Roles of Endoplasmic Reticulum Stress and Unfolded Protein Response Associated Genes in Seed Stratification and Bud Endodormancy during Chilling Accumulation in Prunus persica

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Abstract

Dormancy mechanisms in seeds and buds arrest growth until environmental conditions are optimal for development. A genotype-specific period of chilling is usually required to release dormancy, but the underlying molecular mechanisms are still not fully understood. To discover transcriptional pathways associated with dormancy release common to seed stratification and bud endodormancy, we explored the chilling-dependent expression of 11 genes involved in endoplasmic reticulum stress and the unfolded protein response signal pathways. We propose that endoplasmic reticulum stress and the unfolded protein response impact on seed as well as bud germination and development by chilling-dependent mechanisms. The emerging discovery of similarities between seed stratification and bud endodormancy status indicate that these two processes are probably regulated by common endoplasmic reticulum stress and unfolded protein response signalling pathways. Clarification of regulatory pathways common to both seed and bud dormancy may enhance understanding of the mechanisms underlying dormancy and breeding programs may benefit from earlier prediction of chilling requirements for uniform blooming of novel genotypes of deciduous fruit tree species.

Introduction

The peach (Prunus persica) originated in China and has been cultivated as an ornamental and economic fruit crop in Asia for about 3000 years because of its attractive flowers and delicious fruit. Prunus persica blooms earlier in spring compared with other deciduous fruit crops that usually require a long duration of chilling.

The dormancy and germination of seeds and buds are complex adaptive processes that are affected by a network of genes and environmental elements in tree species native to temperate and boreal regions [1]. Both types of dormancy are characterized by extremely low metabolic activities and a temporary insensitivity to growth-promoting signals. Dormancy is advantageous because it prevents the germination of intact viable seeds during temporarily advantageous conditions in an otherwise undesirable season [2]. Buds only attain ‘deep dormancy’ after a progressive process induces increasing intensity of dormancy during autumn [3]. Subsequently, a chilling accumulation requirement must be fulfilled for seed and bud dormancy release. Insufficient chilling can significantly impact on the time of seed germination and bud break, the duration of blooming, and fruit quality [4]. This phenomenon may result in major economic losses to the agricultural industry.

To understand the mechanisms underlying dormancy, much progress has been made in identification of the genes associated with dormancy. In this regard, a variety of woody species, including peach [5], Japanese apricot [6], grape [7], poplar [8], and raspberry [9], have been studied. Nevertheless, the mechanisms responsible for dormancy in deciduous fruit trees remain to be elucidated.

Proteomic analysis has become an extremely valuable approach with which to investigate the molecular pathways and identify the proteins involved in seed dormancy [10,11]. Proteins that are secreted to the cell surface first enter the endoplasmic reticulum (ER), where they are folded and assembled before their delivery to other compartments in the endomembrane system. A conserved signalling pathway termed the unfolded protein response (UPR) monitors the protein-folding capacity in the ER lumen and transduces information on perturbations in the protein-folding status to the nucleus and cytosol [12]. Plants maintain a balance between protein-folding and -degradation capacity in line with demand either by enhancing the protein-folding or -degradation machinery, or by slowing protein production [13].

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Most previous studies of UPR have focused on yeast and mammalian cells. The UPR signalling pathways in mammalian cells comprise three branches that involve different ER stress transducers and sense the accumulation of misfolded or unfolded proteins and target the stress response genes. One pathway is regulated by the mammalian homolog of inositol-requiring enzyme-1 (IRE1), which splices the mRNA of bZIP-like transcription factor X-box binding protein 1 (XBP1). A second pathway is mediated by activating transcription factor 6 (ATF6), which is transported to the Golgi to be processed by site 1 and site 2 proteases (S1P and S2P). In the third pathway translation is regulated by RNA-activated protein kinase (PKR) - like ER kinase (PERK) [14].

