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Bud dormancy in Japanese pear

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Key words: budbreak, chill units, chilling requirements, climatic change, global warming, Japanese pear, phytohormone.

Abstract: In this paper, after reviewed recent advances in research of endodormancy, research on Japanese pear were reported. In the case of Japanese pear, endodormancy was induced by low temperature of 5°C, without effect of day length. Chilling requirement (CR) of Japanese pear for completion of leaf bud endodormancy shows wide range from below 800 to 1800 chilling units (CU). We investigated the budbreak percent in Taiwanese pear Yokoyama, in Japanese pear strain TH3 and their F1 for 3 years. The percentage of budbreak in TH3 was lower than Yokoyama on any observational days and it gradually increased from early December to early January. The percentage of budbreak in F1 plants was widely distributed between that of Yokoyama and TH3 on all observational days. From results of chi-square test, it was suggested that pear plant had quantitative trait loci (QTL) as genetic factor to decide chilling requirement (CR) for breaking endodormancy. Expression levels of gene encoding GAST-like gibberellin (GA) regulated protein increased with development of endodormancy in Japanese pear cultivars. ABA concentrations in bud of Japanese pear in open-field were increased with the induction of endodormancy by chilling. However, the ABA concentrations in un-chilled plants were lower.

1. Introduction

Bud dormancy in temperate-zone deciduous fruit trees is an adaptive mechanism to survive unfavourable conditions during the winter (Faust *et al*., 1997) and it is classified into three different stages: paradormancy, endodormancy and ecodormancy (Lang, 1987). In autumn, buds enter a dormant state known as endodormancy after the trees stop growing and the leaves fall. During this state the trees cannot start bud growth even if the environmental conditions are favourable. Endodormancy is broken by accumulation of low temperatures, known as chilling requirement (CR), and it depends on the species and cultivar (Westwood, 1978; Saure, 1985). However, a lack of adequate chilling to satisfy the CR to break endodormancy due to climate change (especially global warming) in recent years inhibits the normal growth of new organs in spring (Sugiura *et al*., 2007).

Japanese pear [*Pyrus pyrifolia* (Burm. f.) Nakai] is one of the most important fruits for the Japanese and the earliest records of pear cultivation in Japan date back 1300 years. Commercial Japanese pear cultivation increased with the development of a consumer society during the Edo period (1600-1868). Since then, Japanese pear production underwent from 1920 to 1930, then again from 1950 to a peak in 1970; cultivation is now stable (Tamura, 2006). Figure 1 and Table 1 show the main pear growing areas in Japan. The average annual temperatures of these areas range from 12 to 17°C, and the average growing season temperature (April to September) is 19 to 23°C.

In recent years, Japanese pear cultivation has significantly increased in warm-winter regions (Chen *et al*., 1995). In countries such as New Zealand (Kingston *et al*., 1990; Klinac and Geddes, 1995) and Brazil (Petri and Herter, 2002; Petri *et al*., 2002), a lack of winter chilling has caused problems in promoting bud break in pear during spring. Several recent studies have focused on the breaking of dormancy in grapes (Or *et al*., 2000, 2002; Pang *et al*., 2007; Halaly *et al*., 2008) by using hydrogen cyanamide (HC) as a tool to modify the breaking of endodormancy. In grapes, treatment of buds with HC increased

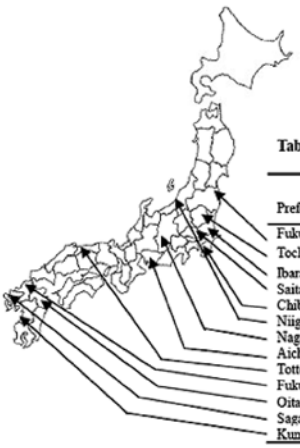

Table 1. Planted area and climate of the main producing prefectures in Japan (2003).

