Cold stress triggers abscission through ABA-dependent signal transduction in early developing apple

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Abstract

Fruit abscission is a complex physiological process that is regulated by internal and environmental factors. During early development, apple fruit are exposed to extreme temperature fluctuations that are associated with premature fruit drop; however, its effect on fruit abscission is largely unknown. We hypothesized that fruit abscission is triggered by cold stress and investigated the molecular basis of premature fruit drop using RNA-seq and metabolomics data from apple fruit undergoing abscission following cold stress in the field. Genes responsive to abscisic acid signalling and cell wall degradation were up-regulated during abscission, consistent with the increased abscisic acid concentrations detected by LCMS. We performed ex-vivo cold shock experiments with excised tree subunits consisting of a branch, pedicel, and fruit. Abscission induction occurred in the cold-stressed subunits with concurrent up-regulation of abscisic acid biosynthesis (MdNCED1) and metabolism (MdCYP707A) genes, and ethylene biosynthesis (MdACS1) and receptor (MdETR2) genes in pedicel. Another key finding was the activation of cytoplasmic streaming of abscission-zone cells detected by electron microscopy. Our results provide a novel insight into the molecular basis of fruit abscission physiology in response to cold stress in apple.

1. Introduction

Fruit abscission is a complex process that is regulated by internal and external factors. During the abscission process, the abscission-zone (AZ) tissues are induced and lead to cell separation. The abscission signals are regulated by hormones, such as ethylene and abscisic acid (ABA), and signal transduction pathways (Botton et al., 2011). The formation of AZ cell layers is induced by the *JOINTLESS* and*MACROCALYX* genes (Nakano et al., 2012) accompanied by the modification of cell wall components, such as lignin and pectin (Kim et al., 2019). Previous studies have mainly focused on elucidating the intrinsic factors of the abscission signal including fruitlet abscission or pre-harvest drop (Dal Cin et al., 2008; Li et al., 2019; Shaya et al., 2019). However, the abscission studies on early developing fruit affected by environmental stresses have rarely been reported.

The cold response of trees includes C-repeat binding factor (CBF)-mediated signalling (Sakuma et al., 2002; Wisniewskiet al., 2011; Yang et al., 2011; reviewed by Miura et al., 2013). During the cold stress response, the hormone ABA is synthesized, which can activate the CBF-mediated signals (Knight et al., 2004). Subsequently, the expression of cold-responsive (*COR*) genes is amplified and as a result, trees undergo various physiological changes involving cell dehydration correlated with dehydrins (Wisniewski et al., 2006).

Apple (*Malus* × *domestica* Borkh.) is one of the major perennial fruit crops, which occupies a large portion of the world fruit industry. Extreme change in weather conditions are expected to increase due to climate change (Lee et al., 2012). Orchard trees are exposed to abnormally low-temperature conditions in the primary

apple production regions in Korea and the frequency of this trend is found to further increase in the prediction model using regional climate change scenarios (Kim et al., 2018). During early stages of development, fruit can be exposed to extreme changes/fluctuations in temperature during spring; however, the mechanism underlying premature fruit drop affected by the environmental stress is largely unknown. Changes in temperature conditions of the orchard may cause fruit drop if the abscission signal transduction is induced. Yuan et al. (2004) demonstrated that exposure to varying temperatures contributes to different responses of leaf and fruit abscission under ethylene application. During the fruit development process, unexpected fruit drop decreases the yield of the orchard. Therefore, from the perspective of the fruit industry, it is important to understand the abnormal fruit abscission mechanism triggered by environmental factors, such as cold stress.

In late May 2018, abnormal fruit drop was reported in many orchards in the apple production areas of South Korea. This damage was also observed in the Apple Research Institute (Gunwi; 36.28°N, 128.47°E) following 2 days of cold stress along with a sharp fluctuation in daily mean temperature compared with the annual mean for the 10 years (Figure 1). In this study, we conducted RNA-seq and metabolomics analyses from young apple fruit collected in-field and hypothesized that cold stress may induce the abscission of early developing fruits undergoing abscission after cold exposure in the field. To elucidate the molecular basis of premature fruit drop, we investigated the morphological traits of AZ cortical cells by electron microscopy and the tissue-specific quantitative reverse transcription PCR (qRT-PCR) of excised tree subunits consisting of a branch, pedicel, and fruit in*ex -vivo* cold shock experiments.

2. Materials and Methods

2.1. Plant material and study sites

6-year-old 'Hongro' apple scions grafted onto 'M9' rootstocks (Hongro/M9) were grown under field conditions at the Apple Research Institute, Gunwi, South Korea. Young 3-cm sized fruit were collected together with the pedicel on 24th (37 days after full bloom, DAFB) of May 2018 for RNA-seq and metabolomics analyses. The following year, the tree subunits consisting of a branch, pedicel, and fruit were excised for *ex-vivo* cold shock experiment and put into a floral foam to prevent dehydration. These subunits were incubated at 25 °C for 168 hr after the 2 hr of initial cold shock treatment. To minimize any effects due to individual variations, we collected our samples from a single Hongro/M9 tree for experiments in both years. In May 2020, we collected tree subunits of Hongro/M9 apple trees at 42 DAFB for the second*ex -vivo* experiment. Subunits were divided into four groups by treatments as follows: Control (water); ABA (125 mg/L of ABA); Cold (initial cold shock at 4 °C for 2 hr); Cold + ABA (125 mg/L ABA followed by initial cold shock at 4 °C for 2 hr). All samples were incubated at 25 °C for 168 hr. The exogenous ABA treatment was applied onto the branch tissues connected to pedicel.

2.2. Weather data

Meteorological data were downloaded from the Korean Meteorological Administration (http://www.kma.go.kr). Data included daily mean, minimum, and maximum air temperature in May 2018 and the annual mean temperature for 10 years at the Apple Research Institute.

