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Purified isolation of vacuoles from *Sedum alfredii* leaf-derived protoplasts*

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This study aims to develop a method for isolating and purifying protoplasts/vacuoles from fresh leaves of the Cd hyperaccumulator plant species, *Sedum alfredii*. The results revealed that preheating cellulase and macerozyme at 50 °C for 5 min significantly accelerated the cell wall degradation. For the most optimal conditions for mesophyll protoplast isolation, the mixture of fresh leaves and cell lysates was followed by a 2-h–long vibration. The protoplast lysate for vacuole isolation was diluted, and 0.675 mmol/L was identified as the most appropriate 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS) level, in which *S. alfredii* large vacuoles are characterized by a high metal and malic acid content. For the best vacuole purification results, we established that 0.8 mol/L was the most optimal mannitol level in the vacuole buffer in terms of vacuole protection during centrifugation, whereas a Ficoll concentration of 0.10 g/ml was adopted in the density-gradient centrifugation.

Sedum alfredii (Crassulaceae) is a rare plant species that hyperaccumulates cadmium (Cd), which is native to China (Yang *et al*., 2004). A better understanding of mechanisms involved in Cd hyperaccumulation by *S. aflredii* may facilitate its usage in phytoremediation of Cd-polluted soils. Previous studies on *S. alfredii* revealed that Cd chelates with malic acid and mainly accumulates in the parenchyma cells consisting of large vacuoles (Tian *et al*., 2011). This strategy of Cd sequestration in *S. alfredii* differs from that employed by most other hyperaccumulators (Leitenmaier and Küpper, 2013), suggesting that vacuolar sequestration of Cd in parenchyma cells could be of great importance for Cd detoxification in shoots of *S. alfredii*. A better understanding of the processes involved in vacuolar sequestration of Cd would contribute to the knowledge on Cd hyperaccumulation mechanisms operating at cellular and subcellular levels in this plant species. Vacuole and protoplast isolation from plant tissues, however, is quite challenging in most cases. To date, purified isolation of vacuoles was successfully achieved in only a few plant species, including barley (*Hordeum vulgare* L. cv. Baraka), *Thlaspi caerulescens* and *Arabidopsis* (Shimaoka *et al*., 2004; Ma *et al*., 2005; Robert *et al*., 2007; Huang *et al*., 2012; Song *et al*., 2014). While these results are certainly beneficial, they cannot be generalized, as properties of cells from different plant species are not identical. As most available cases of vacuole isolation pertain to brassica family plants, the methods used are not applicable to *S. alfredii*, as its properties are distinct from those of brassica plants. Accordingly, we explored several improvements in this study, resulting in highly optimized methods for protoplast and vacuole isolation from the hyperaccumulator *S. alfredii*.

First, protoplast activities were improved by accelerating the cell hydrolysis processes. The results indicated that preheating cellulase and macerozyme in the cell lysate at 50 °C for 5 min significantly accelerated the cell wall degradation rate. In order to facilitate protoplast isolation, we also ripped apart the

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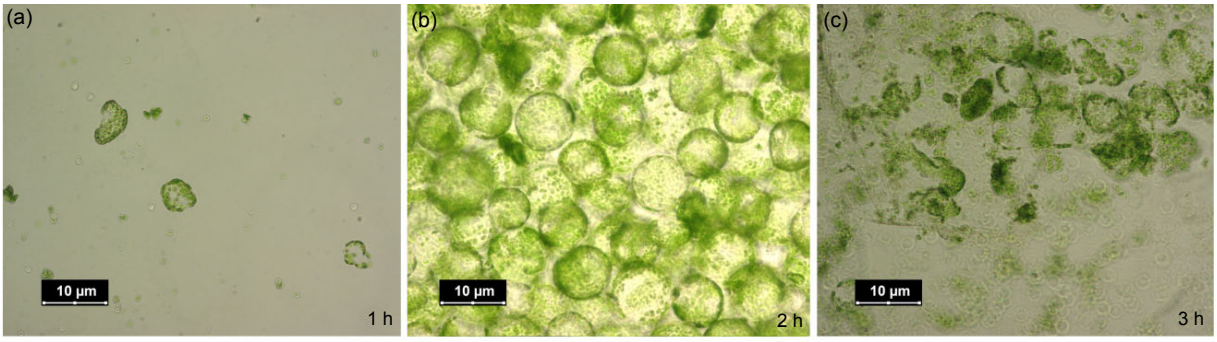

Fig. 1 Microscopic images of resulted protoplasts of *S. alfredii* after vibration
The leaves were mixed with the preheated cell lysate, and then vibrated for 1 (a), 2 (b), and 3 h (c), respectively

epidermis of leaves and sliced leaf tissues to increase the tissue surface area exposed to the cell lysate. Moreover, vibrating the plant tissue and cell lysate mixed solution can improve the contact between tissues and the enzyme solution, thus accelerating the release of the protoplasts from the tissues. Treatments of three different durations (1, 2, and 3 h) were applied when vibrating the plant tissue and cell lysate mixed solutions in the incubator. The results showed that the 2-h vibration was the most optimal for isolating protoplasts from the plant tissue and cell lysate mixed solution (Fig. 1). After being centrifuged twice at 80*g* and 10 °C, suspensions containing a high concentration of protoplasts with the best quality can be obtained (Fig. 2).

