

Comparative metabolomics of developmental alterations caused by mineral deficiency during in vitro culture of *Gentiana triflora*

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Abstract Gentians (*Gentiana triflora*, *G. scabra*, and hybrids of the two) are mainly cultivated as ornamental flowers in Japan. Because gentians are allogamous plants, their diversity and heterozygosity have become a major problem. Recently, explants were clonally cultured to maintain genetic purity, but culture conditions have not been studied systematically, thus the essential nutrients required for gentian culture are unknown. We therefore investigated the effects of potassium (K) and phosphorus (P) deficiency in culture media. Explants grew under K or P deficiency conditions, but P deficiency caused the formation of new structures which are similar to overwintering buds. To elucidate the mechanism behind the gentian response to mineral deficiency, we performed targeted metabolome analyses using capillary electrophoresis-mass spectrometry. Multivariate analysis using metabolite profiles showed that characteristic metabolite patterns arise in response to K or P deficiency. Under P deficiency there is a severe decrease in energy metabolites, which may in turn trigger overwintering bud formation in vitro. These findings may contribute to understanding the horticultural conditions required by gentians to trigger bud formation,

and may provide a new strategy for maintaining genetic purity for long periods.

Keywords *Gentiana triflora* · Mineral deficiency · Capillary electrophoresis mass spectrometry · Principal component analysis · Hierarchical clustering analysis

1 Introduction

Gentians are herbaceous perennials comprised of several globally distributed species. In Japan, *Gentiana triflora*, *G. scabra*, and their hybrids are cultivated as ornamental flowers because of their attractive flower colors and shapes (Yoshiike 1992). Gentians are heterotic and most of the key varieties in Japan are interspecific or intraspecific hybrids. However, since gentians show strong inbreeding depression and there are no homozygous inbred lines, it is difficult to maintain their genetic purity by self-pollination (Doi et al. 2010). Clonal culture of explants was established to solve this problem (Hosokawa et al. 2000). Culturing allowed mass propagation of clones of the same genetic background, and was effective for controlling phenotypes; e.g., gentian plants derived from cultured explants have similar phenotypes, such as growth rate and flower colors (Nakatsuka et al. 2010). Explants of most of parent species and a few F1 hybrids are now subcultured in vitro until needed for whole plant regeneration. Explants have also been used for transformation with *Agrobacterium rhizogenes* (Hosokawa et al. 2000) because of the stable gene integration that is possible with this system. Nakatsuka et al. (2009) also succeeded in producing an early flowering transgenic gentian overexpressing the *Arabidopsis FLOWERING LOCUS T* using *Agrobacterium*-mediated transformation. Thus, in vitro clonal cultures of gentian are

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beneficial for molecular breeding. Unfortunately, only a few varieties are currently suitable for in vitro culture, and there has been no systematic examination of the relationship between cultural conditions and gentian varieties. To establish a broader range of cultural conditions for in vitro gentian explants, it is necessary to identify their metabolic and morphological responses under different conditions.

As a preliminary experiment, we measured the effects of macronutrient deficiency on the growth of cultured gentian explants to ascertain their essential nutrient requirements. Tissue-cultured plants are highly dependent on macronutrients, including potassium (K), phosphorus (P), nitrogen (N), sulfur, calcium, and magnesium, for their normal growth, development, and metabolic functions (Hermans et al. 2006). Among these macronutrients, K plays important roles in several biological processes such as photosynthesis, protein synthesis, leaf and stomatal movement, and osmolarity regulation (Peoples and Koch 1979; Maathuis et al. 1997; Marschner 1995). P is a structural component of nucleotides, and is involved in many plant functions, including molecular energy transfer, photosynthesis, N fixation, flowering, and root growth (Marschner 1995). Thus, deficiencies in these macronutrients induce serious metabolic disorders, resulting in abnormal growth and development. In our experiments, tissue culture explants were studied under mineral nutrition deficiencies to establish the parameters for the optimal growth of gentian. Explants grew as well under K-deficient (-K) conditions as they did under control conditions. However, P-deficiency (-P) induced morphological changes, including formation of buds similar to overwintering buds (OWB), implying that explant response to K or P deficiency is metabolically different, and that the metabolic alterations due to -P conditions which result in in vitro bud (IOWB) formation may be similar to what happens in advance of normal OWB formation.

Since IOWB grew over a prolonged period, much like OWB, the induction of IOWB provides a potential new strategy for extending explant storage periods and preventing the breakdown of parental and hybrid varieties by repeated subculture. Furthermore, during late autumn most field-grown gentians form OWBs, which are highly tolerant to cold and shade (Takahashi et al. 2006; Hikage et al. 2007). OWB formation is therefore one of the most important characters in the cultivation of commercial gentian.