2.3. RNA purification

Total RNA was extracted using a modified CTAB method (Chang et al., 1993) from the pooled fruit and pedicel tissues of a Hongro/M9 apple tree sampled in May 2018 (37 DAFB) with four biological replicates. In the next two years, the tree subunit samples consisting of a branch, fruit, and pedicel were collected in time-course during *ex-vivo*incubation. To extract enough RNA, eight biological replicates were all aggregated and labelled as the same tissue type. The concentration of RNA was measured using BioDrop μLITE spectrophotometer (Cambridge, UK).

2.4. RNA sequencing and bioinformatic analyses

RNA integrity number (RIN) was measured using Agilent 2100 bioanalyzer (Palo Alto, CA, USA) and samples with RIN of 7.5 or above were sent to CnK genomics for sequencing. Libraries were prepared

using the Illumina TruSeq Stranded mRNA sample preparation kit (San Diego, CA, USA), and a total of six libraries were constructed using an Illumina Nexseq 500 platform (San Diego, CA, USA). Raw reads were trimmed by filtering out adaptors with a minimum length of 75 bp using Trimmomatic v0.36 (http://www.usadellab.org/cms/index.php?page=trimmomatic) (Bolger et al., 2014). The quality score was evaluated using fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) both before and after trimming. Trimmed reads were aligned to apple reference genome GDDH13 v1.1 (http://www.iris.angers.inra.fr/gddh13) using HISAT2 software (Kim et al., 2015). Across all the libraries, gene counts were calculated for each predicted coding DNA sequence using FeatureCounts v1.5.2. Read counts in the range of 21,543,441 to 24,876,113 were obtained with Q30 ratio > 0.9. Reads per kilobase per million (RPKM) values were counted from BAM files. Differentially expressed genes (DEGs) with a false discovery rate (FDR) value of $< .05$ and $|\log2 \text{ fold change}| > 1$ were selected using EdgeR Bioconductor software (Empirical analysis of digital gene expression data in R) (Robinson et al., 2010). Functional enrichment analysis of DEGs was performed using InterPro2 (http://www.ebi.ac.uk/interpro) and SwissProt (www.ebi.ac.uk/swissprot). All sequencing data were deposited in National Center for Biotechnology Information Sequence Read Archive database bearing the BioProject ID PRJNA613278.

2.5. Ultra-High-Performance liquid chromatography-mass spectroscopy (UPLC-MS)

The metabolites of lyophilized samples of fruit with its pedicel were measured using UPLC-Quadrupole-Time-Of-Flight MS (UPLC-QTOF-MS). Each 0.05 g ground sample was extracted with a 1 mL of ethanol: water: formic acid (80:20:1) solution and incubated for more than 12 hr at 4 °C. There were four biological replicates and the extracted samples were centrifuged at 15,000 *g* for 5 min. Supernatants were diluted 10-fold with 100 % methanol and analysed in both positive and negative ionization modes. The UPLC-QTOF-MS system consisted of Thermo Scientific Dionex Ultimate 3000 (Sunnyvale, CA, USA) and AB SCIEX Triple TOF 5600 system (Framingham, MA, USA). The analytical column was a U-VDSpher PUR 100 C18-E (2.1 mm x 100 mm, 1.8 µm) column (VDS optilab, Germany) and the solvent composition was A=0.1% formic acid in water, B=0.1% formic acid in acetonitrile. The solvent gradient was: 100 % A 0 % B, 0-10 min; linear gradient to 80 % A 20 % B, 10-12 min; linear gradient to 70 % A 30 % B, 12-15 min; the composition held at 5 % A 95 % B, 15-16 min; linear gradient to 100 % A 0 % B to return to the initial conditions before another sample was injected at 20 min. The flow rate was 0.2 mL/min. The Triple TOF 5600 parameters were: nebulizer N_2, 50 psi; heating gas, 50 psi; curtain gas, 30 psi; temperature, 500 °C; ion spray voltage floating, -4.5 kV for negative mode and 5.5 kV for positive mode; declustering potential was -60 for negative mode and 60 for positive mode; MS scan range, 50-2000 m/z.

2.6. Quantitative reverse transcription PCR (qRT-PCR)

First-strand complementary DNA was synthesized using 1.0 µg of total RNA using oligo dT primer and Transcriptor Reverse Transcriptase (Penzburg, Germany). qRT-PCR analysis was performed in LightCycler 480 SYBR Green I Master mix (Penzburg, Germany) on a Roche 480 LightCycler® (Basel, Switzerland). Complementary DNA (1:20 dilution) was used as a template (5 µL) in a reaction volume of 20 µL. For each sample type, there were four to six technical replicates. PCR cycles were as follows: initial denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 65 °C for 15 s, 72 °C for 12 s, and a final melt curve analysis to determine the amplification of a single product. Primers were designed using Primer-Blast (*http://www.ncbi.nlm.nih.gov/tools/Primer-Blast/*) to span an intron if possible, with 100-150 base pairs product size (Supporting Information Table S2). *MDP0000336547* was selected as the reference gene (Bowen et al., 2014). Primer efficiencies and relative expression were calculated using the Roche 480 Light Cycler software.

2.7. Microscopic analyses

After 168 hr of incubation at 25 °C, pedicel tissues of *ex-vivo*experiments were sectioned vertically, and photographs were taken using Olympus SZX16 microscope under the bright field. For analysis through transmission electron microscopy, AZ containing proximal parts of pedicel tissues were cut into small portions and fixed in Karnovsky's fixative solution (2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium

phosphate buffer, pH 7.4). Samples were washed with 0.05 M sodium cacodylate buffer and post-fixed for 4 hr in 1 % osmium tetroxide diluted in 0.1 M sodium cacodylate buffer. The samples were then washed with distilled water and stained with 0.5 % uranyl acetate buffer for 16 hr at 4°C. Samples were dehydrated in an increasing ethanol gradient (50 %, 60 %, 70 %, 80 %, 90 %, and 100 %) for 20 min and treated with propylene oxide followed by 1:1, 1:2 propylene oxide: Spurr's resin solution (10 g of cycloaliphatic epoxide resin (ERL 4221), 6 g of diglycidyl ether of polypropylene glycol (D.E.R. 736), 26 g of nonenyl succinic anhydride, and 0.3 g of dimethylaminoethanol) for 2 hr each. Then, samples were embedded in 100 % Spurr resin solution and sectioned with EM UC7 ultramicrotome (Leica microsystems, Wetzlar, Germany). We observed samples using Carl Zeiss LIBRA 120 transmission electron microscope (Oberkochen, Germany) operating at an acceleration voltage of 120 kV.

