Tissue Culture as a Source of Replicates in Nonmodel Plants: Variation in Cold Response in Arabidopsis lyrata ssp. petraea

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ABSTRACT While 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 be potentially overcome by using tissue culture techniques. However, plants regenerated from tissue culture may show aberrant phenotypes and “somaclonal” variation. Here, we examined somaclonal variation due to tissue culturing using the response to cold treatment of photosynthetic efficiency (chlorophyll fluorescence measurements for Fv/Fm, Fv'/Fm', and ΦPSII, 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 detect within-family variation in change in chlorophyll fluorescence due to cold shock successfully 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 Fv/Fm, Fv'/Fm', and ΦPSII 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 nonmodel outcrossing plants.

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; Sasaki et al. 2015; Stratton 1998; El-Soda et al. 2014) and Caenorhabditis elegans (Gutting 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 (Mitchell-Olds 2001; Claus and Koch 2006). However, one disadvantage of nonmodel 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 QTL 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 and Bazzaz 1993; Wait and

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Levin 1993; Wu 1998). This method is applicable only 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 and Sherrington 1984). However, tissue culture has been exploited only rarely for studies on the genetic basis of genotype–environment interaction, and the few existing studies (Glock and Gregorius 1986; Glock 1989) focused only on callus characteristics as target phenotypes. One potential issue that should be considered carefully is that tissue-culture derived microshoots can express phenotypic, “somaclonal” variation (Larkin and Scowcroft 1981), or may sometimes show aberrant morphology and physiology in vitro (Joyce et al. 2003). This somaclonal variation resembles that induced by physical mutagens, with elevated levels of chromosome breakage and rearrangement, polyplody, aneuploidy, transposon activation, and point mutation (D’Amato and Bayliss 1985). Therefore, with a view to exploiting the techniques of tissue culture more widely in studies of genotype–environment interaction in outcrossing plants, it is necessary to extend our knowledge on how propagation by tissue culture generates variation in phenotypes that are relevant to adaptation in natural environments, compared to other sources of genetically related replicates such as outbred siblings.

Key plant properties that have attracted marked attention in the field of adaptation to various environments are stress tolerances (e.g., Quesada et al. 2002; Kwon et al. 2007; Zhang et al. 2004; Zhen and Ungerer 2008; Steponkus et al. 1998; Hong and Vierling 2000; Lexer et al. 2003). One trait that can be used to indicate tolerance against various physical stressors in plants is photosynthetic performance. Photosystem II (PSII) activity is sensitive to both biotic and abiotic factors (Murphy and Lawson 2013). Chlorophyll fluorescence can be used to determine the maximum efficiency with which light absorbed by pigments of photosystem II (PSII) is used to drive photochemistry in dark- \( (F_o/F_m) \) or light- \( (F_v/F_m) \) adapted material and the operating efficiency of PSII (\( \Phi_{PSII} \)). It is a reliable indicator of photoinhibition and damage to the photosynthetic electron transport system (Quick and Stitt 1989; Maxwell and Johnson 2000). Changes in chlorophyll fluorescence have been used in \( Arabidopsis thaliana \) to quantify tolerance to cold and freezing temperatures. Ehliert and Hincha (2008) showed that chlorophyll fluorescence imaging detected difference in freezing tolerance between two \( A. thaliana \) lines, both before and after cold acclimation. Mishra et al. (2014) applied chlorophyll fluorescence imaging for nine \( A. thaliana \) lines (both cold and freezing temperature, and suggested that freezing tolerance of lines could be screened by chlorophyll fluorescence under cold \( 4^\circ \)C) condition without exposing plants to subzero temperature. Chlorophyll fluorescence has also been used to study tolerance to drought (Woo et al. 2008; McAusland et al. 2013; Bresson et al. 2015), and salt and heavy-metal stress (Yuan et al. 2013), in \( A. thaliana \), as well as in various other plants for tolerance or response to cold and freezing temperatures (Baldi et al. 2011; Medeiros et al. 2012; Xie et al. 2015; Khanal et al. 2015; Heo et al. 2014), drought (Jansen et al. 2009), and salt (Yuan et al. 2013). If variation in chlorophyll fluorescence can be properly estimated using tissue-culture derived clones, therefore, this method would enhance studies in genotype–environment interaction for stress tolerance in outcrossing plants.

To this end, we have studied change in chlorophyll fluorescence following cold shock in a wild relative of a model plant species. \( A. lyrata \) is a close relative of the model species \( A. thaliana \), but with a different ecology, life history and population genetics (Charlesworth et al. 2003; Davie et al. 2008, 2009; Kuittinen et al. 2008; Kunin et al. 2009). While \( A. thaliana \) is mainly selfing, with a low level of genetic diversity within a population, \( A. lyrata \) is outcrossing, with a high level of genetic diversity even within a population (Clauss and Mitchell-Olds 2006; Kunin et al. 2009; Heidel et al. 2006; 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 \( F_v/F_m \), \( F_o/F_m \), and \( \Phi_{PSII} \) before and after cold shock, as an index of cold response, for seedlings from three families from geographically isolated populations of \( A. lyrata \) 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.