In this study, we focused on how the concentrations of P and K in *G. triflora* tissue culture alter development and metabolite profiles. The data were subjected to principal component and hierarchical clustering analysis, confirming that specific metabolite alterations were associated with morphological changes and IOWB development.

2 Materials and methods

2.1 Plant materials and growth conditions

Gentian (*Gentiana triflora* cv. Albireo) shoot tips were isolated from field-grown gentians and cultured in vitro on solid Murashige and Skoog (MS) medium containing 3% (w/v) sucrose and 0.25% (w/v) gellan gum at 22°C under a 16/8 h light/dark photoperiod with a light intensity of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ after surface sterilization. Explants were subcultured by separating individual shoots from proliferating cultures before transfer to fresh MS medium every 3 months. K-deficient MS medium was prepared by substituting NaNO_3 and NaH_2PO_4 for KNO_3 and KH_2PO_4 , respectively (Bains and Jhoo 1978). P-deficient MS medium was prepared by omitting K_2HPO_4 (Reid and Bielecki 1970). Two-week-old explants were transferred to -K or -P medium and a portion of the shoot mass was harvested after 1, 3, or 12 weeks, frozen with liquid N, and freeze-dried.

2.2 Metabolite analysis

Extraction, separation, and determination of metabolites were done according to the method of Takahashi et al. (2009a, b). Metabolites were extracted from IOWBs and shoots devoid of roots in five replicate pools for each data point. Ten milligram samples of freeze-dried tissue were ground to a fine powder and extracted with 500 μl ice-cold 50% (v/v) methanol containing 50 μM 1,4-piperazinediethanesulfonic acid and 50 μM methionine sulfone as internal standards at 4°C for 5 min. After centrifugation at 15,000 $\times g$ for 5 min to pellet cell debris, the supernatant was filtered through a 5 kD cutoff filter (Amicon), and the filtrate was used for analysis. Separation and quantification of organic acids, phosphates, nucleotides, amino acids, and polyamines were performed by capillary electrophoresis-mass spectrometry (CE-MS) (Agilent Technologies) through a polyethylene glycol-coated capillary (DB-WAX; J&W Scientific) with 20 mM ammonium acetate (pH 9.0) as running buffer for organic acids, phosphates, and nucleotides, and through an uncoated fused-silica capillary (GL Sciences) using 1 M formic acid (pH 1.9) as running buffer for amino acids and polyamines. Quantitative accuracy was determined with known concentrations of standard reference compounds using Agilent ChemStation software (Rev. A.10.01).

2.3 Data analysis

Explant growth data were analyzed with Student's *t*-test of means ($n = 5$), ($P < 0.01$). Metabolome data were processed by means of principal component analysis (PCA)

and hierarchical clustering analysis (HCA) as described previously (Miyagi et al. 2010). Normalization of metabolite data was represented by a Z score. HCA of metabolites based on similarities in their concentrations was performed using the squared Euclidean distance as a similarity metric and the average linkage method; the results were visualized using heat maps and dendrograms. HCA heat maps were created using Microsoft Excel 2007 software. All analyses were carried out using the Statistical Package for the Social Sciences (SPSS v.10.0).

3 Results and discussion

3.1 Phenotypic characterization

Growth of explants was similar in control and nutrient-deficient media up to 3 weeks after transfer (Fig. 1a). The growth observed under -K conditions differs from reports that K deficiency decreases the growth of barley (Drew 1975), bean (Cakmak et al. 1994a, b), tomato (Kanai et al. 2007), Arabidopsis (Armengaud et al. 2004, 2009), and other plants (Pettigrew 2008). On the other hand, growth was significantly decreased after 12 weeks of culture under P deficiency (Fig. 1a and d). Explants grown under -P conditions yielded 1.2- and 1.9-fold lower dry weights after 3 and 12 weeks, respectively (Fig. 1a). Growth inhibition associated with P deficiency is in agreement with a previous report (Hermans et al. 2006).

Explant growth was dramatically restricted, and leaves were discolored after 12 weeks under -P conditions (Fig. 1d). A new type of buds, denoted IOWBs, closely resembling OWB in their morphological appearance and growth characteristics formed under -P conditions (Fig. 1e). In contrast, after 12 weeks, explants grown under -K conditions were morphologically similar to the controls (Fig. 1b and c).