3. Results

3.1. DEGs functionally involved in cell wall modification, drought response, ABA and ethylene hormone signals in fruits undergoing abscission following cold stress

To investigate differences in fruits in-field under the abscission process before any additional physiological changes after falling, we conducted RNA-seq analysis to compare the physiological response of fruits with pedicel under abscission with the control (Supporting Information Figure S1). We identified 439 DEGs using both a FDR value $< .05$ and |log2 fold change| > 1 as cut-offs. 324 genes were up-regulated, and 115 genes were down-regulated (Supporting Information Figure S2). The major gene ontology (GO) classification included the stress response (GO:0006950), process of oxidation-reduction (GO:0055114, GO:0016705, and GO:0016491), protein binding (GO:005515), and flavin adenine dinucleotide binding (GO:0050660). Among 439 DEGs, 269 genes were annotated to the InterPro2 database for the functional enrichment analysis. Based on the annotation, we selected 92 DEGs and classified them into nine functional groups (Supporting Information Table S1). In the cell wall modification class, genes encoding pectinesterase (*MD08G1195600* ,*MD04G1008100* , etc), polygalacturonase (*MD07G1011600* ,*MD10G1179100*), and expansin (*MD06G1195100*) were differentially expressed in the 'Abscission' group.

DEGs related to oxidation-reduction included some down-regulated polyphenol oxidases (e.g. MD10G1298700) and a few up-regulated ribulose biphosphate carboxylases (RuBPC) (e.g. *MD13G1041900*). This category also included genes encoding UDP-glucuronosyl/UDP-glucosyltransferase homologs.

The senescence-related DEGs included the down-regulated senescence regulator 40 (*S40* ; *MD10G1269600* , *MD00G1093200*) and the up-regulated desiccation protectant protein homolog (*MD01G1072000*). The up-regulation of both *dehydrin 1*(*DHN1* ; *MD02G1140100*) and *LEA14* (*MD 00G1127700*) homologs indicated drought stress response of fruits under abscission and the *dehydrin xero 1* (*XERO1*) was found to be down-regulated.

In the DNA-binding group, most DEGs were members of transcription factor families that contained either NAC (NAM, ATAF1/2, and CUC2) or bHLH (basic helix-loop-helix) domain. Interestingly, a *SHORT-ROOT*(*SHR*) homolog (*MDP04G1046000*) was found up-regulated in the tissues under abscission.

In the class of phytohormone signal transduction, genes involved in both ABA and ethylene signal pathways were detected. A few ABA signal-related genes were annotated to either ABA metabolism (*MD15G1082600* ;*ABA 8'-hydroxylase 4*) or downstream response (*MD10G1029100*). The ethylene receptor Reversion-to-Ethylene (*RTE*) *1* homolog (*MD15G1250900*) was up-regulated in the 'Abscission' group.

3.2. High concentration of ABA hormone and lignin precursors in early developing apples undergoing abscission after cold

Next, we performed UPLC-QTOF-MS analysis with the same samples of fruit along with its pedicel collected from 6-year-old Hongro/M9 apple tree. A total of 443 metabolites were detected and labelled by accurate mass m/z and liquid chromatography retention time (Figure 2a). Twenty-five metabolites were selected and annotated fulfilling criteria of $p< .05$ and |log2 fold change| > 1 (Figure 2b). The ABA hormone was highly concentrated in the 'Abscission' group. Additionally, lignin precursors, such as quinic acid and ferulic

acid, were accumulated more in fruit and pedicel tissues of the 'Abscission' group. Other metabolites that significantly differed in concentration between the 'Abscission' and 'Normal' groups included either flavonoids or amino acids which were subsequently related to the oxidative stress responses. These metabolomics data were consistent with the results of DEG analysis.

3.3. Outcome of cold stress followed by a large temperature fluctuation in-field site

In late May 2018, unusually high number of fruit drops occurred after a short period of cold in primary apple production regions of South Korea. In the experimental plots in the Apple Research Institute, Gunwi, we observed similar damage with a distinctive yellow coloured peel compared to normal fruit as well (Supporting Information Figure S1). After the daily minimum temperature reached a peak of over 20 ^{o}C on 17th May, it decreased rapidly to 5 ^{o}C until 20th May (Figure 1a). When the daily mean temperature data was compared to the 10-year annual mean, there was a big fluctuation and showed a maximum gap of +7 degrees on 17th and -4 degrees on the 20th (Figure 1b). This indicated an unusual cold shock starting from 17th to 20th of May, which might have induced an abnormal fruit drop among early developing apple afterward. Therefore, based on these meteorological data combined with the results of both RNA-seq and metabolomics analyses, we hypothesized that cold stress affects the abscission of early developing apple.

3.4. The AZ formation is triggered by cold, as evidenced by the cytoplasmic streaming of AZ cortical cells

In the next year, we conducted an *ex-vivo* experiment to investigate whether cold stress contributes to the activation of early fruit abscission signalling. To determine the origin of early responsive signals, we selected the excised tree subunits consisting of young branch, pedicel, and a fruit from Hongro/M9 apple trees (Supporting Information Figure S3). After 1 week of incubation followed by 2 hr of cold shock at 4 °C, the AZ-containing pedicels were severely damaged compared with control (Figure 3a). We observed the AZ cortical cells by transmission electron microscopy and several vesicles were associated with the cytoplasmic streaming in cold-stressed group (Figure 3b), whereas only a few vesicles were detected in control (Figure 3c). Along with new vesicles detaching from the cell wall, the thickness of the cell wall was more irregular and variable in cold-stressed pedicel.