### MATERIALS AND METHODS

#### Plants

Seeds of \( A. lyrata \) were collected from geographically separated populations in Ardal (Norway) \( (61^\circ 19' 25'' N, 7^\circ 50' 00'' E, alt. 63 m) \), Notsand (Sweden) \( (62^\circ 36' 31'' N, 18^\circ 03' 37'' E, alt. 3 m) \), and Sandfell (Iceland) \( (64^\circ 04' 14'' N, 21^\circ 41' 06'' E, alt. 123 m) \). 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, maintaining 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 two to three seeds (one genotype = cloned plantlets from one seed) of each family.

#### Tissue culture

Seeds were sterilized in 10% commercial bleach for 20 min, washed in sterile water and stored at 4°C overnight. The seeds were then placed on 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 Laboratories). The agar plates were held vertically, allowing for maximum recovery of root tissue. After 4 wk, 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 d, 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 d. The root sections from each plant were resuspended 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-hr day length. Once shoots started to form from...
Table 1 Numbers of plants and blocks in each family (Ardal, Notsand, and Sandfell)

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

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.

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 ($F_{v}/F_{m}$, or $F_{PSII}$) induced by cold shock:

$$P \sim \text{Family/Genotype/Block}$$

for tissue culture material, or

$$P \sim \text{Family/Block}$$

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 = $V(\text{Family}) + V(\text{Genotype}) + V(\text{Block})$

for tissue culture material, or

Total variance = $V(\text{Family}) + V(\text{Block})$

for seedlings.

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

We tested whether variance in the change of $F_{v}/F_{m}$, $F_{PSII}$, or $F_{PSII}$ due to cold shock among tissue-culture derived plantlets within each genotype was different from that in seedlings of half-siblings of the same family using Bartlett tests. Because the number of blocks differed between seedlings and tissue-cultured plantlets (Table 1), we checked first whether the difference in the number of blocks affected the variance, by resampling all possible combinations of four blocks from the 10 blocks of half-siblings in Ardal and Notsand. Reducing block number changed the original variance for 10 blocks only $< \pm 3\%$ without systematic bias.

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

$$CF = \frac{I(B/P+C+T+F+C×T+T×F+C×F+C×T×F)}{P_{PSII}}$$

where $CF$ was a single measurement of either $F_{v}/F_{m}$, $F_{PSII}$, or $F_{PSII}$, and $I(B/P)$ was the intercept with random effects of block, and individual plant nested in each block, $C$ 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 (three 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 (Development Core Team 2008). Akaake’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.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. All
phenotypic data are available in Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.9gs8k.

RESULTS AND DISCUSSION

Variance components in cold-response of Fv/Fm, Fv’/Fm’, and ϕPSII

In the seedlings, the changes in Fv/Fm, Fv’/Fm’, or ϕPSII 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 Fv/Fm, Fv’/Fm’, or ϕPSII 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 Fv/Fm, Fv’/Fm’, and ϕPSII than half-sibs as shown by the Bartlett test (Figure 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 ϕPSII

All single effects of cold shock, tissue culture and family and all possible interaction combinations among them affected Fv/Fm and Fv’/Fm’, and all such effects except the three-way interaction between cold shock, tissue culture and family affected ϕPSII 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.

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 Fv/Fm, Fv’/Fm’, and ϕPSII measurements (Table 2). On the other hand, we showed significant but low somaclonal variation. The within-block (error) variance component for tissue-cultured plantlets was relatively small compared to that for nontissue-cultured seedlings (Table 2). The Bartlett tests showed that somaclonal variation was smaller than, or at least remained within the range of, the within-family variance, which is the smallest naturally observed component of variation in the hierarchy of genetic structure (Figure 1). In A. thaliana, studies of natural variation have focused mainly on between-population variation (e.g., Shindo et al. 2007). In contrast, A. lyrata has substantial within-population variation, for example, in the composition of glucosinolates (Clauss et al. 2006) or self-incompatibility genes (Schierup et al. 2008). In this paper, we showed that there is within-family as well as among-family, and thus among-population, genetic variation in A. lyrata ssp. petraea. Within-family genetic variance was relatively large in Sandfell (Iceland). The observed within-family genetic variances in putatively adaptive traits highlight the wide potential for evolutionary adaptation of the species, and further validate the usefulness of relatives of model organisms in evolutionary biology (Mitchell-Olds 2001; Clauss and Koch 2006).

