# Catabolic reprogramming of Brassica rapa leaf mesophyll protoplasts during the isolation procedure.

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# Abstract

In this study, profiling, clustering, co-expression analysis, and KEGG pathway analysis of primary metabolites and transcriptomes of *Brassica rapa* leaves and leaf mesophyll protoplasts were performed. Primary metabolite profiling indicated that the metabolism of *B. rapa* protoplasts was converted to catabolism. The number of down-regulated differentially expressed genes (DEGs) increased during protoplast isolation. An analysis of GO overexpression revealed activation of genes involved in catabolism and immune system processes and inactivation of chloroplasts and photosynthetic genes. KEGG pathway analysis showed that the activation of autophagy and the proteasome accounted for senescence-associated proteolysis during protoplast isolation. It also revealed activation of the genes involved in endoplasmic reticulum stress and disease resistance responses during the first three hours of isolation. This suggests that elicitor receptor-mediated signal transduction stimulated the pathogen-associated molecular patterns (PAMPs), which triggered immunity.

There were more down-regulated than up-regulated transcription factors during the protoplast isolation. *AT5G39610* (*ANAC092*, *ATNAC2*) gene expression was significantly activated throughout the entire period of protoplast isolation. *ANAC092* was co-expressed with up-regulated *AT5G26340* (*STP13*), which encodes a protein with high affinity, i.e., hexose-specific/H+ symporter activity. *AT1G50030* encodes *TOR* (target of rapamycin) proteins, and the expression of *TOR* decreased during protoplast isolation. Ethylene and photosynthesis-related gene inhibition by glucose and sucrose promoted senescence-associated gene expression of protoplasts. Since a decrease in glucose down-regulates glucose *TOR* signaling, inactivated *TOR* signaling promotes catabolic reprogramming, senescence, and autophagy in protoplasts during protoplast isolation.

We concluded that the initialization of protoplasts requires the blocking of these complicated crosstalk signaling pathways.


# Introduction

Plant protoplasts can provide solutions for improving plants by either DNA transfer or genome editing (Yue et al. 2021). The removal of the plant cell wall allows for the introduction of foreign DNA, RNA, or proteins into protoplasts through either polyethylene glycol (PEG) treatment or electroporation (Reed and Bargmann 2021). Due to the single cell, protoplasts can potentially avoid chimerism in transformation. These characteristic properties provide the advantage of overuse of tissue and calluses for plant transformation.

Since Cocking developed the method for protoplast isolation by enzymatic digestion, protoplasts from many higher plant species and organs have been isolated (Cocking 1960; Roest and Gillissen 1989; Yue et al. 2021). However, most procedures in protoplast isolation and regeneration have been developed using an empirical approach (Davey et al. 2005). Previous studies attempted to optimize the physiological state of donor plants through the foliar appreciation of plant growth regulators, nutrients,

and cycloheximide (Cassells and Tamma 1987; Kaur-Sawhney et al. 1976); preconditioned plants under short periods of low intensity illumination (Shepard and Totten 1977); excised leaves placed in the dark {Johnson, 1981 #44}; and plants grown with calcium nitrate and supplementary light (Cassells and Cocker 1982). It is believed that protoplasts derived from developed organs retain the properties of the original organs (Yue et al. 2021). However, protoplasts are not simply cell wall-free replicates of the original cells, but undergo a lot of abiotic stress and complex metabolic modifications during their isolation procedure (De Marco and Roubelakis-Angelakis 1996). Several factors affect the isolation, culture, and regeneration of plants from protoplasts (Roest and Gillissen 1989; Davey et al. 2005).

There is little understanding of the nutritional and physiological states of leaf mesophyll protoplasts. An increase in RNase activity was accompanied by morphological instability and deterioration of oat leaf protoplasts (Altman et al. 1977). Leaf excision and wounding are prerequisites for leaf protoplast isolation and enhance the activity of senescence-inducing enzymes such as proteases, nucleases, and other hydrolases (Wyen et al. 1971; UDVARDY et al. 1969). An osmotic stress-induced increase in putrescine and a blockage of its conversion to higher polyamines accounts, in part, for the failure of cereal mesophyll protoplasts to divide consistently (Tiburcio et al. 1986). When *Brassica napus* leaves are wounded before the enzymatic digestion-induced senescence of protoplasts before their culture, the free endogenous putrescine, ethylene, and NADH–GDH activity of protoplasts increased (Watanabe et al. 1994). A previous study also showed that leaf protoplasts senesced during the culture, which may account for the recalcitrance of the *B. napus* leaf protoplasts (Watanabe et al. 1998). Propidium iodide (PI) and DAPI staining, transmission electron microscopy, and flow cytometry analysis detected the cleavage of nuclear DNA into oligonucleosomal fragments during the culture of *B. napus* leaf protoplasts. TUNEL also detected the cleavage of nuclear DNA into oligonucleosomal fragments on a southern hybridization membrane; nuclear DNA fragments labeled with DIG-dUTP at the free 3'-OH ends by terminal deoxynucleotide transferase (TdT) were used as a probe for southern hybridization (Watanabe et al. 2002a). These results suggest that senescence of *B. napus* with culture time is accompanied by apoptosis-like cell death.

However, *Petunia hybrida* leaf protoplasts increased their spermidine and spermine contents with little increase in ethylene emission during the culture (Watanabe et al. 2002b). Petunia leaf protoplasts can initiate cell division and form calluses. Their plating efficiency was 0.7% (Watanabe et al. 1992). The reasons why recalcitrant leaf protoplasts are unable to divide remain elusive. Leaf senescence and the associated cell death are developmentally programmed processes. Considering these previous studies, a key to overcoming the recalcitrance of protoplast initialization is to elucidate the underlying molecular mechanism of protoplast isolation-induced senescence. In the present study, we investigated changes in primary metabolites and transcriptome expression during protoplast isolation.

These data indicated that protoplast metabolism was converted to catabolism during the protoplast isolation. This catabolic reprograming supports previous studies in which protoplasts were already senesced at the time of isolation. The transcriptome expression analysis revealed the up-regulation of

genes involved in the pathogen defense immune system process and *TOR* signaling, which account for catabolic reprogramming and senescence during protoplast isolation.