3.5. Tissue-specific response to cold activates ABA signal transduction to potentially induce early fruit abscission

To understand the molecular mechanism underlying early cold response and abscission signals, we conducted qRT-PCR with excised tree subunits and compared tissue-specific responses. Within 6 hr of incubation at 25 °C followed by initial cold, the expression of ABA biosynthesis gene*MdNCED1* was up-regulated in pedicel tissues (Figure 4). However, the expression decreased dramatically within the next 12 hr, whereas there was a delayed up-regulation of *MdNCED1* in neighbouring fruit and branch. The significant up-regulation of *MdCYP707A* , the ABA 8' hydroxylase which is a key regulator of ABA metabolism (Saito et al., 2004), was observed after 18 hr in cold-stressed pedicel.

Next, we investigated the spatial expression pattern of the ABA receptor genes of the PYL family (*MdPYL3* , *MdPYL8*). After 18 hr of incubation at 25 °C, the expression of *MdPYL8* , a mediator of the drought stress response (Lee et al., 2015), increased significantly in cold-stressed pedicel but neither in fruit or branch. In contrast,*MdPYL3* , which is involved in cold and drought stress response (Lenka et al., 2018), was significantly up-regulated in both fruit and branch tissues. In the cold-stressed pedicel, the down-regulation of ABA repressor *MdPP2C* was sustained at both 6 and 18 hr of incubation, whereas the branch tissue did not show any significant difference. The ABA Insensitive 5 gene (*MdABI5*) plays a key role in regulating the core ABA metabolism signalling (Yan et al., 2012; reviewed by Skubacz et al., 2016). *MdABI5* was significantly up-regulated in fruit within 6 hr. On the other hand, its expression was significantly down-regulated in the pedicel after 18 hr. Interestingly, the expression of *MdWRKY40* , the repressor of ABA downstream response, was up-regulated in all tissue types during the early response (6 hr), but it was all significantly down-regulated within the next 12 hr.

The CBF family is a key regulator of cold stress response which can also be activated by ABA (Knight et al., 2004). In Figure 5a, *MdCBF2*gene expression, which is known to play a key role and highly responsive to cold in CBF regulon (Novillo et al., 2007; Tacken et al., 2010), increased significantly after 18 hr in fruit and branch, while, there was no significant change in the pedicel. As a consequence of cold response, not only is the expression of *COR* genes activated by CBF signals, but the dehydration response is induced as well (reviewed by Chinnusamy et al., 2007). We selected CBF downstream targets including one of *COR* genes, Cold shock protein 120 (*CS120*)- like and a couple of drought-responsive genes which differed in expression among tissue types between cold shock treatment and control (Figure 5b). Within 6 hr after the cold shock, the expression of *MdCS120-like* was up-regulated in all tissue types but more extensively in the branch and pedicel. The expression of response to dehydration 22 (*MdRD22*) gene, which is responsive to both ABA and drought stress (Matus et al., 2014), was significantly up-regulated in fruit and the pedicel within 6 hr, as well as in the branch, 18 hr later. In contrast, the *MdWRKY57* gene was up-regulated after 18 hr in the pedicle and rather suppressed in both fruit and branch at the same time point.

3.6. Tissue-specific response to AZ activation through cell wall modification and hormone response signals

Next, we investigated target genes related to cell wall modification, which are substantially associated with AZ formation (Figure 5c). We selected one each of laccase and expansin families as the qRT-PCR target which were also present in the list of DEG analysis in 2018 (Supporting Information Table S1). Laccase 7 (*LAC7*) is involved in the lignin polymerization and discerns the non-separating cells from separating cells when plants undergo cell wall processing accompanied by the lignin brace during the abscission (Lee et al., 2018). The branch tissue showed the biggest gap in the change in expression from 6 to 18 hr after the initial cold shock. Expansins (*EXPs*) are involved in cell wall loosening by disrupting the bond between celluloses and glycans (Ouyang et al., 2013). Here, in our study, both branch and pedicel maintained significantly up-regulated *MdEXPA10* expression compared to that in control. JOINTLESS is responsible for AZ formation (Nakano et al., 2012). Within 6 hr, the expression of *MdJb* , a homolog of JOINTLESS, was significantly down-regulated in cold-stressed branch and pedicel, but it did not show a significant change at 18 hr.

Figure 6 illustrates the spatial relative expression of genes involved in hormone signal transduction including ethylene, auxin, and gibberellin. The expression of ACC synthase (*MdACS1*), a gene regulating ethylene biosynthesis, was significantly up-regulated in the cold-stressed subunits after 18 hr (Figure 6a). Additionally, both the branch and pedicel tissues revealed a significant difference in the expression of the ethylene receptor (*MdETR2*) at 18 hr. The expression of ethylene responsive factor 1 gene (*MdERF1*) was significantly up-regulated in fruit and branch tissues at 6 hr.

Auxin-related genes were down-regulated as a late response (Figure 6b). The expression of auxin efflux carrier *MdPIN1* was significantly down-regulated in fruit and branch after 18 hr. Auxin/indole-3-acetic acid (Aux/IAA) family has been shown to repress the transcription of auxin-responsive factors, and also participate in abiotic stress tolerance (Shani et al., 2017). We selected *MdIAA9* and*MdIAA20* as target genes because *MdIAA9* is thought to be involved in drought tolerance and *MdIAA20* is related to chilling tolerance in apple (Huang et al., 2019). Interestingly, the cold-stressed fruit and pedicel tissues were found to maintain a significantly down-regulated *MdIAA9* expression and *MdIAA20*was significantly down-regulated in branch and pedicel after 18 hr.

We compared the expression of GA 20-oxidase (*MdGA20ox*) which regulates gibberellin (GA) biosynthesis (Figure 6c). The *MdGA20ox*expression was found to be down-regulated in the cold-stressed fruit after 6 and 18 hr. RGLs which encode DELLA protein family are thought to be associated with cold stress response in apple (Wisniewski et al., 2015), and the expression of *MdRGL2* was up-regulated in cold-stress exposed branch and pedicel at 6 hr, however, the pedicel rather maintained the significant up-regulation at 18 hr.

