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RESEARCH NOTE

Optimized protoplast isolation and establishment of transient gene expression system for the Antarctic flowering plant *Colobanthus quitensis* (Kunth) Bartl.

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Abstract

Colobanthus quitensis is one of two terrestrial plants that grow in the maritime Antarctic. Despite its important ecological niche in extreme environments, the molecular mechanisms of its adaptation and tolerance have not been elucidated due to difficulties with genetic or transgenic approaches. For this reason, in many other plant species mesophyll protoplasts as a versatile cell-based system have been developed and used to analyze the biological functions of genes of interest. Here we report an optimized method of protoplast isolation from *C. quitensis* leaves. The main parameters evaluated to reach the highest protoplast yield were the use of a cell wall-degrading enzyme, an osmotic stabilizer, and different pH conditions. Moreover, transient expression and subcellular localization of proteins were validated by an immunoblot assay and a confocal microscopy, respectively, using *C. quitensis* protoplasts. Therefore, these results suggest that protoplasts can provide a useful cell-based system to facilitate the molecular, biochemical, and cellular characterizations of *C. quitensis* genes.

Key message

C. quitensis protoplasts can provide a physiologically relevant cell system to facilitate the molecular, biochemical, and cellular characterization of *C. quitensis* genes.

Keywords Cell-based assay · Low temperature · Molecular adaptation · PEG–$CaCl_2$-mediated transfection · Subcellular localization

The Antarctic continent is well known for exhibiting extreme environments with the coldest, windiest, and driest conditions year-around (Cavieres et al. 2016). Unlike a wide range of species diversity of the Arctic flora (Grundt et al. 2006), only two species of vascular plant, including the Antarctic hair grass *Deschampsia antarctica* Desv. (Poaceae) and the Antarctic pearlwort *Colobanthus quitensis* (Kunth.) Bartl. (Caryophyllaceae), are found and naturally colonized in the maritime Antarctic site (Smith 2003). Because the relatively high diversity of other plant species, such as mosses, liverworts, lichens, and fungi, is revealed in the Antarctic zones (Singh et al. 2018), these facts suggest that the Antarctic vascular plants may have unique characteristics that have allowed them to gradually adapt to such extreme conditions (Cavieres et al. 2016).

Colobanthus quitensis is a cushion-forming perennial plant and has been extensively studied to investigate the adaptation mechanisms via morphological, physiological, and biochemical approaches (Pérez-Torres et al. 2004; Zúñiga et al. 2009; Zúñiga-Feest et al. 2009; Sáez et al. 2017). In addition, the recent advances via 'omics' approaches increase the genomics results. For instance, the

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complete sequence of *C. quitensis* chloroplast genome has been recently provided (Kang et al. 2016), and the genome organization and contents were compared with other chloroplast genomes belonging to Caryophyllaceae (Androsiuk et al. 2018). Moreover, a recent comparative transcriptome analysis using an RNA-seq method showed the reduced expression of thermomorphogenesis-related genes in the maritime Antarctic *C. quitensis* plant, suggesting that it is closely related to the plastic formation of a cushion-like phenotype that is suitable for the Antarctic environment (Cho et al. 2018).

For understanding gradual adaptations of the Antarctic vascular plants, researches based on recent advances in molecular and biochemical approaches should be comprehensively studied with physiological, morphological, and ecological consequences. However, the bottleneck for this process is the lack of functional analysis methods for the myriad genes obtained from "omics" approaches. The Antarctic plants are self-fertilized in the natural habitat and colonized through seed dispersal with the slow vegetative growth in extreme environments. Therefore, it seems that it is not an easy process to collect materials on field sites for functional analysis at the molecular level. For this reason, the Antarctic plants are widely maintained through in vitro propagation or soils in a growth chamber (Pérez-Torres et al. 2004; Zúñiga et al. 2009; Zúñiga-Feest et al. 2009; Cho et al. 2018). Although it has been reported that plants developed from in vitro propagation did not produce viable seeds (Zúñiga et al. 2009), the methodology for reproductive growth and propagation of *C. quitensis* has been recently improved under controlled, limited conditions to study germination ability and the correlation between morphological variation and genetic structure (Cuba-Díaz et al. 2017; Sanhueza et al. 2017). Nevertheless, since *C. quitensis* has a limitation as a non-model plant, it is probably hard to identify the useful functions of novel genes by applying genetic techniques such as transformation methods commonly used in plant genetics, suggesting that a new method for the validation of gene function is practically needed and it is urgent to establish a cell-based approach as a feasible method.

