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# A simple and efficient method for the long-term preservation of plant cell suspension cultures

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**Abstract**

**Background**: The repeated weekly subculture of plant cell suspension is labour intensive and increases the risk of variation from parental cells lines. Most of the procedures to preserve cultures are based on controlled freezing/thawing and storage in liquid nitrogen. However, cells viability after unfreezing is uncertain. The long-term storage and regeneration of plant cell cultures remains a priority.

**Results**: Sycamore (*Acer pseudoplatanus*) and Arabidopsis cell were preserved over six months as suspensions cultures in a phosphate-free nutrient medium at 5°C. The cell recovery monitored via gas exchange measurements and metabolic profiling using *in vitro* and *in vivo* $^{13}$C- and $^{31}$P-NMR took a couple of hours, and cell growth restarted without appreciable delay. No measurable cell death was observed.

**Conclusion**: We provide a simple method to preserve physiologically homogenous plant cell cultures without subculture over several months. The protocol based on the blockage of cell growth and low culture temperature is robust for heterotrophic and semi-autotrophic cells and should be adjustable to cell lines other than those utilised in this study. It requires no specialized equipment and is suitable for routine laboratory use.

## Background

Suspension culture of isolated plant cells is an invaluable tool for providing the material for high-throughput studies such as metabolic analyses, production of secondary plant products, and herbicide discovery. It enables easy experimentation on physiologically and biochemically homogenous population of cells. Different methods for cultivating large quantities of plant cells in liquid nutrient medium (NM) have been described for a long time [1-4]. These methods are based on the subculture of cell suspensions having reached their growth plateau when most of the nutrients initially added to NM, particularly carbohydrates, are metabolised. It leads to more or less homogenous cell populations and usually induces a growth delay (lag phase) following subculture [5]. It has been shown that obtaining homogenous cell suspension cultures requires sophisticated apparatus such as chemostats that optimize NM and cell growth [6]. Alternatively, subcultures every one or two days also yields homogenous cell populations [7], but involve much handling and maintenance that is therefore difficult to perform over long periods of time.

For this reason, alternative procedures to preserve newly optimized cell suspension cultures, ideally for indefinite periods, have been proposed. Apart from the maintenance of cell callus on solid media which lead to appreciable delays to initiate homogenous cell suspension cultures, most of the procedures are based on controlled freezing/thawing and storage in liquid nitrogen [8-12]. However, the viability of the cells after unfreezing is generally low and long lag phases before full recovery of cell culture growth are always mentioned by authors. The highest viability (up to 90 %) was observed by Menges and Murray [13] after cryopreservation of Arabidopsis and tobacco cells in the presence of DMSO and sorbitol. Nevertheless, even in this case, it takes at least one week for cells to

recover normal post-thaw growth and full re-establishment, and there is a risk that preserved cell lines may differ from the original ones [14].

Here, we describe a procedure aiming at preserving higher plant cell populations in their suspension nutrient medium over several months, keeping them homogenous and ready to restart growth after their return to standard culture conditions. The main problem is that, in standard cultures, carbon substrates are consumed within less than two weeks. Afterwards autophagic process and cell death are observed [15,16]. In order to diminish the carbohydrate consumption by cells, cultures were carried out at low temperature in a first series of assays. Cultures were then carried out at low temperature in the absence of phosphate (Pi) so as to induce the arrest of cell growth [17]. In order to validate this procedure, the cell's physiological and biochemical states were monitored both via the measurement of cell growth and gas exchange and by *in vivo* and *in vitro* metabolic profiling utilising ^{31}P- and ^{13}C-NMR [18-20]. Indeed, NMR monitoring permits the detection of early signals of metabolic disruption related to carbon deprivation [21] leading to the expression of autophagy-related genes [22]. Heterotrophic sycamore (*Acer pseudoplatanus* L.) cells and semi-autotrophic Arabidopsis cells (*Arabidopsis thaliana* L., wild type, Columbia ecotype) were used to compare results obtained with non-chlorophylous cells of cambial origin to those obtained with illuminated chlorophylous cells of leaf parenchyma origin. It was expected that, due to photosynthesis activity, the cellular need for exogenous carbohydrate would be lower, thus permitting a reduction in NM renewal.

## Results and discussion

**Growth of sycamore and Arabidopsis suspension-cultured cells at 22°C and 5°C**

At 22°C, the fresh weight (FW) of weekly subcultured sycamore cells increases exponentially with time, without a lag phase ([7] and Figure 1A). A plateau corresponding to a cell FW of $150 \pm 15$ g $l^{-1}$ of culture is reached after two weeks. If cells are subcultured at this stage, a 2-d lag phase attributed to the exhaustion of sucrose supply and to the beginning of the related process of autophagy is observed. At 5°C, the cell density doubling time is much longer: 10 d vs 2.5-2.7 d at 22°C (Figure 1B). Interestingly the pH of their NM, initially adjusted to 5.7, increased progressively up to 7.1-7.3. This is probably due to the presence of nitrate as the only nitrogen source in Lamport's NM and to the lower emission of acidifying $CO_2$, whereas it remains below 6.5 at 22°C.

The growth of Arabidopsis cells cultivated under photomixotrophic conditions at 22°C showed a similar profile (Additional File 1A) and a plateau corresponding to a cell concentration of $200 \pm 20$ mg $ml^{-1}$ culture was reached within ca 10 days. Here too, lowering the temperature resulted in a marked decrease of cell culture growth rate (Additional File 1B). The cell density doubling time increased from 2.1-2.3 d to ca 8 d. Cultures performed in Murashige and Skoog medium use ammonium and nitrate as a nitrogen source, leading the NM's pH to decrease progressively to ca. 5.0.

The growth rates of both types of cells incubated over one month at 5°C recovered standard values as soon as the culture temperature was set back to 22°C (Figure 1 and Additional File 1, arrows). Despite this extended period of very slow growth, no significant lag phase was observed before cell growth restarted at 22°C, suggesting that the preservation of cells at low temperature did not substantially modify their physiological

properties. To confirm this conclusion, measurements of $O_2$-uptake by cells and NMR-based control of their metabolite profile were performed.

**$O_2$-uptake at 22°C and 5°C**

The $O_2$ uptake rates of sycamore and Arabidopsis cells cultivated at 22°C, harvested during the exponential phase of growth, and measured at 22°C in the dark, were $350 \pm 25$ and $480 \pm 40$ nmol $O_2$ min$^{-1}$ g$^{-1}$ cell FW, respectively (Table 1). In contrast, the $O_2$ uptake rate of the same cells cultivated at 5°C for 20 d, and measured at 5°C in the dark, was much lower, averaging $72 \pm 7$ and $105 \pm 10$ nmol $O_2$ min$^{-1}$ g$^{-1}$ cell FW, respectively. Interestingly, the uncoupled rate of $O_2$ consumption measured in the presence of 2 μM FCCP showed a higher relative increase at low temperature. This increase was close to 60% at 22°C and reached 100% at 5°C in sycamore and Arabidopsis cells. This indicates that the decrease of the normal (coupled) cell respiration observed at low temperature was not caused by a limitation of substrate supply to mitochondria or by the intrinsic mitochondria oxidative functioning. It is more likely caused by the general decrease of cell metabolism activity and the correlated decrease of cell need for nucleotide triphosphates (NTP). Increasing the temperature to 22°C after a 20-d incubation at 5°C led to the full recovery of coupled and uncoupled cell respiration within 10 minutes.

The illumination of Arabidopsis cells in the $O_2$-electrode chamber decreased the $O_2$-uptake by cells by ca 30% at 22°C, probably due to the contribution of $O_2$-generating photosynthesis activity (Table 1). Indeed, in the light, these chlorophyllous cells incorporate $^{13}CO_2$ into carbon metabolites [unpublished result; 23]. At 5°C, illuminating Arabidopsis cells also decreased the $O_2$-uptake (Table 1). In addition, this decrease was proportionally greater than that observed at 22°C suggesting that, at low temperature, the photosynthesis activity of illuminated Arabidopsis cells was affected less than their

respiration. Nevertheless, in these semi-autotrophic cells, even at 5°C, the $O_2$ produced by photosynthesis never compensated for the $O_2$ consumed by respiration.