3.7. Exogenous ABA application promotes the development of AZ cells under cold and mediates signals leading to abscission induction

Our results revealed that cold stress impacted different tissue-specific responses of ABA signalling which

may potentially induce AZ formation. If ABA-dependent signals originating from cold truly contribute to abscission induction, exogenous ABA treatment on excised apple subunits would also lead to the responses of AZ development and expression patterns of relevant genes. To elucidate this, we conducted a second *ex-vivo* experiment with ABA and cold stress treatments.

First, we measured the expression of *MdCS120-like* , a member of COR genes responsible for cold response. The expression of*MdCS120-like* was the highest in pedicel with cold and ABA treatment at 24 hr while the branch maintained its significant up-regulation of the gene at all time points in the cold-stressed group without ABA (Figure 7a). Next, we selected *ABCG25* as the target gene, an ABA transporter located at the plasma membrane mediating the homeostasis of ABA. Previously, Park et al. (2016) demonstrated that the activity of *ABCG25* is controlled by stress conditions. If there were differences in response between cold-induced ABA signalling and that from exogenous ABA application, the expression of the ABA transporter would differ among group as well. Indeed, our data showed that *MdABCG25* expression was significantly up-regulated in cold-stressed pedicel at all time points, whereas ABA application maintained its down-regulation regardless of cold stress (Figure 7b). Then, to investigate the gene expression pattern of ethylene biosynthesis, we compared the expression of *MdACS1* . The expression was significantly up-regulated in pedicel under cold at all time points (Figure 7c). Meanwhile, in the branch tissue, the expression gap was higher in ABA treated group compared to that of cold-stressed after 12 hr of incubation. In terms of cell wall modification, we investigated *MdLAC7* expression among groups (Figure 7d). Although the significant up-regulation appeared in the cold-stressed branch early from 2 hr to 12 hr of incubation, the highest expression was shown after 24 hr in ABA treated group.

Moreover, we examined the development of AZ cortical cells at proximal part of pedicel in each group (Supporting Information Figure S4). Interestingly, exogenous ABA application induced the development of cytoplasmic vesicles at similar level to the one in cold stress. The cytoplasmic streaming of vesicles was extensively formed in both groups under cold and ABA treatments. Taken together, these data reflect that ABA signalling induced by cold would possibly mediate the abscission induction of early developing apple.

4. Discussion

4.1. Transcriptomic evidences of cold stress in young apple fruit undergoing abscission

The RNA-seq analysis provided potential evidences that the apple fruit collected in the latter part of May 2018 not only underwent abscission but were cold-stressed as well (Supporting Information Table S1). For example, the expression of *dehydrin 1* (*DHN1*) homolog*MD02G1140100* , a cold stress marker, was down-regulated. Dehydrins are categorized into the LEA family, which contribute to cold tolerance. Although *MdDHN1* was up-regulated in the 'abscission' group,*dehydrin xero 1* (*XERO1*) homolog *MD02G1139900* was rather down-regulated. The earlier study demonstrated that *DHN1*responds to low temperature and *XERO1* is induced by drought, not by cold in peach (Bassett et al., 2009).Therefore, in our study, the up-regulation of *DHN1* seemed to be a result of the cold stress response. Besides, in the oxidation-reduction category, two *RuBPC*homolog genes were up-regulated in the 'abscission' group. *RuBPCs*are known to make plants more susceptible to thermal variation in response to chilling stress (Gao et al., 2013).

4.2. Cold response and the emergence of spatial patterns of ABA signal induction in apple subunits

Unlike mature fruit, early stage developing apple fruit are mostly seedless or have very few incomplete seeds. Assuming that endogenous ABA is mainly generated from seeds during fruit development (Yuan et al., 2003; Zhang et al., 2009), ABA-dependent signals for either cold response or abscission induction may not originate merely from fruit and it is likely that other tissues also contribute to the amplification of abscission induction signals.

Indeed, there was a difference in ABA signal induction among tissue types in cold-stressed apple subunits. Figure 5 indicates the tissue-specific response of ABA signal transduction. As a consequence of early cold stress response, the ABA biosynthesis gene (*MdNCED1*) was activated within 6 hr in the pedicel but not

in branch or fruit. After 18 hr, delayed up-regulation of ABA biosynthesis gene were observed in these two tissues, whereas the pedicel showed the down-regulation of the gene. ABA can be transported to neighbouring tissues and possibly amplify its biosynthesis after being delivered from one tissue to another (Nonogaki et al., 2014). Therefore, the up-regulation of *MdNCED1* signals in branch and fruit could be stimulated later by ABA which originated from pedicel after 6 hr as a result of the primary cold response.

The branch seems to exhibit a relatively high level of cold response among tissues. Initially, it showed high up-regulation of both*MdCS120-like* and *MdERF1* . *CS120-like* is a member of COR signalling (Fu et al., 2018) and *ERF1* is also responsive to cold and is associated with accelerated abscission (Gao et al., 2019; Ma et al., 2014; Zhang et al., 2010). The CBF signal was found at a later time point, and there might be a previous signal to activate COR signalling at some time earlier than 6 hr, considering that*MdCS120-like* expression was up-regulated at 6 hr in all tissue types.

Furthermore, we observed that the pedicel not only developed AZ cells during 168 hr of incubation but was also severely damaged by desiccation as a result of *ex-vivo* cold treatment (Figure 3). The response of this cold-driven dehydration had occurred at early time points with the increased expression of responsive genes (Figure 5b). Pedicel showed a significant up-regulation of *MdRD22,* which is responsive to both ABA and drought stress (Matus et al., 2014) in 6 hr, and *MdWRKY57*in 18 hr. *WRKY57* is also known to activate ABA biosynthesis by binding directly to the NCED promoter (Jiang et al., 2014).