Since the first isolation of mesophyll protoplasts from plant tissues, a versatile cell system via DNA transfection has been developed and used for molecular and cellular studies, such as the subcellular localization of proteins, protein–protein interactions, transcriptional activities, signal transduction, and gene silencing, to systematically determine functional characteristics of genes of interest (Yoo et al. 2007; Burris et al. 2016). Heterologous transient expression system using mesophyll protoplasts isolated from model plant *Arabidopsis* (Yoo et al. 2007) has been commonly used to investigate the biological functions of genes of interest in valuable non-model plants such as *C. quitensis*. However, because these heterologous systems often provide inaccurate information due to the different genetic background (Zhang et al. 2011), new methods for such plants are perpetually needed; to this end, optimized methods for protoplast isolation have recently been established for many non-model plant species, such as *Liriodendron* (Huo et al. 2017), cassava (Wu et al. 2017), and switchgrass (Burris et al. 2016). Although protoplast isolation from the Antarctic sea ice alga *Chlamydomonas* sp. ICE-L was also reported (Liu et al. 2006), no methodological study has used protoplasts of Antarctic vascular plants.

To isolate mesophyll protoplasts from fully differentiated *C. quitensis* leaves (Fig. 1a), various enzyme cocktails were empirically applied to digest plant cell walls that possess rigid polysaccharide compounds such as cellulose, hemicellulose, and pectin. Although different concentrations and proportions of widely used Cellulase RS and Macerozyme R-10 (Table 1, No. 1–3) were tested, the protoplast yield unexpectedly showed a relatively low range of 10^3–10^4 protoplasts per gram of fresh weight (g FW), indicating that *C. quitensis* likely requires further optimization of enzyme cocktails. For this, we additionally employed a different cellulolytic enzyme called Viscozyme. Applying a proper combination of this and two other digestive enzymes with a relatively short digestion time (3 h) led to a significantly increased yield of protoplasts, namely, $5.8 \pm 0.8 \times 10^5$ protoplasts/g FW (one-way ANOVA test, $p < 0.05$) (Fig. 1b; Table 1, No. 4). Longer incubations, such as 6 h or 12 h, for enzyme digestion decreased protoplast yield, viability or both (Supplementary Fig. S1).

Next, based on the important role of an osmotic stabilizer such as mannitol in the isolation and maintenance of viable protoplasts, we examined whether different concentrations (0.4, 0.5, 0.6, or 0.7 M) of mannitol would affect protoplast

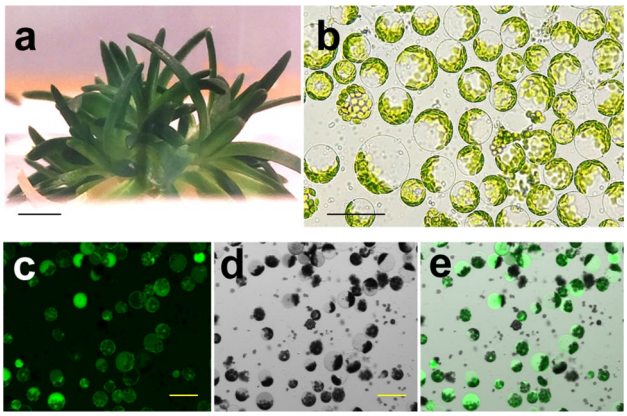

Fig. 1 Protoplast isolation from *C. quitensis* leaves. **a** A plant used in this study. Bar, 2 mm. **b** Isolated protoplasts from *C. quitensis* leaves. **c**–**e** Viability assay of isolated protoplasts. Protoplasts were treated by 19.2 µM of fluorescein diacetate (FDA). FDA-signal (**c**), Differential interference contrast (DIC) (**d**) and merged images of FDA-signal and DIC (**e**). Bars, 50 µm (**b**–**e**)

Table 1 Effects of important parameters, such as enzyme cocktails, mannitol, and pH conditions, on the yield of protoplasts isolated from leaves of *Colobanthus quitensis*