**Metabolite profile of cells cultivated at 22°C and 5°C**

The metabolic profiles of sycamore and Arabidopsis cells grown at 22°C and 5°C were analysed from perchloric acid (PCA) extracts using $^{13}C$- and $^{31}P$-NMR spectroscopy. $^{13}C$-NMR spectra show that lowering the temperature in sycamore cells induces significant change in their carbohydrate and organic acid stores (Figure 2). For example, sucrose decreased from 70-80 µmol g$^{-1}$ cell FW to 30-35 µmol g$^{-1}$ cell FW, whereas citrate increased from 5.5-6.5 µmol g$^{-1}$ cell FW to 40-48 µmol g$^{-1}$ cell FW and proline increased from 2.5-3.5 µmol g$^{-1}$ cell FW to 8.5-9.5 µmol g$^{-1}$ cell FW. Arabidopsis cells did not contain $^{13}C$-NMR-detectable carbohydrates, neither at 22 °C nor at 5°C (Additional File 2). This means that their intracellular concentration was below 0.5 µmol g$^{-1}$ cell FW, whatever the culture temperature. Concerning organic acids, glutamine concentration increased fourfold at low temperature, reaching 25-30 µmol g$^{-1}$ cell FW, and aspartate twofold, reaching 9-10 µmol g$^{-1}$ cell FW. On the contrary, citrate and malate, but not fumarate, decreased significantly. The very low level of soluble carbohydrates present in Arabidopsis cells and the low amount of starch [24] could contribute to why a dramatic fall of physiological activity and extensive transcriptomic responses are observed a few hours only after the onset of sugar deprivation leading to cell death within 24 h [25]. Indeed, in suspension-cultured Arabidopsis cells more than one third of the proteins content is degraded during the first 24 h of sucrose starvation [22], whereas it takes 2-3 d in sycamore cells [26,27].

Increasing the temperature to 22°C after a one-month incubation at 5°C led to the recovery of standard $^{13}C$-NMR profiles for both types of cells within a couple of hours. For

example, proline and glutamine which accumulate at low temperature and/or in the absence of Pi [28-30] were metabolised within a few hours. The accumulation of citrate and malate in sycamore cells could be attributed to the fact that the pH of Lamport's NM alkalizes with time [31]. The contrary was observed with Arabidopsis cells grown in Murashige and Skoog's NM which acidifies.

$^{31}$P-NMR spectra (Figure 3) indicate that sycamore cells accumulate nearly twice as much hexoses phosphate and nucleotides at 5°C than at 22°C (0.70-0.80 µmol glucose 6-P g$^{-1}$ cell FW at 5°C vs 0.45-0.50 µmol at 22°C, and 140-160 nmol ATP g$^{-1}$ cell FW at 5°C vs 80-90 nmol at 22°C). Similar results were obtained with Arabidopsis cells with the exception of glycerophosphoglycerol (GPG) accumulating at low temperature (Additional File 3). GPG is a phosphodiester involved in the metabolism of phosphatidylglycerol, the only abundant phospholipid in chloroplast membranes. Importantly, in both cell lines, neither P-choline nor asparagine accumulated, indicating the absence of cytosplasmic autophagy processes known to occur in cells lacking carbon supply [15,16].

Increasing the temperature to 22°C after 20 days of incubation at 5°C led to the recovery of standard cell hexose-P and nucleotide concentrations within 10-15 minutes.

**Carbohydrate stores**

The consumption of sucrose in a nutrient medium is proportional to the number of cells growing in this medium and, consequently, it increases exponentially in accordance with the exponential growth of cell populations. At 22°C, the rate of carbohydrate consumption by sycamore cells was 40-50 mg sucrose d$^{-1}$ g$^{-1}$ cell FW (Figure 4 and Table 2). It took ca 10 d for the sucrose initially present in the NM, where cells were subcultured, to be exhausted. At 5°C, the rate of sucrose consumption per gram of cell FW was 4-5 times lower (Figure 4), and it took 5-6 weeks for the sucrose initially present in the culture medium to be exhausted.

Similar rates of sucrose uptake by Arabidopsis cells cultivated in the light were measured. In the dark it was slightly higher (50-60 mg sucrose $d^{-1}$ $g^{-1}$ cell FW at 22°C). At 5°C, the sucrose initially added in NM was consumed within only 4-5 weeks. This indicates that the contribution of photosynthesis to sugar supply nearly compensated for the slightly higher growth rate of this cell strain. After exhaustion of the sucrose present in NM, the intracellular stores of carbohydrates are consumed and cell autophagy starts [15]. For this reason, it was necessary to subculture cells grown at 5°C at least every 4-5 weeks.

Subculturing cell cultures monthly might nevertheless still look as an excessive constraint. Thus, the question was to determine whether it was possible to further diminish the consumption of carbohydrates by cells whilst at the same time keeping cell suspensions physiologically safe. Since nearly one half of incorporated sugar is consumed by respiration and the other half by different metabolic pathways involved in cell growth [32], we stopped cell growth before lowering the temperature. For this we incubated cells in a Pi-free NM.

**Cell culture at 5°C in Pi-starved media and recovery**

At 22°C, provided sucrose is added regularly to NM, it is possible to keep cell cultures alive in a Pi-free nutrient medium for several months. After a few days, the cell concentrations of Pi, phosphorylated intermediates of glycolysis, and nucleotides dramatically decrease [17,32], whereas the phospholipid/galactolipid ratio is reduced by ca. 70% [33,34]. Under these culture conditions, the growth of sycamore and Arabidopsis cells stops within one week (Figure 5 and Additional File 4). Afterwards, both the cell concentration and the rate of carbohydrate uptake from NM remained constant (Figure 4). Surprisingly, the cell respiration rate of Pi-deprived cells is only 30% lower (Table 3) than that of cells cultivated in standard NM (Table 1). In addition, the uncoupled respiration rates of P- deprived and Pi-supplied cells were similar, indicating that Pi-starvation did not modify the maximum potential

activity of mitochondria. After the addition of Pi to NM, cell respiration recovered to normal values within minutes and cell ^{31}P-NMR spectra showed a normal profile within a couple of hours [17].

When the incubation temperature of Pi-deprived cells was dropped to 5°C, a fivefold decrease of cell respiration was observed (Table 3), as mentioned above in the case of Pi-supplied cells, and the sucrose uptake from the culture medium diminished accordingly (see Figure 4 for sycamore cells). The mean sucrose uptake by sycamore cells was close to 3.4 mg d$^{-1}$ g$^{-1}$ cell FW (Table 2). This suggests, since their culture medium initially contains 20 g l$^{-1}$ sucrose, that it should be possible to keep alive 20 g of cells incubated at 5°C in one litre of a Pi-free NM culture over ca 10 months without renewing the culture medium.

After adding Pi to NMs and returning the temperature to 22°C, the cell respiration of cells stored during 6 months at 5°C in Pi free medium returned to standard values within minutes (Table 3). This suggests that cell suspensions remained physiologically homogenous after months of preservation at low temperature in Pi-free NMs. In particular, no appreciable cell death was measured. Accordingly, Figures 6 and Additional File 5 show examples of sycamore and Arabidopsis cell metabolite profile recovery after 6 months of incubation at 5°C in Pi-free NMs. At time zero, the concentration of cell's soluble P-compounds was close to the threshold for *in vivo* ^{31}P-NMR detection of ca 20 nmol. Thirty minutes after the addition of phosphate in the perfusing NMs, cytoplasmic Pi (cyt-Pi), and NTP recovered standard values, rapidly followed by glucose 6-P and UDP-glucose, and one hour later by vacuolar Pi. After two hours, the *in vivo* ^{31}P-NMR profiles of sycamore and Arabidopsis cells were close to those of standard cells. In fact, the time course recovery of the metabolite profile of Pi-starved cells incubated over 6 months at 5°C resembles that observed by Pratt et al. [17] for cells incubated 5 days in Pi-free NMs at 22°C. The analysis of *in vivo* spectra also indicated that the cytoplasmic pH of both types of cells, as measured from the chemical shift

of cyt-Pi as soon as it was unambiguously identified (30 min), remained close to 7.4. This suggests that the regulation of the cytoplasmic pH was maintained by Pi-starved cells incubated at low temperature, as is the case at 22°C [32]. *In vivo* $^{13}$C-NMR cell profiles of Pi-starved cells cultivated at 5°C were comparable to those of corresponding Pi-supplied cells. Similarly, a full recovery was observed within a few hours: hexoses were metabolised as well as accumulated amino acids.

Finally, the growth rates of cells incubated at low temperature in Pi-free NM rapidly recovered to standard values after the return to standard culture conditions. In particular, no appreciable delay was noticed (Figure 5) and cells can be further normally subcultured. This confirms that the preservation of culture cells at low temperature in a Pi-free medium not permitting cell growth, and without the addition of growth inhibitors, did not generate long-lasting physiological and metabolic changes. Importantly, *in vitro* and *in vivo* $^{13}$C- and $^{31}$P-NMR analyses showed that the metabolic changes observed at low temperature in the absence of Pi in NM were rapidly reversed following the return to standard cell culture conditions. In addition, the absence of lag phase during the recovery of full cell respiration and growth rates suggests that the cell population remains physiologically homogenous during the preservation period.

