Combined signal sequence trap and macroarray analysis identifies genes associated with differential fruit softening characteristics during ripening in European and Chinese pears

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Abstract

During ripening, European pear (*Pyrus communis* L. cv. 'La France') fruit undergo dramatic softening in response to increased ethylene production, whereas Chinese pear (*Pyrus bretschneideri* Rehd. cv. 'Yali') fruit remain firm, despite producing large amounts of ethylene. The molecular basis of this differential softening behavior is not well understood. In this study, we combined a yeast-based signal sequence trap (YSST) and macroarray gene expression analysis to identify putative genes encoding secreted proteins that control pear fruit softening. We identified 22 cDNAs annotated as encoding proteins with diverse cell wall-associated functions that were up- or down-regulated during fruit ripening in 'La France'. Gene expression analysis in fruit that were treated with the ethylene perception inhibitor 1-methylcyclopropene (1-MCP) at 4 d after the onset of ripening revealed that 16 of the targeted genes are ethylene-regulated, while the others appear to be ethylene independent. Comparative gene expression analyses of 'La France' and 'Yali' fruit during ripening suggested that four ethylene-regulated cDNAs encoding cell wall modifying proteins, *contig 2* (*polygalacturonase 3*), *contig 15* (*expansin*), *contig 19* (*expansin*) and *contig 55* (*pectate lyase*) contribute to the different softening behaviors of 'La France' and 'Yali' fruit. Additionally, one ethylene-independent cell wall related gene, *contig 36* (*expansin*), and three genes encoding proteins of unknown function, *contigs 1*, *13* and *contig 75* showed differential expression between 'La France' and 'Yali' fruit during ripening. The results presented herein represent promising candidates for future functional analysis and elucidation of softening mechanisms.

Keywords:

YSST, 'La France', 'Yali', Polygalacturonase, Expansin, Pectate lyase

1.0. Introduction

Softening is an integral part of fruit ripening, and is a key contributing factor to the edibility and palatability of many fruits (Seymour et al., 2002). However, softening can also increase susceptibility to physical damage and postharvest pathogen-induced infection, which in turn may limit fruit transportation, storage practices and postharvest shelf life (Brownleader et al., 1999; Brummell and Harpster, 2001; Yang et al., 2017). An understanding of the molecular processes that underlie fruit softening is therefore of major economic importance.

Fruit softening is largely influenced by major alterations in the structure and composition of cell wall components, which result in loss of intercellular wall adhesion, wall loosening and disintegration (Uluisik et al., 2016; Wang et al., 2018). Plant primary cell walls are generally composed of cellulose microfibrils embedded in a matrix of hemicellulose and pectic polymers (Brummell and Harpster, 2001). During softening, pectins and hemicellulose undergo solubilization and depolymerization (Rose et al., 1997; Fischer and Bennett, 1991; Wakabayashi, 2000). Such cell wall modifications are complex and involve the coordinated and interdependent action of families of various enzymes and proteins, including polygalacturonase (PG), pectate lyase (PL), pectin methylesterase, β-galactosidase (β-GAL), expansin (EXP), and xyloglucan endotransglucosylase hydrolase (XTH) among others (Guo et al., 2018; Wang et al., 2019; Xiao et al., 2019). The contribution of individual cell wall modifying proteins to fruit softening may vary among fruit species, and indeed the abundance and composition of pectin, cellulose and hemicellulose in cell walls differs between and within fruit species (Redgwell et al., 1997; Wakabayashi, 2000).

In climacteric fruit, ripening-associated softening is typically regulated by the phytohormone ethylene, whose role in the induction of cell wall modifying enzymes has been extensively investigated in multiple fruit species, using natural mutants, genetic engineering techniques and treatments with ethylene inhibitors, such as Ag^{+} or 1-methylcyclopropene (1-

MCP) (Alexander and Grierson, 2002; Hiwasa et al., 2003; Cara and Giovannoni, 2008; Pech et al., 2008). For instance, PG-encoding genes were shown to be transcriptionally regulated by ethylene during ripening in tomato (*Solanum lycopersicum*; Sitrit and Bennett, 1998), kiwifruit (*Actinidia chinensis*; Mworia et al., 2012; Atkinson et al., 2011; Asiche et al., 2018), European pear (*Pyrus communis*; Hiwasa et al., 2003; Hiwasa et al., 2004), and melon (*Cucumis melo*; Pech et al., 2008). Ethylene induction of genes encoding other cell wall modifying proteins, such as β-GAL, PL, EXP and XTH, has also been previously reported in different fruit species (Shen et al., 2017; Asiche et al., 2018; Ban et al., 2018; Mitalo et al., 2019*a*, *b*). However, ethylene-independent regulation of one or more family members of each type of cell wall modifying enzymes has been reported (Hiwasa et al., 2003; Tacken et al., 2010; Mworia et al., 2012; Mitalo et al., 2019*a*).

Recently, other ethylene-independent mechanisms such as oxidative or hydrolytic degradation have been associated with ripening-related fruit softening. Oxidative processes have been especially linked to fruit softening in summer pears (Lindo-García et al., 2019; Lindo-García et al., 2020), where high lipid peroxidation levels and fruit softening preceded the climacteric rise in ethylene production. In addition, separate studies have clearly demonstrated the ascorbate-induced oxidative scission of xyloglucan (Fry,1998), as well as solubilization of tomato pectins (Dumville and Fry, 2003).

European pear (*Pyrus communis* L.) 'La France' fruit and Chinese pear (*Pyrus bretschneideri* Rehd.) exhibit differential softening patterns during ripening. While 'La France' fruit undergo rapid and extensive softening in response to increased ethylene production (Hiwasa et al., 2003), 'Yali' fruit remain firm throughout ripening, despite a large increase in ethylene production (Hiwasa et al., 2004; Mwaniki et al., 2007; Hao et al., 2018). At present, little is known about the mechanisms that underlie the differential softening behaviors of these two cultivars. An exception is the study of Hiwasa et al. (2004) who reported that *PC-PG1* and

PC-PG2 expression increased during ripening in 'La France' fruit, whereas neither gene was expressed during ripening in 'Yali' fruit. Therefore, a more extensive comparative analysis to identify candidate genes involved in the differential softening of these two pear cultivars and the role of ethylene in their regulation has the potential for enhancing understanding of the fruit softening mechanism.

A high throughput approach to identify secreted cell wall/apoplastic proteins is the yeast-based signal sequence trap (YSST) (Klein et al., 1996; Jacobs et al., 1997). This involves ligating a library of cDNAs from the target tissue to the 5' end of a DNA sequence encoding a truncated yeast invertase gene that lacks both an initiator methionine and an N-terminal signal peptide. The resulting library plasmids are then transformed into an invertase-deficient yeast mutant which is grown on a medium with sucrose as the sole carbon source. Any yeast transformed with a cDNA encoding secreted proteins can utilize sucrose, as cell wall or apoplastic proteins have the capacity to rescue the invertase mutant. Although YSST screens have been used previously to study various biological processes in different plants (Hugot et al., 2004; Yamane et al., 2005; Lee et al., 2006), few studies have used this strategy to unravel fruit ripening mechanisms.

In the present study, a YSST screen was used characterize the cell wall proteome of ripening 'La France' pear fruit. Subsequent macroarray analysis was then used to identify cDNAs that were differentially expressed during fruit ripening and those that were differentially expressed between 'La France' and 'Yali' fruit. Furthermore, 1-MCP treatments were used to probe the role of ethylene in regulating the target genes. The objective of this study was to identify key genes involved in the differential softening behaviors of 'La France' and 'Yali' pears.

2.0. Materials and methods

2.1. Plant material, treatments and RNA extraction

'La France' fruit samples used in this study were similar to those of Hiwasa et al. (2003). Fruit were harvested at a pre-climacteric stage from a commercial orchard in Yamagata, Japan. Chilling treatment (1 ºC, 21 d) was carried out to initiate climacteric ethylene production, followed by ripening at 20 ºC for 13 d. At 4 d during ripening, the fruit were divided into two groups; one set were treated with 20 $\mu L L^{-1}$ 1-MCP (Rohm and Haas, Philadelphia, PA, USA) for 12 h to block ethylene action while the other set were non-treated. As for 'Yali' cultivar, fruit samples used in this study were similar to those of Hiwasa et al. (2004). Fruit were harvested at a pre-climacteric stage from a commercial orchard in Tottori, Japan and stored at 20 ºC for 23 d.

Rates of ethylene production and flesh firmness were determined as previously described (Hiwasa et al., 2003) at appropriate intervals, with each sample containing three independent biological replicates. At each sampling point, flesh tissues were frozen in liquid nitrogen and stored at -80 ºC until further use for RNA extraction.