3.2 Multivariate analysis

To determine the effects of K and P deficiency on growth and IOWB formation, 47 metabolites, including organic acids, phosphates, nucleotides, amino acids, polyamines, and others, were measured after 1, 3, and 12 weeks of treatment under -K or -P conditions (Supplementary Table I, II, and III). Metabolite data were subjected to PCA and major differences among control, -K, and -P treatments were identified using a PCA scores plot. The PCA revealed two principal components, PC1 and PC2 that explained 33.2 and 13.4% of the total variance, respectively (Fig. 2a). PC1 distinguished -P with a positive coefficient from both control and -K. PC1 scores tended to increase with time under -P conditions. An examination of PC1 loadings

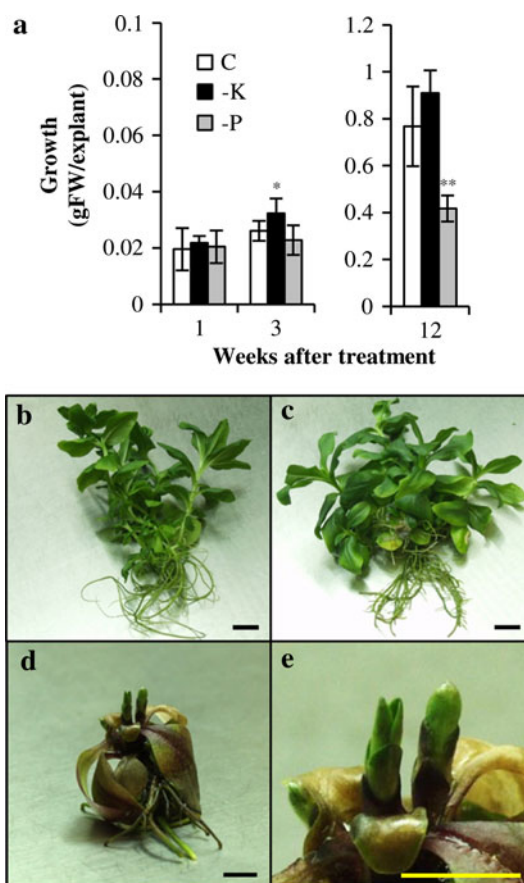
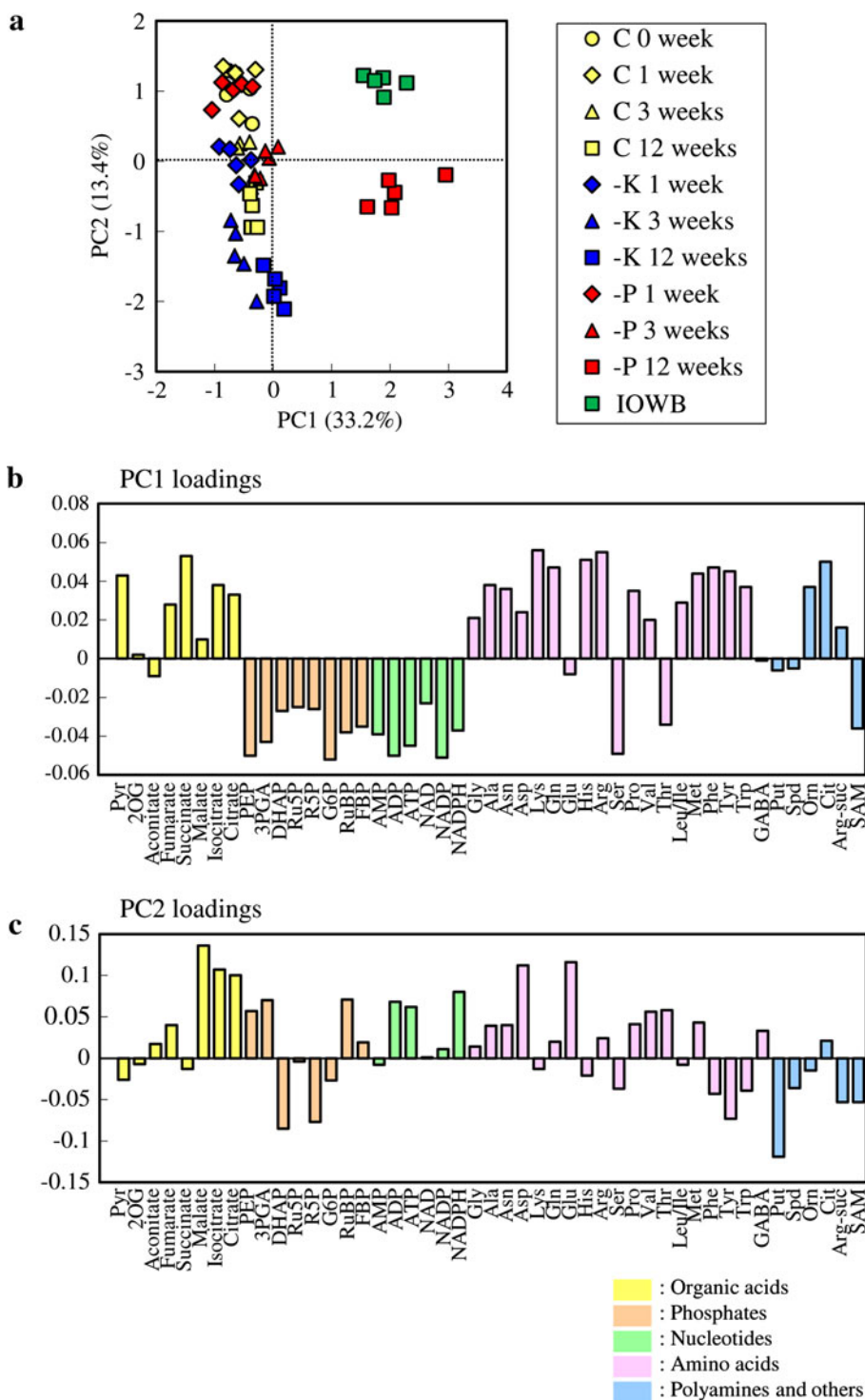