# Materials And Methods

Plant Materials

Seeds of *B. rapa* cv. himehassen-chingensai were purchased from Nakahara Seed Product Co. Ltd. (Hukuoka, Japan). Plants were grown under a 14 h photoperiod at a photon fluence density of 50 μmol $m^{-2}$ $s^{-1}$ beneath LED tubes and under a day/night temperature cycle of 24/20°C.

Water-soluble metabolites and mRNA were extracted from *Brassica rapa* leaves as control and leaf mesophyll protoplasts; 3 h after treatment of cell wall digestion enzymes, water-soluble metabolites and mRNA were extracted; 6 h after the treatment, mRNA was extracted.

Protoplast isolation and purification

Protoplasts were prepared by incubating leaf strips in an enzyme solution containing 0.5% Cellulose Y-C (Kyowa Kasei), 0.05% Pectolyase Y-23 (Kyowa Kasei), 5 mM MES (pH 5.8), and 0.6 M sorbitol (Watanabe et al. 2002a). Cell debris was removed by floating the intact protoplasts on 30% (w/w) sucrose with 5 mM MES (pH 5.8) solution for RNA extraction. For the extraction of primary metabolites of protoplasts, 12% (w/v) Ficoll® PM400 (Sigma–Aldrich) in 118 mM KCl, 81.7 mM MgCl2, 8.50 mM CaCl2, and 5 mM MES (pH 5.8) was used for protoplast purification.

Preparation of RNA sequencing samples

The *B. rapa* leaves were immersed in 0.5% sodium hypochlorite and rinsed several times with sterilized distilled water. The sterilized *B. rapa* leaves were cut into narrow strips and incubated in a filter-sterilized enzyme solution containing 1.0% Cellulase Y-C (Kyowa Kasei, Osaka, Japan), 0.05% Pectolyase Y-23 (Kyowa Kasei, Osaka, Japan), 5 mM MES (pH 5.8), and 0.6 M sorbitol. Then, 3 h after incubation, excised leaves were filtered through nylon mesh and rinsed several times with sterilized distilled water. The leaves were placed on filter paper and dried. Then, 14 h after incubation, protoplasts were isolated as previously described (Watanabe et al. 2002a). The dried leaves and the isolated protoplasts ($5 \times 10^5$) were placed in Eppendorf tubes, frozen by liquid nitrogen, and stored in a -80 ℃ freezer until RNA extraction.

RNA extraction and RNA-sequencing

Two independent biological replicates were performed for the transcriptome analysis. Total RNA extraction from leaves and protoplasts was performed using the NucleoSpin RNA Plant (TAKARA BIO INC, Japan) according to the manufacturer's instructions. The quantity and quality of the extracted RNA were assayed by a 2100 Bioanalyzer and RNA6000 Nano kit (Agilent Technologies Japan, Ltd.). A total amount of 1 μg total RNA per sample was used as input material for RNA sequencing library preparations. Sequencing libraries were generated using a TruSeq Stranded mRNA library kit (Illumina

K.K.) following the instruction manual. Library quality was assessed with a 2100 Bioanalyzer DNA1000 kit and Kapa Library Quantification Kit (Roche). The libraries were subjected to 50-base single read sequencing using the Illumina HiSeq 2500 System, and 22–26 million reads were obtained for each sample. Clean reads were generated by removing reads containing adapter sequences and/or low quality sequences from the raw data using CLC Genomics Workbench v10.0.0 (QIAGEN).

STAR 2.5.3a (https://github.com/alexdobin/STAR) was used to align the reads against the *B. rapa* reference genome downloaded from the Ensembl genome database (ftp://ftp.ensemblgenomes.org/pub/plants/release-32/fasta/brassica_rapa/dna/Brassica_rapa.IVFCAASv1.dna.toplevel.fa). The transcriptome abundance count data were generated by htseq-count (Anders et al. 2014), and the TAIR ID of the annotation file was added (Brassica_rapa.IVFCAASv1.34.gff3). As there are many *B. rapa* genes that have the same TAIR ID, the duplicated *B. rapa* counts were summed as the TAIR ID counts. The read count data were normalized, and differentially expressed genes (DEGs) were identified with edgeR. Genes with a CPM value higher than 0.5 in at least two libraries are listed in Supplementary Table 1/Count.all.AT1.

All of the DEGs were annotated in the NCBI NR database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the Arabidopsis information resource (TAIR) website (https://www.arabidopsis.org/), and the DAVID bioinformatics resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp).

GO, KEGG Pathway, and co-expression analyses

The genes whose expression ratio increased more than 2-fold or decreased to less than 1/2 with FDR less than 0.01 were analyzed. GO and KEGG pathway analyses were performed by the clusterProfiler R package (Yu et al. 2012) using the above data. Cytoscape 3.9.0 software (cytoscape.org) with the GeneMANIA application was used to visualize co-expression networks.

Primary metabolite extraction and GC–TOF–MS analysis

Leaves and protoplasts ($n$ = 6, biological replicates) were immediately frozen in liquid nitrogen to quench enzymatic activity. After lyophilization, each sample was extracted with a concentration of 2.5 mg dry weight of tissues per 1 ml of extraction medium (methanol/chloroform/water [3:1:1 v/v/v]) containing 10 stable isotope reference compounds using a Retsch mixer mill MM310 at a frequency of 15 Hz for 10 min at 4°C. The extracts were evaporated to dryness in a Savant SPD2010 SpeedVac Concentrator (Thermo Electron Corporation, USA). For methoximation, 30 µl of methoxyamine hydrochloride (20 mg/ml in pyridine) was added to the sample. After 15 h of derivatization at room temperature, the sample was trimethylsilylated for 1 h using 30 µl of MSTFA with 1% TMCS at 37°C with shaking. Thirty microliters of *n*-heptane was added following silylation. All derivatization steps were performed in a vacuum glove box VSC-100 (Sanplatec, Japan) filled with 99.9995% (G3 grade) dry nitrogen.