The UPR in plants involves the transcriptional regulation of genes that function in protein folding and degradation [15]. In plants only two arms of the UPR signalling pathway have been identified: one arm involves the proteolytic processing of membrane-associated basic leucine zipper domain (bZIP) transcription factors (ATFs) and the second arm involves the RNA splicing factor IRE1 [16]. PERK has not been detected in Arabidopsis [16,17]. In Arabidopsis, ER stress is sensed and stress signals are transduced by the membrane-bound IRE1-like (IRE1a and IRE1b) and ATF6-like (bZIP28) transducers. Plant cells contain IRE1a and IRE1b. IRE1a and IRE1b are similar in structure and function to mammalian counterparts, which target bZIP mRNA [10,19]. In response to ER stress, protein kinase and endoribonuclease activities of IRE1a are activated through the dissociation of binding immunoglobulin protein (BiP) from IRE1a and subsequent dimerization. Activated IRE1a removes an unconventional intron from unspliced bZIP60 mRNA in the cytosol. The bZIP60 translocates to the nucleus and controls transcription of the genes that encode ER chaperones. In response to ER stress, IRE1b induces translational repression through 28S ribosomal RNA cleavage. Similar to ATF6 in response to ER stress, the bZIP28 transcription factor is activated and relocated from the ER to Golgi where it is processed by S1P and S2P successively [20–22]. The bZIP28 assembles a larger transcriptional activation complex by interaction with the heterotrimERIC CCAAT binding factors composed of subunits NF-YA4, NF-YB3, and NF-YC2. Thus, IRE1-bZIP60 and bZIP28-NF-Y UPR signalling pathways are known in plants.

Recent studies show that in plants the UPR is closely associated with response to adverse environmental stresses, such as salt stress [20,23], heat stress [24–26], and drought stress [27,28]. Thus, ER stress and UPR genes are involved in the response to abiotic stress. However, the genotype-specific period of seed stratification and bud endodormancy are elicited by ER stress and the UPR by molecular mechanisms that are poorly understood.

To elucidate characteristics common to seed and bud dormancy, we explored the expression of ER stress- and UPR-associated genes involved in transcriptional regulation during chilling of seeds and buds in peach. Clarification of the regulatory pathways of ER stress and the UPR in both seeds and buds will enhance understanding of the mechanisms of dormancy and may benefit breeding programs through earlier prediction of chilling requirements for uniform blooming of novel genotypes of deciduous fruit tree species.

### Materials and Methods

#### Plant material

Trees of peach (*Prunus persica* (L.) Batsch var. *nectarina* (Suckow) C. K. Schneid.) were cultivated for five years under standard conditions. In 2011 budding material from 15 October to 1 January 2012 was collected, and the date of bud break was recorded. The data indicate variation in mean time to bud break (MTB) values during dormancy from 15 October 2011 to 1 January 2012. Values are means (n = 3) and error bars represent the standard deviation.

![Figure 1. Progression of bud dormancy.](image)

The data indicate variation in mean time to bud break (MTB) values during dormancy from 15 October 2011 to 1 January 2012. Values are means (n = 3) and error bars represent the standard deviation. doi:10.1371/journal.pone.0101808.g001

#### Table 1. Sequences of primer pairs used for qRT-PCR.

| Accession number (Gene) | Forward Primer (5’ - 3’) | Reverse Primer (5’ - 3’) |
|-------------------------|--------------------------|-------------------------|
| ppa002489m (BiP1-like) | ACCAGGGTAACGATTCATCAC   | GTTATTCTTCACCTGCCTTT    |
| ppa002527m (BiP2-like) | TTGCTCGGCTCATAAGTGGT    | ATAGCTGTCGCTGATGCGG    |
| ppa001126m (IRE1a-like) | GTAGTTGAAGAGAGAAATG     | ATATAATAGAGTGACAGCAA   |
| ppa001418m (IRE1b-like) | ATAATGGCTGCTTGTGATG     | GTTACTGTATGTATGTTGAT    |
| ppa002181m (bZIP28-like) | AGTGATGAGGACAGAACACG    | CTTATGCTCAAGTCTTCACAT   |
| ppa008311m (bZIP60-like) | AGGATGTCGAGAGGATCGAG    | GCAGACGTGACGCAACAGG    |
| ppa000662m (SIP-like) | TTGTATGGCTCCTGAGGAA     | ACAGTATGTTGCTTCTCCT    |
| ppa0024254m (SIP-like) | GCTTAACTCTTCTGATGGA     | TTTCTGCTGATGATGATG      |
| ppa001627m (NF-YA-like) | GTTCTGATAAATGGAATCTGATG | GTCGTTTTCCAACCTGTG      |
| ppa011174m (NF-YB3-like) | GACATGACATGACATCACA     | AACCAACATTCCACTACA      |
| ppa010147m (NF-YC2-like) | CAACACACTACGACCAACAG    | TCTCATCAGGCTTCTA       |
| ppa002399m (ACTIN) | GTATTGCTCATGCCTGCCTCG    | CTTCACCATTCCAGTCCATTGTC |

*ppa000662m (SIP-like) Forward Primer (5′-3′) Reverse Primer (5′-3′)*

- **Table 1.** Sequences of primer pairs used for qRT-PCR.
agricultural practices at the horticulture experimental station of Shandong Agricultural University, Tai’an, China. Experiments were carried out between June 2011 and February 2012.