Fig. 1 Effects of K or P deficiency on the growth and morphology of gentian explants. (a) Dry weight (DW) of explants grown under control (white bars), K deficient (-K; black bars), and P deficient (-P; gray bars) conditions at 22°C for 1, 3, and 12 weeks. Explants were cultured on MS medium for 2 weeks and then on -K or -P media. Control explants were transferred to fresh MS medium. Data are means \pm SD ($n = 5$). Significant differences from the control explants were evaluated by a Student's *t*-test and are represented by asterisks (** $P < 0.01$). Photographs of gentian explants grown for 12 weeks under control (b), K deficient (c), and P deficient conditions (d). (e) Bud resembling overwintering bud (IOWB). Scale bar 0.5 cm

indicated that differences mainly involved pyruvate and the TCA cycle metabolites fumarate, succinate, isocitrate, citrate, and most of the amino acids on the positive side, and phosphates and nucleotides on the negative side (Fig. 2b). PC2 separated -K from control and -P conditions with a negative coefficient, and the PC2 scores of -K decreased with an increase in exposure time, whereas the PC1 scores were almost unchanged. Examination of PC2 loadings suggested that the differences mainly involved malate, isocitrate, citrate, and acidic amino acids on the positive side, and dihydroxyacetone phosphate, ribose-5-phosphate, aromatic amino acids, and putrescine on the negative side (Fig. 2c). The loadings of malate showed the highest positive values, and putrescine the most negative. Furthermore, PCA also revealed a significant difference

Fig. 2 PCA of metabolites in shoots of gentian explants. (a) PCA score plots of explants grown under control (c), K deficient (-K), or P deficient (-P) conditions are represented at three or four different time points, and the plot of IOWBs is represented as *green-colored squares*. Assignment of colors: yellow, control shoots; blue, -K shoots; red, -P shoots. PC loading values of metabolites on PC1 (b) and PC2 (c) are also represented



between shoots and IOWBs. The PC2 score clearly separated IOWBs on the positive side from shoots after 12 weeks of -P treatment on the negative side, whereas their PC1 scores were similar. Therefore, metabolites that had a high value of PC2 loading contributed to the difference between -P shoots and IOWBs. On the basis of

these PCA results, it is clear that targeted metabolome analysis can be used to distinguish the characteristics of gentian explants grown under -K and -P conditions.

The metabolite data were also subjected to HCA (Fig. 3). These analyses indicated that there were two major clusters, which could be further divided into a set of