2.2. Construction of the pTEF-YSST vector

This study used a pTEF426-YSST vector (Fig. 1), derived from the pSMASH plasmid (Goo et al., 1999). Transcription elongation factor (TEF), a strong promoter of fusion protein expression in yeast (Mumberg et al., 1995), was subcloned as *Not*I-*Eco*RV fragments into pSMASH that contained a mutated yeast invertase (Suc2) lacking its own signal peptide (SP) and initiator methionine. The pTEF426-YSST vector also contained URA3; an additional selectable marker that confers ability to grow in the absence of uracil.

2.3. Construction of pear fruit cDNA libraries

To construct cDNA libraries, total RNA was extracted as described by Wan and Wilkins (1994) from 'La France' pear fruit at harvest (0 d) and 21 d of cold storage as well as after 4 d of post-cold storage ripening at 20 ºC in triplicate. Poly(A) mRNA was purified from the total RNA using Oligotex-dT30 (TaKaRa Shuzo Co., Shiga, Japan), and then pooled for construction of both mRNA and sRNA libraries. To create the mRNA library, first strand cDNAs were synthesized from poly(A) mRNA using a random hexamer primer (5'-AAGCAGTGGTATCAACGCAGAGCGGCCGCCNNNNNN-3'; the underlined bases correspond to *NotI* site), and a SMART PCR cDNA Synthesis Kit (Clontech Laboratories, Osaka, Japan) according to the manufacturer's instructions. The sRNA library was constructed using the random hexamer primer indicated above, and a SMART mRNA Amplification Kit (Clontech Laboratories), according to the manufacturer's instructions. In both cases (mRNA and sRNA libraries), the cDNAs were prepared for cloning by introducing *Eco*RI and *Not*I sites at the 5' and 3' ends, respectively.

The cDNA fragments (≥ 300 bp) were recovered, following agarose gel electrophoresis, with an Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and ligated to pTEF426-YSST vectors that previously had been digested with *Eco*RI and *Not*I. The ligations were transformed into *Escherichia coli* strain DH5α competent cells (Invitrogen, Carlsbad, CA, USA) by electroporation and the transformants selected on an ampicillin-containing medium. cDNA library plasmid DNA was purified from a pool of the transformed bacterial colonies using Qiagen Plasmid Midi Kit (Qiagen, Germantown, MD, USA).

2.4. Yeast transformation and selection

Fifty micrograms of cDNA library plasmids were transformed into the native invertase mutated yeast (*Saccharomyces cerevisiae*) strain DBYα2445 (MATα, *suc2*Δ*-9*, *lys2-801*,

ura3-52, *ade2-101*) (Goo et al., 1999), using the dimethyl sulphoxide (DMSO)/ lithium acetate high efficiency yeast transformation method (Hill et al., 1991). Yeast transformants appeared on glucose agar plates lacking uracil (0.67 % Yeast Nitrogen Base Without Amino Acids, 0.192 % Uracil Dropout Supplement, 2 % glucose and 1.7 % agar) after incubation at 30 ºC for 6 d. Colonies were replica plated on sucrose agar plates lacking uracil (0.67 % Yeast Nitrogen Base Without Amino Acids, 0.192% Uracil Dropout Supplement, 2 % sucrose and 1.7 % agar) and incubated at 30 ºC for 2–3 d. Yeast transformants were selected based on the ability to grow in the absence of uracil. The resulting colonies were restreaked on sucrose agar plates for further use.

2.5. Yeast colony PCR

Yeast transformants generated as above were subjected to colony PCR to confirm the gene inserts. Primers were designed based on the 3' end of TEF promoter sequence and the 5' end of *Suc2* gene. The PCR reaction mixture contained 1x Taq polymerase buffer, 200 μmol/L dNTPs, 0.25M of each oligonucleotide and 1-unit *Taq* polymerase in a 50 μl reaction volume. Colony PCR was performed using a program of 95 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s with an initial denaturation at 95 ºC for 60 s and a final extension at 72 ºC for 7 min. Resultant PCR products were purified with Illustra GFX-96 PCR Purification Kit (GE Healthcare Life Sciences) before being used for DNA sequencing and macroarray analysis.

2.6. Sequence analysis

Plasmid inserts were sequenced by Eurofin Genomics, Tokyo, Japan. The sequences were aligned using ATGC software and adjusted manually. Sequences of the pTEF426-YSST clones were used to search GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and the UC Davis Apple Expressed Sequence Tags (EST, http://cgf.ucdavis.edu/home) databases. After

checking the sequences for frame shifts, signal peptides and putative cleavage sites were predicted using SignalP (version 3.0, http://www.cbs.dtu.dk/services/SignalP-3.0/). Functional classification of the sequences was based on the Munich Information Center for Protein Sequences (MIPS) functional catalogue database (http://mips.gsf.de).

2.7. Macroarray analysis

2.7.1. Preparation of cDNA macroarray filters

PCR products (15 µL) obtained in section 2.5 above were spotted onto nylon membrane filters in triplicate using a Biomek 200 (Beckman, Fullerton, CA, USA) according to the procedure described by Ishihara et al. (2004). DNA samples were then fixed on the nylon filters by UV cross-linking. Fragments of *Actin* (BAL14271) and λDNA were also spotted as controls.

2.7.2. Probe synthesis and macroarray hybridization

Both 'La France' and 'Yali' fruit samples were used in this analysis. 'La France' probes were synthesized from total RNA extracted from fruit at harvest (0 d), after cold storage (21 d), and at 4 and 13 d post-cold storage ripening. Total RNA from 1-MCP-treated 'La France' fruit at 13 d post-cold storage ripening stage were also used to construct additional probes. 'Yali' probes were also synthesized using total RNA from fruit after 0, 11 and 15 d storage at 20 ºC. Each RNA sample was heat-denatured by heating at 65 ºC for 5 min in a reaction mixture containing 5 µg RNA and oligo dT primer. The reaction mixture was then incubated at 42 ºC for 2 min following addition of 2.5 µL of 10X SuperScript II Buffer (Gibco BRL, Gaithersburg, MD, USA), 2.5 µL of 0.1 M DTT and 1 µL of RNaseOUT in the presence of 5 µL of ^{33}PdCTP (Amersham Pharmacia Biotech, UK). An additional 1 µL of 10X SuperScript II Buffer (Gibco BRL) was added and the mixture was re-incubated at 42 ºC for 50 min to generate radio-

labelled probes. The reaction was terminated by heating at 70 ºC for 15 min and the reaction mixture purified with QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

Prehybridization of the previously prepared nylon filters was carried out at 60 ºC for 12 h in a solution containing 0.5 M Church Phosphate Buffer (pH 7.2), 1 mM EDTA and 7 % SDS. Radiolabeled cDNA probes were then added to the prehybridization solution and hybridization was carried out at 60 ºC for 16 h. After incubation, the filters were washed twice with 0.2X SSC and 0.1 % SDS at 60 ºC for 30 min, and then exposed to an imaging plate (Fuji Film, Tokyo, Japan) for detection.

2.7.3. Identification of differentially regulated cDNAs during ripening

Radioactive images were obtained with a high-resolution scanner FXPro (Bio-Rad, Hercules, CA, USA) and quantification of the signal intensity was carried out using an Array Vision 5.1 software (Imaging Research Inc., Ontario, Canada). The intensity of all the spots on the filter was averaged, normalized as in Ishihara et al. (2004), and relative expression values were calculated as the ratio of each signal to the average intensity of each filter. Additionally, ratios of ripening (4 d post-cold storage at 20 ºC) *versus* pre-ripening (21 d storage at 1 ºC) were calculated for 'La France' samples and genes with a ratio ≥ 3 or ≤ 0.33 were defined as upregulated or downregulated, respectively. Ethylene dependence was calculated for 'La France' samples based on the response to 1-MCP using the formula $1 - \frac{M34 - La21}{La34 - La21} x\ 100$ (Yan et al., 2013), where La21 represents cold-stored fruit at 21 d, La34 represents post-cold storage ripening control fruit 13 d and M34 represents 1-MCP treated post-cold storage ripening fruit at 13 d. Based on this parameter, clones that showed values ≥ 70 % were categorized as strongly ethylene-dependent, 30–70 % as partially ethylene-dependent and < 30 % as ethylene-independent.

2.8. Statistical analysis

Data obtained in this study were subjected to statistical analysis using R version 3.4.0 software package (R Project). ANOVA followed by post-hoc Tukey's tests ($p < 0.05$) were used to detect differences in ethylene production and flesh softening rates and gene expression patterns for 'La France' and 'Yali', respectively.