Metabolites were analyzed according to the previously established methods (Kusano et al. 2007a) (Kusano et al. 2007b) by using a GC–TOF–MS system, which consisted of an Agilent 7890N gas

chromatograph (Agilent Technologies, Wilmington, USA) hyphenated with a Pegasus IV TOF mass spectrometer (LECO, USA). One microliter of each sample was injected in the splitless mode by a CTC CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) into an Agilent 7890N GC equipped with a 30 m × 0.25 mm inner diameter fused-silica capillary column with a chemically bound 0.25 µl film Rtx-5 Sil MS stationary phase (RESTEK, USA) for metabolite profiling. Helium gas (G1 grade) was used as the carrier gas at a constant flow rate of 1 ml/min. The temperature program for the metabolome analysis started with a 2 min isothermal step at 80°C and was followed by temperature ramping at 30°C to a final temperature of 320°C, which was maintained for 3.5 min. Data acquisition was performed on a Pegasus IV TOF MS with an acquisition rate of 30 spectra/s in the mass range of a mass-to-charge ratio of $m/z$ = 60–800. Alkane standard mixtures (C8–C20 and C21–C40) purchased from Sigma–Aldrich were used to calculate the retention index (RI).

The calculation of the signal intensity of each metabolite was performed by a method described previously (Kusano et al. 2007b). The extracted MS spectra were finally identified or annotated according to their RI and comparison with the reference mass spectra in our libraries in RIKEN CSRS. The data were normalized using the CCMN algorithm (Redestig et al. 2009). All raw data in the NetCDF format were pre-processed by MATLAB (Mathworks, Natick, MA, USA). Data processing was described by (Kusano et al. 2020).

Primary metabolite extraction for LC–MS–MS and determination of the content

Six biological replicates of leaves and protoplasts were used for extraction and analysis. The metabolites were extracted with 60°C 70% ethanol twice and rinsed with cold ethanol. The extract was evaporated by a vacuum evaporator (EYELA CVE-100, Japan) and dissolved in 500 µl ultra-pure water filtered through a 0.22 µm membrane. Determination of sugars (arabinose, fructose, galactose, and trehalose) and organic acids (3PGA, galacturonate, lactate, malonate, pyruvate, malate, OAA, and salicylate) was performed by LC–MS–MS (LCMS-8030, Shimadzu, Japan) with a normal phase TSKgel $NH_2$-100 column (2.0 mm X 15 cm, 3 µm particles, TOSOH, Japan) at a flow rate of 0.5 ml/min at 40°C. The sample was analyzed in a positive mode. Solvent A was 20 mM ammonium acetate in 95:5 (V/V) water/acetonitrile. Solvent B was acetonitrile. The gradient was t = 0, 85% B; t = 12, 0% B; t = 24, 0% B; t = 26, 85% B; t = 35, 85% B. The injection volume was set to 1 µl.

Mass spectrometry parameters were set to default and were determined through autotuning. The multiple reaction monitoring (MRM) mode was used in the metabolite analysis. The Q3 m/z of each targeted metabolite was determined based on the standard compounds. Supplementary Table 5_Target ions lists the precursor and Q3 m/z of productions.

The contents of ammonium ion, ascorbic acid, ethylene, dehydroascorbic acid, GSH, GSSG, salicylic acid, and spermine are referenced in our previous reports (Masami Watanabe et al. 2011; Watanabe et al. 2002b). The combined data were used for principal component analysis (PCA) and correlation analysis as $\log_2$ transformed data by R packages.

# Results

Normalization and clustering of RNA-Seq-based transcriptomes

A comparative statistical analysis of gene expression was performed with the edgeR program (v3.36.0) (Robinson et al. 2009). A total of 16386 differentially expressed genes (DEGs) were detected (Supplementary Table 1/ All_EdgeR.LogData). Of this total, 5083 were down-regulated (log2 <= -1) and 3872 were up-regulated (log2 > = 1) in the protoplasts. The remaining 7431 genes did not fluctuate.

Figure 1.1 shows a heat map of the DEGs of leaves, leaves 3 hours after the protoplast isolation procedure, and the protoplasts. The heat map analysis shows that the expression data of DEGs were almost identical to each duplicate sample. The DEG expression data were analyzed by principal component analysis (PCA). The PCA (Fig. 1.2) shows that the variability of gene expression data in leaves increased three hours after the protoplast isolation procedure (Leaf3h).

MultiExperiment Viewer (MeV, v4.8.1) is based on the k-means method and produced the hierarchical clustering analysis of the DEGs. The clustering analysis of co-regulated gene expression patterns was performed with MeV. The 16386 DEGs were clustered by MeV (v4.8.1) based on the k-means method (k = 9). Clustering of the DEG expression data by k-means cluster analysis (k = 9) showed a temporal change of the gene expression from leaves to protoplasts (Supplementary Fig. 1). The data for the six sampling points were clustered into nine groups. The cluster number and corresponding expression values for each DEG in the six sampling point clusters are shown in Supplementary Table 2.

Comparison between two different time points during the process of protoplast isolation

The number of genes with a CPM greater than 0.5 between the two samples was 20,286 (Supplementary Table 1/Count.all.AT1). Between the leaves three hours after the onset of the protoplast isolation procedure (Leaf3h) and the untreated leaves as controls (Leaf0h), 8223 DEGs with an FDR less than 0.01 were detected from 20286 genes (Supplementary Table 1/Leaf0h_Leaf3h_significant); 3741 genes were up-regulated and 4482 genes were down-regulated (Supplementary Table 1/Leaf0h_Leaf3h_induction/Leaf0h_Leaf3h_repression). A total of 4537 DEGs were found (Supplementary Table 1/Leaf3h_Protoplast_significant) between Protoplasts and Leaf3h; 2132 were up-regulated and 2405 were down-regulated (Supplementary Table 1/Leaf3h_Protoplast_induction/Leaf3h_Protoplast_repression). Moreover, 8734 DEGs were found between Protoplasts and controls (Leaf0h) (Supplementary Table 1/ Leaf0h__Protoplast_significant ); 3846 were up-regulated and 4888 were down-regulated (Supplementary Table 1/Leaf0h__Protoplast_induction/ Leaf0h__Protoplast_repression). These results indicated that there were more down-regulated DEGs than up-regulated ones during the protoplast isolation.