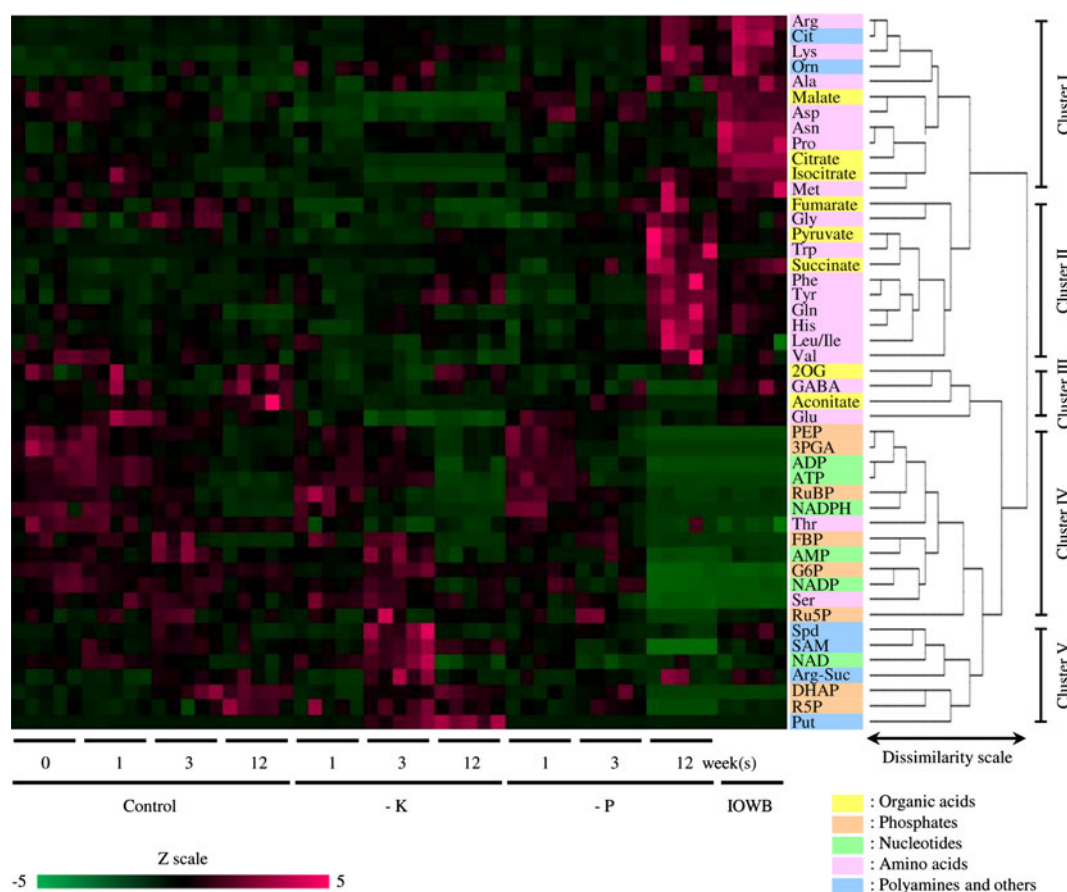


Fig. 3 Metabolite profiling of gentian shoots under K or P deficiency. Heat maps of metabolites extracted from shoots grown under control, K deficient (-K), and P deficient (-P) conditions are compared.

two subclusters (clusters I and II) and a set of three subclusters (clusters III to V). Cluster I mainly consisted of amino acids and organic acids, including arginine, asparagine, malate, and citrate, which each had relatively high concentrations. Cluster II mainly consisted of branched-chain amino acids and aromatic amino acids. The metabolite patterns of clusters I and II were fundamentally similar, but the metabolites of cluster I were prominent in IOWBs, and those of cluster II were prominent in shoots grown for 12 weeks under -P conditions. In cluster II, pyruvate and some amino acids, which are adjacent to pyruvate on the metabolic pathway (Fig. 5), localized to a small group, implying that pyruvate-related pathways are affected by P deficiency and might be related to IOWB formation. Cluster III was composed of four metabolites: aconitate, 2-oxoglutarate (2OG), glutamate and γ -aminobutyric acid, which were abundant in control shoots, but only moderate changes were observed under the other conditions. Cluster IV mostly consisted of phosphates and nucleotides. Although metabolites in cluster IV decreased during the 12 week culture period under all conditions, the most severe decrease was observed under -P

Hierarchical clustering of metabolites according to a dissimilarity scale is shown on the right. Normalized data of metabolite concentrations are represented by the Z scale

conditions. Since P is an essential element of the phosphates and nucleotides used in plant energy metabolism, P deficiency would be expected to selectively decrease the levels of metabolites in cluster IV. Furthermore, threonine and serine also fell into cluster IV. These amino acids are synthesized in part from TCA cycle precursors, including 3-phosphoglycerate (3PGA) and pyruvate. HCA revealed that threonine and serine are in the same group as 3PGA, but not pyruvate, suggesting that these amino acids are synthesized from 3PGA via several enzymatic steps. Cluster V mostly consisted of polyamines and their precursors, and these metabolites increased in response to -K deficiency. Thus, HCA indicates the salient characteristics of each metabolite group, allowing identification of the metabolites and metabolic pathways that specifically respond to K or P deficiency.