3.0. Results

3.1. Changes in ethylene production and flesh firmness

In 'La France' pears, ethylene production was detected after 21 d cold storage, after which it increased rapidly (to ~179 nmol kg^{-1} s^{-1}) during ripening at 20 ºC (Fig. 2A). Concomitant with the increased ethylene production, flesh firmness decreased substantially (Fig. 2C), resulting in a melting texture at 13 d post-cold storage. Application of 1-MCP at 4 d after the onset of ripening inhibited both ethylene production and further fruit softening. In contrast, 'Yali' fruit produced large amounts of ethylene (a maximum of 2300 nmol kg^{-1} s^{-1} after 15 d) during ripening (Fig. 2B) but flesh firmness did not change throughout the experiment (Fig. 2D).

3.2. Identification and classification of pear fruit cDNAs using YSST screen

We identified a total of 600 transformants, which were assembled into 239 unigenes (Supplementary Table 1), comprising 96 contigs and 143 singletons (yeast clones isolated only once). Subsequent analysis using SignalP software was used to predict the existence and sequences corresponding to N-terminal signal peptides in the isolated clones. A few clones (16 contigs and 65 singletons) lacked a predicted signal peptide in their sequences (Fig. 3A) and

predicted signal peptides were detected mostly in clones that were isolated more than twice, consistent with cell wall-associated functions.

To elucidate gene functions associated with the isolated clones, derived amino acid sequences were used to conduct a BLASTX searches of the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), and taking advantage of the GenBank and UC Davis Apple Expressed Sequence Tags (EST) databases. The resulting predicted proteins had diverse annotated functions, including cell wall modification (17 %), metabolism (14 %), defense (11 %), stress response (11 %), cellular transport (9 %), cellular signaling (3 %), cellular interaction (3 %) and a group of other family proteins (22 %) (Fig. 3B). Additionally, we identified a small group of unclassified proteins (10 %) that had no suitable match in the reference databases, and thus their putative functions remain unpredicted.

3.3. Transcriptional dynamics of pear fruit YSST genes during ripening

We conducted a macroarray analysis of the YSST clones using RNA from 'La France' fruit at ripening (4 d after the onset of ripening) and pre-ripening (21 d cold storage) stages as probes (Fig. 4). This analysis revealed that only twenty-two of the YSST genes were differentially expressed during ripening, of which 16 were upregulated (Table 1) and the other six were downregulated (Table 2). Eight of the upregulated cDNAs were predicted to encode cell wall modifying proteins, of which six, *PG3* (*contig 2*), *EXP* (*contigs 15*, *19* and *36*), *PL* (*contig 55*) and *β-GAL1* (*contig 402*) were involved in fruit softening while two *β-1,3-glucanase* (*contig 97*) and *PG-inhibiting protein* (*PGIP*, *contig 3*) were associated with defense against pathogens (Table 1). The analysis also revealed six cDNAs, including *contig* 32 (cysteine protease), *contig 35* (*thaumatin-like protein precursor*), *contig 83* (*proteinase inhibitor se60-like protein*), *clone 672* (*aspartic proteinase*), *clone 876* (*stellacyanin*) and

contig 1025 (*lectin-like receptor kinase*) that were upregulated during fruit ripening. In addition, two cDNAs that were predicted to encode proteins of unknown function (*contigs 1* and *13*) were found to be upregulated during ripening. Of the six cDNAs that were downregulated during ripening, five were predicted to encoded known proteins: *contig 11* (*dehydrin-like protein*), *contig 28* (*bifunctional UDP-glucose-epimerase*), *contig 86* (*rapid alkalinization factor precursor*), *clone 263* (*fasciclin-like protein*) and *clone 550* (*putative endomembrane protein 70*), while one sequence (*contig 75*) had no suitable match in the reference protein databases.

Responses to 1-MCP, a well-known ethylene antagonist, were then used to determine the effect of ethylene on the differentially expressed cDNAs. Nine of the sixteen upregulated cDNAs exhibited an ethylene dependence value of 75–100 % (Table 1), indicating that they were strongly regulated by ethylene. Additionally, five cDNAs that were upregulated during ripening (*contigs/clones 3*, *36*, *55*, *97* and *672*) showed ethylene dependence values of ~40–55 %, suggesting that ethylene had a slight effect on their expression. In contrast, ethylene dependence values of the remaining two upregulated cDNAs (*contigs 32* and *1025*) were 16–19 %, indicating no ethylene regulation. Of the six downregulated cDNAs, four (*contigs 11*, *86*, *263* and *550*) appeared to be strongly regulated by ethylene (ethylene dependence value was 100 %), while the remaining two (*contigs 28* and *75*) were ethylene independent (Table 2).

3.4. Expression patterns of YSST genes during fruit ripening in ‘La France’ and ‘Yali’

To further focus on candidate genes that contribute to softening in pears, gene expression patterns were compared between ripening ‘La France’ and ‘Yali’ fruit using a macroarray. Hybridized spots were detected in cDNA macroarray filters of ‘La France’ pears, suggesting that homologues of the genes also exist in the ‘Yali’ genome.

The expression of five cDNAs encoding cell wall modifying proteins increased with ripening in 'La France' (Fig. 5), and 1-MCP treatment significantly reduced their expression, indicating an ethylene regulation. In 'Yali' fruit, however, four of these genes, including *contig 2* (*PG3*), *contig 15* (*EXP*), *contig 19* (*EXP*) and *contig 55* (*PL*), showed no significant changes in expression during ripening (Fig. 5A–D). The remaining cDNA, *contig 402* (*β-GAL1*) was upregulated during ripening in both 'La France' and 'Yali' pears (Fig. 5E). Lastly, *contig 3* (*PGIP*), which is associated with inhibition of pathogen PG (Powell et al., 2000), was also upregulated during ripening in both 'La France' and 'Yali' pears.

Five of the other ethylene-regulated genes, including *contig 1* (*hypothetical protein*), *contig 13* (*unnamed protein product*), *contig 83* (*proteinase-inhibitor se60-like protein*), *clone 672* (*aspartic proteinase*) and *clone 876* (*stellacyanin*), were upregulated during ripening in 'La France' (Fig. 6A, C, E, I, J). However, they either showed no change in expression or were downregulated during ripening in 'Yali'. Three other ethylene-regulated genes, *contig 11* (*dehydrin-like protein*), *contig 86* (*rapid alkalization factor*) and *clone 550* (*putative endomembrane protein*), were downregulated during ripening in 'La France' (Fig. 6B, F, H), but showed no expression changes during 'Yali' fruit ripening. *Clone 263* (*fasciclin-like protein*) was downregulated with ripening in 'La France', while its expression increased in 'Yali' fruit (Fig. 6G). The expression of *contig 35* (*thaumatin-like protein precursor*) increased with ripening in both pear cultivars (Fig. 6D).

Although *contig 36* (*EXP*) and *contig 97* (*β-1,3-Glucanase*), were initially thought be regulated by ethylene (Table 1), subsequent analysis indicated that 1-MCP treatment did not significantly inhibit their upregulation during ripening in 'La France' (Fig. 7). In 'Yali' fruit, *contig 36* (*EXP*) was instead downregulated (Fig. 7A), whereas *contig 97* (*β-1,3-Glucanase*) was also upregulated in a similar pattern with ''La France' (Fig, 7B).

The other ethylene-independent clones showed varied expression patterns during ripening in 'La France' and 'Yali' pears (Fig. 8). *Contig 28* (*bifunctional UDP-glucose 4-epimerase*) was downregulated with fruit ripening in 'La France' with no expression changes in 'Yali' (Fig. 8A). *Contig 1025* (*lectin-like receptor kinase*) was upregulated during ripening in 'La France' (Fig. 8B), but downregulated in 'Yali'. *Contig 32* (*cysteine protease*) was upregulated with ripening in both pear cultivars (Fig. 8C), and *contig 75* (*no suitable match*) was downregulated during ripening in 'La France' (Fig. 8D), but showed no changes in expression during ripening in 'Yali'.

4.0 Discussion

The two types of pears used in this study exhibited different softening patterns during fruit ripening. 'La France' fruit showed increased ethylene production (Fig. 2A) and softened rapidly to a melting texture (Fig. 2C). In contrast, 'Yali fruit showed no significant changes in flesh firmness throughout the ripening period (Fig. 2D), despite producing large amounts of ethylene (Fig. 2B). These findings agree with previous reports (Hiwasa et al., 2004; Mwaniki et al., 2007; Hao et al., 2018). Hiwasa et al. (2004) associated the different softening behaviors between 'La France' and 'Yali' fruit with different expression patterns of genes encoding the polygalacturonase genes *PcPG1* and *PcPG2,* which are presumed to hydrolyze cell wall pectin. In this study, the objective was to broaden the perspective and profile the cell wall proteomes of 'La France' and 'Yali' pears, using a coupled YSST screen and macroarray analysis.