Figures 2.1a and b show Venn diagrams of the up- and down-regulated genes between the two treatments. The number of down-regulated genes was higher than the number of up-regulated genes in all treatments; 391 were up-regulated and 1164 were down-regulated. The most up-regulated gene in the

protoplasts was the serine protease inhibitor gene (AT5G43570: 11792.48-fold), followed by a peroxidase (AT2G38390: 9538.36-fold). The expression levels of the two genes in Leaf3h decreased 63.257-fold and 122.31-fold. A gene with significant down-regulation was an MYB transcription factor (AT5G07700, *MYB76*) that was down-regulated 0.0002595-fold in protoplasts and 0.009194-fold in Leaf3h.

GO over-representation analysis

The up- or down-regulated DEGs overlapping in the sample treatment of Leaf0h, Leaf3h, and Protoplast were analyzed through GO over-representation analysis by using a web application, PANTHER (http://geneontology.org) (Fig. 2.2). Supplementary Table 3 includes the data. The protoplast isolation procedures activated genes associated with catalytic activity (GO:0003824, molecular function category) and metabolic process (GO:0008152, biological process category), while the procedures also inactivated genes involved in metabolic process (GO:0008152, biological process category).

A Venn diagram was created from the GO analysis data. The four GOs overlapped in the three treatments, and one of the four GOs was related to ethylene. Transcription factor ATG5G39610 (*ANAC092*, *ATNAC2*) was found in the ethylene-related GO. DEGs were assigned to biological process (BP), cellular component (CC), and molecular function (MF) categories based on the enrichGO package of the R program.

As a result of the GO over-representation analysis by enrichGO, various transport systems in the BP category were enhanced in both Leaf3h and Protoplasts compared with Leaf0h (Fig. 3a). *STP13* (AT5G26340) encodes a protein with high affinity, i.e., hexose-specific/H + symporter activity. The study of the expression of this gene in PCD mutants demonstrated a tight correlation between this gene's expression and PCD (Norholm et al. 2006). The expression levels of this gene in Leaf3h and Protoplasts exhibited a 160.65-fold and 39.840-fold increase compared to Leaf0h, but decreased to 0.24799-fold in Protoplasts compared to Leaf3h. The protoplast isolation procedure led to enhanced expression of proteasome-related genes, as there were many DEGs involved in the proteasome assembly in the BP category in Protoplasts compared with Leaf0h and Leaf3h (Fig. 3a).

The gene expression associated with oxidative stress and fungus GO increased significantly (Fig. 3a). GO terms for endoplasmic reticulum (CC category) were enhanced in both Leaf3h and Protoplasts. Mitochondrial-related GO terms were also enhanced in Protoplasts (Fig. 3b). The results of the GO over-representation analysis for the MF category are presented in Supplementary Fig. 2. Gene groups associated with chloroplast and photosynthesis in BP and CC categories were repressed in both Leaf3h and Protoplasts compared with Leaf0h (Figs. 3c and d). When DEGs in Protoplasts were compared with DEGs in Leaf3h, GO terms of various transport systems to the nucleus were inhibited (Fig. 3c). The genes encoding protein kinases in the MF category were repressed in Protoplasts compared with Leaf0h (Supplementary Fig. 2).

DEGs Related to Senescence

Plant cell death is closely related to cellular senescence and photosynthesis. A total of 182 genes were listed by PODC (http://bioinf.mind.meiji.ac.jp/podc/) as senescence-associated genes (Supplementary Table 4). A total 64 genes were up-regulated and 40 were down-regulated in Leaf3h, whereas 68 and 38 genes were up- and down-regulated in protoplasts, respectively. When the gene expression of protoplasts was compared with Leaf3h, 52 genes were up- and down-regulated. These results suggest that the expression of senescence-associated genes was up-regulated relatively soon after the protoplast isolation treatment.

Temporal distribution of primary metabolites during protoplast isolation

Water-soluble primary metabolites measured by GC–MS (Supplementary Table 5/GC–MS normalized data) were analyzed by principal component analysis (Fig. 4.1). The analysis shows that the primary component separated metabolites of protoplasts from other leaf metabolites. Figure 4.2 shows a heat map and clustering analysis of log2 fold ratios between the two samples. The content of most metabolites decreased during the first 6 h of protoplast isolation and slightly increased from the intermediate point to the end of protoplast isolation. However, the metabolite content in protoplasts hardly recovered, except for proline.

Figure 4.3 shows correlation matrices of water-soluble primary metabolites extracted from leaves 3 and 6 h after the onset of protoplast isolation and from protoplasts. The metabolites with a high correlation coefficient are shown in red in the panels. There was a drastic metabolical conversion from 3 to 6 h, which then partially returned to the control (Leaf0h) level at the end of the protoplast isolation.

Sugar and organic acid contents were measured via LC–MS/MS to obtain more quantitative data (Table 1). The galactose, arabinose, fructose, glucose, and sucrose contents decreased in leaves 3 h after protoplast isolation but increased more than 10-fold in protoplasts. Organic acids measured in the LC–MS/MS experiments showed almost the same result as GC–MS data except for galacturonate, whose content increased in protoplasts.

These figures indicated that sucrose and amino acids increased in protoplasts compared with Leaf3h or Leaf6h. Considering these results and the increase in catabolic activity shown by the GO over-representation analysis (Fig. 2.2), the protoplast isolation procedures induced protein degradation.

Carbon and oxidative phosphorylation metabolic pathways based on KEGG pathway analysis

The expression profiles of all DEGs and the primary metabolites were color-coded in each pathway with expression data from the three time points in the following order: Leaf3h vs. Leaf0h, Protoplasts vs. Leaf3h, and Protoplasts vs. Leaf0h.