3.3 Metabolite profiles of K and P deficiency

Absolute concentrations of aconitate, fumarate, malate, isocitrate, and citrate were lower under -K conditions at 1,

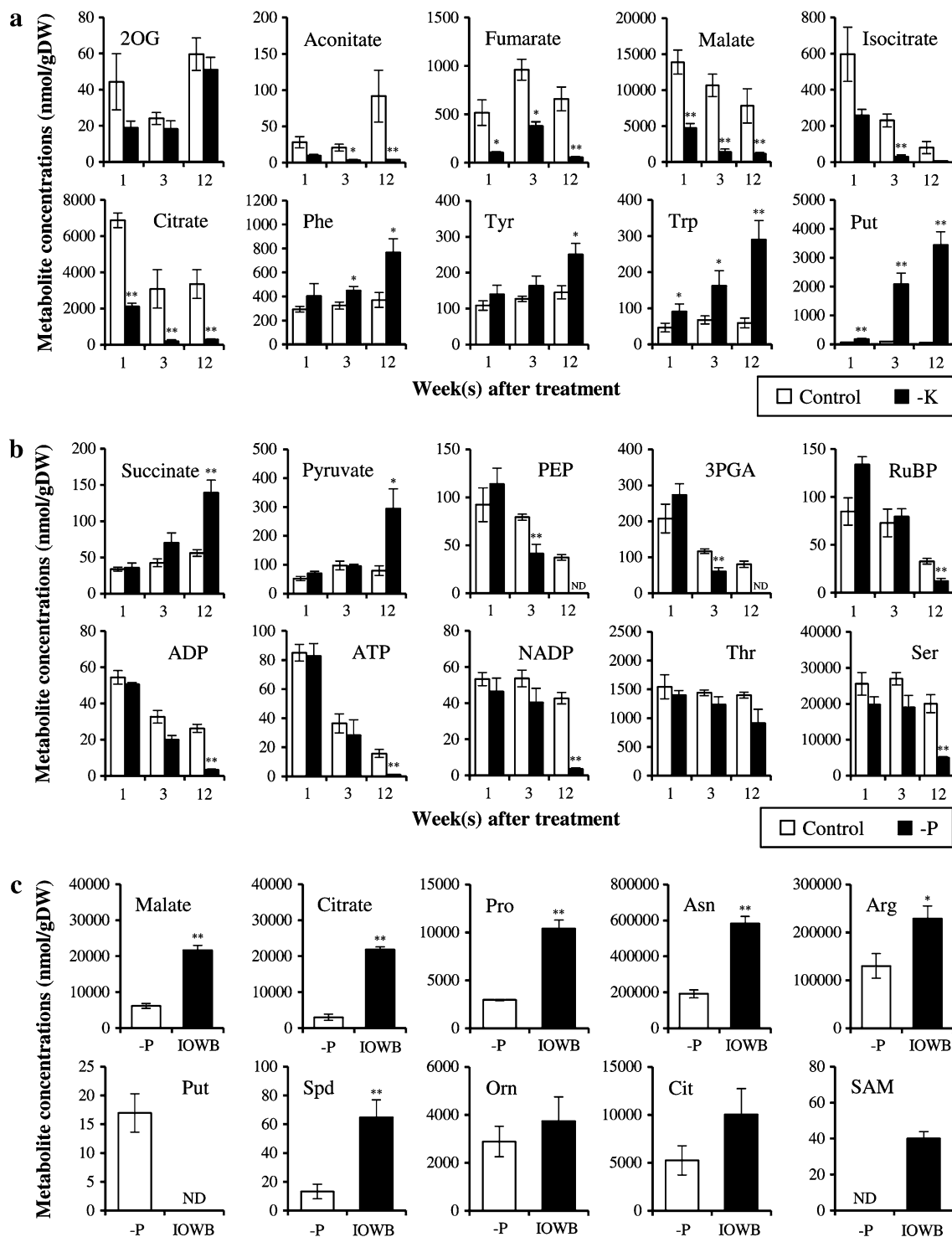


Fig. 4 Metabolite concentrations in shoots of gentian grown under K or P deficiency. Absolute concentrations of selected metabolites in shoots grown under K deficient (-K) conditions (a) or P deficient (-P) conditions (b) are indicated on the Y axis. White bars represent concentrations in control shoots and black bars represent concentrations in -K or -P shoots. (c) Comparison of the concentrations of selected metabolites between shoots grown for 12 weeks under -P

conditions and IOWBs. Data are means \pm SD ($n = 5$); significant differences from the control shoots were evaluated by Student's t -test and are represented by asterisks (* $P < 0.05$ or ** $P < 0.01$). 2OG 2-oxoglutarate, Put putrescine, PEP phosphoenolpyruvate, 3PGA 3-phosphoglycerate, RuBP ribulose-1,5-bisphosphate, Spd spermidine, Orn ornithine, Cit citrulline, SAM S-adenosylmethionine, DW dry weight