Most of the YSST clones isolated in this study corresponded to sequences with predicted N-terminal signal peptides (Fig. 3A). However, a few, particularly the singletons, lacked predicted signal peptides. This discrepancy may be attributed to either an inherent low level error rate in the SignalP (v3.0) software (Bendtsen et al., 2004; Armenteros et al., 2019) or the existence of an alternative secretion pathway (Jacobs et al., 1997; Chivasa et al., 2002;

Lee et al., 2006). Nevertheless, sequences with confirmed signal peptides were found to encode proteins with diverse cell wall-associated functions (Fig. 3B), and 17 % were related to cell wall modification. We note that 10 % of the clones had no sequence identity or similarity to previously identified pear genes, suggesting the utility of YSST screen in gene discovery.

4.1. Identification of genes that are differentially expressed during fruit ripening and the role of ethylene in their regulation

Following the isolation by YSST, macroarray analysis was used to pinpoint genes that showed differential expression patterns during fruit ripening in 'La France' and that thus have potential roles in fruit softening. Six of the upregulated genes were predicted to encode various cell wall modifying proteins including *PG*, *EXP*, *PL*, and *β-GAL1* (Table 1), which would account for the softening observed in 'La France' fruit (Fig. 2C). Previous studies have also reported increased expression of different families of cell wall modification-associated genes during ripening in 'La France' fruit (Hiwasa et al., 2003; 2004; Sekine et al., 2006), and in other pear fruit that soften during ripening (Fonseca et al., 2004; 2005; Song et al., 2016). Five other cDNAs encoding proteins of assorted functions and two with unknown functions, *contig 1* (*hypothetical protein*) and *contig 13* (*unnamed protein product*), (Table 1), were upregulated during ripening although their roles in softening have yet to be established. Most of the cDNAs that were downregulated during ripening encoded proteins with predicted functions (Table 2), although *contig 75* corresponds to a novel protein.

While 1-MCP treatment may have various effects on the fruit physiology and transcriptome, it is clear in the present study that it inhibited ethylene production and subsequently, fruit softening in 'La France' pears (Fig. 1A, C). 1-MCP is a well-known inhibitor of ethylene action (Kamiyoshihara et al., 2012), and treatments with this antagonist have been previously used to identify ethylene-regulated and ethylene-independent genes and

molecular responses during fruit ripening in various pear cultivars (Hiwasa et al., 2003; Nham et al., 2017; Charoenchongsuk et al., 2018; Mitalo et al., 2019*b*). Therefore, in this study, we used responses to 1-MCP to investigate the role of ethylene in regulating the genes we identified by YSST which showed differential expression during fruit ripening in 'La France'. We found that most of the genes (16 out of 22 differentially expressed genes) were regulated by ethylene (Tables 1, 2), which agrees with previous reports demonstrating that ethylene plays a crucial role in initiation and progression of fruit ripening in 'La France' fruit (Hiwasa et al., 2003; Mwaniki et al., 2005). However, one cell wall modification-associated genes, that is, *contig 36* (*EXP*) and as well as five other cDNAs encoding proteins of various functions (*contigs 28*, *32*, *75, 97* and *1025*), were not ethylene regulated (Tables 1, 2). Other studies have also demonstrated the existence of ethylene-independent aspects of fruit ripening in various pear cultivars (Nham et al., 2017; Lindo-García et al., 2019; Mitalo et al., 2019*b*; Lindo-García et al., 2020), and in different climacteric fruit species (Dumville and Fry, 2003; Tacken et al., 2010; Mworia et al., 2012; Asiche et al., 2018; Mitalo et al., 2019*a*).

4.2. Genes associated with differential softening during ripening in 'La France' and 'Yali' pears

The involvement of predicted cell wall modifying proteins identified through the YSST screen in pear fruit softening was investigated through comparison of expression patterns between 'La France' and 'Yali'. Increased expression of four ethylene-regulated genes, including *contig 2* (*PG3*), *contig 15* (*EXP*), *contig 19* (*EXP*) and *contig 55* (*PL*) correlated with softening in 'La France' fruit (Fig. 5A–D), and they showed no expression changes in 'Yali' fruit, suggesting consistent with a role in the softening process. *Contig 2* (*PG3*) has 99 % amino acid sequence homology with *PcPG2* (AX392013) and *PC-PG2* (AB084462), which were found to be associated with softening during ripening in 'Rocha' (Fonseca et al., 2005) and 'La

France' pears (Hiwasa et al., 2003; 2004), respectively. Ethylene-induced expression of several *EXP* genes has also been associated with fruit ripening-related softening in pears (Fonseca et al., 2005), peach (Hayama et al., 2006), kiwifruit (Yang et al., 2007), and tomato (Minoia et al., 2016). Additionally, *PL* genes have also been implicated in ethylene-induced fruit softening in various fruit species (Yang et al., 2017; Uluisik and Seymour, 2020).

Fruit softening has also been shown to correlate with increased expression of several *β-GAL* genes (Smith et al., 1998; Tateishi et al., 2001; Mwaniki et al., 2005). However, in this study, *contig 402* (*β-GAL*) was highly expressed with fruit ripening in 'Yali' fruit (Fig. 5E), even though softening was not observed (Fig. 2D). This finding suggests that this gene may not be critical for fruit softening in pears.

Although most of the isolated genes were ethylene-regulated, it is noteworthy that ethylene-independent genes (Fig. 7, 8) were also identified through the 1-MCP treatment. 1-MCP is thought to irreversibly bind to ethylene receptors (Kamiyoshihara et al., 2012), resulting in stable complexes that inhibit ethylene signaling. Previous studies have also used 1-MCP to differentiate between ethylene-regulated and ethylene-independent pathways in pears (Nham et al., 2017; Mitalo et al., 2019*b*;) and other fruit species (Golding et al., 1998; Mworia et al., 2012; Yang et al., 2013). Interestingly, differential expression patterns were also observed between 'La France' and 'Yali' fruit for the ethylene-independent cell wall modification-associated gene *contig 36* (*EXP*) (Fig. 7A). This suggests that the differential softening behavior of 'La France' and 'Yali' fruit involves the differential expression of both ethylene-regulated and ethylene-independent processes.

It is also worthy to acknowledge the role that cold acclimation may play in fruit softening in 'La France', given that the fruit were initially stored at 1 ºC for 21 d. In our previous report, we demonstrated that low temperature treatment potentiates ethylene responsiveness of ripening-related genes in the European pear 'Passe Crassane' via a unique

set of low temperature-specific genes (Mitalo et al., 2019*b*). Additionally, genes associated with low temperature-induced pathways were found to be differentially expressed during chilling-induced ripening in 'Bartlett' pear fruit (Nham et al., 2017). Since these LT-induced genes may not be captured by YSST, further research should be carried out using higher throughput strategies such as RNA-seq to provide a more comprehensive understanding of fruit softening in general, as well as the different softening patterns between 'Yali' and European pear cultivars.

5.0. Conclusion

The coupled YSST screen and macroarray strategy has advanced understanding of the genes involved in different softening behaviors exhibited by 'La France' and 'Yali' pear fruit during ripening. Genes encoding proteins associated with cell wall modification featured prominently in this group. Subsequent macroarray analysis revealed subsets that were differentially expressed during ripening, and gene expression analysis in fruit treated with 1-MCP helped to reveal the role of ethylene in their regulation. Additionally, comparative expression analysis revealed that different softening behaviors during fruit ripening in 'La France' and 'Yali' pears may be associated with different expression of both ethylene-regulated and ethylene-independent genes.

Author contributions

Mwaniki MW and Kubo Y designed the project. Mwaniki MW, Mworia EG and Ushijima K performed most of the experiments under the supervision of Kubo Y. Hiwasa-Tanase K performed the initial phenotyping experiments. Rose JKC and Ushijima K performed the YSST analysis. Aoki K performed the macroarray analysis. Nakano R, Esumi T and Kawai T were involved in data analysis. Mitalo OW and Owino OW took part in data interpretation and

writing of the manuscript. All authors have read and approved the final version of the manuscript.

Declaration of competing interests

The authors declare that they have no known competing interests.

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Figure Legends

Fig. 1. Schematic diagram of the YSST vector used in this study for cDNA library construction. Restriction enzyme sites for cDNA insertions and positions of the promoter (P_{TEF}) and terminator (T_{TEF}) of the transcription elongation factor gene are indicated. suc2 represents a yeast invertase gene lacking its own signal peptide (SP) and initiator methionine.