Seed sampling
Two hundred and forty ripe fruits were collected from 60 distinct trees (4 fruits each tree, respectively) on 26 June, 2011, which were divided randomly into three groups. The endocarp was dissected from the fruit immediately. Seeds were sterilized and flamed with alcohol, and then the coats were removed under sterile conditions. Embryos were cultured on 20 ml aliquots of sterile WPM medium solidified with 0.8% bacteriological agar in culture tubes. The tubes were stored at 4°C under continuous darkness for 0, 15, 30, 45, and 60 days, respectively [29]. Thirty randomly selected, untreated embryos were frozen in liquid nitrogen and stored at −80°C for RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR) analysis.

Bud sampling
Buds were sampled before leaf abscission, and during the dormancy and dormancy-release periods, on 15 and 23 October, 1, 8, 15 and 23 November, 1, 8, 15 and 23 December, and 1 January. At each time point, 60 flower buds with the scales removed were harvested from first-year branches of different vigorous individual trees, immediately frozen in liquid nitrogen, and stored at −80°C for RNA extraction and qRT-PCR analysis.

Measurement of bud endodormancy status
Bud endodormancy was measured on 120 first-year branches incubated in 5% sucrose solution after leaf abscission on 15 and 23 October, 1 and 15 November, 1, 15 and 23 December, and 1 January. Trials were conducted in a completely randomized design with three replicates of 40 cuttings per treatment. The branches were incubated in a growth chamber under a daily cycle of 25°C with artificial fluorescent light (200 mol m⁻² s⁻¹) for 16 h and 18°C in darkness for 8 h with constant 70% relative humidity. Bud break was considered to have occurred when a green shoot tip was observed among the bud scales. The mean time to bud break (MTB) was calculated as described by Leida et al. [30]. The period before the date that corresponded to an MTB interval of 6 weeks was considered to represent paradormancy, and the same interval after this date represented endodormancy. The basal ends of the shoots were cut weekly, and sucrose solution was replaced daily. The number of sprouting buds was recorded weekly. The time to bud break of a group of shoots was the time in days required for at least 50% of the flower buds to break. The results were expressed as the mean time to sprout for the three replicates.

Genes associated with ER stress and UPR in stratified seeds and buds
To explore changes in gene expression during seed stratification and bud endodormancy under chilling treatment, the expression of ER stress-related genes in experimentally chilled embryos and buds were analysed by qRT-PCR. BiP, a member of the HSP70
family, is the most abundant chaperone in the ER and includes BiP1 and BiP2. Ppa002489m and ppa002572m code for putative BiP1 and BiP2 proteins in peach, respectively. The relative expression of transcripts that code for IRE1a-like (peach transcript model ppa001128m), IRE1b-like (ppa001418m), bZIP28-like (ppa002181m), bZIP60-like (ppa008311m), S1P-like (ppa000662m), and S2P-like (ppa024254m) protein in stratified seeds and buds during endodormancy were analysed. The bZIP28 interacts with the heterotrimeric CCAAT binding factors composed of subunits NF-YA4, NF-YB3, and NF-YC2, therefore expression of NF-YA4-like (ppa011627m), NF-YB3-like (ppa011714m) and NF-YC2-like (ppa010147m) transcripts were also analysed.

Isolation of RNA and qRT-PCR analysis

Total RNA from 100 mg seeds (at least four seeds every time) with their coats removed or 300 mg buds was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer’s instructions. Chloroform (200 µl) was included in the extraction buffer for seeds. Three duplicates of approximately 9 µg total RNA extracts were reverse-transcribed with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) in a total volume of 20 µl. The qRT-PCR was performed with SYBR Premix Ex Taq (TaKaRa Biotechnology, Dalian, China). Composition of the reaction solution and conditions followed the manufacturer’s instructions. The primers employed are listed in Table 1. The cycling protocol comprised 10 min at 95°C, then 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing and extension. Specificity of the PCR was assessed by the presence of a single peak in the dissociation curve after the amplification and by size estimation of the amplified product. The comparative threshold cycle CT (2^(-ΔΔCT)) method was used to quantify cDNAs with amplification efficiencies equivalent to that of the reference actin gene. The standard curve regression was applied when amplification efficiencies were not equivalent to that of the reference actin gene. Results presented are the average of three independent biological replicates repeated three times.