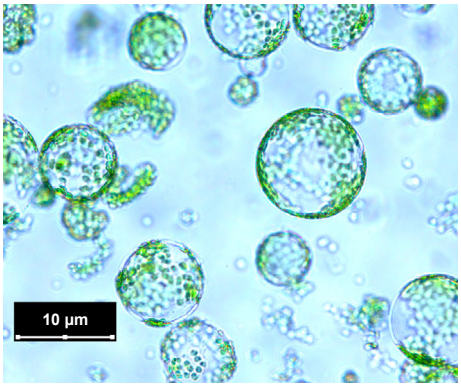

Fig. 2 Microscopic image of resulted protoplasts from young leaves of *S. alfredii* after centrifugation
The *S. alfredii* leaves were mixed with the preheated cell lysate, and then vibrated for 2 h. To get rid of the residues in the mixed solution, the mixed solution was centrifuged twice at 80*g* and 10 °C

To accelerate the vacuole isolation, we optimized the CHAPS concentration, a substance used to break down cell membrane structures (Schuck *et al*., 2003). Three different CHAPS levels (0.450, 0.675, and 0.900 mmol/L) in protoplast lysate were applied to release the vacuoles from the protoplast. The results revealed that the CHAPS concentration of 0.675 mmol/L was the best for obtaining a sufficient number of intact vacuoles from protoplasts. After being isolated by protoplast lysate, the vacuoles should be suspended in a specific buffer to remove CHAPS for their membrane protection. An appropriate concentration of mannitol in vacuole buffer is important to avoid damage during centrifugation. We tried a range of mannitol concentrations (0.5 to 1.0 mol/L), and indicated 0.8 mol/L mannitol as the optimal choice for vacuole purification of *S. alfredii*. In order to purify the vacuoles effectively, another major change was conducted as compared to the previous method (Ma *et al*., 2005). During vacuole purification, Ficoll was used as the medium of density-gradient centrifugation. We investigated the effects of three Ficoll concentration treatments (0.05, 0.10, and 0.15 g/ml) on vacuole isolation during centrifugation. The findings revealed that many intact protoplasts (green) and the cell residues were aggregated in the 0.05 g/ml Ficoll concentration treatment group (Fig. 3a). In the 0.15 g/ml Ficoll group (Fig. 3c), some protoplasts could still be observed and the vacuoles were not purified completely. However, purified vacuoles (red) were clearly observed under the microscope in the 0.10 g/ml Ficoll group (Fig. 3b). Therefore, a Ficoll concentration of 0.10 g/ml is the most optimal choice and should be adopted for vacuole purification in the density-gradient centrifugation.

In conclusion, the present study provides an optimized method of isolating protoplasts and vacuoles

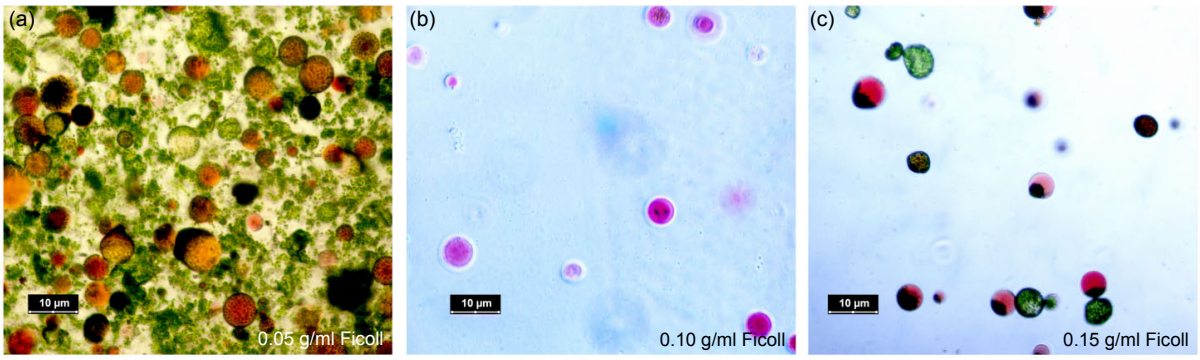

Fig. 3 Microscopic images of vacuoles after being centrifuged at 1500*g* and 10 °C for 5 min

To purify the vacuole solution, 0.05 (a), 0.10 (b), and 0.15 g/ml (c) Ficoll were added to the bottom of the vacuole solution. The purified vacuoles were collected from the Ficoll layer after centrifugation. The vacuoles were marked using a neutral red (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

from *S. alfredii* leaves. The isolated protoplasts and vacuoles can be used for physiological and biological investigations of metal sequestration in the hyperaccumulator *S. alfredii* at the cellular or subcellular level.

