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Optimization of EDTA Exudation Technique for Proteome Study of the Phloem

Zaidah Rahmat[a*], Colin Turnbull[b]

[a]*Department of Biotechnology and Medical Engineering, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia*
[b]*Division of Cell and Molecular Biology, Imperial College London, SW7 2AZ, United Kingdom*

*Corresponding author: zaidahrahmat@utm.my

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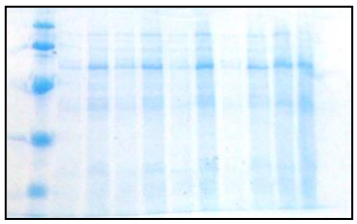
Graphical abstract



Abstract

Phloem proteome study is gaining recognition due to the phloem important function to a plant. Identification of proteins within the phloem with varying function could help understand the regulation of growth and development. EDTA exudation technique applied to obtain *Arabidopsis* phloem protein was optimized. It was found that plants grown up until 6 weeks in SD (short day) condition, exposed to 3 days LD (long day) condition, the phloem sap collected in 20 mM EDTA with overnight incubation in LD and precipitated using Amicon filter device gave the optimum amount of protein with good quality. Sufficient amount of protein was obtained using the optimized EDTA exudation technique for protein identification.

Keywords: EDTA exudation; phloem; proteomics; *Arabidopsis*; 1D-PAGE, 2D-PAGE

Abstrak

Kajian proteomik berkaitan floem semakin dikenali disebabkan fungsi penting floem kepada tumbuhan. Pengenalpastian protein dalam floem dengan pelbagai fungsi boleh membantu pemahaman regulasi pertumbuhan dan perkembangan. Teknik pengaliran keluar EDTA yang diaplikasikan untuk memperoleh protein daripada floem *Arabidopsis* telah dioptimumkan. Tumbuhan yang tumbuh sehingga 6 minggu dalam keadaan hari pendek (SD), didedahkan 3 hari kepada keadaan hari panjang (LD), gubal floem dikumpul dalam 20 mM EDTA dengan inkubasi di dalam keadaan LD and dimendakkan menggunakan *Amicon filter device* menghasilkan amaun protein yang optimum dengan kualiti yang baik. Amaun protein yang mencukupi diperolehi menggunakan teknik penagaliran keluar EDTA yang telah dioptimumkan untuk pengenalpastian protein.

Kata kunci: Pengaliran keluar EDTA; floem; proteomic; *Arabidopsis*; 1D-PAGE, 2D-PAGE

1.0 INTRODUCTION

The accumulation of photoassimilates occurs in the phloem. Through a process known as translocation, the products of photosynthesis are then transported from the phloem across the plant. Phloem proteome study has been the focus of many plants such as *Arabidopsis*,[2,6] *Brassica napus*,[5] rice,[1] cucurbit[8] and many more. Identification of proteins with diverse functions from the phloem proteome has enabled further understanding not only of the phloem but overall regulation of plant growth and development.

The phloem can be found in more than one location in most plants such as the leaves, stems, and roots. It is said to be in abundance in the leaves owing to the role it plays in collecting photosynthates from photosynthesis for distribution throughout the plant. Driven by positive pressure, the nutrients are pushed up and down the plant through the plant vasculature system. Hence, phloem sap can be easily collected by either injuring the plant at the stem or cutting the stem to allow exudation of the sap.

It is important to justify the protocols for proteomics for *Arabidopsis* because good quality and a large amount of proteins are needed as the method used to obtain protein is by wounding. Sufficient protein without or with less contaminant that could cater for the whole phloem proteome and be used to reproduce would be beneficial to ensure a satisfactory result. Unlike other phloem proteome studies such as in *Brassica* and *Cucurbita* where a large amount of pure phloem protein could be obtained by wounding plants with a hypodermic needle,[5,8] small plants such as *Arabidopsis* require an exudation technique in which wounded plants are soaked into a chelating agent to collect the phloem sap. Optimization in terms of chelating agent

concentration and time among other factors played important role to ascertain sufficient amount and good quality protein for analysis.

Developing these methods and optimizing the necessary parameters to ensure good quality, ease of use and reproducibility would be advantageous especially for proteomics work. Further work following the outcome of this study could be accelerated using methods developed not only to identify certain protein but also to take *Arabidopsis* phloem proteomics work one step further particularly in relation to systemic signaling and regulation of flowering.