3, and 12 weeks (Fig. 4a). Even though the concentration of TCA metabolites decreased, neither 2OG nor succinate were affected (Supplementary Table II). On the other hand, the concentrations of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan increased in -K shoots, whereas their levels in controls were almost unchanged. Putrescine, the simplest polyamine, increased the most in this experiment, to levels 2.8-, 20.6-, and 56.2-fold that of controls at 1, 3, and 12 weeks, respectively, under -K conditions. Polyamines are involved in development, senescence, and stress responses of plants (Galston and Sawhney 1990; Kumar et al. 1997). Additionally, putrescine accumulation in response to K deficiency has been reported in other plant species (Klein et al. 1979; Smith et al. 1982; Adams et al. 1990; Sung et al. 1994). Thus, our results indicate that -K stress response is similar in gentian, even in explants culture. However, there was almost no effect on the growth of gentian explants despite putrescine accumulation, suggesting that gentians have some mechanism to maintain growth under -K conditions, and the putrescine accumulation reported in other plants may not be completely responsible for their growth inhibition.

The concentration of pyruvate increased under -P conditions, whereas phosphoenolpyruvate (PEP) concentrations decreased (Fig. 4b). These changes are presumably due to activation of PEP carboxylase and PEP phosphatase to produce Pi under Pi starvation conditions (Juszczuk and Rychter 2002; Hammond et al. 2004). Therefore, the pyruvate accumulation observed in gentian shoots might also be involved in compensating for Pi deficiency under -P conditions. The concentration of succinate increased under both conditions, but was significantly higher in -P shoots than in control shoots after 12 weeks. This may reflect movement of precursors into the TCA cycle to alter relative metabolite concentrations in either direction. On the other hand, 3PGA, ribulose-1,5-bisphosphate, ADP, ATP, and NADP concentrations were all negatively affected by P deficiency over the 12 weeks. Many reports have provided evidence that P deficiency represses photosynthesis (Reid and Bielecki 1970; Terry and Ulrich 1973; Sawada et al. 1982; Brooks et al. 1987; De Groot et al. 2003), probably caused by downregulation of genes involved in photosystem I, photosystem II, and the Calvin cycle, as well as decreased activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Wu et al. 2003). The respiratory chain is also repressed under P deficiency (Juszczuk and Rychter 1997). On the basis of these reports, our results indicate that P deficiency inhibits energy metabolism via the respiration chain and Calvin cycle, resulting in succinate accumulation and a decrease in phosphate and nucleotide concentrations. The concentrations of threonine and serine, neutral amino acids bearing hydroxyl groups, tended to decrease under -P conditions, whereas most amino acids increased under

these conditions. The reason for this difference is unknown, but enzymes using these amino acids as a substrate might be activated under -P conditions.

IOWB metabolite levels were also compared to those of shoots derived from explants grown for 12 weeks under -P conditions (Fig. 4c). Significant increases in malate, citrate, proline, aspartate, arginine, and spermidine were observed in IOWBs. There was a trend toward increased concentrations of ornithine, citrulline, and S-adenosylmethionine (SAM), whereas putrescine concentrations decreased, implying that polyamine synthesis through the urea cycle is repressed. Since polyamine synthesis often occurs in actively growing tissues (Kumar et al. 1997), cell growth and proliferation might be arrested in IOWBs. Additionally, energy metabolites ATP, ADP, AMP, NADP, and NADPH were dramatically lower in IOWBs compared to control shoots (Supplementary Table III). Lower levels of energy metabolites were commonly observed in shoots and IOWBs of explants grown for 12 weeks under -P conditions, but TCA cycle intermediates were only lower in shoots. This metabolic alteration is one of the characteristics of cell death (Takahashi et al. 2008; Ishikawa et al. 2010). Importantly, field-grown gentian plants produce OWBs before winter, implying that energy consumption in these buds is reduced by arresting their growth as a survival mechanism under severe environmental conditions that may include both extreme cold and drought. The IOWB had high amounts of TCA cycle intermediates and appeared healthy despite low levels of energy metabolites, indicating the possibility that, like OWBs, IOWB maintain a low energy metabolism. We also found that IOWB grew like control explants, after subculturing to media containing normal amounts of P (data not shown), suggesting that a limitation of energy metabolism under P deficiency is necessary for the explants to differentiate into IOWBs. This stringency may be valuable information for gentian breeding methodologies that require long term maintenance of genetic resources. Furthermore, since the mechanism of OWB formation remains largely unknown, this artificial induction of IOWB may prove to be useful for elucidating the physiological characteristics of OWBs as well as for commercial applications.

