ Family/Genotype/Block

for tissue culture material, or

P ~ Family/Block

for seedlings, where P is the difference in each type of chlorophyll fluorescence for a plant individual between two measurements (i.e. value after cold shock minus that before cold shock), the '/' symbol implies nesting and terms were fitted as fixed effects. Variance in P was partitioned such that:

Total variance = V (Family) + V (Genotype) + V (Block)

231 for tissue culture material, or

232 Total variance = V (Family) + V (Block)

233 for seedlings.

234 We did this analysis separately for the tissue-cultured plants and seedlings, in order to
235 evaluate variation in each natural and tissue-cultured condition. We conducted these
236 variance component analyses using the varcomp function in the ape library and the
237 lme function in *R* 2.8.0 (*R* Development Core Team 2008).

238

239 We tested whether variance in the change of F_v/F_m, *Fv'/Fm'* or Φ_{PSII} due to cold
240 shock among tissue-culture derived plantlets within each genotype was different from
241 that in seedlings of half-siblings of the same family using Bartlett tests. Because the
242 number of blocks differed between seedlings and tissue-cultured plantlets (Table 1),
243 we checked first whether the difference in the number of blocks affected the variance,
244 by re-sampling all possible combinations of 4 blocks from the 10 blocks of half-
245 siblings in Ardal and Notsand. Reducing block number changed the original variance
246 for 10 blocks only < ±3 % without systematic bias.

247

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

252 $CF = I|B/P + C + T + F + C\times T + T\times F + C\times F + C\times T\times F$

253 where CF was a single measurement of either F_v/F_m, *Fv'/Fm'* or Φ_{PSII} and I|B/P was
254 the intercept with random effects of block, and individual plant nested in each block,
255 C was a categorical variable of cold shock (cold-shocked or not), T was a categorical

variable of tissue culture (tissue-cultured or not) and F was a categorical variable of family (3 families), followed by the interaction terms among those variables. The effect of each term was estimated by the lme function using the statistical software *R* 2.8.0 (*R* Development Core Team 2008). Akaike's Information Criterion (AIC) was compared between the full model and a model lacking each term in a stepwise manner and the best model with the lowest AIC was selected, followed by testing the significance of each selected parameter using the Wald test.

Results

Variance components in cold-response of Fv/Fm Fv'/Fm'and Φ_{PSII}

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

Evaluation of somaclonal variation in comparison to within-family variation

Variances in the change of F_v/F_m, *Fv'/Fm'* or Φ_{PSII} among clones within genotype were clearly smaller than those among half-siblings of the same family in the Sandfell family. Most genotypes had significantly smaller variances in F_v/F_m, F_v'/F_m' and Φ_{PSII} than half-sibs as shown by the Bartlett test (Fig. 1). Similar patterns were observed in

Notsand and Ardal. No studied genotype had larger variance among clones than the variance among half-siblings in any family.

Effects of cold shock, tissue culturing and family on Fv/Fm Fv'/Fm'and Φ_{PSII}

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

Discussion

Among-genotype variance

We were able to test for among-genotype variance using replicates generated by tissue culture within genotypes and we detected such variance in F_v/F_m, F_v'/F_m' and Φ_{PSII} measurements (Table 2). On the other hand, we showed significant but low somaclonal variation. The within-block (error) variance component for tissue-cultured