Fig. 2. Changes in ethylene production rate (A, B) and flesh firmness (C, D) in 'La France' and 'Yali' pear fruit during ripening with or without cold storage. 'La France' fruit were harvested at commercial maturity and stored at 1 ºC for 21 d, followed by ripening at 20 ºC. Fruit were then separately treated with 20 $\mu L L^{-1}$ 1-MCP for 12 h at 4 d after onset of ripening. 'Yali' fruit at commercial maturity were stored at 20 ºC for up to 23 d.

Fig. 3. Identification of secreted proteins during fruit ripening in 'La France' pears. (A) Putative translation products of the isolated YSST clones were analyzed for the presence of signal peptide sequence. (B) Functional classification of the isolated YSST clones based on Munich Information Center for Protein Sequences functional catalogue database.

Fig. 4. Scatter plot of the signal intensity corresponding to each YSST clone isolated from 'La France' fruit. Logarithmic values of expression ratios of pre-ripening and ripening fruit were plotted on the x-axis and y-axis, respectively.

Fig. 5. Expression patterns of ethylene-regulated YSST cDNA isolates associated with cell wall modification during fruit ripening in 'La France' and 'Yali' pears (A–E), and defense against pathogens (F). For 'La France', fruit at harvest (0 d) and after 21 d storage at 1ºC, as

well as after 4 and 13 d post-cold storage ripening at 20 ºC were used for the analysis. 1-MCP treated fruit at 13 d post-cold storage were used to confirm ethylene-dependence of the cDNAs. For 'Yali', fruit after 0, 11 and 15 d storage at 20 ºC were used for the analysis. Data points represent the mean (± SE) of three macroarray replications and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$). Note that the scales for y-axis are different

Fig. 6. Expression patterns of other ethylene-regulated YSST cDNA isolates during fruit ripening in 'La France' and 'Yali' pears. For 'La France', fruit at harvest (0 d) and after 21 d storage at 1ºC, as well as after 4 and 13 d post-cold storage ripening at 20 ºC were used for the analysis. 1-MCP treated fruit at 13 d post-cold storage were used to confirm ethylene-dependence of the cDNAs. For 'Yali', fruit after 0, 11 and 15 d storage at 20 ºC were used for the analysis.

Fig. 7. Expression patterns of ethylene-independent YSST cDNA isolates associated with cell wall modification during fruit ripening in 'La France' and 'Yali' pears (A), and defense against pathogens (B). For 'La France', fruit at harvest (0 d) and after 21 d storage at 1ºC, as well as after 4 and 13 d post-cold storage ripening at 20 ºC were used for the analysis. 1-MCP treated fruit at 13 d post-cold storage were used to confirm ethylene-independence of the cDNAs. For 'Yali', fruit after 0, 11 and 15 d storage at 20 ºC were used for the analysis. Data points represent the mean (± SE) of three macroarray replications and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$). Note that the scales for y-axis are different.

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List of Tables

Table 1. Pear YSST clones that were upregulated during fruit ripening along with their degrees of response to ethylene.

Table 2. Pear YSST clones that were downregulated during fruit ripening along with their degrees of response to ethylene.

Supplementary Tables

Supplementary Table 1. List of contigs and singletons that were assembled from the YSST clones isolated from ripening 'La France' fruit.

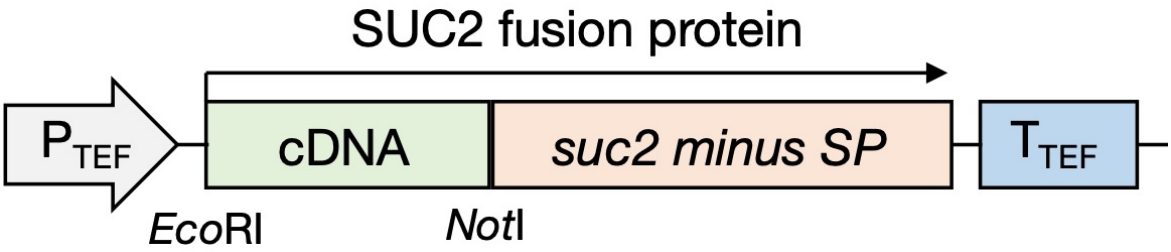


Fig. 1. Schematic diagram of the YSST vector used in this study for cDNA library construction. Restriction enzyme sites for cDNA insertions and the positions of the promoter (P_{TEF}) and terminator (T_{TEF}) of the transcription elongation factor gene are indicated. suc2 represents a yeast invertase gene lacking its own signal peptide (SP) and initiator methionine.

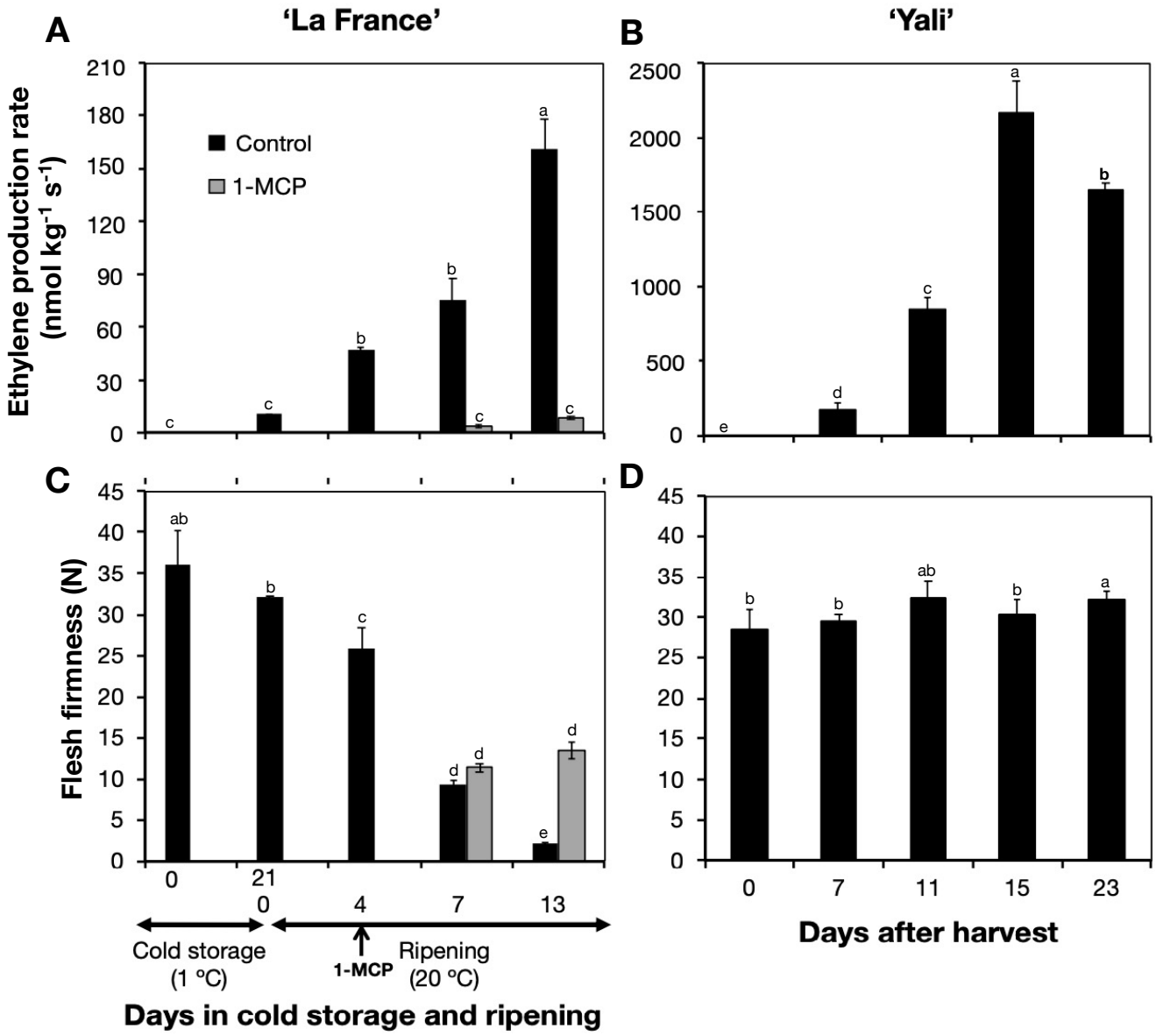


Fig. 2. Changes in ethylene production rate (A, B) and flesh firmness (C, D) in 'La France' and 'Yali' pear fruit during ripening with or without cold storage. 'La France' fruit harvested at commercial maturity were stored at 1 ºC for 21 d to induce ethylene production and then transferred to 20 ºC for ripening. At 4 d after the onset of ripening, fruit were separately treated with 20 μLL^{-1} 1-MCP for 12 h to block ethylene signaling. 'Yali' fruit at commercial maturity were stored at 20 ºC for up to 23 d. Data points represent the mean (± SE) of three fruit and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$). Note that the scales for y-axes in (A) and (B) are different.