4.3. Responses in cell wall modification contribute to AZ formation with the cytoplasmic stream of vesicles

From 2019 *ex-vivo* experiment, we observed that both branch and pedicel showed the up-regulation of the cell wall loosening (*MdEXPA10*) and the lignin polymerization (*MdLAC7*) signals for the substantial AZ formation after 18 hr of initial cold shock (Figure 5c). This is possible because the abscission signal is affected by the cold response. A similar tendency was observed in the RNAseq and metabolomics data from the previous year. Based on LCMS result, metabolites related to lignin biosynthesis (quinic acid and ferulic acid) accumulated more in fruit/pedicel mixed tissues (Figure 2). Transcriptomics data revealed an up-regulated *SHR* homolog*MDP04G1046000* , which is related to the lignin signalling pathway for abscission induction (Supporting Information Table S1). *SHR*mediates the formation of Casparian strips in endodermis (Lee et al., 2018). When the neighbouring cells undergo organ separation during abscission, the lignin brace is constructed in the secession cells. Therefore, such *SHR* gene, which mediates the formation of lignin structure, would be positively regulated in fruit pedicel as a consequence of abscission induction following cold stress.

Another member participating in the AZ development is pectin, one of the main cell wall components. A number of pectinesterases were differentially expressed, as shown in Supporting Information Table S1. Pectin is accumulated during AZ formation, and changes in its methylesterification occur before cell separation (Roongsattham et al., 2016). Furthermore, AZ cells show the association of cytoplasmic vesicles during cell separation process (reviewed by Roberts et al., 2002). We observed the accumulation of vesicles in the cytoplasm of AZ cortical cells as a result of cold shock in this study (Figure 3b). Several of them appeared to be bound to the cell wall, and the cytoplasmic streaming of vesicles seemed strongly associated with the modification of cell wall by carrying molecules to be used in cell wall metabolism or by removing these from the cell wall.

4.4. Spatial responses leading to complex downstream signal transduction for AZ formation

Phytohormone signal transduction affects the abscission process which includes ethylene, ABA, jasmonic acid as positive regulators and auxin, gibberellin as negative regulators (Cin et al., 2005; Estornell et al., 2013). We found different signal responses among tissue types in terms of abscission induction by cold, but the gene expression data were difficult to understand clearly. Overall, the initial key activator for the cold-inducible abscission signal seems to be ABA as its biosynthesis was mainly amplified after cold stress (Figure 4). Given that ABA hormone, being cold-inducible (Knight et al., 2004), mediates early abscission induction of apple fruitlet (Giulia et al., 2013), it is no wonder that ABA-dependent signalling may contribute to abscission

induction of early developing fruit as a result of cold response.

Initially, we found different responsive time for the expression of phytohormone signals, such as ABA and ethylene, in the pedicel. Though ABA biosynthesis (*MdNCED1*) was up-regulated in the early stage, it was soon inhibited (Figure 4) and the signals for ethylene biosynthesis (*MdACS1*) and reception (*MdETR2*) increased significantly in the later stage (Figure 6a). This may imply that change from ABA to ethylene signalling in the pedicel explains the abscission induction signals and the fact that AZ cells also acquire the competence to respond to ethylene signal during AZ development (Meir et al., 2019). After cold stress, fruit showed an overall down-regulation of gene expressions including both GA biosynthesis (*MdGA20ox*) and polar auxin transport (*MdPIN1*), whereas the other branch and pedicle tissue did not exhibit such change (Figure 6). We believe that this down-regulation may also contribute to signals of AZ formation by potentially increasing the sensitivity to ethylene in the pedicel, considering that the auxin signal in fruit affects the ethylene sensitivity for AZ formation (Botton et al., 2011). Finally, the branch showed more pronounced changes in gene expression after cold compared with the other two tissues with regard to AZ formation as well as cold response. Although all tissues might potentially respond to cold stress, it seemed that the branch showed maintenance of the up-regulation of downstream cold responses. After 18 hr of cold shock, the expressions of*MdCBF2* and *MdLAC7* were the highest in branch than in other tissues. The expression of these genes increased significantly from 6 to 18 hr in cold-stressed branch compared with that in the control.

4.5. Exogenous ABA application impacted the responses for abscission induction but in a different manner from those of cold response

Our *ex-vivo* experiments with apple subunits demonstrated that cold stress induced ABA-dependent signals, especially in branch and pedicel tissues. Although previous studies have shown that ABA biosynthesis and signalling are controlled under environmental stress signals, such as cold or drought in plants (Ji et al., 2011; Seiler et al., 2011), it is worth noting that the responsive pattern of ABA transporter quite differed between cold and exogenous ABA treatments. In particular, *MdABCG25* expression was significantly up-regulated in branch and pedicel under cold stress while exogenous ABA treatment rather maintained its down-regulation (Figure 7b). Perhaps this is attributed to the ABA homeostasis through its negative feedback regulation (Liu et al., 2016; Ma et al., 2018). In terms of ethylene biosynthesis, cold stress contributed to increase *MdACS1*expression earlier in pedicel whereas exogenous ABA application resulted in the delayed up-regulation in branch and fruit conversely. On the other hand, the intensity of AZ development became greater when apple subunits were treated together with cold stress and exogenous ABA (Supporting Information Figure S4). These results imply that cold-induced signals leading to abscission induction may occur in a different manner than those typically amplified by ABA itself.

Furthermore, the correlation analysis of qRT-PCR genes indicated that the expression of *MdACS1* was consistently positively correlated to *MdLAC7,* which mediates lignin polymerization in all branch and pedicel, under cold stress regardless of exogenous ABA (Supporting Information Figure S5). Among them, cold-stressed group without exogenous ABA showed the significant correlations of cold responsive gene *MdCS120-* like with other genes, such as *MdABCG25* and*MdLAC7* , in branch and pedicel. In particular, *MdABCG25*expression was positively correlated with *MdCS120-like* and*MdLAC7* was rather negatively correlated. Besides, cold-stressed fruit did not show the significant relationship between*MdCS120-like* and *MdABCG25* while there were negative correlations in fruits from exogenous ABA treatment and control. These particular patterns of gene correlation may reflect the characteristics of cold-inducible ABA-dependent signals leading for abscission induction.