Materials and methods

Plant culture

S. alfredii seeds were collected from an old Pb/Zn mine area in Zhejiang, China, and were germinated on a mixture of perlite and vermiculite moistened with deionized water. Four weeks after germination, plants were subjected to 4 d exposure to 1/4, 1/2, or full strength nutrient solutions according to Tian *et al*. (2011).

Protoplast isolation

After a 60-d preculture period, fresh leaves were cut from the plants of *S. alfredii*, resulting in 5.0 g of leaf samples. The epidermis of the fresh leaves was ripped apart. In order to determine the effect of preheating on cell hydrolysis, the leaf slices were mixed with a cell lysate containing 15 g/L cellulase, 4 g/L macerozyme, 0.4 mol/L mannitol, 20 mmol/L KCl, 20 mmol/L 2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mmol/L $CaCl_2$, and 5 mmol/L β-mercaptoethanol. They were separated into two groups, one of which was preheated at 50 °C for 5 min. Protoplast concentration was obtained. The isolated protoplasts were observed using a microscope (NIKON-ECLIPSE-E600).

Vacuole isolation

For this stage of our study, we used the protoplast lysate containing 0.5 mol/L mannitol, 0.5 mmol/L ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*',*N*' tetraacetic acid (EGTA), 0.5 mmol/L CHAPS, 20 mmol/L MES, 125 mmol/L $CaCl_2$, 5 mmol/L KCl, and pH 8.0, as reported previously (Yang *et al*., 2004; Ma *et al*., 2005; Robert *et al*., 2007). To determine the concentration of the protoplast lysate that yields the vacuoles with the most optimal characteristics, protoplast lysate was diluted with deionized water at different ratios (protoplast lysate/water, 9:1 and 4:1 (v/v)). Next, 15 ml of the diluted protoplast lysates were added to three protoplast suspensions, together with a 75 μl neutral red solution (3.46 mmol/L neutral red, 0.33 μmol/L acetic acid, 0.05% chloroform) used to dye the vacuoles. The protoplast lysis processes and the released vacuoles were observed under a microscope. After vacuole isolation for 30 min, the above solution was centrifuged at 600*g* and 10 °C for 3 min, whereby the supernatant was gently removed. Next, 15 ml of vacuole buffer containing 20 mmol/L MES, 113.63 mmol/L $CaCl_2$, 4.55 mmol/L KCl, pH 8.0, and different mannitol levels (0.5, 0.6, 0.8, and 1.0 mmol/L) was added to the solution in order to resuspend the isolated vacuoles. The solution was once again centrifuged at 600*g* and 10 °C for 3 min to completely remove EGTA and CHAPS. In order to purify the vacuole solution, 15 ml of 0.05, 0.10, and 0.15 g/ml Ficoll solutions, containing 20 mmol/L MES, 0.8 mol/L mannitol, 113.63 mmol/L $CaCl_2$, 4.55 mmol/L KCl, and pH 8.0, were added to the bottom part of the

vacuole solutions derived from the previous centrifugations, respectively. The vacuole solutions were centrifuged at 1500*g* and 10 °C for 5 min (Thermo-S00264).

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Compliance with ethics guidelines

Xiao-yu GAO, Xing-cheng LIAO, Ruo-lai WU, Ting LIU, Hai-xing WANG, and Ling-li LU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：东南景天叶肉细胞原生质体和液泡的分离与纯化技术

目　的：在超积累植物东南景天对镉的区隔化过程中，叶肉细胞等内含大型液泡的薄壁细胞起重要作用。本文旨在建立并优化其叶肉细胞原生质体和液泡的提取和纯化技术，在技术层面上为东南景天的镉区隔化机理研究奠定基础，有助于深入探明其超积累镉的生理与分子机理。

创新点：优化了东南景天叶片原生质体的提取和纯化技术，并建立了能较高效率获得膜完整性好、数量多、纯度高的液泡提取方法。

方　法：主要包括原生质体提取、液泡粗提和液泡纯化。原生质体提取：取东南景天叶片，切成 1~2 mm 的细条状后浸入经预热过的细胞裂解液中，震荡 2 h 后过滤，离心清洗后获得原生质体。液泡粗提：采用 1-丙磺酸浓度为 0.675 mmol/L 的原生质体裂解液裂解原生质体，离心后获得粗提的液泡，并加入含 0.8 mol/L 甘露醇的液泡保护液。液泡纯化：往初提液泡的悬浮液下层加入质量体积比浓度为 0.10 g/ml 的 Ficoll 溶液，进行密度梯度离心，获取纯化的液泡。

结　论：细胞裂解液的预热处理可加速细胞壁降解，裂解时间设置为 2 h 有利于原生质体的高效提取；通过对原生质体裂解液浓度、细胞保护液浓度和梯度离心等参数的改良，可有效提取叶片细胞原生质体中的液泡。

关键词：超积累植物；东南景天；原生质体；液泡；提取与纯化