Prefecture	Area (ha)	Average temp (°C)		Rainfall (mm)	
		Annual	Apr.-Sept.	Annual	Apr.-Sept.
Fukushima	1160	12.8	19.5	1105	743
Tochigi	882	13.4	20.0	1443	1076
Ibaraki	1600	14.2	20.3	1154	742
Saitama	678	14.6	21.0	1338	917
Chiba	1660	15.4	21.2	1294	790
Niigata	546	12.9	19.7	1828	820
Nagano	1010	12.5	19.4	1607	1094
Aichi	421	14.5	21.1	1448	1002
Tottori	1390	14.4	20.3	1754	962
Fukuoka	559	16.0	22.3	1919	1450
Oita	450	15.1	21.8	1843	1365
Saga	413	15.5	21.6	2207	1640
Kumamoto	629	16.5	22.9	1993	1514

Fig. 1 - Main producing prefectures in Japan.

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hydrogen peroxide concentration and inhibited catalase activity (Perez *et al*., 2008). This is the first step in a cascade that up-regulates several signaling proteins such as transcription factors, protein phosphatases, and protein kinases (Neill *et al*., 2002). Application of HC has also been shown to result in transcriptional up-regulation of grape dormancy-breaking related protein kinase (GDBRPK), a sucrose non-fermenting protein kinase (SNF-like protein), pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), thioredoxin h (Trxh), glutathione S-transferase (GST), ascorbate peroxidase (APX), glutathione reductase (GR), and sucrose synthase (SuSy) (Or *et al*., 2000; Pérez and Lira, 2005; Keilin *et al*., 2007; Halaly *et al*., 2008; Pérez *et al*., 2008). However, the mechanism regulating induction and breakage of endodormancy in Japanese pear remains unknown.

In this paper, we review the progress of our research on endodormancy related problems, e.g. CR of cultivars, genetic factors of CR, endodormancy induction, gene expression in Japanese pear over the past 10 years, and future trends.

2. Materials and Methods

Environmental factors of endodormancy induction

The effects of temperature and day length during autumn on induction of endodormancy in Japanese pear were examined. The experiment was conducted using two Japanese pear cultivars [(*Pyrus pyrifolia* (Burm.f.) Nakai)], 'Nijisseiki', grafted onto *P. betulaefolia* Bunge seedlings planted at Tottori University, Tottori, Japan (35.5°N, 134.2°E). Potted 'Nijisseiki' pear trees were placed before endodormancy induction in a greenhouse kept at a minimum temperature above 18°C in September 2004 or in an open field; the photoperiod was controlled for 16 hrs or kept under natural conditions in each place, respectively. Then, one year old shoot was collected and the percentage of budbreak was defined. The apical flower bud was cut, and shoots were divided into five-node cuttings containing five continuous lateral leaf or floral buds. The basal part of the cuttings was submerged in 0.03% (v/v) aluminum sulfate and 0.3% (v/v) 8-hydroxyquinoline. The cuttings were then maintained in a growth chamber at 23±1°C and 24-h photoperiod for four weeks. Bud break is defined as a developmental stage characterized by swelling of the buds and the emergence of a green tip between scales (Tamura *et al*., 1992). The incidence of bud break in each bud type was determined on five shingle shoots having five buds for 28 days.

In addition to this experiment, 'Nijisseiki' pear shoots were collected in late September 2005. Shoots were submerged in 0.003% (v/v) aluminum sulfate and 0.3% (v/v) 8-hydroxyquinoline and treated at 5 and 15°C for 5, 7 and 14 days. After that bubreak percentage was defined.

CRs and forecasting model for endodormancy breaking

In order to estimate the CR for breaking endodormancy in wild pear species and pear cultivars, cultivars grown in the orchard of Tottori University and Tottori Horticultural Experiment Station (35.5°N, 133.7°E) during the 2008-2009 season were used. Leaf bud break on the cuttings prepared from these trees was determined as previously described. The CR was calculated as chill unit (CU) values using the Saitama method (Asano and Okuno, 1990) as described by Tamura *et al*. (1997). Effective chilling hours were calculated after October 31 when the largest negative accumulation was attained.