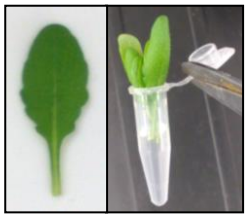
2.0 EXPERIMENTAL

2.1 Plant Growth and Conditions

Seeds of *Arabidopsis* wild type (WT) plant (Col-0) and ft-null mutant (ft-10) were grown on soil to vermiculite ratio of 4:1 and kept at 4°C for 2-3 days. Subsequent growth was done in growth chamber in short day (SD) condition at 23°C with 10 h light (160 µmol) and 60% humidity. Plants that need to be grown in LD condition were exposed to light for 16 h with the same intensity and humidity.

2.2 Exudation Technique

Prior to exudation, the 6 weeks-old plants which were previously grown in SD condition were exposed to LD condition for 3 days.[4] In the initial method development experiments, after 77 h exposure to LD, the petiole was cut close to the base (Figure 1 (a)). Up to 7 leaves was then arranged and soaked into 10 mM EDTA pH 7.0 (Figure 1 (b)) overnight in LD condition. The collected exudates were then frozen in liquid nitrogen, concentrated and stored at -20°C prior to use. Later experiments were conducted with a slight modification. The leaves were first immersed in 20 mM EDTA for 30 min in LD and later transferred to a fresh solution for overnight exudation, the exudates were later ultracentrifuged at 100,000 rpm for 1 h at 4°C before being purified and concentrated.



(a) (b)

Figure 1 Mature and healthy leaves of 6 weeks old plants were used for exudation where (a) it is cut at the base of the petiole and (b) up to 7 leaves were located into 1.5 mL tube filled with 20 mM EDTA

For optimization purposes, these parameters were explored; 1) comparison of exudates from two growth conditions, 2) concentration of EDTA (10 mM and 20 mM), 3) incubation period (3 h and overnight), 4) LD exposure (2 days and 3 days), 5) Incubation condition (SD and LD), 6) plants' age (6 weeks and 7 weeks) and 7) purification method (acetone precipitation and using Amicon filter device [Millipore]).

Prior to SDS-PAGE analysis, Bradford assay was performed to determine protein concentration against BSA as standard. Quality of protein was examined by running 5 µg of protein on 18% SDS-PAGE at 150 V. The gel was later stained in Coomassie stain followed by destaining in 30:10 methanol: acetic acid and distilled water. For silver staining, the gel was fixed with 40:10 methanol: acetic acid, washed in 30% ethanol, soaked in reductant solution and prior to silver stain, was washed in deionized water. After soaking the gel in silver stain, the gel was washed in deioized water before being left in developer solution until bands became visible followed by washing in deionized water and treatment with stop solution. The gels were later viewed under gel documentation system

3.0 RESULTS AND DISCUSSION

3.1 Establishing Exudation Technique

Optimization of phloem sap protein exudation was carried out on these parameters with varying result; 1) EDTA concentration (10 & 20 mM), 2) plant growth condition (SD & LD), 3) exposure to LD prior to exudation (2 & 3 days), 4) exudation incubation period (3 hours & overnight), 5) incubation condition (SD & LD) and 6) plants age (6 & 7 weeks). Sufficient amount of protein for further analysis were obtained from protein collected in 20 mM EDTA for overnight in LD condition from plants grown for 6 weeks in SD and exposed to LD for 3 days prior to exudation (Figure 2). These parameters were selected based on the quantity and quality of phloem sap protein obtained. Meanwhile, efficient protein precipitation was achieved using Amicon filter device as compared to acetone precipitation which causes protein degradation (Figure 3).