Among-family variance

There was significant or marginally significant among-family variance in the change of Fv/Fm, Fv’/Fm’, and ϕPSII values following cold treatment for seedlings (Table 2). We used different growth chambers for plant growth and for cold shock, and therefore light condition for cold shock inevitably differed from that for growth. Light and temperature are difficult to disassociate in such a study system, and both the single effect of cold treatment, and the light–temperature interaction, can be involved in the effect of cold shock. In A. thaliana, the change in
chlorophyll fluorescence from before to after cold shock correlates with tolerance to subzero temperatures measured by electrolyte leakage, and, therefore, this is regarded as an indicator of cold tolerance or response (Ehlert and 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 $\Phi_{PSII}$ (Table 3, and Supplemental Material, Table S1). This is consistent with a previous report of a genotype-specific effects on callus characteristics (Glock and Gregorius 1986; Glock 1989). 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 $\Phi_{PSII}$), 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 $\Phi_{PSII}$ 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 and 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 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 interaction effect between tissue culture and family was much smaller in $F_v/F_m$ or $F_{v^*}/F_m^*$ (the ranges between maximum and minimum estimates were 0.043 (−0.005) = 0.048, 0.082 − 0 = 0.082 and 0.181 − 0 = 0.181, respectively; Table 3). An interaction between cold shock, tissue culture, and family was detected only in $F_v/F_m$ and $F_{v^*}/F_m^*$. Also, the relative impact of

| Table 3 The best linear mixed models for $F_v/F_m$, $F_{v^*}/F_m^*$ and $\Phi_{PSII}$ based on AIC |
|---------------------------------------------------------------|
| Estimates   | SE   | DF | t    | P    |
| $F_v/F_m$ |
| 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 × Tissue culture | −0.007 | 0.011 | 311 | −0.7 | 0.506 |
| Cold shock × Fam A | −0.035 | 0.011 | 311 | −3.2 | 0.002 |
| Cold shock × Fam S | −0.007 | 0.012 | 311 | −0.5 | 0.584 |
| Tissue culture × Fam A | 0.029 | 0.020 | 302 | 1.5 | 0.147 |
| Tissue culture × Fam S | 0.082 | 0.021 | 302 | 3.9 | <0.001 |
| Cold shock × Tissue culture × Fam A | 0.043 | 0.014 | 311 | 3.0 | 0.003 |
| Cold shock × Tissue culture × Fam S | 0.015 | 0.015 | 311 | 1.0 | 0.327 |
| $F_{v^*}/F_m^*$ |
| 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 × Tissue culture | 0.011 | 0.015 | 311 | 0.8 | 0.446 |
| Cold shock × Fam A | 0.015 | 0.015 | 311 | 0.9 | 0.345 |
| Cold shock × Fam S | 0.068 | 0.017 | 311 | 4.0 | <0.001 |
| Tissue culture × Fam A | 0.070 | 0.025 | 302 | 2.8 | 0.006 |
| Tissue culture × Fam S | 0.181 | 0.026 | 302 | 6.9 | <0.001 |
| Cold shock × Tissue culture × Fam A | −0.013 | 0.020 | 311 | −0.7 | 0.514 |
| Cold shock × Tissue culture × Fam S | −0.077 | 0.021 | 311 | −3.7 | <0.001 |
| $\Phi_{PSII}$ |
| 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 × Tissue culture | 0.034 | 0.006 | 313 | 5.8 | <0.001 |
| Cold shock × Fam A | −0.004 | 0.007 | 313 | −0.5 | 0.610 |
| Cold shock × Fam S | 0.028 | 0.007 | 313 | 3.9 | <0.001 |
| Tissue culture × Fam A | −0.005 | 0.021 | 302 | −0.2 | 0.822 |
| Tissue culture × Fam S | 0.043 | 0.022 | 302 | 2.0 | 0.051 |

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.

Figure 1 Change in chlorophyll fluorescence ($F_v/F_m$, $F_{v^*}/F_m^*$, and $\Phi_{PSII}$) in seedlings or plantlets originating from Norway (Ardel), Sweden (Notsand), and Iceland (Sandfell) after cold-treatment (values after shock – values before shock). * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$ (Bartlett test) indicate a significantly lower variance of the genotype than among half-siblings in the same family. Three $F_v/F_m$ values (0.340, 0.375, and 0.592), and an $F_{v^*}/F_m^*$ value (0.354) in Sandfell half-siblings were out of the vertical ranges shown, but were included in the statistical tests.
among-genotype variance was smaller for $\Phi_{PSII}$ (8.5% of the total variance, Table 2) than for $F_{v}/F_{m}$ (31.5%) or $F_{v}^{i}/F_{m}^{i}$ (10.9%). These results imply that, although the maximum efficiencies of photosynthesis for dark- ($F_{v}/F_{m}$) and light-adapted leaves ($F_{v}^{i}/F_{m}^{i}$) were affected by tissue culturing in genotype-specific ways, the actual electron transport operating efficiency ($\Phi_{PSII}$) was less affected by tissue culture.

**Conclusion**

Overall, we successfully detected among-genotype variance, with low somaclonal variation, indicating that the advantage of tissue culturing in generating genetically isogenic replicates exceeded its disadvantage in amplifying somaclonal variation in our study system. We detected interaction effects of tissue culture with genotype for a putatively adaptive trait, cold response; however, such variation would not mask the single effect of genotype. Therefore, although one should consider effects of tissue culturing carefully when interpreting any results relying on the technique, tissue culturing is a useful method for obtaining genetically homogenous replicates in this, and probably other, nonmodel organisms. It can provide critical additional power 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 QTL accounting for phenotypes.

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