No	Cellulase RS (%)	Macerozyme R-10 (%)	Viscozyme (%)	Mannitol (M)	pH	Protoplast yield (mean ± SD × 10^5/gFW)	Viability (%)
1	1.5	0.3	–	0.5	5.7	0.036 ± 0.019^{g}	91.34 ± 9.87
2	2.0	0.6	–	0.5	5.7	0.077 ± 0.029^{g}	93.79 ± 6.83
3	3.0	1.2	–	0.5	5.7	0.12 ± 0.04^{g}	94.78 ± 5.59
4	3.0	1.2	1.5	0.5	5.7	5.8 ± 0.8^{b}	89.00 ± 5.40
5	3.0	1.2	1.5	0.4	5.7	4.6 ± 0.3^{cd}	85.64 ± 5.88
6	3.0	1.2	1.5	0.6	5.7	3.2 ± 0.5^{e}	93.29 ± 7.22
7	3.0	1.2	1.5	0.7	5.7	2.6 ± 0.2^{e}	92.14 ± 4.92
8	3.0	1.2	1.5	0.5	4.0	8.7 ± 0.5^{a}	91.50 ± 5.82
9	3.0	1.2	1.5	0.5	5.0	5.2 ± 0.6^{bc}	91.10 ± 5.97
10	3.0	1.2	1.5	0.5	6.0	4.2 ± 0.3^{d}	87.12 ± 8.27
11	3.0	1.2	1.5	0.5	7.0	1.7 ± 0.2^{f}	93.35 ± 7.30

Enzyme digestion was performed for 3 h with each enzyme cocktail. SD, standard deviation ($n = 7$–9); Multiple comparisons among different enzyme cocktails were analyzed by one-way ANOVA followed by post hoc Tukey HSD test. Different letters indicate significant differences ($p < 0.05$) according to Tukey HSD test. Pairwise comparisons of yield and viability among different conditions of enzyme cocktails were presented at Supplementary Table S1 and S2, respectively

yield (Table 1, No. 4–7). Among them, the highest yield of protoplasts was found at a concentration of 0.5 M mannitol. Moreover, to decide the optimum pH for digestion enzymes, we isolated and counted protoplasts under different pH levels (Table 1, No. 8–11; Supplementary Fig. S2). Indeed, the best protoplast yield ($8.7 \pm 0.5 \times 10^5$ protoplasts/g FW; one-way ANOVA test, $p < 0.05$) was obtained at pH 4.0 buffered by MES applying the aforementioned enzyme cocktail and mannitol conditions (Table 1, No. 8), whereas the efficiency of protoplast yield was gradually decreased toward higher pH levels (Table 1, No. 8–11; Supplementary Fig. S2A). The pH effect on protoplast yield was consistently observed under the condition without Viscozyme in the enzyme cocktail (Supplementary Fig. S2B), while viability was not changed by different pH levels (Supplementary Fig. S2C). Although optimal pH conditions can vary depending on the composition of the particular enzyme mixture, acidic pH tends to maintain integrity of protoplasts in the enzymatic digestion process (Lung et al. 2011). The viability of isolated protoplasts was almost 90% regardless of the composition in enzyme cocktails, as determined by fluorescein diacetate staining (Fig. 1c–e and Table 1). Taken together, these optimized conditions for the isolation of viable protoplasts provide a sufficient methodological resource for performing a variety of experiments with *C. quitensis* genes via a transient expression system.

To examine the possibility of creating a transient expression system, we practically transfected six *C. quitensis* orthologous genes (see Supplementary Materials and Methods) into *C. quitensis* protoplasts. In our protoplast system, protein expression of all genes was sufficient after 6 h incubation at room temperature. Because we could observe that independent transfection events were reproducible (Fig. 2 lanes 2–5) and expression levels of proteins varied depending on each gene (Fig. 2 lanes 2–9), these results indicate that protoplasts prepared from *C. quitensis* can act as a useful system for cell-based assays based on protein expression.

To address whether this system would be an also useful tool for studying the subcellular localization of proteins, we expressed green fluorescent protein (*GFP*) alone and *AtWRKY29*, *CqERF1*, *CqHVA22*, and *CqTSPO* fused with *GFP* in *C. quitensis* protoplasts. GFP alone showed its green fluorescent signals in the plasma membrane, cytoplasm, and nucleus (Fig. 3a), whereas the nuclear localization signal was clearly found in protoplasts transfected