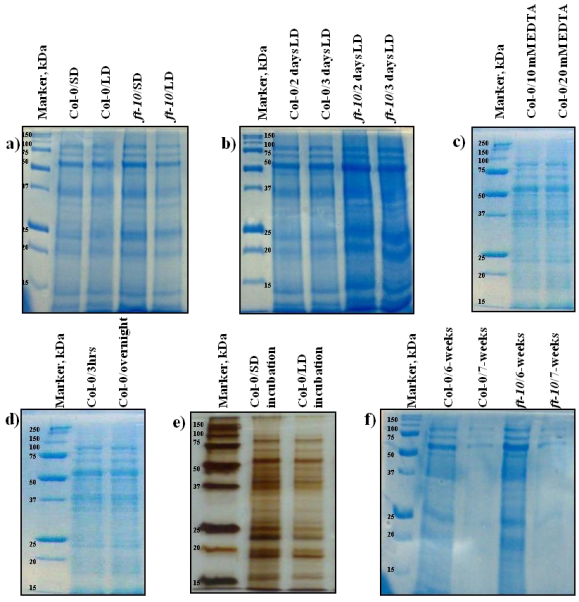


Figure 2 Optimization of *Arabidopsis* phloem sap protein under different parameters. (a) Protein from plants grown under full SD and plants exposed to LD 3 days prior to exudation, (b) protein from plants exposed to 2 days and 3 days LD prior to exudation, (c) protein collected in 10 mM and 20 mM EDTA, (d) protein from 3 h and overnight incubation, (e) protein incubated in SD and LD condition and (f) protein from 6-weeks and 7-weeks old plants. 5 µg of protein from each sample was loaded onto 17% SDS-PAGE and stained with coomassie blue or silver for (d). Both coomassie blue and silver stain were used for comparison of the protein bands as silver stain is known to have high sensitivity in detecting less abundant protein

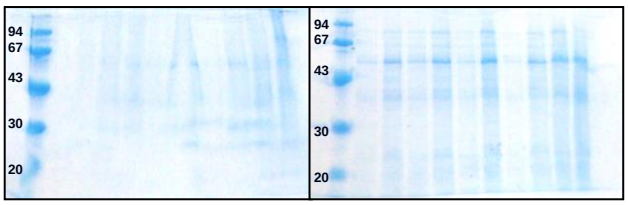


Figure 3 Protein purification using acetone precipitation (left) and Amicon filter (right). Equal amount of proteins (5 µg) were loaded onto each well and the marker sizes are in kDa

EDTA is a chelating agent that had been found able to form stable complexes with divalent cations. Chabarek and Martell (1959)[3] reported the efficiency of EDTA as a chelating agent decreased with decreasing pH when the pH was decreased from 7.0 to 4.4. EDTA binds divalent cations on an equimolar basis, resulting in freely available ions in the presence of a salt with greater concentration than EDTA. An excess of cation (Ca^{2+}) will lead to abolition.[3] Thus, EDTA was thought to permit continuous exudation by maintaining the phloem in an unblocked state in the tissue near the base of the cut petiole .[9]

In this study comparison was also made between two different concentration of EDTA; 10 and 20 mM. In terms of quantity, both concentrations produced similar amount of proteins from exudation based on quantification done using Bradford Assay (data not shown). Furthermore, the pattern of protein bands obtained via SDS-PAGE was identical indicating the quality was not affected by different concentrations of EDTA. In addition to EDTA concentration, exudation process itself is influenced by temperature, humidity and light intensity. Tully and Hanson (1979)[12] reported that leaf exudates must be mainly of phloem origin since they found evidence that rate of exudation is inhibited by cyanide and Ca^{2+}.

In the phloem, sieve tubes are equipped with defense mechanisms and once wound is inflicted, protein clogging and callose sealing are activated. However, as both mechanisms are calcium dependent,[14] the presence of calcium chelating agents such as ethylenediaminetetraacetate (EDTA) or ethylene-glycol-bis-(b-aminoethyl-ether) N, N9-tetraactetate (EGTA) decreases the abundance of free Ca^{2+} in the sieve elements[10] allowing collection of phloem exudate from excised stems.[9] Hence, EDTA enables collection of phloem sap from the wound at the petiole which would otherwise be sealed as part of the plant defense mechanism.

Exudation technique is a good method of obtaining phloem sap from *Arabidopsis*. It provides an alternative to stylet-cutting techniques[15] which may pose problems in terms of finding suitable insects, collecting sufficient amount of sample for analysis and especially during cutting of stylet. Furthermore, samples from stylet-cutting technique are only obtained from sieve elements accepted and fed by the aphids.[11,13]

4.0 CONCLUSION

A suitable exudation method utilizing EDTA to study *Arabidopsis* phloem proteome was developed by optimizing previous protocols used in other plants.[9,7] The optimization carried out to establish EDTA exudation technique produced quantifiable protein samples for further proteomics work. Similar pattern of bands were also reproduced on 1D SDS-PAGE.

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