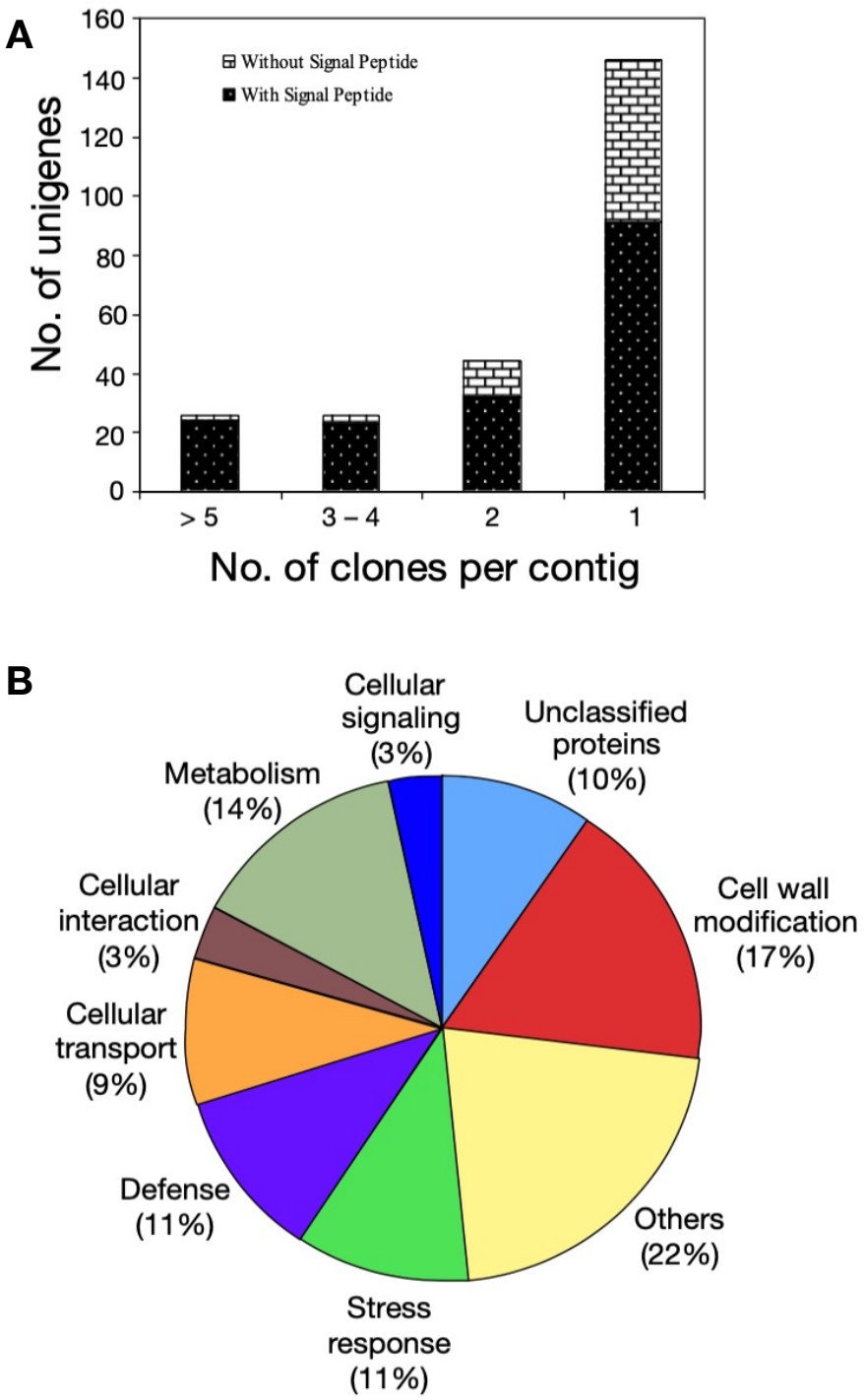


Fig. 3. Identification of secreted proteins during fruit ripening in 'La France' pears. (A) Putative translation products of the isolated YSST clones were analyzed for the presence of signal peptide sequence. (B) Functional classification of the isolated YSST clones based on Munich Information Center for Protein Sequences functional catalogue database.

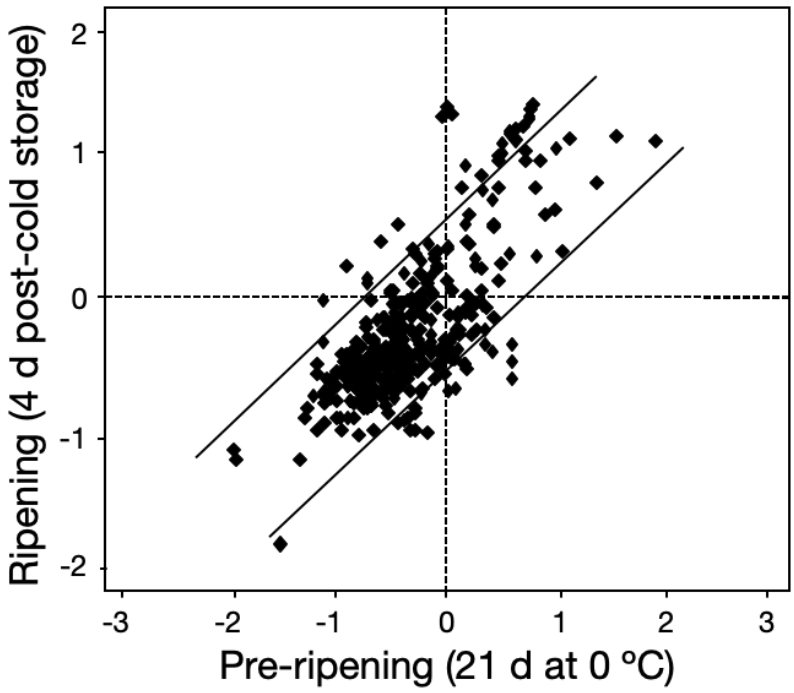


Fig. 4. Scatter plot of the signal intensity corresponding to each YSST clone isolated from 'La France' fruit. Logarithmic values of expression ratios of pre-ripening and ripening fruit were plotted on the x-axis and y-axis, respectively. Each point represents a single clone; the slanting lines represent the 3-fold increase or decrease in expression which was use as a cut-off for differentially expressed clones.

Table 1. Pear YSST clones that were upregulated during fruit ripening along with their degrees of response to ethylene.