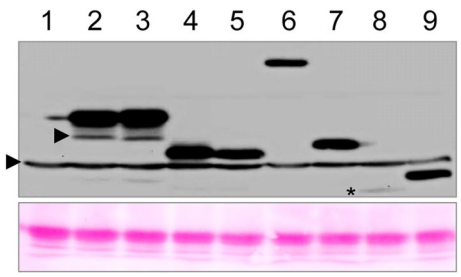

Fig. 2 Establishment of a transient gene expression system in *C. quitensis* protoplasts. Translational expression of human influenza hemagglutinin (HA) epitope-tagged proteins was detected by an anti-HA-Peroxidase antibody. Lane 1: Control expression of empty vector; lanes 2, 3: independent expression of CqMKK6; lanes 4, 5: independent expression of CqNPK1; lane 6: CqADC2; lane 7: CqERF1; lane 8: CqHVA22; lane 9: CqTSPO. Arrowheads indicate nonspecific expression. An asterisk indicates a lower level of protein expression. An equal amount of total proteins was loaded per lane (lower, Ponceau S staining)

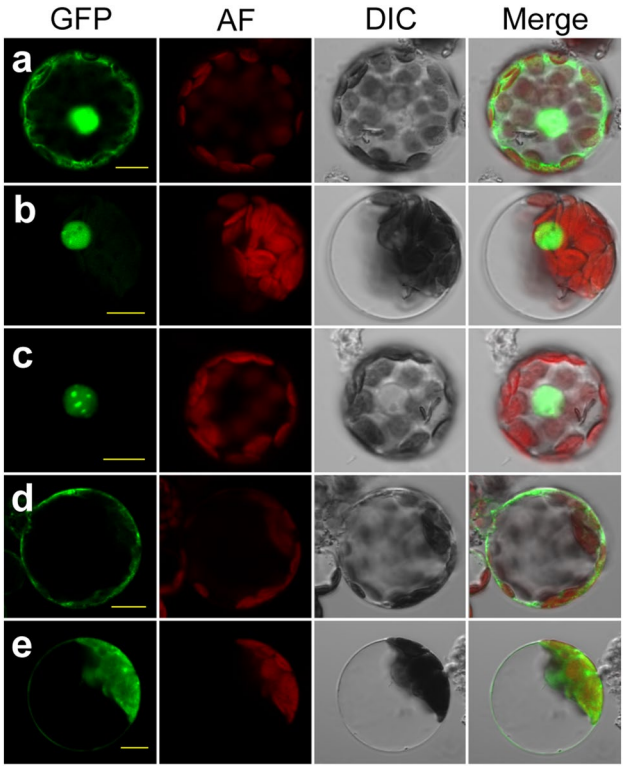

Fig. 3 Subcellular localization of *C. quitensis* GFP-fusion proteins using protoplasts. **a**–**e** Subcellular localization of proteins: GFP alone (**a**), AtWRKY29 (**b**), CqERF1 (**c**), CqHVA22 (**d**), and CqTSPO (**e**). GFP, green fluorescence protein. AF, chlorophyll autofluorescence. DIC, differential interference contrast. Merge, merge of three images. Bars, 10 µm (**a**–**e**)

by *AtWRKY29-GFP* (Fig. 3b), which is a key transcription factor involved in a defense signaling pathway of *Arabidopsis*. Furthermore, we also determined the subcellular localizations of orthologous proteins of *C. quitensis*. Similar to previous results obtained using other plant mesophyll protoplasts, the fluorescent signal of CqERF1-GFP was found in the nucleus (Fig. 3c), whereas CqHVA22-GFP and CqTSPO-GFP proteins were broadly distributed throughout the cytoplasm and plasma membrane (Fig. 3d, e). These results exhibited the first evidence of protein expression and subcellular localization of *C. quitensis* genes of interest, supporting the use of *C. quitensis* protoplasts as a tool for determining gene functions.

In this study, we established a simple, fast, and reproducible transient gene expression system for the Antarctic flowering plant *C. quitensis*. We also optimized the procedure of protoplast isolation through vigorous applications of important parameters such as digestion enzyme, mannitol, and pH conditions. Compared to well-established protoplast isolation techniques derived from *Arabidopsis* (Yoo et al. 2007), the relatively higher concentrations of Cellulase (3.0%) and Macerozyme (1.2%) with an additional digestion enzyme Viscozyme were required for obtaining the highest yield of protoplasts from *C. quitensis* (Table 1). Intriguingly, this is in line with a previous study that also required higher concentrations of digestion enzyme cocktails to isolate protoplasts from Antarctic sea ice algae (Liu et al. 2006). Different compositions of cell wall components often require newly improved, optimized combinations of digestive enzyme cocktails in protoplast isolation (Huo et al. 2017). For example, the commonly used combination of Cellulase and Macerozyme for model plants was revealed to be inefficient to obtain reliable protoplasts from wax-enriched *Liriodendron* leaves, requiring improved combinations with additional digestive enzymes (Huo et al. 2017).