4 Concluding remarks

Here, we showed that in vitro cultures of *G. triflora* could grow healthy under -K conditions but formed IOWBs under -P conditions. To determine why, we investigated the effects of K or P deficiency on the explants at the metabolome level. To visualize the effects of the nutrient deficiencies described in this study, the quantified metabolites were mapped onto primary metabolic pathways (Fig. 5). Although K deficiency gave relatively limited effects, P deficiency induced severe

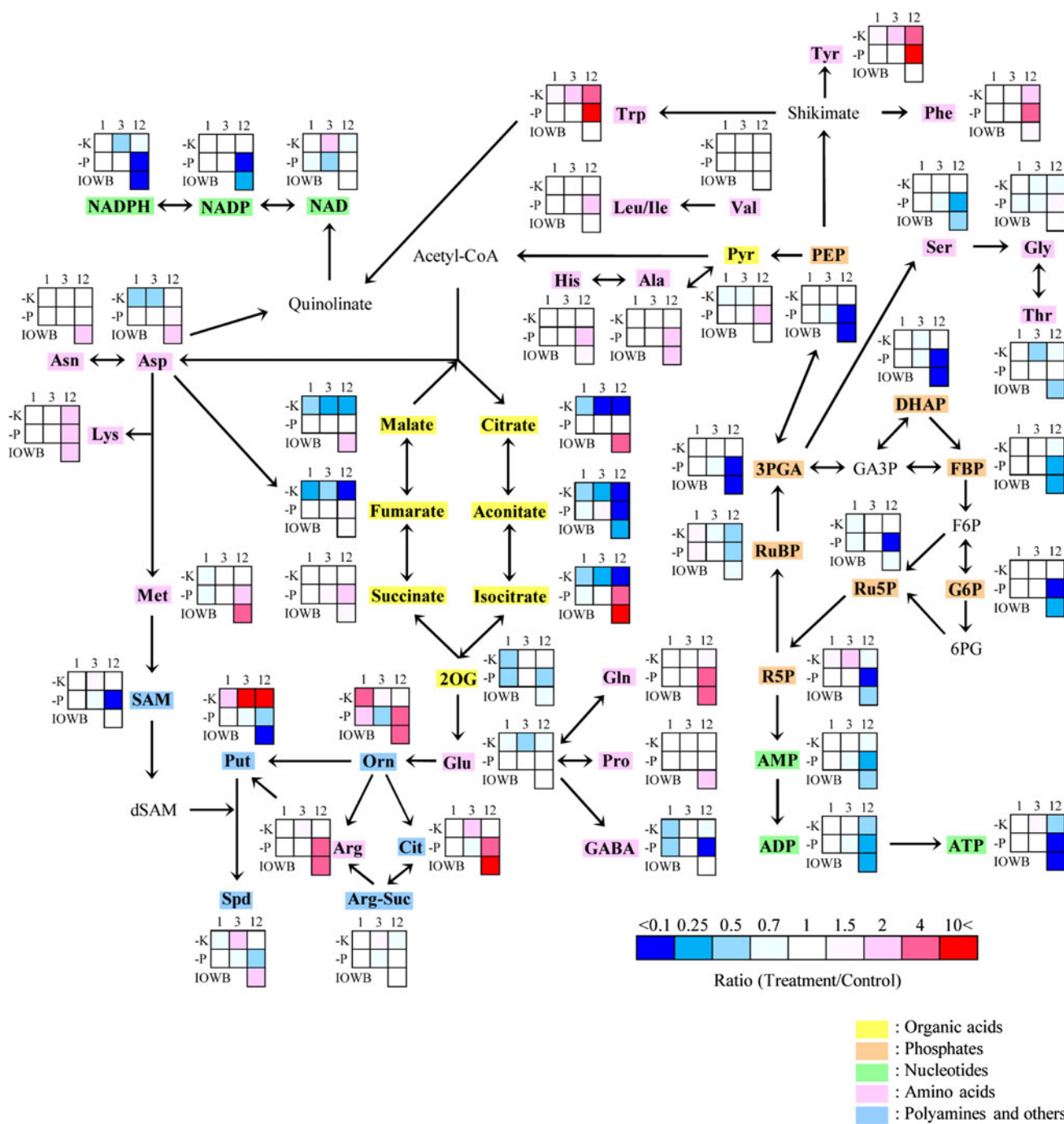


Fig. 5 Mapping of measured metabolites onto primary metabolic pathways. Ratios of changes in metabolite concentrations in shoots subjected to K or P deficiency, as well as in IOWBs, are shown as heat maps

modification of metabolites, including the concentrations of organic acids and amino acids. These results indicate that a sufficient level of P, but not K, is essential for maintaining gentian explant growth and metabolism. Importantly, P deficiency also decreased the levels of energy metabolites, which might have resulted in the formation of IOWBs, which

we expect to be similar to the OWBs produced in field-grown plants. This is the first report establishing the conditions for IOWB formation from in vitro cultured gentians. Investigating the metabolic changes during IOWB induction may provide further insights into the process and the inducing conditions for OWB formation.

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