The protocol for preserving plant cells without subculture over several months is described step-by-step in Additional file 6.


## Conclusion

The incubation of sycamore and Arabidopsis cells in a Pi-free nutrient medium at 5°C allowed the cell lines to stay alive for several months. This was due to the arrest of cell growth resulting from Pi starvation, which was initiated 10 days before lowering the temperature, and to the large decrease of cell metabolic activity at low temperature as

indicated by the drop of respiration. Importantly, no cell death was observed and cells recovered a normal physiological and biochemical activity without a long delay or lag period. To summarize, the procedure of cell preservation described in this paper opens the possibility of storing plant cells lines for several months whilst retaining their capacity to restart growing homogenously and without delay. It can be performed easily and routinely, without requiring any specific equipment, freezing procedure, or addition of growth inhibitors. This method for the preservation of suspension cultured cell lines, with a medium renewal every 6 months only, is now routinely used in our laboratory.

## Material and Methods

### Plant material and growth conditions

Sycamore (*Acer pseudoplatanus* L.) and Arabidopsis (*Arabidopsis thaliana* L.) cells were respectively grown in Lamport [2] and Murashige and Skoog [35] nutrient media as described by Bligny and Leguay [36]. Both types of cells were aerated on orbital shakers monitored at 120 rpm in either a cell culture room at 22°C, or in a cold room at 5°C. Arabidopsis cells received continuous illumination delivering 100 µmol m$^{-2}$ s$^{-1}$ photosynthetic photon flux density (PPFD) whatever the temperature. Nutrient media contained 20 g l$^{-1}$ sucrose as carbohydrate source. At 22°C, cell suspensions were subcultured each 7 days, i.e. before sucrose present in NM was exhausted, by adding 40 ml of old cell culture to 200 ml of fresh NM in 800-ml flasks in order to obtain an initial cell concentration of nearly 5-10 mg FW ml$^{-1}$. At 5°C cells were subcultured when 90% of the initially added sucrose was consumed.

**Metabolomic analyses using NMR**

Analyses were performed either *in vitro* from perchloric acid (PCA) cell extracts or *in vivo* using freshly harvested cells. Extracts were prepared from 10-g (wet wt) cells quickly filtered, washed with pure water at 0°C, and thrown into liquid nitrogen. Frozen samples with 0.7 ml of 70% (v/v) PCA were ground to a fine powder with a mortar and pestle at liquid nitrogen temperature. The frozen powder was then thawed at 0°C and the resulting thick suspension was centrifuged at 15,000 $g$ for 10 min at 0°C to remove particulate matter. The supernatant was neutralised to pH 5.0 with 2 M $KHCO_3$ to precipitate PCA as $KClO_4$, centrifuged at 15,000 $g$ for 5 min and lyophilised. The freeze-dried material was dissolved in 2.0 ml water containing 10% $^2H_2O$ for further NMR adjustment and 1µM sodium azide to avoid fermentation when unfrozen, and it was stored at -20°C.

*In vitro* NMR analyses were performed on a Bruker AMX 400 wide bore spectrometer (Bruker Instruments, Inc., Billerica, MA, USA) equipped with a 10-mm multinuclear-probe. The probe was tuned at 100.6 and 162.0 MHz for $^{13}$C- and $^{31}$P-NMR, respectively. The deuterium resonance of $^2H_2O$ was used as a lock signal. Spectra were recorded at 295 K. $^{13}$C-NMR spectra were the result of 3600 transients with a 6-s repetition time (6 h) recorded with 90° pulses (11 µs), a 20 kHz spectral width, and a Waltz-16 $^1$H decoupling sequence with 2.5 W and 0.5 W during acquisition time and delay, respectively. Free induction decays were collected as 32,000 data points, zero-filled to 64,000 and processed with a 0.2-Hz exponential line broadening. $^{31}$P-NMR spectra were the result of 1000 transients with a 3.6-s repetition time (1 h) recorded with 70° pulses (15 µs), a 8.2 kHz spectral width, and a Waltz-16 $^1$H decoupling sequence with 1 W during acquisition and 0.5 W during delay, respectively. Free induction decays were collected as

16,000 data points, zero-filled to 32,000 and processed with a 0.2-Hz exponential line broadening.

For ^{13}C-NMR analyses, 4 µmol 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA) was added to the PCA extract to chelate $Mn^{2+}$, 150 µmol of maleate was added for calibration, and the pH was adjusted to 7.4. Spectra were referenced to the -CH=CH- peak of maleate positioned at 131.4 ppm. For ^{31}P-NMR analyses, all divalent cations were chelated by the addition of sufficient amounts of CDTA, 2 µmol methylphosphonate was added for calibration, and the samples were buffered by addition of 150 µmol Hepes at pH 7.4. Spectra were referenced to the peak of methylphosphonate positioned at 22.67 ppm. The identification of the peaks of resonance was done by comparing the spectra of standard solutions of known compounds at pH 7.4 with that of the PCA extracts. The definitive assignments were made after running a series of spectra of the extracts spiked with authentic compounds, at different pHs to separate potentially overlapping peaks [18]. To accurately quantify compounds identified on spectra, the intensities of their different resonance peaks were referred to those of the reference compounds added to samples before grinding. Twenty seconds recycling time was used to obtain fully relaxed spectra. The integration function of the spectrometer was utilized to compare the intensity of resonance peaks.

*In vivo* NMR analyses were performed on the same spectrometer equipped with a 25-mm multinuclear-probe. The deuterium resonance of $^2H_2O$ was used as a lock signal. Cells (10 g FW) were placed in a 25-mm NMR tube and oxygenated as described by Aubert et al. [18] with a perfusion flux of 20 ml min^{-1} sufficient for a perfect oxygenation of all cells at 22°C. The 4 l of perfusion NM contained the macro-nutrients (sucrose, $KNO_3$, $NH_4NO_3$, KCl, $Ca[NO_3]_2$, and $MgSO_4$) normally present in 200 ml of Lamport's or Murashige and Skoog's media, according to cell strains, which is sufficient for the growth

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of 10 g of cells over several days and limits decoupling-related temperature elevation at the level of analyzed cells. To further improve the signal-to-noise ratio, micro-nutrients, and in particular $Mn^{2+}$, were not added to NM. The temperature of the perfusing NM was adjusted in a thermoregulated water bath outside the magnet.

$^{13}$C-NMR spectra were acquired by accumulating 900 scans recorded with 90° pulses (70 µs) at 5.6-s intervals, a 20.7 kHz spectral width, and a Waltz-16 $^1$H decoupling sequence with 4 W and 0.5 W during acquisition time and delay, respectively. Free induction decays were collected as 16,000 data points, zero-filled to 32,000, and processed with a 2-Hz exponential line broadening. Spectra were referenced to hexamethyldisiloxane contained in a capillary inserted inside the central outlet perfusion tube at 2.7 ppm. $^{31}$P-NMR spectra were acquired as described by Pratt et al. [17]. Spectra were referenced to methylenediphosphonate (MDP, pH 8.9) contained in the same capillary, at 17.38 ppm.

The identification of the resonance peaks was performed by comparing the *in vivo* spectra of perfused cells to those of PCA extracts prepared from these cells and adjusted at different pHs, as described above. *In vivo* quantification was performed by comparing the spectra of analyzed cells with those of the extracts prepared from the same amount of cells, and using the MDP reference. The concentration of metabolites in the cytoplasm and in the vacuole were calculated as indicated in Pratt et al. [17]. Cytoplasmic and vacuolar pH (cyt- and vac-pH) was estimated from the chemical shift of the pools of Pi (cyt- and vac-Pi) present in these two compartments as described by Gout et al. [21].

**Other analytical methods**

The cell samples FW and the growth of cell suspensions were measured as described by Bligny and Leguay [36]. The oxygen uptake by cells was measured at 5°C and 22°C in their respective cell culture media. $O_2$-uptake was monitored polarographically using a

Clark-type oxygen-electrode (Hansatech Ltd King's Lynn, UK). 50 mg cell (FW) was stirred in the 1 ml measurement chamber filled with NM. The $O_2$ concentration in the air-saturated NM was taken as 250 µM at 22°C and 360 µM at 5°C according to Truesdale and Downing [37]. Uncoupled respiration was measured after adding 2 µM cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). For photosynthesis measurements, Arabidopsis cells were illuminated with 500 µmol m$^{-2}$ s$^{-1}$ PPFD.