Figure 5.1 shows changes in sugar metabolic pathways during the process of protoplast isolation. DEGs involved in photosynthesis were down-regulated as in the GO over-representation analysis, but the process of protoplast isolation hardly affected the DEGs involved in oxidative phosphorylation (Supplementary Fig. 3). Most of the DEGs involved in the TCA cycle were up-regulated, as predicted by the

results of the GO over-representation analysis. The DEGs involved in the glycolysis and pentose phosphate pathways were up- or down-regulated during the process of protoplast isolation. The sucrose and glucose ratio of Leaf3h and Leaf0h decreased to 0.33 and 0.00, respectively, but increased to 13.74 and 24.28 at the end of the protoplast isolation.

The content of organic acids for TCA cycle components decreased during the first 3 h of the protoplast isolation procedure and slightly increased until the end of the protoplast isolation, but the content in protoplasts remained at a lower level than in the leaves.

DEGs involved in protein degradation machinery, plant–pathogen interaction, and plant hormone signal transduction

The GO over-representation analysis and primary metabolite analysis by GC–MS revealed that catabolic activity was enhanced throughout the protoplast isolation (Fig. 2.2, Fig. 4.2). Therefore, the KEGG pathway analyzed DEGs related to autophagy and the proteasome for protein degradation (Fig. 5.2). These results showed that most of the DEGs related to autophagy and the proteasome were significantly up-regulated, suggesting that a stimulus generated by the process of protoplast isolation triggered proteolysis. *AT4G01610* encodes a cathepsin B-like protease (*ATCATHB3*). The cathepsin gene expression increased throughout the protoplast isolation, i.e., 1.04-fold in Leaf3h and 2.30-fold in Protoplasts compared to Leaf0h as a control (Supplementary Table 1/All_EdgeR.LogData).

Since the GO over-representation analysis also revealed that the immune system process in the biological function category was up-regulated (Fig. 2.2), DEGs were analyzed by the KEGG pathway of plant–pathogen interaction (Fig. 5.3). The results shown in Fig. 2.2 and Fig. 5.3 indicate that elicitors initiated a cascade of defense response events and senescence-related syndromes, such as activation of catabolism and proteolysis.

Gene expressions of the chitin elicitor receptor, *CERK1*, bacterial flagellar protein (flg22) receptor, *FLS2*, bacterial translational elongation factor (elf18) receptor, EFR, and another pattern-recognition receptor, *SERK4*, were up-regulated during the first 3 h of protoplast isolation and decreased or remained constant thereafter. These elicitor receptor-mediated signal transductions stimulated the immunity triggered by pathogen-associated molecular patterns (PAMPs). Interestingly, hypersensitive response genes were down-regulated or remained constant except for the *CR88* gene. Up-regulation of *WRKY22* and *WRKY29* accounted for defense-related gene induction.

The KEGG pathway analysis assigned DEGs for plant hormone signal transduction (Fig. 5.4). These results indicated ethylene, jasmonic acid, and salicylic acid-activated *ESR2*, *JAZ5*, and *TGA9* gene expression during the first 3 h of protoplast isolation and at the end of protoplast isolation. These genes were the initial steps for endoplasmic reticulum stress responses, proteolysis, and disease resistance responses. The KEGG pathway analysis suggested that some unknown elicitors and plant growth substances promoted senescence-related syndromes and pathogen defense immune systems.

Transcriptome profiles of transcription factors

Transcription factors play essential roles in many biological aspects of life cycles. Supplementary Fig. 4 shows a heat map for the temporal distribution profile of differentially expressed transcription factor genes during the protoplast isolation. There were more down-regulated transcription factors than up-regulated ones. These results indicated that the protoplast isolation procedure triggered transcriptional reprogramming during the protoplast isolation.

The Plant TFDB (plant transcription factor database v4.0) lists 1726 transcription factor genes (Supplementary Table 6). The number of DEGs on the list was 1079. Leaf3h vs Leaf0h had genes with significantly up-regulated ($p < 0.05$) ERF, HFS, Myb, Trihelix, and WRKY families. Many other transcription genes showed no significant difference between Protoplasts and Leaf0h and suggested relatively early responses. On the other hand, the late-response TF families that significantly increased in Protoplast vs Leaf0h were ARF, bHLH, C2H2, CO-like, ERF, GRAS, Myb-related, MFY-A, TCP, YABYY, and the ZFHD family.

Two TF families (HSF and Trihelix) contained significantly up-regulated genes in Leaf3h, but not in the comparison between Protoplasts and Leaf0h. On the other hand, several TF families (C2H2, CO-like, GATA, GRAS, SBP, TCP, YABBY, and ZF-HD), containing genes whose expression was suppressed, were found in Leaf3h but not in the comparison between Protoplasts and controls. The TF families containing genes that were significantly suppressed in Leaf3h were b-HLH, C2H2, CO-like, G2-like, GATA, GRAS, HD-ZIP, SBP, TCP, YABBY, and the ZFHD family. Among these, there were many TF families that were highly expressed in Protoplasts. In other words, the gene expression of some TF families was once suppressed by Leaf3h but then restored in Protoplasts, but the expression was higher than that under Leaf0h.

A total of 391 up-regulated DEGs in the Venn diagram overlapped among Leaf0h, Leaf3h, and Protoplasts (Fig. 2.1). There were 26 transcription factors in these DEGs (Supplementary Table 1); five WRKY, six MYB, four MAC, and four ERF transcription factors accounted for more than 70% of the 26 up-regulated transcription factor genes that overlapped in the Venn diagram of Leaf0h, Leaf3h, and Protoplasts. Figures 6a, b, and c show the co-expression of these up-regulated transcription factor genes.

AT5G39610 (*ANAC092*, *ATNAC2*) encodes an NAC-domain transcription factor and positively regulates senescence-induced cell death and senescence in leaves. *ANAC092* gene expression was significantly activated throughout the entire period of protoplast isolation. There was a 277.97-fold increase in the leaves three hours after protoplast isolation (Leaf3h) and a 2442.40-fold increase in Protoplasts, compared with the control leaves (Leaf0h). Therefore, to explore the regulatory network administered by *ANAC092*, co-expression of *ANAC092* and other up-regulated genes extracted from the overlapping Venn diagram in Leaf0h, Leaf3h, and Protoplasts was analyzed by Cytoscape (cytoscape.org), which is an open-source software platform for visualizing complex networks (Figs. 6d, e and f). These figures indicate that the NAC and WRKY transcription factors were closely co-expressed during the process of protoplast isolation. Supplementary Fig. 5 shows the co-expression of *STP13* genes and transcription factor genes found as DEGs. *ANAC092* was co-expressed with *STP13* and was significantly up-regulated throughout the protoplast isolation.