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 19 (SPSS, Chicago, IL, USA). Categorical variables were expressed as frequencies, and percentages and continuous variables as the mean ± standard deviation, as appropriate. The data were analysed by ANOVA and, when appropriate, Duncan’s test was used. A significance level of p<0.05 was applied.

Results

Measurement of bud endodormancy status

Seasonal progression of bud MTB followed an oscillating pattern during the experimental period (Figure 1). A marked increase in MTB occurred in early winter until late November, when the highest MTB value was recorded. A slight decrease in MTB was observed in early December and thereafter MTB continued to decrease in December and January until dormancy release. The minimum MTB observed was acquired by early January, just before blooming. The interval of 6 weeks in MTB before 23 October was considered to represent paradormancy,
and the same interval in MTB between 23 October and 23 November represented endodormancy.

**BiP transcript accumulation and IRE1-bZIP60 pathway activation**

During seed dormancy, BiP1-like transcripts accumulated during the first month and attained a peak that was three-fold higher than the baseline level (Figure 2A). Thereafter, the expression level was lower but remained relatively stable until dormancy release. In contrast, expression of BiP2-like transcripts drastically declined after 15 days and subsequently remained stable at an extremely low level. Expression of BiP1-like and BiP2-like transcripts in dormant buds showed similar trends to those observed in stratified seeds, with transcript expression showing a significant increase during the first month and subsequently decreasing to a lower, significantly different level (Figure 2C, D).

Expression of IRE1a-like and IRE1b-like transcripts significantly increased within the first 30 days of chilling (Figure 3A, B). Expression of both genes subsequently decreased significantly. Transcription of both genes during bud dormancy showed the same trends as those observed during seed dormancy (Figure 3D, E). A significant decrease in expression of IRE1a-like and IRE1b-like in buds was observed after 23 November.

In seeds, bZIP60-like transcription was significantly up-regulated and maximum expression was observed after chilling for 15 days. The peak expression level was 3.63-fold higher than the baseline level (Figure 3C). Subsequently, the expression level declined and after chilling treatment for 45 days did not differ significantly from the baseline level. Expression of in buds showed similar trends, although the changes were less marked compared with those observed in seeds (Figure 3F).

**Up-regulation of bZIP28-NF-Y transcription factors**

Expression of bZIP28-like in seeds and buds was significantly up-regulated after 15 days of chilling, but thereafter bZIP28-like was down-regulated and the expression level was significantly lower than the baseline level (Figure 4A, D).

A gradual increase in S1P-like and S2P-like expression levels was observed after deep dormancy was formed during the first 30 days of chilling in both seeds and buds. The peak expression level was significantly higher than the baseline level (0 day, 23 October) (Figure 4B, C, E, F). Subsequently, expression decreased to a level similar to, or significantly lower than, the baseline level.

In seeds NFYA4-like and NFYB3-like transcripts showed a continuous, significant decline in expression from the baseline level throughout the experimental period. After chilling for 60 days, the relative expression level was only about one-fourth that of the baseline (Figure 5A, B). In contrast, the NFYC2-like expression level in seeds increased significantly to a maximum after chilling for 30 days, and thereafter declined to a level similar to the initial baseline values (Figure 5C).

The changes in NFYC2-like expression in buds were similar to those observed in seeds (Figure 5F). However, trends in the expression of NFYA4-like and NFYB3-like transcripts in buds differed from those observed in seeds. NFYA4-like and NFYB3-like transcripts were both up-regulated but at different time points (NFYA4-like, 23 November; NFYB3-like, 8 November) (Figure 5D, E).
Discussion

Dormancy of seeds and buds in deciduous fruit trees are complex phenomena that provide a protective mechanism against the impact of low winter temperatures on plant survival, development and architecture [31]. The ability to manipulate these processes is important to increase the yield and seasonal availability of many fruit crops. In many cases, release of dormancy results in increased cell division and changes in developmental programs. Much can be learned about dormancy regulation by identifying interactions of signals in these crucial processes.

Studies of the regulation of bud dormancy have resulted in some of the most fundamental discoveries in plant science. Related studies of plant growth and shoot development have identified many genes involved in meristem initiation and organ formation, and many genes and signals that control cell division in plants [4]. Despite these findings, gaps remain in our knowledge of the induction and release of bud and seed dormancy. Endogenous dormancy, which is regulated by endogenous physiological factors, plays a pivotal role in bud and seed dormancy. The temperature in autumn and early winter contributes to the intensity and progression of dormancy. Analogous observations are reported in apple, in which the chilling accumulation affects the progression of bud dormancy [32]. Chilling accumulation has long been considered to be the major factor leading to endogenous dormancy [33]. The present research is aimed at determining whether the fundamental process of dormancy release in seeds and buds dependent on chilling accumulation is regulated by a common signalling pathway.