306 plantlets was relatively small compared to that for non-tissue-cultured seedlings
307 (Table 2). The Bartlett tests showed that somaclonal variation was smaller than, or at
308 least remained within the range of, the within-family variance, which is the smallest
309 naturally observed component of variation in the hierarchy of genetic structure (Fig.
310 1). In *A. thaliana*, studies of natural variation have focused mainly on between-
311 population variation (e.g. Shindo *et al.* 2007). In contrast, *A. lyrata* has substantial
312 within-population variation, for example in the composition of glucosinolates (Clauss
313 *et al.* 2006) or self-incompatibility genes (Schierup *et al.* 2008). In this paper, we
314 showed that there is within-family as well as among-family, and thus among-
315 population, genetic variation in *A. lyrata* ssp. *petraea*. Within-family genetic variance
316 was relatively large in Sandfell (Iceland). The observed within-family genetic
317 variances in putatively adaptive traits highlight the wide potential for evolutionary
318 adaptation of the species and further validate the usefulness of relatives of model
319 organisms in evolutionary biology (Clauss & Koch 2006; Mitchell-Olds 2001).
320
321 *Among-family variance*
322
323 There was significant or marginally significant among-family variance in the change
324 of F_v/F_m, F_v'/F_m' and Φ_{PSII} values following cold treatment for seedlings (Table 2).
325 We used different growth chambers for plant growth and for cold shock and therefore
326 light condition for cold shock inevitably differed from that for growth. Light and
327 temperature are difficult to disassociate in such a study system, and both the single
328 effect of cold treatment and the light–temperature interaction can be involved in the
329 effect of cold shock. In *A. thaliana*, the change in chlorophyll fluorescence from
330 before to after cold shock correlates with tolerance to sub-zero temperatures measured

by electrolyte leakage and, therefore, this is regarded as an indicator of cold tolerance or response (Ehlert & Hincha 2008; Mishra *et al.* 2014). Therefore, our result also represents evidence for among-family (thus possibly among-population) variance in cold response.

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, Supplementary Table 1). This is consistent with a previous report of a genotype-specific effects on callus characteristics (Glock 1989; Glock & Gregorius 1986). The three measured parameters of chlorophyll fluorescence all decreased after the cold shock (the effects of cold shock in Table 3 are all negative for F_v/F_m, F_v'/F_m' and Φ_{PSII}), 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 with a recent study showing 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

356 to, or much smaller than the single effect of family (Table 3), indicating that such
357 interactions would not mask the single effect of genotype. The interaction effect
358 between tissue culture and family was much smaller in Φ_{PSII} than in F_v/F_m or F_v'/F_m'
359 (the ranges between maximum and minimum estimates were 0.043 − (−0.005) =
360 0.048, 0.082 − 0 = 0.082 and 0.181 − 0 = 0.181, respectively; Table 3). An interaction
361 between cold shock, tissue culture and family was detected only in F_v/F_m and F_v'/F_m'.
362 Also, the relative impact of among-genotype variance was smaller for Φ_{PSII} (8.5% of
363 the total variance, Table 2) than for F_v/F_m (31.5 %) or F_v'/F_m' (10.9 %). These results
364 imply that, although the maximum efficiencies of photosynthesis for dark- (F_v/F_m)
365 and light-adapted leaves (F_v'/F_m') were affected by tissue culturing in genotype-
366 specific ways, the actual electron transport operating efficiency (Φ_{PSII}) was less
367 affected by tissue culture.
368
369 *Conclusion*
370 Overall, we successfully detected among-genotype variance, with low somaclonal
371 variation, indicating that the advantage of tissue culturing in generating genetically
372 isogenic replicates exceeded its disadvantage in amplifying somaclonal variation in
373 our study system. We detected interaction effects of tissue culture with genotype for a
374 putatively adaptive trait, cold response; however, such variation would not mask the
375 single effect of genotype. Therefore, although one should consider effects of tissue
376 culturing carefully when interpreting any results relying on the technique, tissue
377 culturing is a useful method for obtaining genetically homogenous replicates in this,
378 and probably other non-model organisms. It can provide critical additional power
379 when studying phenotypes such as cold response related to adaptation in natural

environments, the variation in the phenotypes among families or populations, the reaction norms of a genotype or the QTLs accounting for phenotypes.

Acknowledgements

We are grateful to Prof. M. Burrell for advice, Dr. P. Vergeer for providing seeds and Dr. C. Lilley and Ms. J. Hibbard for providing tissue culture protocols. This research was funded by the Natural Environment Research Council Post-Genomics and Proteomics programme (NE/C507837/1) in UK; the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (MEXT); and research exchange program between Japan and UK by Japan Society for the Promotion of Science (10037611-000065).