Based on our results, we propose a hypothetical scheme for early induction of fruit abscission with spatial signals induced by cold stress (Figure 8). As a primary cold response, ABA biosynthesis is promoted first in pedicel and its biosynthesis is activated in neighbouring branch and pedicel tissues, possibly as a consequence of positive feedback, concurrent with the up-regulation of ABA transporter. The cold-induced drought responses also appear to contribute to the up-regulation of abscission signals. The signals for AZ formation are stimulated largely from branch rather than pedicel, in terms of cell wall modification. Fruit coordinates

to the abscission induction with an increase in ethylene sensitivity in the pedicel by inhibiting auxin flow and gibberellin signals.

However, the molecular mechanisms of cold-induced abscission process under ABA inhibition still remains to be further elucidated.

5. Conclusions

Premature fruit drop is often associated with abiotic stresses. During the early development, apple fruit are exposed to abnormal cold conditions following temperature fluctuations. Our results indicate that apple trees having early developing fruit produce signals for abscission induction in response to cold. Once the initial elevation of ABA biosynthesis in the pedicel spreads to adjacent organs, increased ABA signalling orchestrates cold response. AZ development is coordinated with the signals for cell wall modification produced in the branch and inhibition of negative hormones in the fruit. Based on these findings, we suggest that ABA-dependent responses to cold contribute to abscission induction signalling in early developing apple.

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Figure legends

Figure 1. Meteorological data of the experimental plot in Apple Research Institute, Gunwi (lat. 36.28°N, long. 128.47°E). (a) Change in daily minimum, mean, maximum temperature during May of 2018. (b) Comparison of daily mean temperature to 10-year annual mean.

Figure 2. Results of UPLC-QTOF-MS analysis of young apple fruit/pedicel mixed tissues undergoing abscission. (a) Volcano plot (positive : top, negative : bottom) (b) List of metabolites detected with a criteria of$p < .05$ and |log2 fold change| > 1.

Figure 3. The morphological change of an excised Hongro/M9 apple tree subunit after *ex-vivo* cold shock followed by 168 hr of incubation at 25 °C. (a) Comparison of AZ containing pedicel tissues. (left: normal (before the incubation), middle: treatment (cold shock followed by the incubation), right: control (after the

incubation). (b, c) Transmission electron microscopic images of the AZ cortical cells at proximal tissues in pedicel. Samples were observed with a LIBRA 120 transmission electron microscope at an acceleration voltage of 120 kV, magnification with 4 k (left, middle) and 6.3 k (right). (b) Treatment (168 hr of incubation following initial 2 hr of cold shock). (c) Control (168 hr after incubation without cold shock). Arrows indicate the development of cytoplasmic vesicles.

Figure 4. Spatial expression patterns of genes involved in ABA hormone biosynthesis, catabolism, and signal transduction in the cold-stressed apple subunit consisting of fruit, branch and pedicel.*MDP0000336547* was used for reference gene. Data are mean ± standard error (6 [?] n [?] 8). * indicate significant difference in relative expression between cold treatment and control at $p<$ .05.

Figure 5. Spatial expression patterns of genes involved in (a) cold, (b) drought, and (c) the AZ formation in the cold-stressed apple subunit consisting of a branch, pedicel and fruit. *MDP0000336547* was used for reference gene. Data are mean +- standard error (6 [?] n [?] 8). * indicate significant difference in relative expression between cold treatment and control at $p < .05$.

Figure 6. Spatial expression patterns of genes involved in hormone metabolism: (a) ethylene, (b) auxin, and (c) gibberellin in the cold-stressed apple subunit consisting of branch, pedicel and fruit.*MDP0000336547* was used for reference gene. Data are mean +- standard error (6 [?] n [?] 8). * indicate significant difference in relative expression between cold treatment and control at $p<$ .05.

Figure 7. Spatial expression patterns of genes involved in the metabolic responses: (a) cold response, (b) ABA transport, (c) ethylene biosynthesis, and (d) cell wall modification in apple subunits consisting of a branch, pedicel and fruit. Samples were grouped by treatments as follows: Control (water); ABA (125 mg/L of ABA); Cold (initial cold shock at 4 degC for 2 hr); Cold + ABA (125 mg/L ABA followed by initial cold shock at 4 degC for 2 hr). *MDP0000336547* was used for reference gene. Data are mean +- standard error ($n = 6$). Values with different letters differ significantly from each other at$p < .05$.

Figure 8. A scheme of the abscission signals induced by cold-stress in early developing apple.

Supporting Information

Figure S1. Early developing fruits collected from a 6-year-old Hongro/M9 apple tree in May 2018. (a) 3cm-sized fruits undergoing abscission. (b) Control.

Figure S2. DEG profile of Hongro/M9 young apple fruit/pedicel mixed tissues undergoing abscission. (a) 439 DEGs filtered by a criteria of |log2 fold change| > 1 and FDR value $< .05$. 324 were upregulated and 115 were downregulated. (b) Gene ontology category lists with a criteria of $p < .05$.

Figure S3. The excised subunits collected from a 6-year-old Hongro/M9 apple tree in May 2019. (a) Apple subunit consisting of branch, pedicel, and fruit. (b) Apple subunits exposed to 2 hr of ex-vivo cold shock were severely damaged and more dehydrated after 168 hr of incubation at 25 degC compared to the control.

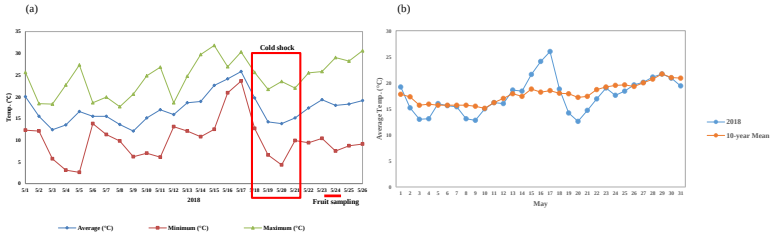
Figure S4. Transmission electron microscopic images of the AZ cortical cells at proximal tissues in pedicel. Samples were grouped by treatments as follows: Control (water); ABA (125 mg/L of ABA); Cold (initial cold shock at 4 degC for 2 hr); Cold + ABA (125 mg/L ABA followed by initial cold shock at 4 degC for 2 hr). AZ cortical cells were observed with a LIBRA 120 transmission electron microscope at an acceleration voltage of 120 kV, magnification with 4 k (left) and 6.3 k (right). Arrows indicate the development of cytoplasmic vesicles.