Endoplasmic reticulum stress agents that affect Ca^{2+} homeostatic balance are surrogates for environmental stresses, and all of these agents induce the UPR. As yet, there is no clear understanding as to how endogenous dormancy induces ER stress and the UPR in plants and how the membrane-associated transcription factor system discriminates during dormancy. BiP has been demonstrated to act as a multifunctional protein in animal systems [34]. In addition to its role as molecular chaperone, in mammalian cells BiP has been shown to function as a sensor of alterations in the ER environment that activate the cytoprotective UPR [34]. Like BiP from animal systems, plant BiP has been shown to regulate UPR negatively [35], which have been identified in a variety of processes, such as the regulation of endosperm proliferation in *Arabidopsis thaliana* and the accumulation of seed storage proteins in rice, and are up-regulated in response to abiotic stresses such as salt, drought and other ER stress agents [17,19,21,36–39]. Therefore, it will provide an improved understanding of folding capacity in the ER and more accurate assessment of the stress status of the plant by monitoring BiP transcription [27,37,40]. In the present study, two marker genes of ER stress, *BiP1* and *BiP2*, were up-regulated by fulfilment of the chilling requirement. BiP accomplishes two main functions. During accumulation of unfolded or misfolded proteins induced by endodormancy in the ER, BiP appears to alleviate the stress by binding to misfolded proteins in the ER to prevent their abnormal aggregation during refolding processes. Secondly, BiP is one of the main transcription targets of active bZIP transcription factors [20,21,41]. BiP regulates the activity of the ER stress sensors or transducers, bZIP28 and bZIP60, as well as IRE1a and IRE1b, through targeting increased expression of the chaperones of BiP or decreasing the transcriptional expression of unfolded or misfolded

![Figure 5. Relative expression levels of *NF-YA4-like*, *NF-YB3-like*, and *NF-YC2-like*. Expression was measured in seeds (A–C) and buds (D–F). Expression levels were normalized against that of the actin gene. Values are means (*n* = 9) and error bars represent the standard deviation. Different letters above bars indicate a significant difference among chilling periods according to ANOVA and Duncan’s test. doi:10.1371/journal.pone.0101808.g005](#)
proteins. Collectively, the UPR is a critical approach to establish homeostasis and also plays a crucial role in mediating the release of endodormancy by processing misfolded or unfolded proteins.

The present qRT-PCR analysis revealed a parallel pattern of gene expression related to dormancy in seeds and buds. Most of the genes down-regulated during bud endodormancy release after fulfillment of the chilling requirement were also repressed by cold stratification in seeds. This suggests that common regulatory signalling pathways are involved in the mechanisms of endogenous dormancy and its release in seeds and buds.

The transcriptional similarities between bud and seed dormancy highlighted in the present study may also be relevant for plant breeding purposes. The selection of early- and late-flowering genotypes from a segregating population usually requires thearduous evaluation of large collections of individuals, which could be improved by previous selection of the desirable trait at the seed level. Previous studies found a positive correlation between the chilling requirements for seed germination and blooming in almond and apple [42]. The present work contributes tocharacterization of the molecular basis for these and other physiological traits of interest to plant breeders.

**Conclusions**

The current research provides an overview of the essential processes associated with seed stratification and bud endodormancy, and a description of possible impediments that may result in dormancy. The results show that ER stress and the UPR in seeds and buds elicit BiP chaperones. Thus, both seed and bud endodormancy involve a complex network of signalling. We conclude that endogenous dormancy of seeds and buds is associated with ER stress and the UPR, and the ER stress signal pathway could play a pivotal role in the complex network of endogenous dormancy regulation. The emerging interplay between seed stratification and bud endodormancy status suggests that these two fundamental processes are probably regulated by common signalling pathways. The identification of ER stress and UPR signal pathways provides a new perspective for future research to evaluate their significance in dormancy. The elucidation of general regulatory pathways in both seeds and buds may contribute to an improved knowledge of dormancy mechanisms, and may be valuable for plant breeding programs that will profit from early prediction of chilling requirements for blooming of novel genotypes.

**Author Contributions**

Conceived and designed the experiments: XLF DSG. Performed the experiments: XLF WX LL MC. Analyzed the data: XLF MC LL DSG. Contributed reagents/materials/analysis tools: QPT WX DLW XDC. Wrote the paper: XLF DSG. Revised the article critically for intellectual content: XLF DSG. Final approval of the completed article: XLF WX DLW MC QPT LL DSG.

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