The sucrose present in NM was measured as described by Bergmeyer [38], using invertase, hexokinase, and glucose 6-P dehydrogenase, and by $^{13}$C-NMR.

Neutral red was be used as a vital stain to detect the presence of dead cells in cultures.

When means ± SD are given, the statistical Student's *t*-test was applied to the data with *P* values ≤ 0.05.

**List of abbreviations**

Pi: inorganic phosphate; PCA: perchloric acid; NM: nutrient medium; NMR: nuclear

magnetic resonance

**Author's contribution**

RB and CR conceived the study and drafted the manuscript. A-M B and EG carried out the

analysis and contributed to draft the manuscript. All authors approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1.  Tulecke W, Nickel LG: **Methods, problems and results of growing plant cells under submerged conditions.** *Transactions of New York Academy of Sciences* 1960, **22:**196-206.

2.  Lamport DTA: **Cell suspension cultures of higher plants: isolation and growth energetics.** *Experimental Cell Research* 1964, **33:**195-206.

3.  Short KC, Brown EG, Street HE: **Studies on the growth in culture of plant cells. V. Large-scale culture of *Acer pseudoplatanus* L. cell suspensions.** *Journal of Experimental Botany* 1969, **20:**572-578.

4.  Wilson JB, King PJ, Street HE: **Studies on the growth in culture of plant cells. XII. A versatile system for the large scale batch or continuous culture of plant cell suspensions.** *Journal of Experimental Botany* 1971, **22:**177-207.

5.  Henshaw GG, Jha KK, Mehta AR, Shakeshaft DJ, Street HE: **Studies on the growth in culture of plant cells. I . Growth  patterns in batch propagated suspension cultures.** *Journal of Experimental Botany* 1966, **17:** 362-377.

6.  Monod J: **La technique de culture continue. Théorie et applications.** *Annales de l'Institut Pasteur* 1950, **79:**390-410.

7.  Bligny R: **Growth of suspension-cultured *Acer pseudoplatanus* L. cells in automatic units of large volume.** *Plant Physiology* 1977, **59:**502-505.

8.  Nag KK, Street HE: **Freeze preservation of cultured plant cells. Part I. The pretreatment phase.** *Physiologia Plantarum* 1975a**, 34:**254-260.

9.  Nag KK, Street HE: **Freeze preservation of cultured plant cells. Part II. The freezing and thawing phase.** *Physiologia Plantarum* 1975b**, 34:**261-265.

10. Maddox AD, Gonsalves F, Shields R: **Successful preservation of suspension-cultures of 3 *Nicotiana* species at the temperature of liquid-nitrogen.** *Plant Science letters* 1983, **28**:157-162.

11. Jain S, Jain RK, Wu R: **A simple and efficient procedure for cryopreservation of embryogenic cells of aromatic Indica rice varieties.** *Plant cell reports* 1996, **15**:712-717.

12. Kim SI, Choi HK, Son JS, Yun JH, Jang MS, Kim HR, Song JY, Kim JH, Choi HJ, Hong SS: **Cryopreservation of *Taxus chinensis* suspension cell cultures.** *Cryo-letters* 2001, **22**:43-50.

13. Menges M, Murray JAH: **Cryopreservation of transformed and wild-type *Arabidopsis* and tobacco cell suspension cultures.** *Plant Journal* 2004, **37**:635-644.

14. Zeliang PK, Pattanayak A, Inagrai B, Khongwir EA, Sarma BK: **Fertile plant regeneration from cryopreserved calli of *Oryza rufipogon* Griff. and assessment of variation in the progeny of regenerated plants.** *Plant Cell Report* 2010, **29**:1423-1433.

15. Roby C, Martin J-B, Bligny R, Douce R: **Biochemical changes during sucrose deprivation in higher plant cells. Phosphorus-31 nuclear magnetic resonance studies.** *Journal of Biological Chemistry* 1987, **262**:5000-5007.

16. Aubert S, Gout E, Bligny R, Mazars-Marty D, Barrieu F, Alabouvette J, Marty F, Douce R: **Ultrastructural and biochemical characterization of autophagy in higher plant cells submitted to carbon deprivation; control by the supply of mitochondria with respiratory substrates.** *Journal of Cell Biology* 1996a, **133**:1251-1263.

17. Pratt J, Boisson A-M, Gout E, Bligny R, Douce R, Aubert S: **Phosphate (Pi) starvation effect on the cytosolic Pi concentration and Pi exchanges across the**

tonoplast in plant cells: an in vivo ^{31}P-nuclear magnetic resonance study using methylphosphonate as Pi analog. *Plant Physiology* 2009, **151**:1646-1657.

18. Aubert S, Bligny R, Douce R: **NMR studies of metabolism in cell suspensions and tissue cultures.** In *Nuclear Magnetic Resonance in Plant Physiology*. Edited by Shachar-Hill Y, Pfeffer P. Rockville, USA: American Society of Plant Physiologists; 1996b:109-154.

19. Fan TW-M: **Recent advances in profiling plant metabolites by multi-nuclear and multi-dimensional NMR.** In *Nuclear Magnetic Resonance in Plant Physiology*. Edited by Shachar-Hill Y, Pfeffer P. Rockville, USA: American Society of Plant Physiologists; 1996:181-254.

20. Krishnan P, Kruger NJ, Ratcliffe RG: **Metabolite fingerprinting and profiling in plants using NMR.** *Journal of Experimental Botany* 2005, **56**:255-265.

21. Gout E, Bligny R, Douce R, Boisson A-M, Rivasseau C: **Early response of plant cell to carbon deprivation:** *in vivo* [31]**P-NMR spectroscopy shows a quasi-instantaneous disruption on cytosolic sugars, phosphorylated intermediates of energy metabolism, phosphate partitioning, and intracellular pHs.** *New Phytologist* 2011, **189**:135-147.

22. Lundgren Rose T, Bonneau L, Der C, Marty-Mazars D, Marty F: **Starvation-induced expression of autophagy-related genes in Arabidopsis.** *Biology of the Cell* 2006, **98**:53-67.

23. Chen W-P, Yang X-Y, Harms GL, Gray WM, Hegeman AD, Cohen JD: **An automated growth enclosure for metabolic labeling of *Arabidopsis thaliana* with ^{13}C-carbon dioxide – an *in vivo* labeling system for proteomics and metabolomics research.** *Proteome Science* 2011, 9:9-22.

24. Williams TCR, Miguet L, Masakapalli SK, Kruger NJ, Sweetlove LJ, Ratcliffe RG: **Metabolic network fluxes in heterotrophic Arabidopsis cells: stability of the flux distribution under different oxygenation conditions.** *Plant Physiology* 2008, **148**:704-718.

25. Contento AL, Kim S-J, Bassham DC: **Transcriptome profiling of the response of Arabidopsis suspension culture cells to suc starvation.** *Plant Physiology* 2004, **135**:2330-2347.

26. Journet E-P, Bligny R, Douce R: **Biochemical changes during sucrose deprivation in higher plant cells.** *Journal of Biochemical Chemistry* 1986, **261**:3193-3199.

27. Genix P, Bligny R, Martin J-B, Douce R: **Transient accumulation of asparagine in sycamore cells after a long period of sucrose starvation.** *Plant Physiology* 1990, **94**:717-722.

28. Pollock CJ, ap Rees T: **Effect of cold on glucose metabolism by callus and tubers of *Solanum tuberosum*.** *Phytochemistry* 1975, **14**:1903-1906.

29. Withers LA, King PJ: **Proline: a novel cryoprotectant for the freeze preservation of cultured cells of *Zea mays* L.** *Plant Physiology* 1979, **64**:675-678.

30. Dorne A-J, Bligny R: **Physiological adaptation to subantarctic climate by the Kerguelen cabbage, *Pringlea antiscorbutica* R. Br.** *Polar Biology* 1993, **13**:55-60.

31. Gout E, Bligny R, Pascal N, Douce R: **[13]C nuclear magnetic resonance studies of malate and citrate synthesis and compartmentation in higher plant cells.** *Journal of Biological Chemistry* 1993, **268**:3986-3992.

32. Gout E, Boisson A-M, Aubert S, Douce R, Bligny R: **Origin of the cytoplasmic pH changes during anaerobic stress in higher plant cells. Carbon-13 and phosphorus-31 nuclear magnetic resonance studies.** *Plant Physiology* 2001, **125**:912-925.

33. Jouhet J, Maréchal E, Baldan B, Bligny R, Joyard J, Block M A: **Phosphate deprivation induces transfer of DGDG galactolipid from chloroplast to mitochondria.** *Journal of Cell Biology* 2004, **167**:863-874.