*AT3G11020* (*DREB2B*) encodes a member of the DREB subfamily in A-2 of the ERF/AP2 transcription factor family, which includes four major subfamilies: the AP2, RAV, ERF, and dehydration-responsive element-binding protein (*DREB*) subfamilies (Sakuma et al. 2002). Plant abiotic stress-induced DREB subfamily members regulate gene expression via the cis-acting dehydration-responsive element/C-repeat (DRE/CRT) element. ERF subfamily members that bind to ethylene response elements (EREs) respond to abiotic stress. Co-expression analysis indicated that 120, 122, and 96 transcription factor genes that were either up- or down-regulated during the protoplast isolation were identified as the first neighbors of DREB2B (Supplementary Fig. 6).

The three overlapped treatments resulted in 1163 down-regulated DEGs (Fig. 2.1). There were 48 transcription factors, which amounted to about 4% in these overlapped treatments. In contrast to up-regulated transcription factors, only one ethylene-responsive factor (ERF), two MYB, and four bHLH transcription factors were found in the three overlapped treatments. Up-regulated DEGs in the three overlapped treatments did not indicate bHLH transcription factors.

The *AT1G50030*-encoded TOR (target of rapamycin) protein belongs to the family of phosphatidylinositol 3-kinases and is the target of the antiproliferative drug rapamycin. Figure 9 shows the co-expression of *TOR* and other first neighbor DEGs analyzed by Cytoscape and indicates that the expression of *TOR* decreased during the protoplast isolation. There was no transcription factors co-expressed with *TOR*. Almost all of these co-expressed genes were down-regulated. However, AT5G54620 encoding an ankyrin repeat-containing repeat protein was up-regulated during the early stage of protoplast isolation.

# Discussion

To use cell-wall-free protoplasts in the production of genetically modified plants, the protoplasts must be initialized before transferring useful genes and undergoing genome editing. The cell wall must be digested by cellulase and pectinase in a relatively hyperosmotic medium. Therefore, protoplasts are exposed to a great deal of stress during their isolation procedure.

Up- and down-regulated genes were counted during the first three hours of the protoplast isolation procedure. There were 3741 up-regulated genes and 4482 down-regulated genes (Supplementary Table 1/ Leaf0h_Leaf3h_induction/Leaf0h_Leaf3h_repression). The abundance of down-regulated genes means a decrease in viability. Therefore, the stresses generated by the early stage of protoplast isolation will play an essential role in the fate of subsequent protoplasts. There were more than 1000 down-regulated genes compared with up-regulated genes at the end of the protoplast isolation (Supplementary Table 1/Leaf0h__Protoplast_induction/Leaf0h__Protoplast_repression), which indicates that protoplast viability is reduced throughout the isolation procedure.

GO over-representation analysis using PANTHER (Fig. 2.2) showed that the protoplast isolation procedure activated genes involved in catabolic activity and metabolic process, while the procedure also inactivated genes involved in the metabolic process. Figures 3.2a and b show that many chloroplast and photosynthesis-related genes were repressed during the protoplast isolation. On the other hand, the

protoplast isolation procedure activated the expression of many mitochondrial electron transport-related genes (Supplementary Fig. 3). A decline in mitochondrial inner membrane potential triggers apoptosis in animals (Gottlieb et al. 2003; Hengartner 2000). However, the mitochondrial inner membrane potential of protoplasts was high immediately after isolation and decreased with culture time (Rajesh Kumar Tewari et al. 2012). These data suggest that chloroplasts, rather than mitochondria, are preferentially involved in signal transduction of senescence and the apoptotic-like cell death of protoplasts.

Numerous physiological and biochemical studies previously suggested the end-product inhibition of photosynthesis (Azcón-Bieto 1983; Blechschmidt-Schneider et al. 1989; Quick et al. 1989; Krapp et al. 1991; Jeannette et al. 2000). An inhibition of Calvin cycle enzymes and activation of glycolytic enzymes were attributed to photosynthesis inhibition (Stitt et al. 1991). Photosynthetic end products of sucrose and starch in maize mesophyll protoplasts specifically and coordinately repressed photosynthetic gene expression via their promoters (Sheen 1990). Jang et al. proposed a novel role of hexokinase (HK) as a putative sugar sensor and signal transmitter in higher plants (Jang and Sheen 1994; Jang et al. 1997; Xiao et al. 2000).

Leaf senescence seems to be induced by the accumulation of carbohydrates in leaves (Rolland et al. 2002; Yoshida 2003; Quirino et al. 2000). Metabolic profiles showed that leaves and protoplasts had different metabolisms (Fig. 4). The protoplast isolation stress triggered the induction of catabolic metabolism at 3 to 6 hours during the isolation procedure. The sucrose content in Protoplasts increased compared to Leaf0h, while the glucose content decreased during the first three hours of protoplast isolation (Fig. 5.1, Table 1). The gene expression of hexokinase (ATHKL1), known as a sugar signal (Jang et al. 1997), increased about 2.68-fold in Protoplasts. These data suggest that the photosynthetic product of sucrose promotes the senescence of protoplasts.

The NADPH content of *B. napus* protoplast chloroplasts increased more than 6-fold (Rajesh Kumar Tewari 2014). These data suggest that NADPH accumulation in protoplast chloroplasts results from the inactivation of the Calvin–Benson cycle in protoplasts. Because thiol enzymes in the Calvin–Benson cycle are susceptible to ROS (Foyer 2018; Wise 1995), it is quite possible to inhibit the Calvin–Benson cycle under the stressful protoplast isolation procedure.

Consequently, glucose decreased during the early stage of the protoplast isolation procedure. Glucose that is not used for catabolism in protoplasts is synthesized into sucrose. Since sucrose is not loaded onto phloem, protoplasts will accumulate sucrose.