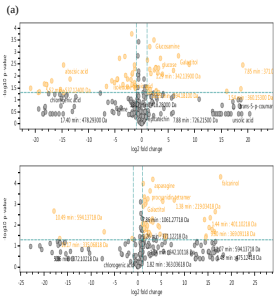
Figure S5. Correlation matrix for qRT-PCR expressions of target genes shown in Figure 7. Correlation coefficient r was calculated between the relative expression levels of genes from all time points. * indicate significant difference at $p < .05$.

Table S1. A list of selected DEGs of Hongro/M9 young apple fruit/pedicel mixed tissues undergoing abscission. 92 DEGs were categorized into nine groups: cell wall modification, oxidation-reduction, senescence,

DNA binding, phytohormone signal transduction, dehydration, degradation, phosphorylation, and hydrolysis.

Table S2. List of primers for qRT-PCR used in this study.





(b)

Metabolite	Adduct	Formula	Annotation	ID confidence†	Classification	Comment	p-value	|log2 fold change| 'Abscisicin'/'Normal'	ion detection mode
17.04 min : 487.35 m/z	[2M-H]-	C12H24N2O3	(Iso)Leucyl-leucine	3	Dipeptide	Protein catabolism	0.000	17.71	neg
15.49 min : 247.133 m/z	**[M+H-H2O]+**	**C15H28O4**	**Abscisic acid**	**2**	**Terpene**	**ABA biosynthesis, metabolism abscission, cold stress response**	**0.003**	**14.71**	**pos**
1.26 min : 183.086 m/z	[M+H]+	C6H14O6	Sorbitol	2	Sugar alcohol	Product of sugar (galactose) breakdown	0.001	6.91	pos
3.07 min : 268.104 m/z	[M+H]+	C10H13N5O4	Adenosine	2	Nucleic acid	DNA, RNA metabolism	0.006	5.99	pos
2.81 min : 132.102 m/z	[M+H]+	C6H13NO2	(Iso)Leucine	2	Amino acid	Glucose homeostasis, amino acid metabolism	0.016	5.61	pos
1.45 min : 198.097 m/z	[M+NH4]+	C6H12O6	Hexose	2	Sugar	Starch degradation	0.002	3.46	pos
1.26 min : 133.060 m/z	[M+H]+	C4H8N2O3	Asparagine	3	Amino acid	Interconversion of aspartate and aspartagine	0.007	2.87	pos
1.30 min : 118.086 m/z	[M+H]+	C5H11NO2	Valine		Amino acid	Amino acid metabolism	0.007	2.87	pos
14.76 min : 871.267 m/z	[2M-H]-	C21H24O10	Phloridzin	2	Polyphenol	Oxidative stress	0.006	2.51	neg
1.27 min : 148.060 m/z	[M+H]+	C5H9NO4	Glutamic acid	2	Amino acid	NAD biosynthesis, oxidative stress	0.005	2.41	pos
10.48 min : 1153.261 m/z	[M-H]-	C60H50O24	Procyanidin tetramer	3	Polyphenol	Oxidative stress	0.000	2.39	neg
1.64 min : 180.086 m/z	[M+H]+	C6H13NO5	Glucosamine	3	Sugar	-	0.000	2.04	pos
1.44 min : 193.070 m/z	**[M+H]+**	**C7H12O6**	**Quinic acid**	**2**	**Organic acid**	**Lignin biosynthesis, cell wall component**	**0.014**	**1.60**	**pos**
8.24 min : 305.065 m/z	[M+H]+	C15H12O7	Dihydroquercetin	2	Flavonoid	Oxidative stress	0.000	1.42	pos
9.02 min : 195.065 m/z	**[M+H]+**	**C10H10O4**	**Ferulic acid**	**2**	**Phenol**	**Lignin component**	**0.022**	**1.28**	**pos**
16.03 min : 230.248 m/z	[M+NH4]+	C14H28O	Tetradecanal	3	Aldehyde	-	0.008	1.24	pos
10.83 min : 867.213 m/z	[M+H]+	C45H38O18	Procyanidin trimer	2	Polyphenol	Oxidative stress	0.007	1.23	pos
17.04 min : 487.342 m/z	[M-H]-	C30H48O5	Trihydroxyurs-12-en-28-oic acid	2	Terpene	Triterpene	0.001	-1.01	neg
11.46 min : 1729.388 m/z	[M-H]-	C90H74O36	Procyanidin hexamer	3	Polyphenol	Oxidative stress	0.014	-1.08	neg
14.34 min : 447.093 m/z	[M-H]-	C21H20O11	Quercetin 3-rhamnoside	2	Flavonoid	Oxidative stress	0.040	-1.09	neg
1.39 min : 133.014 m/z	[M-H]-	C4H6O5	Malic acid	2	Organic acid	-	0.018	-2.24	neg
14.02 min : 433.077 m/z	[M-H]-	C20H18O11	Quercetin 3-xyloside	2	Flavonoid	Oxidative stress	0.014	-2.28	neg
10.09 min : 865.198 m/z	[M-H]-	C45H38O18	Procyanidin trimer	2	Polyphenol	Oxidative stress	0.013	-3.80	neg
14.94 min : 417.082 m/z	[M-H]-	C20H18O10	Kaempferol 3-arabinoside	2	Flavonoid	Oxidative stress	0.038	-4.72	neg
8.57 min : 577.134 m/z	[M-H]-	C30H26O12	Procyanidin B2	2	Polyphenol	Oxidative stress	0.017	-6.14	neg

† Metabolites are annotated with three confidence levels (1 = compared to standard; 2 = exact mass/formula + ms/ms or retention time; 3 = exact mass/formula).