34. Misson J, Raghothama KG, Jain A, Jouhet J, Block MA, Bligny R, Ortet P, Creff A, Somerville S, Rolland N, Doumas P, Nacry P, Herrerra-Estrella L, Nussaume L, Thibaud M-C: A **genome-wide transcriptional analysis using** *Arabidopsis thaliana* **Affymetrix gene chips determined plant responses to phosphate deprivation.** *Proceedings of the National Academy of Sciences USA* 2005, **102**:11934-11939

35. Murashige T, Skoog F: **A revised medium for rapid growth and bioassay with tobacco tissue cultures.** *Physiologia Plantarum* 1962, **15**:473–497.

36. Bligny R, Leguay J-J: **Techniques of cell suspension culture.** *Methods in Enzymology* 1987, **148**:3-16.

37. Truesdale GA, Downing AL: **Solubility of oxygen in water.** *Nature* 1954, **173**:1236.

38. Bergmeyer HU: *Methods of Enzymatic Analysis. Volume 3*. New York: Academic press; 1974.

# Figure legends

**Figure 1 Growth of suspension-cultured sycamore cells at 22°C and 5°C.** Cells were cultivated in Lamport's nutrient medium as described in "Material and Methods". They originated from an exponentially growing cell suspension maintained at 22°C and repeatedly subcultured before the growth plateau. The cell concentration (FW) at time zero was 5.0 mg ml$^{-1}$ culture. At 22°C **(a)**, cells grew exponentially for 10 d (linear on semi-logarithmic plot) before reaching a plateau. At day 14, the dilution of cell suspension to their initial concentration (subculture) restarted the exponential cell growth after a 2-d lag. At 5°C **(b)**, cells grew exponentially for 40 d before reaching a plateau. In separate experiments (arrow in Figure 1b), the temperature was set back to 22°C after 20 d of culture at 5°C. This experiment was repeated five times. In semi-logarithmic plot, standard deviation is of the order of the symbol size.

**Figure 2 Proton-decoupled $^{13}$C-NMR spectra of perchloric acid extracts of sycamore cells grown at 22°C and 5°C.** Cells were harvested 5 d after subculture at 22°C (**a**) and 20 d after subculture at 5°C (**b**). Peak assignments are as follows: ref, reference (maleate) used for quantification; s, sucrose; g, glucose; f, fructose; cit, citrate; mal, malate; suc, succinate; Pro, proline.

**Figure 3 Proton-decoupled $^{31}$P-NMR spectra of perchloric acid extracts of sycamore cells grown at 22°C and 5°C.** Cells were harvested 5 d after subculture at 22°C (**a**) and 20 d after subculture at 5°C (**b**). Peak assignments are as follows: ref, reference (methylphosphonate) used for quantification; glc-6-P, glucose 6-phosphate; fru-6-P,

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fructose 6-P; Pi, inorganic phosphate; GPC, glycerophosphocholine; UDP-glc, uridine-5'-diphosphate-$\alpha$-D-glucose.

**Figure 4 Temperature and phosphate effect on sucrose decrease in the nutrient medium of sycamore cell culture.** Time course decrease of sucrose was measured in the nutrient medium of sycamore cell cultures at 22°C and 5°C, in the presence or absence of inorganic phosphate (Pi). Cells were initially incubated for 5 d in Pi-supplied or Pi-free NM. The cell concentration (FW) at time zero was 20 mg ml$^{-1}$ culture in Pi-supplied NM and 30 mg ml$^{-1}$ culture in Pi-free NM. Sucrose was quantified as described in "Material and Methods". Values are given as means $\pm$ SE (n=3).

**Figure 5 Growth of sycamore cells under different culture conditions.** Growth of suspension-cultured sycamore cells was measured in a Pi-free nutrient medium at 22°C followed by cell preservation at 5°C, and recovery in a Pi-supplied medium at 22°C. Cells were cultivated as described in "Material and Methods". They originated from an exponentially growing cell suspension repeatedly subcultured in Pi-supplied NM before the growth plateau. At time zero, cells were incubated at 5.0 mg ml$^{-1}$ in Pi-free Lamport's NM at 22°C. After a short period of exponential growth, a growth plateau corresponding to a cell concentration of only 20 mg cell FW ml$^{-1}$ culture was attained due to Pi starvation. At that stage, the temperature was set to 5°C. Six months later preserved cells were incubated under standard culture conditions and subcultured (day 190). This experiment was repeated five times. In semi-logarithmic plot, standard deviation is of the order of the symbol size.

**Figure 6 *In vivo* proton-decoupled $^{31}$P-NMR spectra showing the recovery of sycamore cells preserved over 6 months.** Cells were preserved over 6 months at 5°C in a Pi-free nutrient medium. They were then placed in the perfusion system dedicated to *in vivo* NMR analyses as described in "Material and Methods". At time zero, 100 µM Pi was added to NM and the temperature was set to 22°C. Spectra are the result of 1500 transients with a 0.6-s repetition time (15 min). Peak assignments are as follows: ref, reference (methylenediphosphonate) used to measure chemical shifts and for quantification; glc-6-P, glucose 6-phosphate; fru-6-P, fructose 6-P; cyt-Pi, cytoplasmic-Pi; vac-Pi, vacuolar Pi; NTP, nucleoside triphosphate; UDP-glc, uridine-5'-diphosphate-$\alpha$-D-glucose.

**Table 1. O$_2$-uptake by sycamore and Arabidopsis cells incubated at different temperatures**

| Temperature | sycamore | + 2 µM FCCP | Arabidopsis | + 2 µM FCCP | + light |
|---|---|---|---|---|---|
| 22°C | 350 ± 25 | 550 ± 50 | 480 ± 40 | 780 ± 70 | 330 ± 30 |
| 5°C | 72 ± 6 | 145 ± 12 | 105 ± 10 | 200 ± 15 | 30 ± 3 |
| 5°C→22°C | 360 ± 30 | 560 ± 50 | 470 ± 40 | 750 ± 70 | 290 ± 25 |

The O$_2$-uptake by Sycamore and Arabidopsis cells harvested 5 d after subculture at 22°C and 20 d after subculture at 5°C was measured in their respective culture media at 22°C or 5°C. In addition, the O$_2$-uptake by cells cultivated for 20 d at 5°C was measured at 22°C after a 10-min recovery (5°C→22°C). O$_2$-uptake rates were measured in the dark, except for Arabidopsis cells when specified (+ light), as indicated in "Material and Methods". They are expressed as nmol O$_2$ consumed min$^{-1}$ g$^{-1}$ cell FW. Values are means ± SD ($n = 5$). FCCP, cyanide $p$-trifluoromethoxyphenylhydrazone.

**Table 2. Sucrose-uptake by sycamore cells incubated in Pi-supplied and Pi-deprived NM at 22°C and 5°C**

| | Pi-supplied NM | | Pi- starved NM | |
|---|---|---|---|---|
| Temperature | 22°C | 5°C | 22°C | 5°C |
| Sucrose uptake (mg d$^{-1}$ g$^{-1}$) | 51 ± 5 | 9.7 ± 0.9 | 17 ± 1.5 | 3.4 ± 0.3 |

Cells were initially incubated for 5 d at 22°C in either Pi-supplied or Pi-free Lamport's NM. At day 5, incubation was pursued at 22°C or 5°C and sucrose-uptake expressed as mg d$^{-1}$ g$^{-1}$ cell FW was measured as indicated in "Material and Methods". Values are means ± SD ($n$ = 5).

**Table 3. O$_2$-uptake by sycamore and Arabidopsis cells incubated in Pi-free nutrient media at different temperatures**

| Temperature | sycamore | | Arabidopsis | |
|---|---|---|---|---|
| | | + 2 µM FCCP | | + 2 µM FCCP |
| 22°C | 250 ± 25 | 510 ± 50 | 320 ± 30 | 690 ± 60 |
| 5°C | 59 ± 6 | 135 ± 12 | 70 ± 7 | 190 ± 15 |
| 5°C→22°C | 370 ± 30 | 530 ± 50 | 470 ± 40 | 720 ± 60 |

Sycamore and Arabidopsis cells were initially incubated for 5 d at 22°C in their respective NM devoid of Pi in order to stop cell growth. At day 5, incubation was pursued at 22°C and 5°C in the same nutrient media. O$_2$-uptake by Pi-deficient cells was measured after 5 d incubation at 22°C and 6 months incubation at 5°C. In addition, the O$_2$-uptake by cells incubated for 6 months at 5°C was measured at 22°C in standard Pi-supplied media after a 10-min recovery (5°C→22°C). All cells were incubated in the dark. O$_2$-uptake rates measured as indicated in "Material and Methods" are expressed as nmol O$_2$ consumed min$^{-1}$ g$^{-1}$ cell FW. Values are means ± SD ($n = 5$). FCCP, cyanide *p*-trifluoromethoxyphenylhydrazone.