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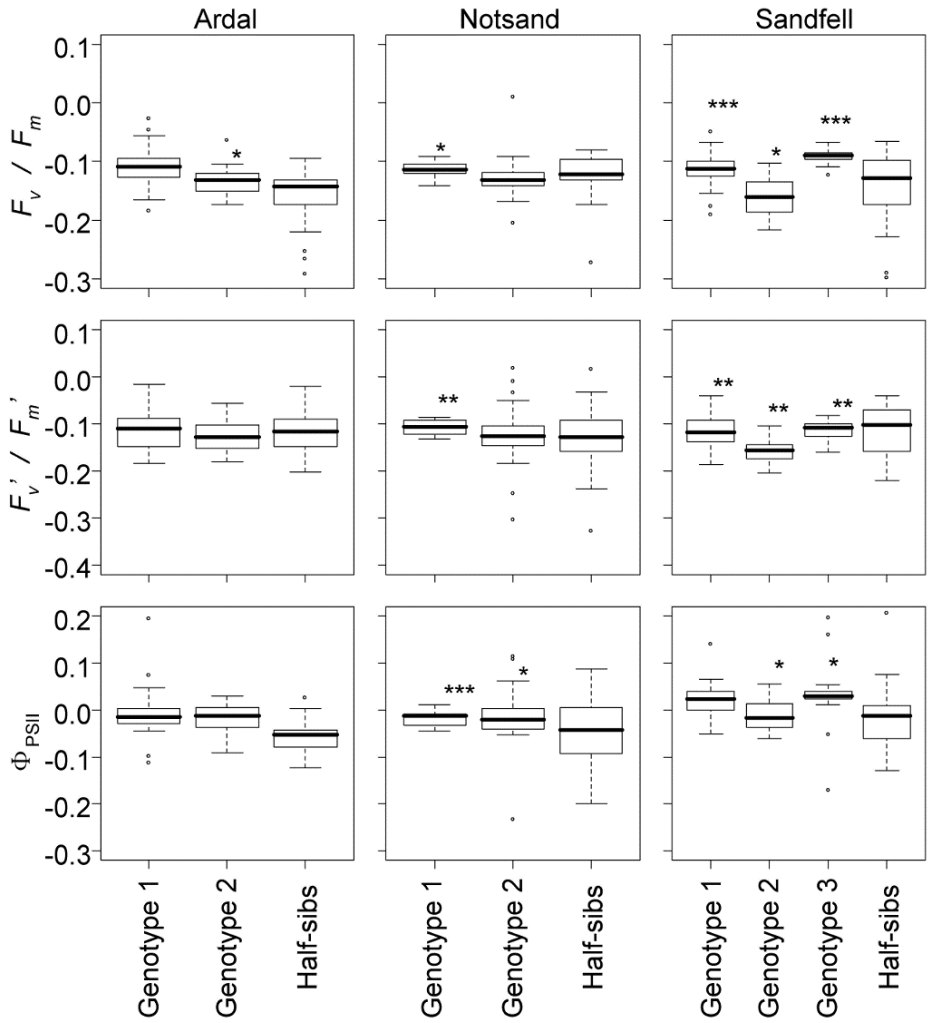
550 Figure



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

558 Sandfell half-siblings were out of the vertical ranges shown but were included in the
559 statistical tests.
560

Tables

Table 1. Numbers of plants and blocks in each family (Ardal, Notsand and Sandfell). Plants were either seedlings in a half-sibling family or tissue-cultured clonal plantlets from genotypes derived from a seed from each family. Block refers to the groups of plantlets from each genotype, or groups of seedlings from the same family for half-sibling families, that were treated and measured at the same time.