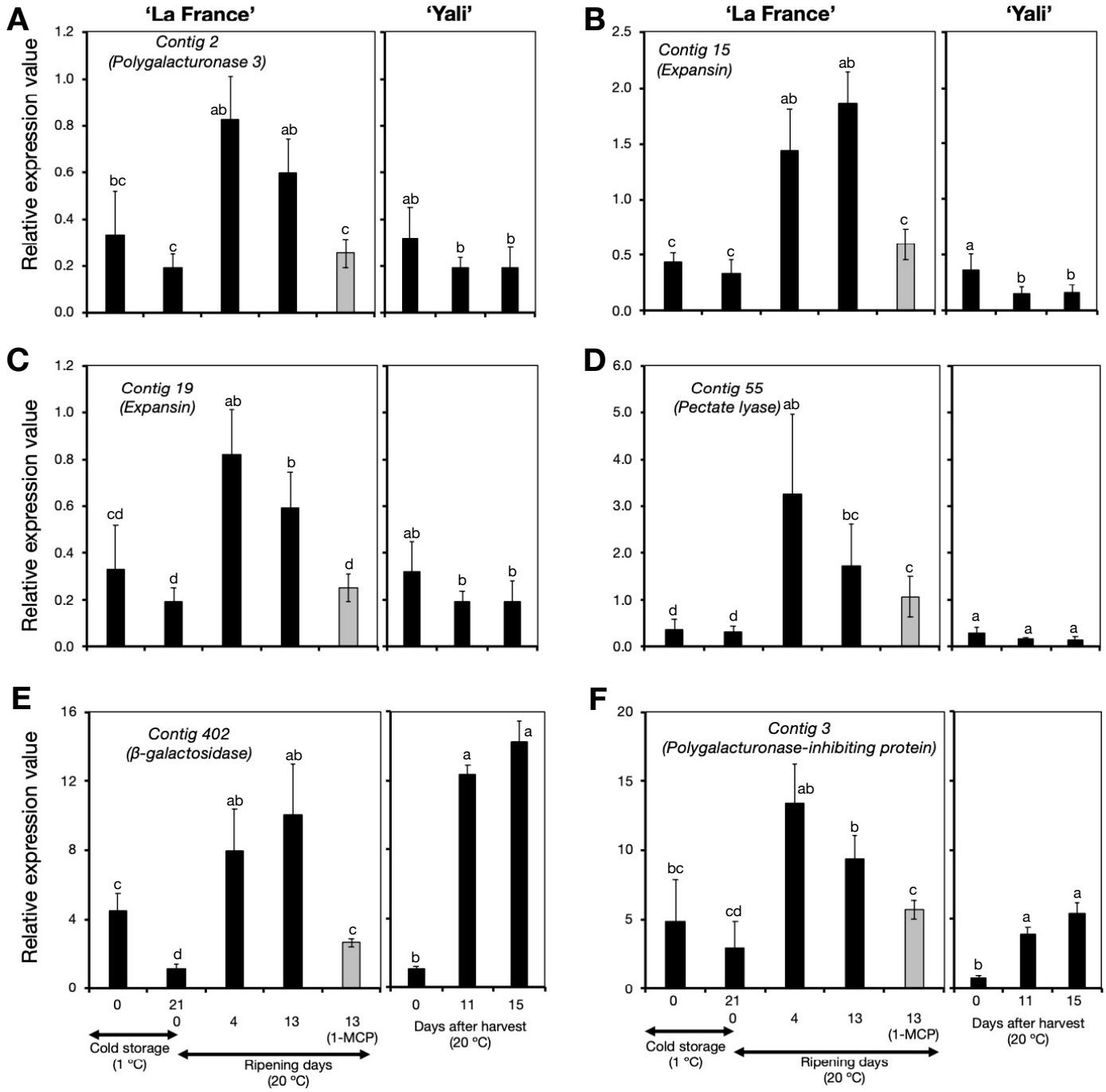
	Database annotation			SignalP 3.0 score[b]			
Contig/Clone (acc. no.)[a]	**Protein name**	**Annotated function**	**E value[c]**	**Species**	**Signal peptide probability**	**Signal anchor probability**	**Ethyleme Dependence[d] (%)**
1 (CAN77517)	Hypothetical protein	Unclassified proteins	2.00e-23	*Vitis vinifera*	1.000	0.000	100
2 (BAF42034)	Polygalacturonase 3	Cell wall modification	3.00e-135	*Pyrus communis*	0.994	0.005	100
13 (CAO45301)	Unnamed protein product	Unclassified proteins	2.00e-137	*Vitis vinifera*	0.999	0.001	100
15 (BAC67190)	Expansin	Cell wall modification	3.00e-131	*Pyrus communis*	0.999	0.000	86.78
19 (BAC67189)	Expansin	Cell wall modification	2.00e-53	*Pyrus communis*	0.999	0.000	75.08
83 (AAG38520)	Proteinase inhibitor se60-like protein	Defense	1.00e-14	*Citrus x paradisi*	0.996	0.004	97.03
876 (AAC32421)	Stellacyanin	Others	6.00e-12	*Cucumis sativus*	0.935	0.035	88.05
35 (AAX19847)	Thaumatin-like protein precursor	Defense	2.00e-101	*Malus x domestica*	1.000	0.000	91.08
402 (BAE72073)	Beta-galactosidase 1	Cell wall modification	6.00e-31	*Pyrus communis*	1.000	0.000	100
36 (BAC67191)	Expansin	Cell wall modification	3.00e-50	*Pyrus communis*	0.999	0.001	50.14
55 (BAF43573)	Pectate lyase	Cell wall modification	1.00e-53	*Prunus persica*	0.959	0.041	48.04
672 (AAB03843)	Aspartic proteinase	Metabolism	0.015	*Vigna unguiculata*	0.999	0.001	40.13
1025 (AAR11300)	Lectin-like receptor kinase 7;3	Stress response	1.00e-13	*Medicago truncatula*	0.999	0.000	19.87
3 (AAR15145)	Polygalacturonase-inhibiting protein	Defense	9.00e-75	*Eucalyptus grandis*	0.997	0.002	54.55
32 (AAB97142)	Cysteine protease	Stress response	4.00e-153	*Prunus armeniaca*	1.000	0.000	16.51
97 (BAC66141)	Beta-1,3-glucanase	Defense	7.00e-59	*Fragaria x ananassa*	0.997	0.003	52.25

[a] Contig/clone names with GenBank and UC Davis Apple expressed sequence tag accession numbers.
[b] SignalP analysis based on hidden Markov Model (HMM) and neutral networks (NN) methods.
[c] E value from protein-protein blast in NCBI database.
[d] Based on response to the ethylene perception inhibitor, 1-methylcyclopropene.

Table 2. Pear YSST clones that were downregulated during fruit ripening along with their degrees of response to ethylene.

	Database annotation				SignalP 3.0 score[b]		
Contig/Clone (acc. no.)[a]	**Protein name**	**Annotated function**	**E value[c]**	**Species**	**Signal peptide probability**	**Signal anchor probability**	**Ethyleme Dependence[d] (%)**
11 (BAA96447)	Dehydrin-like protein	Stress response	2.00e-06	*Prunus persica*	0.000	0.000	100
263 (BAE71278)	Putative fasciclin-like arabinogalactan protein FLA2	Cellular interaction	2.00e-63	*Trifolium pratense*	1.000	0.000	100
86 (ABS72341)	Rapid alkalinization factor precursor	Cellular signaling	3.00e-08	*Litchi chinensis*	0.982	0.018	100
550 (NP_179994)	Putative endomembrane protein 70	Others	3.00e-08	*Arabidopsis thaliana*	0.994	0.006	100
28 (BAF51705)	Bifunctional UDP-glucose-epimerase	Metabolism	3.00e-61	*Malus x domestica*	0.996	0.004	27.03
75	No suitable match	Unclassified proteins	–	–	0.997	0.002	5.23

[a] Contig/clone names with GenBank and UC Davis Apple expressed sequence tag accession numbers.
[b] SignalP analysis based on hidden Markov Model (HMM) and neutral networks (NN) methods.
[c] E value from protein-protein blast in NCBI database.
[d] Based on response to the ethylene perception inhibitor, 1-methylcyclopropene.



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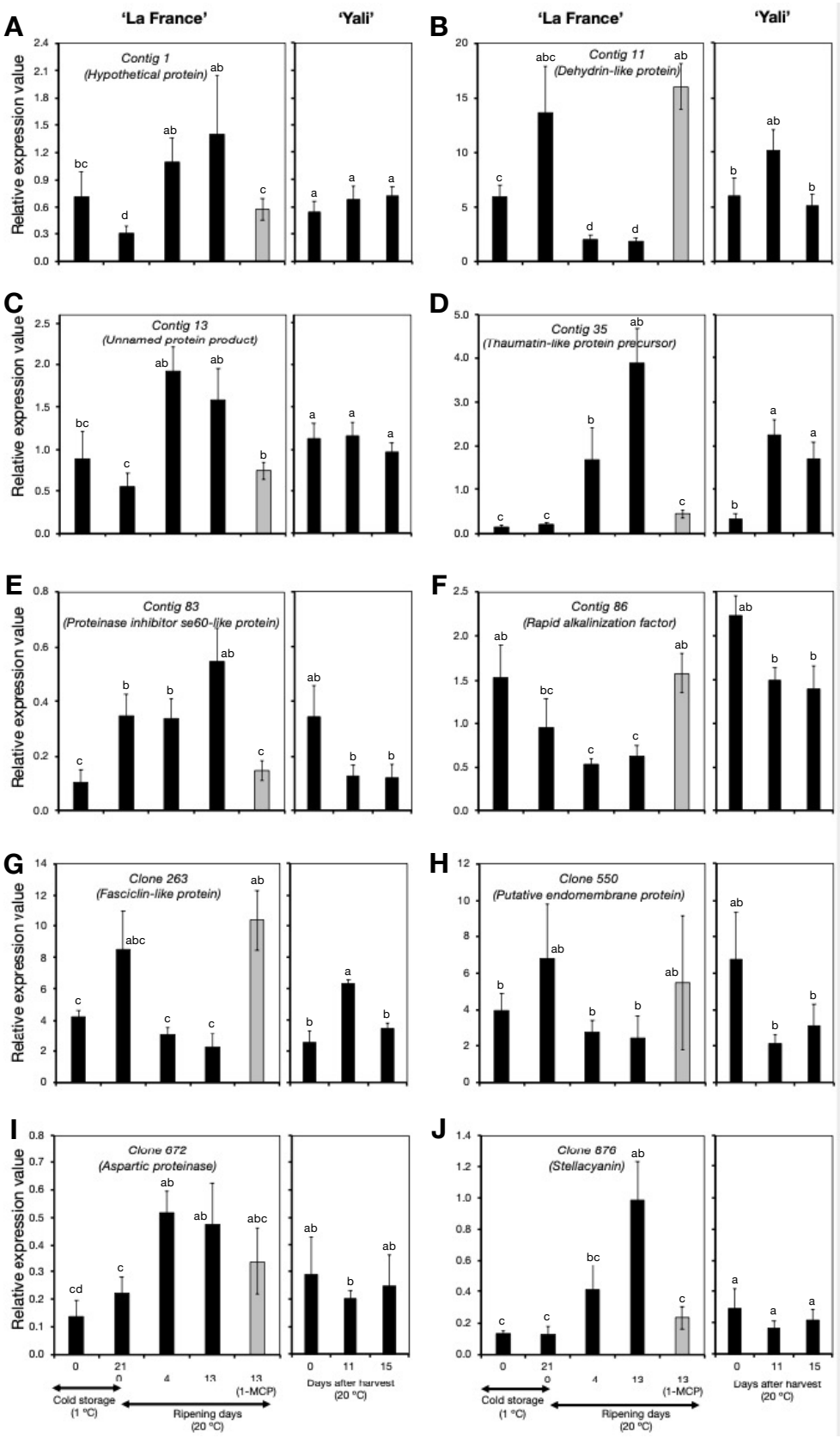