*STP13* (*AT5G26340*) encodes a protein with high affinity, i.e., hexose-specific/H + symporter activity. The study of the expression of this gene in programmed cell death (PCD) mutants demonstrated a tight correlation between the *STP13* gene's expression and PCD (Norholm et al. 2006). Compared to Leaf0h, this gene's expression significantly increased during the early stages of protoplast isolation: 160.65-fold in Leaf3h and 39.84-fold in Protoplasts. *STP13* was up-regulated in cell suspension cultures undergoing PCD (Swidzinski et al. 2002), senescing leaves (Buchanan-Wollaston et al. 2005), and in the two- to six-

leaf stage in the accelerated cell death 11 (acd11) mutant that develops cotyledons normally but subsequently undergoes spontaneous PCD (Brodersen et al. 2002).

Up-regulation of *STP13* was also observed in Arabidopsis plants treated with fungal toxin fumonisin B1 (FB1) and pathogen *Pseudomonas syringae* (Norholm et al. 2006). Our previous study reported that ROS were released outside of protoplasts and also accumulated in the chloroplasts of protoplasts (Rajesh Kumar Tewari 2014; Rajesh Kumar Tewari et al. 2012; Yasuda et al. 2007). These previous results also indicated that unknown elicitors accounted for the up-regulation of *STP13* and accumulation of ROS in protoplasts during protoplast isolation.

In *Arabidopsis thaliana*, *ANAC029*, also called *AtNAP* (*AT1G69490*) (Guo and Gan 2006), and *ANAC092*, also called *ATNAC2* and *ORE1* (*AT5G39610* ) (He et al. 2005), play a regulatory role in senescence. *ANAC092* expression is up-regulated simultaneously with leaf senescence by *EIN2* (ethylene insensitive 2) but is negatively regulated by micro-RNA *miR164*. The ein2 mutant delayed miR164 abundance with senescence, supporting the conclusion that ethylene signaling is essential for the age-dependent regulation of senescence through the *ANAC092* transcription factor (Kim et al. 2009). Comparative gene expression analysis revealed six senescence-associated NAC transcription factors (*ANAC019*, *AtNAP*, *ANAC047*, *ANAC055*, *ORS1*, and *ORE1*) as candidate downstream components of *ETHYLENE-INSENSITIVE2* (*EIN2*) (Kim et al. 2014).

Our results showed that expression *ein2* was down-regulated throughout the protoplast isolation; 1.249 Leaf3h/Leaf0h, 0.676 Protoplast/Leaf3h, 0.8445484 Protoplast/Leaf3h (Supplementary Table 1, All_EdgeR.LogData_Description).

Ethylene is known as a senescent hormone of plants. In order to penetrate the cell wall digestion enzymes into leaf mesophyll tissue, leaves were cut into narrow strips. This wounding manipulation induces ethylene in wounded leaves (Watanabe et al. 2002b). Wounded leaves release jasmonic acid and oligosaccharide before ethylene production (Watanabe et al. 2007). Salicylic acid is also produced in wounded leaves and protoplasts (Masami Watanabe et al. 2011). These plant hormone molecules seem to promote senescence during protoplast isolation. These results suggest that the ethylene released from the wounded leaves during protoplast isolation induces senescence in a different regulatory network than the *ANAC029/ EIN2/ miR164* network.

Figure 5.3 shows that the gene expression of acceptors for chitin and a bacterial flagellar protein increased during the early stages of protoplast isolation. Since chitin is a kind of oligosaccharide and uses cellulases/pectinases as crude enzymes, oligosaccharides derived from cell wall digestion and any unknown protein contained in the cellulase/pectinase enzyme seem to be elicitor candidates for ROS-inducing hypersensitive responses and immune defense systems. The ROS-inducing defense responses finally lead to hypersensitive response (HR)-associated cell death. These results imply that sucrose, oligosaccharides, and protein elicitors contained in the cellulase/pectinase crude enzymes promote senescence and immune defense responses during the early stage of protoplast isolation. In preparation of protoplasts, cells were disrupted and formed cell debris, which was removed by a sucrose density

gradient. The cell debris resulted from sucrose trigger senescence or HR-associated cell death. Therefore, the surviving protoplasts already induced senescence, which seems to be the difficulty of initializing leaf protoplasts in many plant species.

*TOR*-related proteins from yeast and mammals are cell growth regulators in response to nutrient availability. While *AtTOR* is involved in embryogenesis and is expressed in embryos, endosperm, and meristems, it negatively modulates ethylene signals. The molecular mechanism of *AtTOR* is likely involved in the regulation of ethylene biosynthesis by affecting 1-aminocyclopropane-1-carboxylic acid synthase (ACS) in transcription and protein levels. The regulation of autophagy by *TOR* and sucrose non-fermenting related protein kinase 1(*SnRK1*) is conserved in the plant kingdom. *Tor* RNAi, raptor1b, and tap46 mutants, as well as inactivation of *TOR* by the inhibitor AZD8055 induce autophagy, as indicated by the increased numbers of autophagosomes and ATG8e expression levels (Shi et al. 2018). *TOR* inhibition up-regulated senescence-related gene expression, accompanied by up-regulation of the expression of ethylene biosynthetic and response genes (Zhuo et al. 2020).

Our results showed that the gene expression of *TOR* was down-regulated during the isolation of protoplasts and strongly supported the previous reports that *TOR* negatively regulated autophagy in yeast and animals (Liu and Bassham 2010). This *TOR* down-regulation would cause ethylene-induced senescence and autophagy in protoplasts.

Cathepsin B shows a strong implication in PCD induced by abiotic stress such as UV-C, oxidative stress, and ER stress, as well as biotic stress such as HR caused by *Pseudomonas* infections (Ge et al. 2016). The gene expression of cathepsin B-like protein (ATCATHB3, AT4G01610 ) increased throughout the protoplast isolation (Supplementary Table 1/All_EdgeR.Log.Data). The cathepsin B-like protease is a candidate of proteolysis involved in cellular protein catabolic processes, especially autophagy.