**Additional material**

**Additional file 1: Growth of suspension-cultured lightened Arabidopsis cells at 22°C (a) and 5°C (b)**. Legend as in Figure 1.
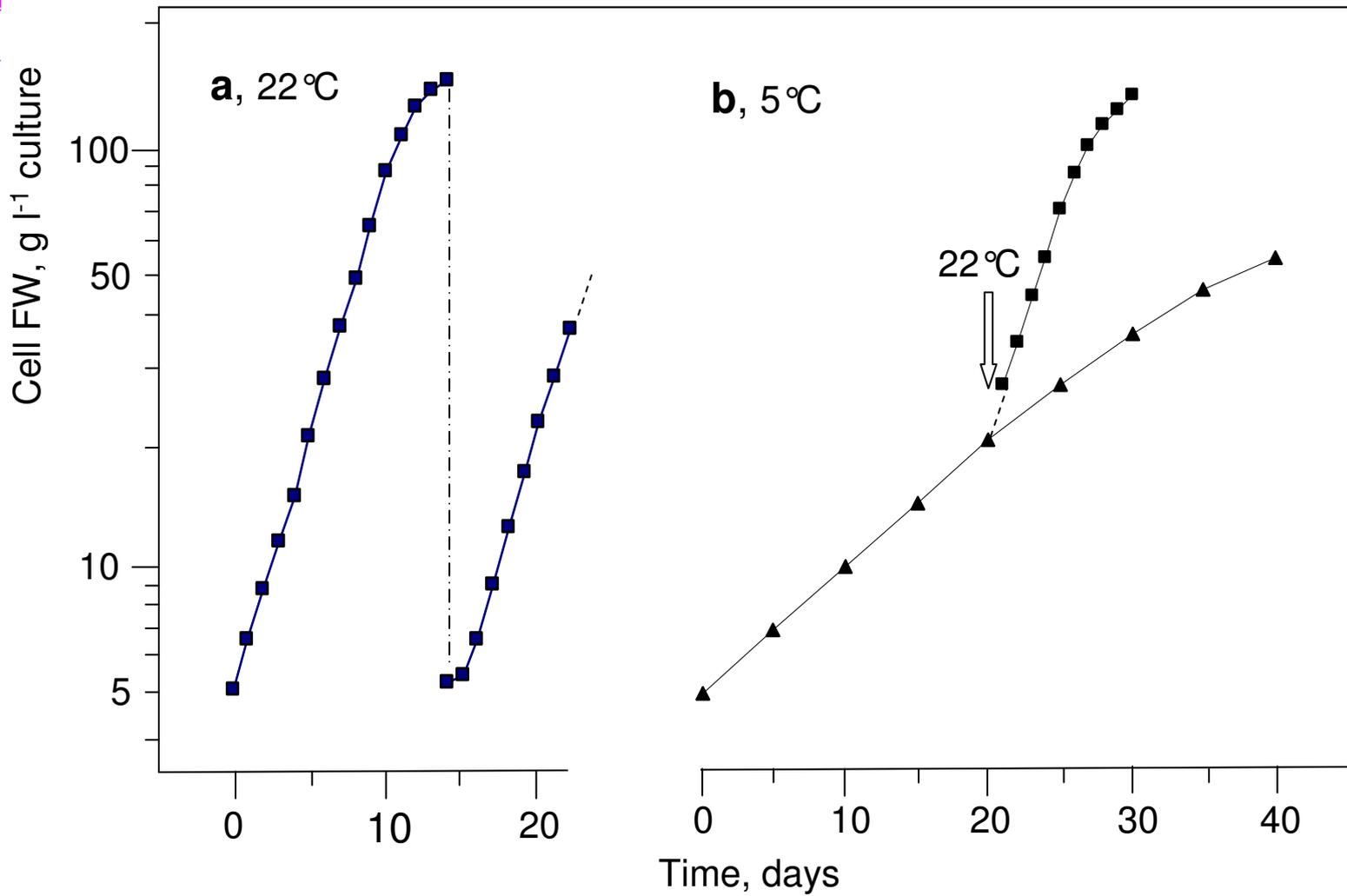
**Additional file 2: Proton-decoupled $^{13}$C-NMR spectra of perchloric acid extracts of Arabidopsis cells grown in the light at 22°C (a) and 5°C (b)**. Legend as in Figure 2; fum, fumarate; Asp, aspartate; Glu, glutamate; Gln, glutamine.
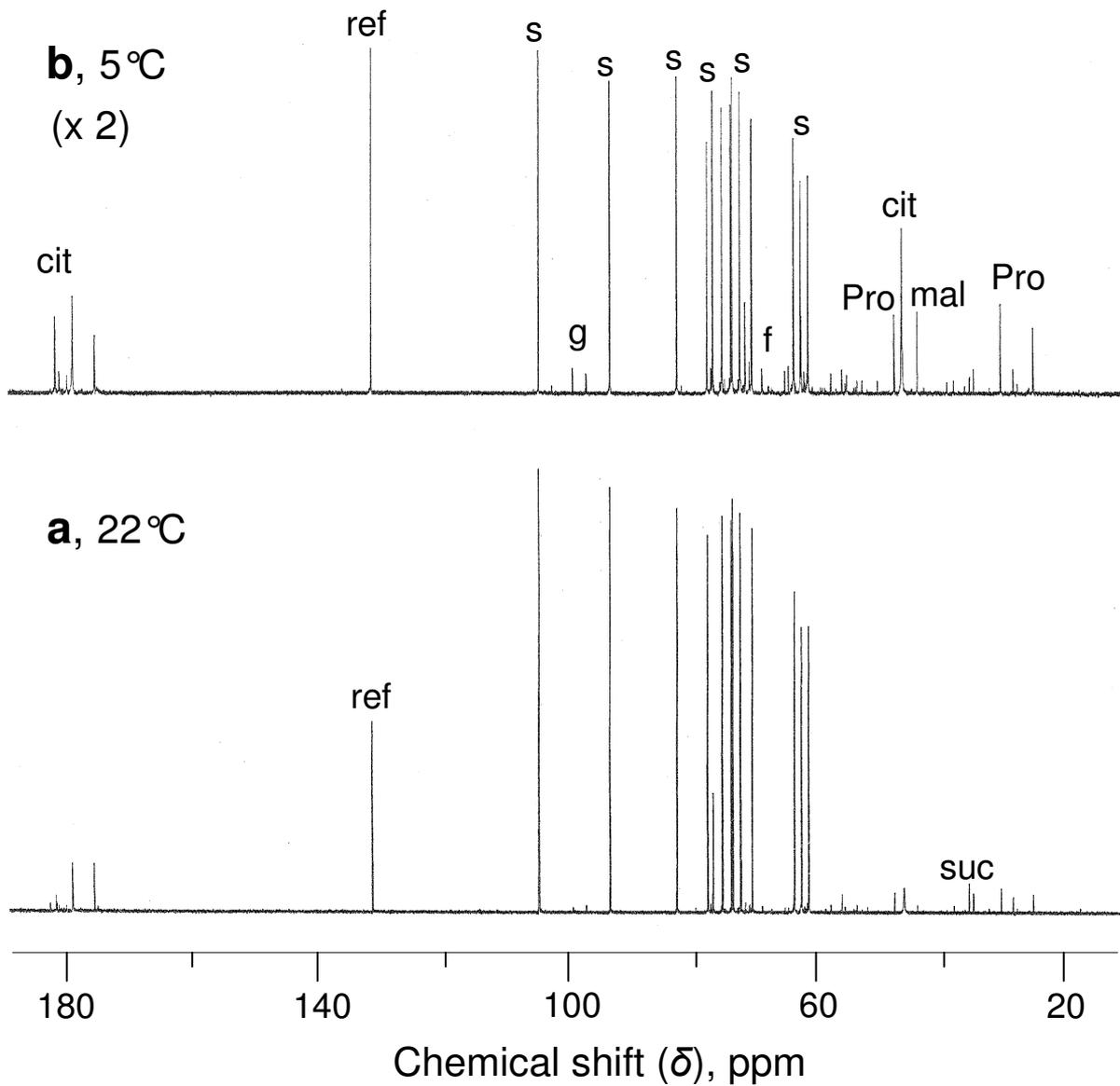
**Additional file 3: Proton-decoupled $^{31}$P-NMR spectra of perchloric acid extracts of Arabidopsis cells grown in the light at 22°C (a) and 5°C (b)**. Legend as in Figure 3; GPG, glycerophosphoglycerol; spectra are the result of 250 transients.