Using our optimized method, we first established a transient expression system in *C. quitensis*. Interestingly, because all proteins were expressed using the constitutive *35S* promoter-driven vector system as described previously (Chiu et al. 1996; Yoo et al. 2007) (Figs. 2 and 3), it demonstrates that the universal expression system used in model plants can also be applied to *C. quitensis* protoplasts. Furthermore, we examined the subcellular localization of orthologous proteins of *C. quitensis*. Based on previous reports (Cheng et al. 2013), CqERF1 encoding a homologous *ERF1* transcription factor in *Arabidopsis* was clearly localized in the nucleus (Fig. 3c), but not CqHVA22 and CqTSPO (Fig. 3d, e). HVA22 is known to be an abscisic acid/stress-induced protein and it is first isolated from barley aleurone cells. Previous report provides that HVA22 is localized in an endoplasmic reticulum (ER) and Golgi organelles to inhibit vesicular trafficking involved in gibberellin-induced vacuolation under abiotic stress conditions (Guo and Ho 2008). Moreover, TSPO protein as a multi-stress regulator has known to physically interact with aquaporin at the ER and Golgi membrane to reduce the level of aquaporin in the plasma membrane (Hachez et al. 2014). Consistently, the subcellular localization of CqHVA22 and CqTSPO in *C. quitensis* protoplasts were found at the cytosol and the plasma membrane without the nucleus (Fig. 3d, e). In Fig. 3b, we showed that the transient protein expression of AtWRKY29 was localized in the nucleus in *C. quitensis* protoplasts similar to that in *Arabidopsis* protoplasts (Asai et al. 2002). Therefore, these results suggest that heterologous analyses of genes of interest are also capable in *C. quitensis* protoplasts. However, in the case of CqERF1, because we observed several nuclear speckles in *C. quitensis* protoplasts (Fig. 3c) unlike the nuclear localization of AtERF1 in *Arabidopsis* protoplasts (Cheng et al. 2013), this supports the need of corresponding *C. quitensis* protoplasts for *C. quitensis* genes of interest.

In conclusion, our results demonstrate that *C. quitensis* protoplasts can be rapidly isolated and prepared from differentiated vegetative leaves, and used as a valuable system for functional study of *C. quitensis* genes via protein

expression and subcellular localization. This simple and efficient method using protoplasts will be very useful to elucidate gene functions involved in the environmental adaptation of Antarctic flowering plants.

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Author contributions JL and HL developed concept and supplied plant materials. JL, HSL, and HL designed the research. HL wrote the manuscript and OKC performed all experiments.

Data availability All sequence information of *C. quitensis* orthologous genes used in this study were submitted to GenBank under accession numbers MH0033823–MH0033828. All data are stored at Korea Polar Data Center (KPDC; http://kpdc.kopri.re.kr).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

Androsiuk P, Jastrzębski JP, Paukszto Ł, Okorski A, Pszczółkowska A, Chwedorzewska KJ, Koc J, Górecki R, Giełwanowska I (2018) The complete chloroplast genome of *Colobanthus apetalus* (Labill.) Druce: genome organization and comparison with related species. PeerJ 6:e4723

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature 415:977–983

Burris KP, Dlugosz EM, Collins AG, Stewart CN Jr, Lenaghan SC (2016) Development of a rapid, low-cost protoplast transfection system for switchgrass (*Panicum virgatum* L.). Plant Cell Rep 35:693–704

Cavieres LA, Sáez P, Sanhueza C, Sierra-almeida A, Rabert C, Corcuera LJ, Alberdi M, Bravo LA (2016) Ecophysiological traits of Antarctic vascular plants: their importance in the responses to climate change. Plant Ecol 217:343–358

Cheng MC, Liao PM, Kuo WW, Lin TP (2013) The *Arabidopsis ETHYLENE RESPONSE FACTOR1* regulates abiotic stress-responsive gene expression by binding to different cis-acting elements in response to different stress signals. Plant Physiol 162:1566–1582

Chiu WL, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr Biol 6:325–330

Cho SM, Lee H, Jo H, Lee H, Kang Y, Park H, Lee J (2018) Comparative transcriptome analysis of field- and chamber-grown samples of *Colobanthus quitensis* (Kunth) Bartl, an Antarctic flowering plant. Sci Rep 8:11049