	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

569 Table 2. Analysis of variance for change in F_v/F_m, F_v'/F_m' and Φ_{PSII} by cold treatment
570 for non-tissue-cultured seedlings and tissue-cultured plantlets. Family and Block refer
571 to variation among families and among blocks within families, respectively. Each
572 Block was a group of seedlings from the same family for Seedling or group of
573 plantlets from the same genotype for Tissue cultures. Error refers to variation among
574 plants within blocks.
575

	Seedlings						**Tissue cultures**					
	Df	Sum Sq	Mean Sq	*F*	*P*	Variance component (%)	Df	Sum Sq	Mean Sq	*F*	*P*	Variance component (%)
Fv/Fm												
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
Error	84	0.353	0.004			89.1	183	0.167	0.001			61.7
F_v'/F_m'												
Family	2	0.081	0.041	10.01	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
Error	84	1.048	0.012			92.2	183	0.225	0.001			74.9
Φ_{PSII}												
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
Error	84	0.282	0.003			90.9	183	0.349	0.002			81.8

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Table 3. The best linear mixed models for F_v/F_m, F_v'/F_m' and Φ_{PSII}, based on AIC. Fam A and Fam S refer to families Ardal and Sandfell, respectively. Intercepts represent the combination of background conditions, i.e. not cold shocked, not tissue cultured, and family Notsand. All effects are for family Notsand unless another family name was shown. Effects for the other families are shown as differences from the background effect of family Notsand.

	Estimates	SE	DF	*t*	*P*
Fv/Fm					
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
Fv'/Fm'					
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

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Supplementary Table 1. Akaike's Information Criterion (AIC) of each examined linear mixed models for F_v/F_m, F_v'/F_m' and Φ_{PSII} with effects specified by "+". The best model (top) with the lowest AIC was selected for each of F_v/F_m, F_v'/F_m' and Φ_{PSII} and their details are shown in Table 3. C: cold shock (cold-shocked or not), T: tissue culture (tissue-cultured or not), and F: family. "×" indicates interaction effects between two or three variables. Delta indicates difference in AIC from the best model.

C	T	F	C×T	C×F	T×F	C×T×F	AIC	Delta
Fv/Fm								
+	+	+	+	+	+	+	-1846.6	0.0
+	+	+	+		+		-1842.3	4.3
+	+	+	+	+	+		-1841.1	5.5
+	+	+			+		-1838.6	8.0
+	+	+		+	+		-1837.8	8.7
+	+	+	+				-1826.0	20.6
+	+	+	+	+			-1824.7	21.8
+	+	+					-1822.3	24.3
+	+	+		+			-1821.5	25.1
+	+		+				-1814.8	31.7
+		+					-1812.5	34.1
+		+		+			-1811.7	34.9
+	+						-1811.1	35.4
+							-1805.6	40.9
	+	+			+		-1192.8	653.8
	+	+					-1177.7	668.8
		+					-1168.5	678.0
	+						-1167.2	679.4
							-1162.0	684.6
Fv'/Fm'								
+	+	+	+	+	+	+	-1503.2	0.0
+	+	+	+		+		-1493.0	10.2
+	+	+			+		-1491.6	11.6
+	+	+	+	+	+		-1491.5	11.7
+	+	+		+	+		-1488.9	14.3
+	+	+	+				-1463.2	40.0
+	+	+					-1461.8	41.4
+	+	+	+	+			-1461.7	41.5
+	+	+		+			-1459.1	44.1
+	+		+				-1454.5	48.7
+	+						-1453.1	50.1
+		+					-1432.4	70.9
+							-1430.2	73.0
+		+		+			-1429.7	73.5
	+	+			+		-1069.7	433.5

		+	+					-1039.9	463.4
		+						-1031.2	472.1
			+					-1010.5	492.8
								-1008.3	495.0
Φ_{PSII}									
	+	+	+	+	+	+		-1768.6	0.0
	+	+	+	+	+			-1766.5	2.1
	+	+	+	+	+	+	+	-1766.3	2.2
	+	+	+	+		+		-1745.7	22.9
	+	+	+	+				-1743.6	25.0
	+	+	+		+	+		-1738.2	30.4
	+		+		+			-1738.0	30.5
	+	+	+		+			-1736.1	32.5
	+	+		+				-1730.7	37.8
	+	+	+			+		-1705.8	62.7
	+		+					-1705.7	62.8
	+	+	+					-1703.8	64.8
	+							-1692.6	75.9
	+	+						-1690.9	77.6
		+	+			+		-1683.9	84.7
			+					-1683.8	84.8
		+	+					-1681.8	86.7
								-1670.6	97.9
		+						-1669.0	99.6

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