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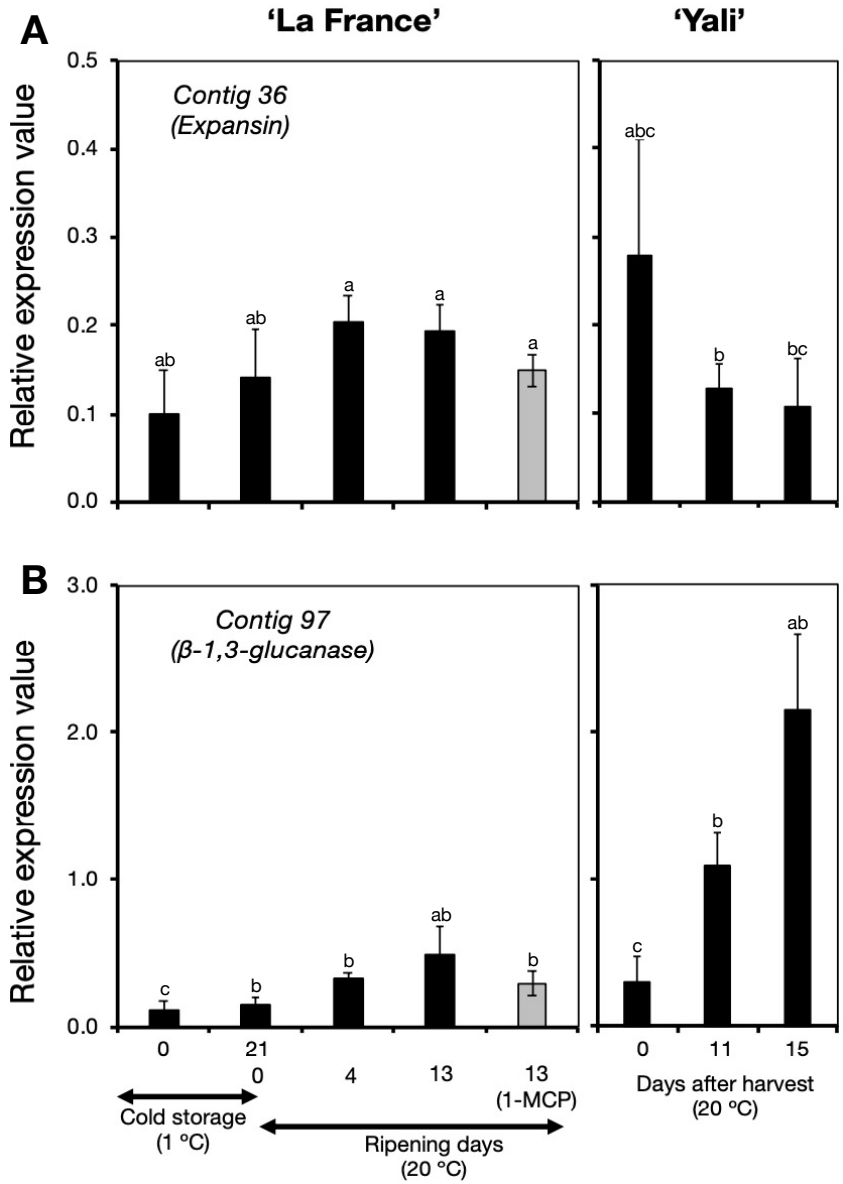

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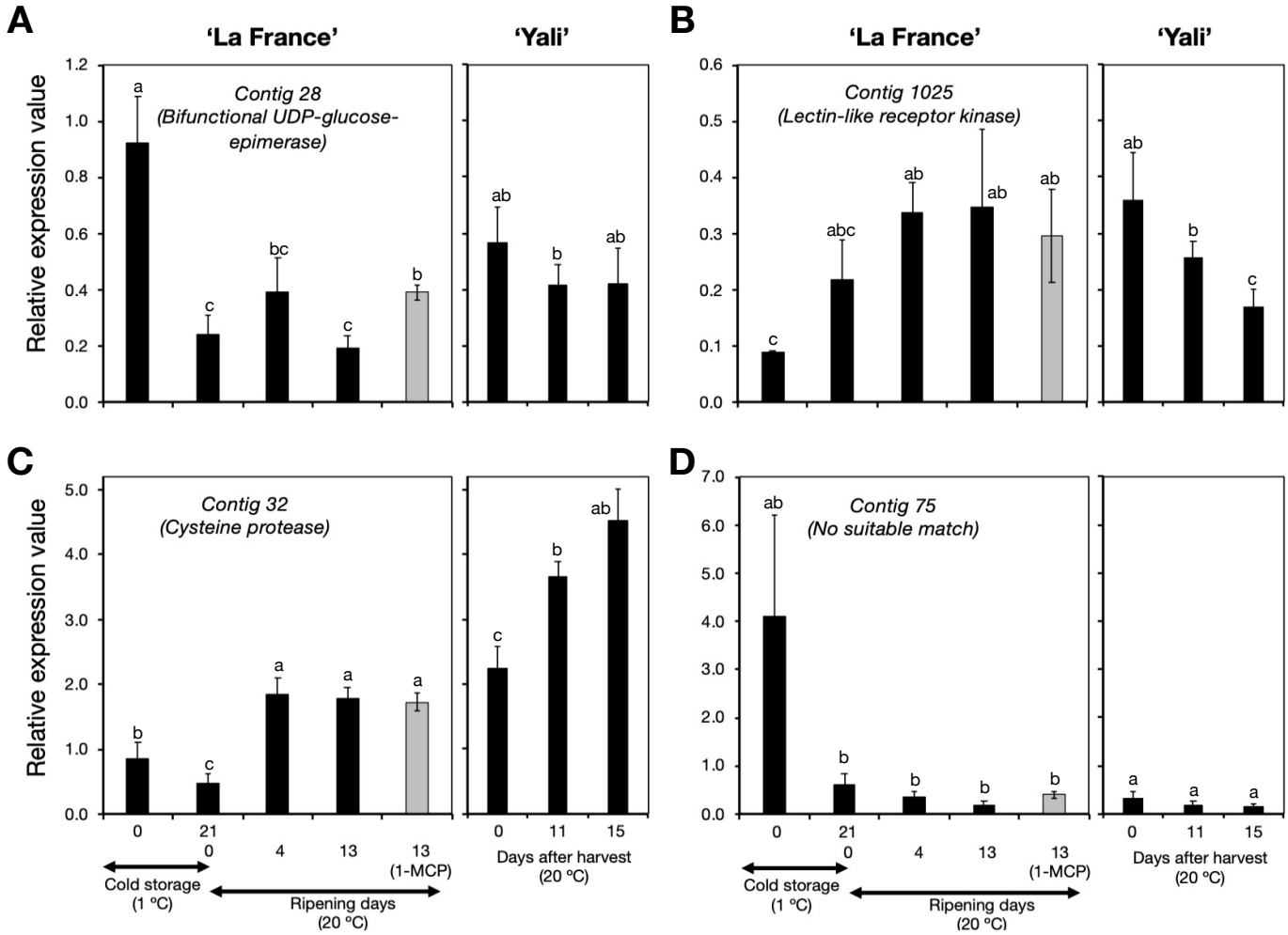

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