AT5G54620 encodes ankyrin repeat-containing proteins universally present in eukaryotes, prokaryotes, and some viruses. They function in protein–protein interactions. A total of 509 ankyrin repeats are present in 105 proteins in Arabidopsis, and the most abundant group contains proteins with ankyrin repeats and transmembrane domains (AtANKTM, cluster A) (Becerra et al. 2004). The AT5G54620-encoded protein is classified as AtANKTM. Gene expression of AT5G54620 was significantly activated during the early process of protoplast isolation (Fig. 9). Although there are no reports that AtANKTM accepts protein elicitors, AtANKTM is a transmembrane protein. Therefore, it likely binds unknown protein elicitors contaminated in the crude cellulase/pectinase enzyme. The AT5G54620-encoded protein may down-regulate TOR.

Recently, glucose-*TOR* signaling has been shown to be involved in the transcriptional reprogramming of a broad range of genes (Shi et al. 2018; Xiong and Sheen 2015). The glucose content decreased during the first 3 h of the protoplast isolation (Fig. 5.1, Supplementary Table 1). The decrease in glucose will inactivate glucose-TOR signaling. Consequently, the inactivated TOR signaling activates the catabolic reprogramming signals and leads to ethylene-induced senescence and autophagy in protoplasts. Since Altman et al. (Altman et al. 1977) reported that protoplasts isolated from *Avena sativa* L. leaves

underwent progressive senescence, our study provides the molecular basis for protoplast senescence via *TOR* signaling during protoplast isolation. However, protoplast senescence seems to involve complex crosstalk signaling pathways such as *TOR* signaling, ROS, elicitor signaling, wound-induced ethylene signaling, etc. Therefore, the protoplast initialization may be required to obstruct these senescence signaling pathways individually.

# Declarations

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Data Availability

All data are available in the manuscript and its supplementary files.

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# Tables

Table 1 is available in the Supplementary Files section.

# Figures

## Figure 1

RNA-Seq-Based transcriptome profiling of the temporal distribution of *B. rapa* leaf mesophyll protoplasts during the isolation procedure.

1.1. Heat map of DEGs of *B. rapa* leaves, leaves three hours after the protoplast isolation treatment, and leaf mesophyll protoplasts.

1.2. Principal component analysis (PCA) of DEGs in *B. rapa* leaves (Leaf), leaves three hours after the protoplast isolation procedure (Leaf3h), and leaf mesophyll protoplasts (Protoplast). The PCA is presented as the combination of the first two dimensions.

## Figure 2

Venn diagrams and pie charts for the overlapping DEGs of *B. rapa* leaves, leaves three hours after the protoplast isolation procedure, and leaf mesophyll protoplasts.

2.1. Venn diagrams showing the overlapping DEGs in the *B. rapa* leaf mesophyll protoplasts during the isolation procedure.

2.2. Pie charts showing the overlapping DEGs categorized by the GO over-representation analysis. Up-regulated DEGs (a) and down-regulated DEGs (b).

## Figure 3

Functional classification of DEGs of *B. rapa* leaves (Leaf0h), leaves three hours after the protoplast isolation procedure (Leaf3h), and leaf mesophyll protoplasts (Protoplast). The DEGs of the three treatments were classified into three gene ontology (GO) categories: cellular components (CC), molecular functions (MF), and biological processes (BP). The top 20 induction (a, c) and repression (b, d) subcategories in BP and CC categories are shown. The top 20 induction and repression subcategories in the MF category are shown in Supplementary 1.

## Figure 4

Temporal distribution profiles of metabolites during the *B. rapa* leaf mesophyll protoplast isolation procedure.

4.1. Principal component analysis (PCA) of Leaf0h, Leaf3h, Leaf6h, and Protoplast metabolites obtained by GC–MS. The PCA is presented as the combination of the first two dimensions.

4.2. Heat map representation of 54 metabolites in 24 samples (six independent biological replicates for each treatment: Leaf0h, Leaf3h, Leaf6h, and Protoplast). Values were expressed as z scores of log2 fold changes: Leaf3h/Leaf0h, Leaf6h/ Leaf0h, Leaf6h/Leaf3h, Protoplast/Leaf0h, Protoplast/Leaf3h, Protoplast/Leaf6h.

4.3. Heat map showing Pearson's correlation coefficients for log2 fold changes of the primary metabolites quantified by GC–MS. Correlations are colored either in red (positive) or blue (negative) hues, while correlations that were not significant are shown in white.

## Figure 5

Overview of the metabolic pathway from the KEGG pathway analysis. The expression profiles of all genes and metabolites were color-coded in each pathway with log2 fold change data in the order of left, center, and right as Leaf3h/Leaf0h, Protoplast/Leaf3h, and Protoplast/Leaf0h. Expression profiles of primary metabolic pathways ( 5.1), catabolic pathways ( 5.2), plant–pathogen interaction ( 5.3), and plant hormone signal transduction ( 5.4)-related genes and metabolites visualized on a KEGG pathway diagram using the Pathview package. Red and green indicate genes induced or suppressed, respectively, by protoplast isolation procedures. Red and blue indicate metabolites increased or decreased, respectively, by protoplast isolation procedures.

## Figure 6

Co-expression of differentially expressed transcription factor genes extracted from the overlapping Venn diagram in Leaf0h, Leaf3h, and Protoplast with the first direct neighbor genes analyzed by Cytoscape (cytoscape.org). Co-expression of the *ANAC092* transcription factor gene and the first direct neighbor genes analyzed by Cytoscape (cytoscape.org). The gene expression is compared with Leaf0h and Leaf3h a, d), Protoplast and Leaf0h b, e), and Protoplast and Leaf3h c, f). Red and blue indicate genes induced or suppressed, respectively.

## Figure 7

Co-expression analysis of the *TOR* (target of rapamycin, *AT1G50030*) gene and the first direct neighbor differentially expressed genes analyzed by Cytoscape (cytoscape.org). The gene expression is compared

with Leaf0h and Leaf3h, Protoplast and Leaf0h, and Protoplast and Leaf3h. Red and blue indicate genes induced or suppressed, respectively.

## Supplementary Files

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- SupplementaryFig.1.pdf
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- SupplementaryFig.3.pdf
- SupplementaryFig.4.pdf
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- SupplementaryTable3.xlsx
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