**Additional file 4: Growth of suspension-cultured lightened Arabidopsis cells in a Pi-free nutrient medium at 22°C followed by cell preservation at 5°C, and recovery in a Pi-supplied medium at 22°C**. Legend as in Figure 5.

**Additional file 5: *In vivo* proton-decoupled $^{31}$P-NMR spectra of Arabidopsis cells**. The recovery of preserved cell was followed *in vivo* after the return to standard perfusion conditions (Pi-supplied NM, 22°C) as indicated in the legend of Figure 6.

**Additional file 6: Step-by-step description of the protocol for preserving plant cells without subculture over several months.**
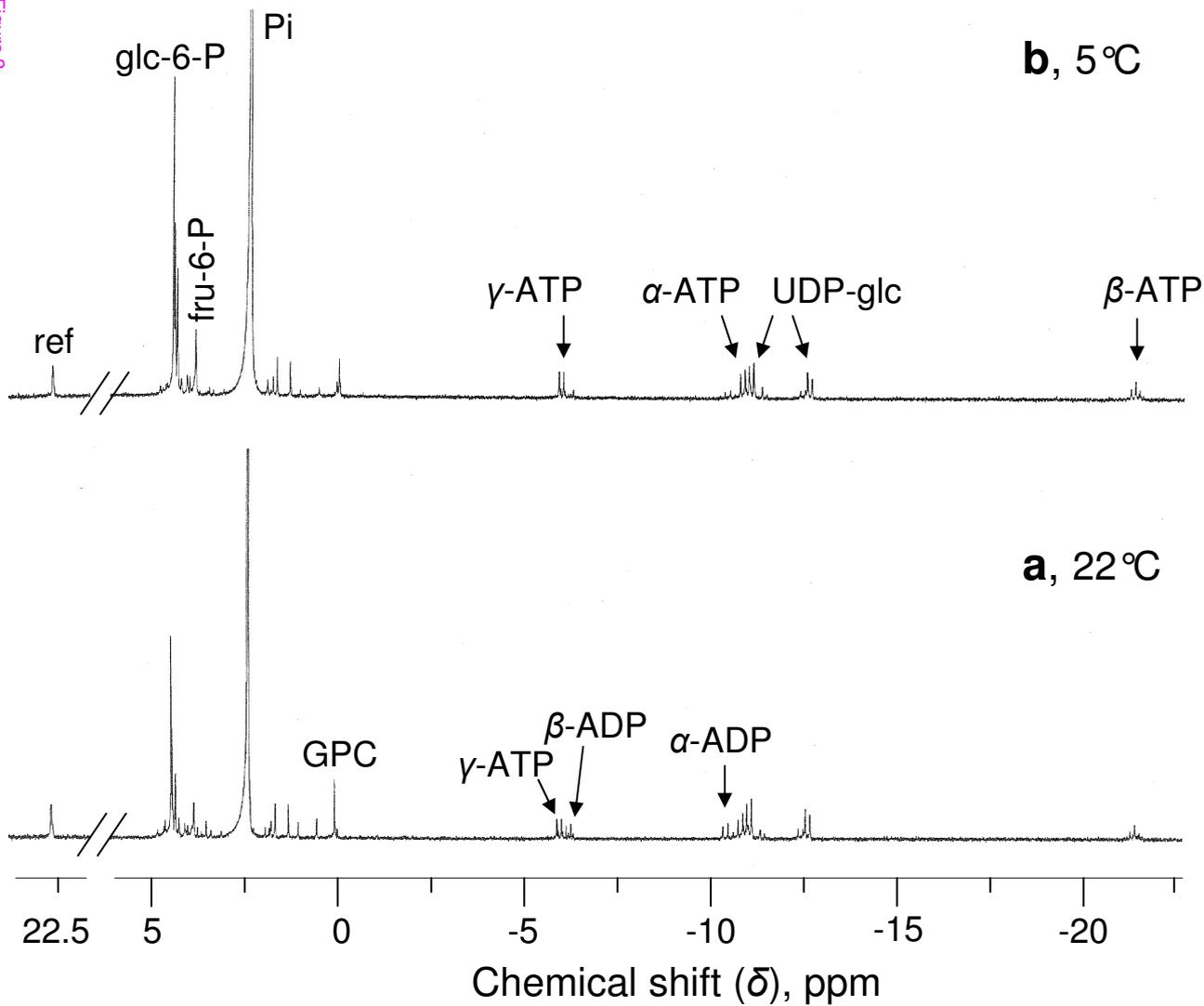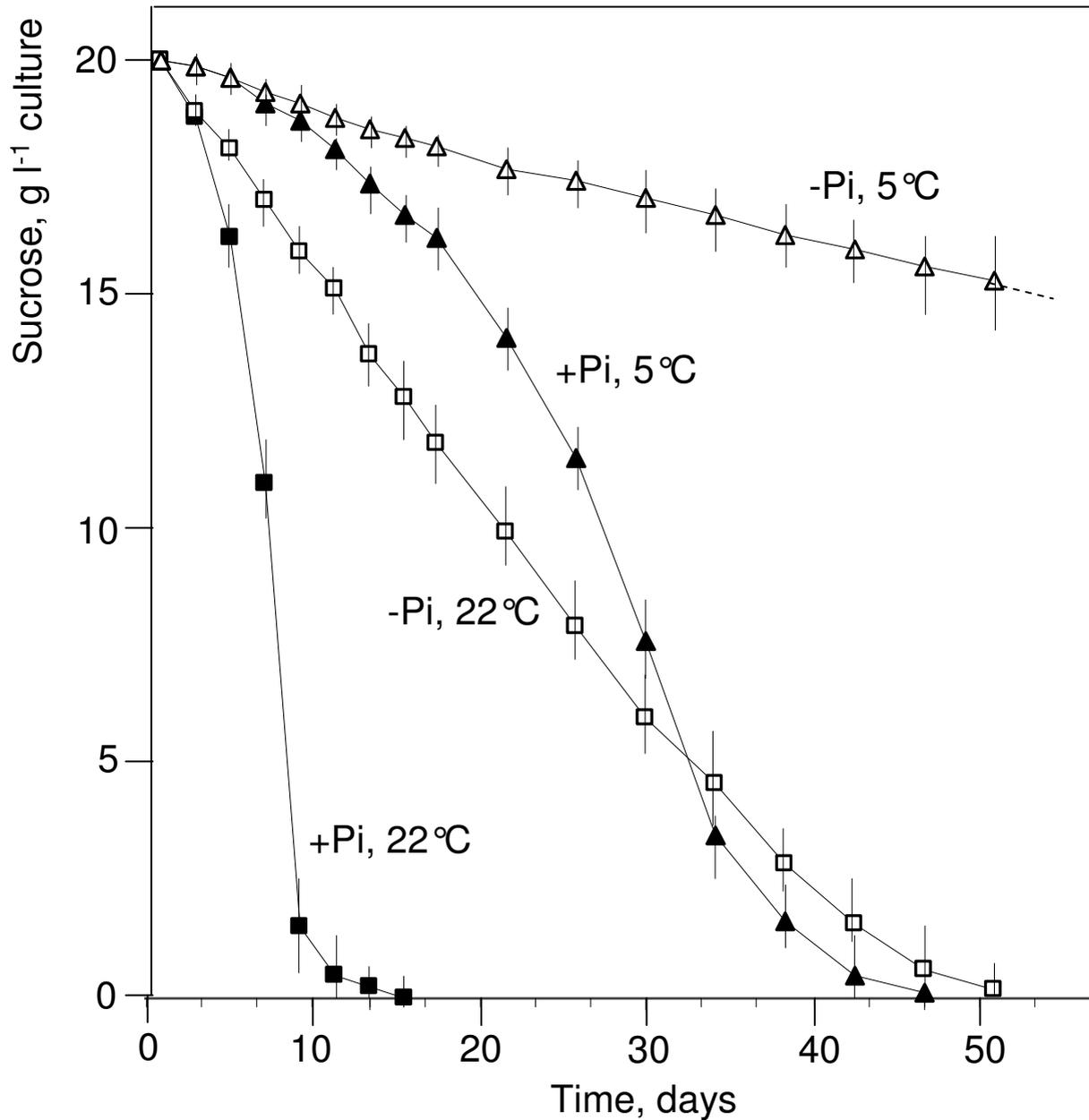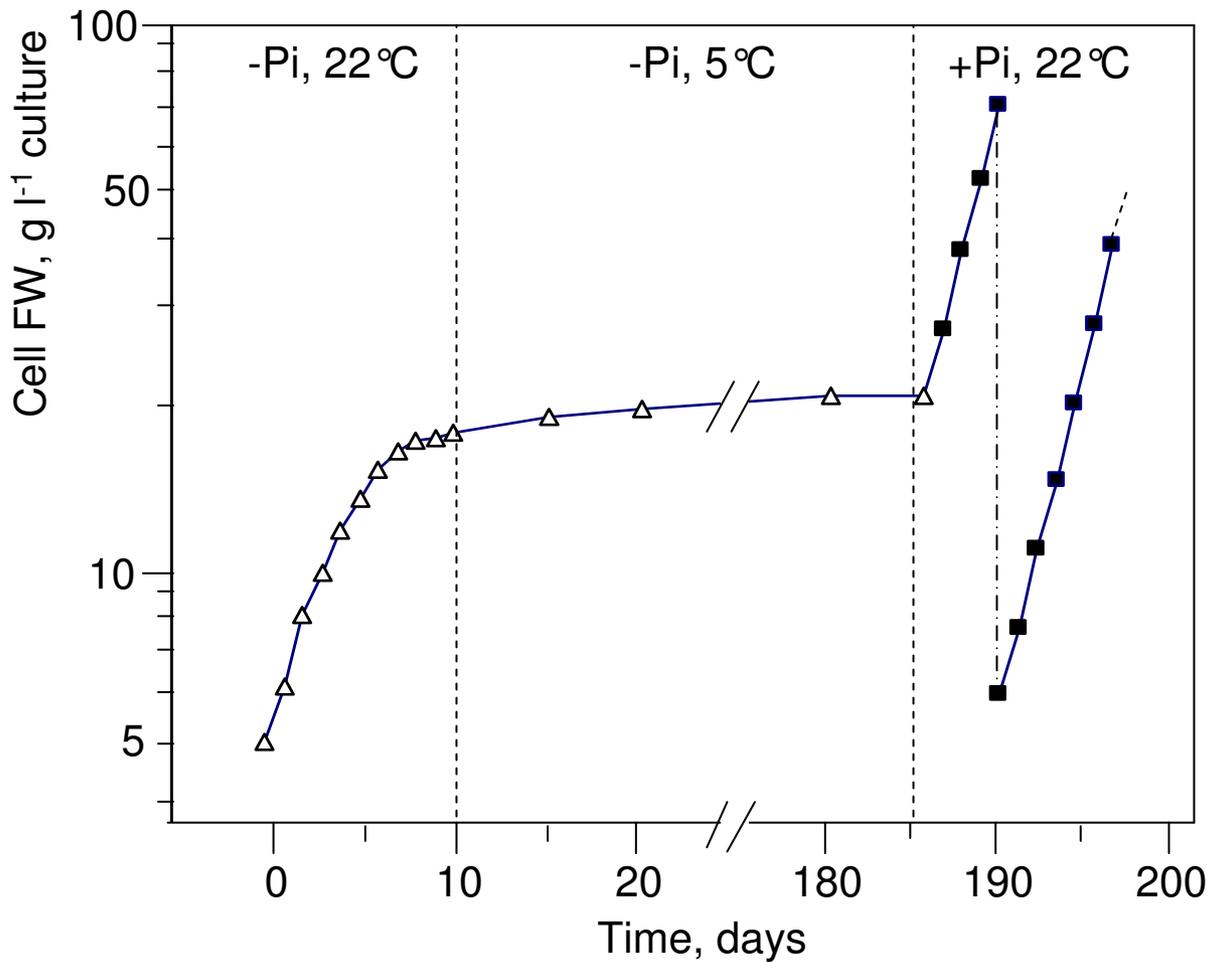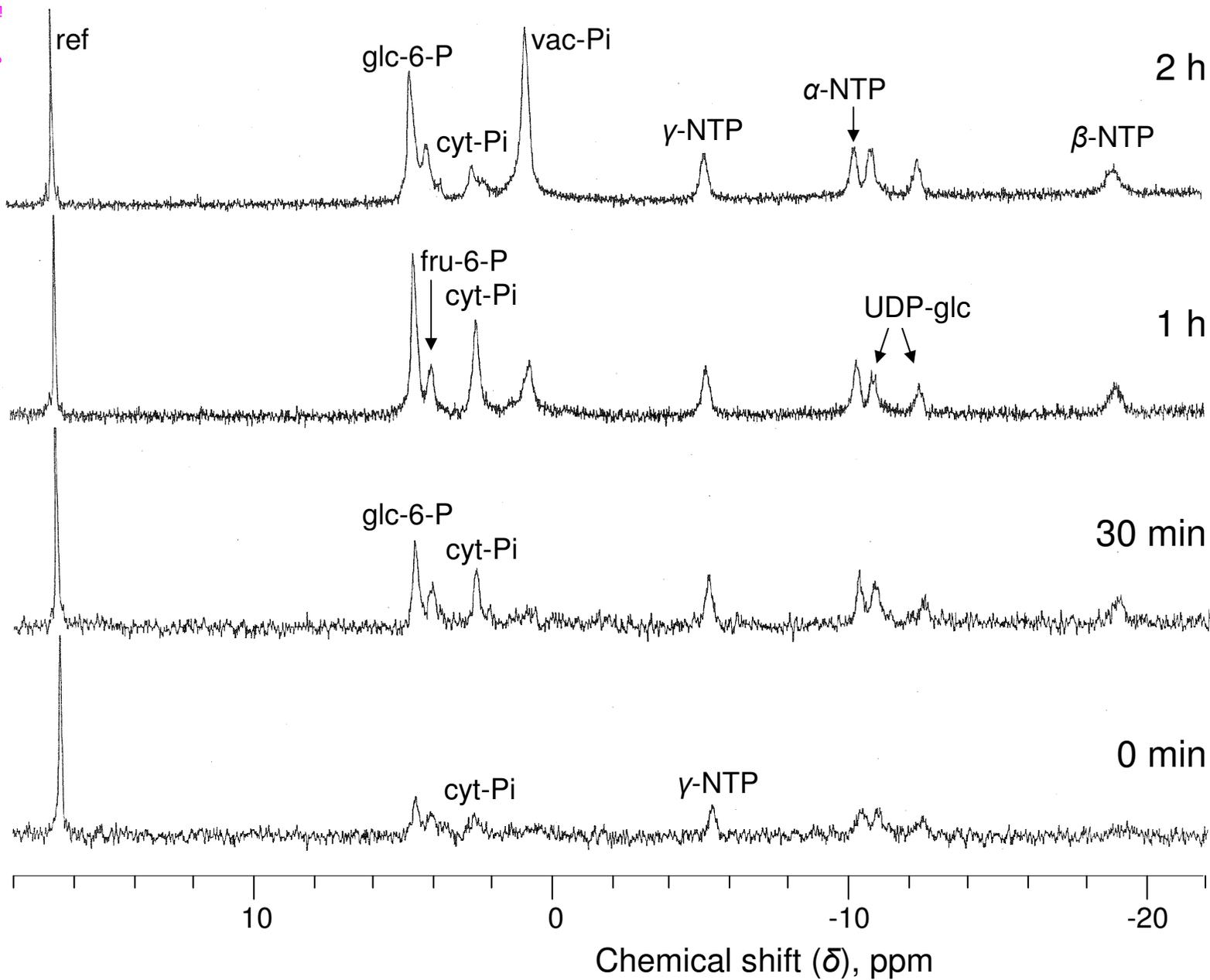
Figure 1

Figure 2

Figure 3

Figure 4

-Pi, 5 ℃

+Pi, 5 ℃

-Pi, 22 ℃

+Pi, 22 ℃

Sucrose, g l⁻¹ culture

Time, days

Figure 5

Figure 6

**Additional files provided with this submission:**

Additional file 1: Additional_File_1.pdf, 6K
http://www.plantmethods.com/imedia/8587640046633332/supp1.pdf
Additional file 2: Additional_File_2.pdf, 41K
http://www.plantmethods.com/imedia/1264344258663333/supp2.pdf
Additional file 3: Additional_File_3.pdf, 39K
http://www.plantmethods.com/imedia/5780544866633347/supp3.pdf
Additional file 4: Additional_File_4.pdf, 5K
http://www.plantmethods.com/imedia/3665146366333477/supp4.pdf
Additional file 5: Additional_File_5.pdf, 108K
http://www.plantmethods.com/imedia/1002493952663335/supp5.pdf
Additional file 6: Additional_File_6.pdf, 15K
http://www.plantmethods.com/imedia/1937508601663330/supp6.pdf