Cuba-Díaz M, Klagges M, Fuentes-Lillo E, Cordero C, Acuña D, Opazo G, Troncoso-Castro JM (2017) Phenotypic variability and genetic differentiation in continental and island populations of *Colobanthus quitensis* (Caryophyllaceae: Antarctic pearlwort). Polar Biol 40:2397–2409

Grundt HH, Kjølner S, Borgen L, Rieseberg LH, Brochmann C (2006) High biological species diversity in the arctic flora. Proc Natl Acad Sci USA 103:972–975

Guo WJ, Ho THD (2008) An abscisic acid-induced protein, HVA22, inhibits gibberellin-mediated programmed cell death in cereal aleurone cells. Plant Physiol 147:1710–1722

Hachez C, Veljanovski V, Reinhardt H, Guillaumot D, Vanhee C, Chaumont F, Batoko H (2014) The *Arabidopsis* abiotic stress-induced TSPO-related protein reduces cell-surface expression of the aquaporin PIP2;7 through protein-protein interactions and autophagic degradation. Plant Cell 26:4974–4990

Huo A, Chen Z, Wang P, Yang L, Wang G, Wang D, Liao S, Cheng T, Chen J, Shi J (2017) Establishment of transient gene expression systems in protoplasts from *Liriodendron hybrid* mesophyll cells. PLoS ONE 12:e0172475

Kang Y, Lee H, Kim MK, Shin SC, Park H, Lee J (2016) The complete chloroplast genome of Antarctic pearlwort *Colobanthus quitensis* (Kunth) Bartl. Mitochondrial DNA Part A 27:4677–4678

Liu S, Liu C, Huang X, Chai Y, Cong B (2006) Optimization of parameters for isolation of protoplasts from the Antarctic sea ice alga *Chlamydomonas* sp. ICE-L. J Appl Phycol 18:783–786

Lung S-C, Yanagisawa M, Chuong SDX (2011) Protoplast isolation and transient gene expression in the single-cell C_4 species, *Bienertia sinuspersici*. Plant Cell Rep 30:473–484

Pérez-Torres E, Dinamarca J, Bravo LA, Corcuera LJ (2004) Responses of *Colobanthus quitensis* (Kunth) Bartl. to high light and low temperature. Polar Biol 27:183–189

Sáez PL, Bravo LA, Cavieres LA, Vallejos V, Sanhueza C, Font-Carrascosa M, Gil-Pelegrín E, Peguero-Pina JJ, Galmés J (2017) Photosynthetic limitations in two Antarctic vascular plants: importance of leaf anatomical traits and Rubisco kinetic parameters. J Exp Bot 68:2871–2883

Sanhueza C, Vallejos V, Cavieres LA, Saez P, Bravo LA, Corcuera LJ (2017) Growing temperature affects seed germination of the antarctic plant *Colobanthus quitensis* (Kunth) Bartl (Caryophyllaceae). Polar Biol 40:449–455

Singh J, Singh RP, Khare R (2018) Influence of climate change on Antarctic flora. Polar Sci 18:94–101

Smith RIL (2003) The enigma of *Colobanthus quitensis* and *Deschampsia antarctica* in Antarctica. In: Huiskes AHL, Gieskes WWC, Rocema J, Schorno RML, Van Der Vies SM, Wolff WJ (eds) Antarctic biology in a global context. Backhuys, Leiden, pp 234–239

Wu JZ, Liu Q, Geng XS, Li KM, Luo LJ, Liu JP (2017) Highly efficient mesophyll protoplast isolation and PEG-mediated transient gene expression for rapid and large-scale gene characterization in cassava (*Manihot esculenta* Crantz). BMC Biotechnol 17:29

Yoo SD, Cho YH, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2:1565–1572

Zhang Y, Su J, Duan S, Ao Y, Dai J, Liu J, Wang P, Li Y, Liu B, Feng D, Wang J, Wang H (2011) A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. Plant Methods 7:30

Zúñiga G, Zamora P, Ortega M, Obrecht A (2009) Micropropagation of Antarctic *Colobanthus quitensis*. Antarct Sci 21:149–150

Zúñiga-Feest A, Bascuñán-Godoy L, Reyes-Diaz M, Bravo LA, Corcuera LJ (2009) Is survival after ice encasement related with sugar distribution in organs of the Antarctic plants *Deschampsia Antarctica* Desv. (Poaceae) and *Colobanthus quitensis* (Kunth) Bartl. (Caryophyllaceae)? Polar Biol 32:583–591