In addition to this experiment, we also investigated the budbreak percentage in Taiwanese pear Yokoyama, Japanese pear strain TH3 which is a selected strain from the seedlings of self-pollinated 'Osa-Nijisseiki' having homozygote of S4sm gene and their F1 for three years (2008, 2009 and 2010 seasons).

Mechanisms involved in induction and breaking of endodormancy

We isolated the candidate gene related to endodormancy breaking by suppression subtractive hybridization (SSH) method. Total RNA was isolated from 'Nijisseiki' pear buds on 12 November 2005 (deepest period of endodormancy: DP) and 12 January 2006 (breaking period of endodormancy: BP). SSH was carried out between bud in BP ('tester') and bud in DP ('driver') using the PCR-Select Subtractive Hybridization kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions, except for the modification of the first- and second-round PCR conditions. Genes isolated as candidate genes related to bud endodormancy were analyzed by Northern blot analysis using total RNA isolated from buds of 'Nijisseiki' and 'Kousui' pear.

3. Results and Discussion

Endodormancy is induced in buds of deciduous fruit trees in autumn, and then it is broken by the accumulation of low temperature in winter. The report focused on endodormancy induction are few, but the necessary environment condition to induce endodormancy is reported to be short day condition (Kawase, 1961) or low temperature (Tohbe *et al*., 1998). In 'Delaware' grape before endodormancy induction, buds treated long-days inhibited the induction of endodormancy than buds on natural condition (Horiuchi *et al*., 1981). On the other hands, Tamura *et al*. (1997) showed that the induction period of endodormancy in Japanese pear varies greatly as calendar days from year to year.

Thus, the effects of temperature and day length during autumn on induction of endodormancy in Japanese pear were tested. As a result, green house grown 'Gold Nijisseiki' trees kept high level of budbreak even though on mid-December, with no effect of photoperiod (Table 2) (Takemura *et al*., 2011). In contrast, the percentage of budbreak of trees decreased in both open field. Thus, a lack of chilling by the heating treatment above 18°C inhibited endodormancy induction, but a 16 hours photoperiod did not prevent it. In addition to this experiment, we collected 'Nijisseiki' pear shoots on late September, and then were

exposed at 5 and 15 °C for 5, 7 and 14 days. Then percentage of budbreak was detected. As a result in the experiment, it was cleared that temperature of 5°C was effective for inducing bud endodormancy in the cuttings, whereas a temperature of 15°C was ineffective (Fig. 2) (Takemura *et al*., 2011). From these results, the endodermancy of the Japanese pear was induced by low temperatures in autumn rather than short-day.

The CU and Developmental Index (DVI) models were earlier developed to predict the day of breaking bud endodormancy in the Japanese pear (Asano and Okuno, 1990; Sugiura and Honjo, 1997; Tamura *et al*., 1997). The start day of the calculation and the low temperature accumulation on both models are decided as the day perceived low temperature or calendar day. To decide environmental factors of endodormancy induction is important to reduce the gap of start day in both models by climate change in the future.

Next, the effects of temperature and day length during autumn on induction of endodormancy in Japanese pear was tested. From these results, the chilling requirement for completion of leaf bud endodormancy in pear plants ranged from below 400 to 1800 CU (Table 3) (Takemura *et al*., 2013). Among the pear plants examined, 'Yokoyama', which is originated in Taiwan, had the lowest chilling requirement (below 400 CU). In wild pear species, *P. fauriei* showed the lowest chilling requirement (400-800 CU), followed by *P. calleryana* and *P. dimorphophylla*. In addition, Akibae cultivar had the lowest CR among the Japanese pear cultivars examined, and their CR was between 800 and 1000 CU (Table 3) (Takemura *et al*., 2013). This cultivar has the same level of CR of 'Hosui' and 'Ninomiya', the lowest CU cultivar observed among Japanese pear varieties (Tamura *et al*., 2001). Therefore, these three cultivars may serve as suitable cultivars for propagation in warm-winter areas.

On the other hand, there is no Japanese pear which required low-chilling as shown in 'Anna' apple, having 300 CU as chilling requirement. From these results, 'Yokoyama' was selected as gene resource for breeding Japanese pear requiring low-chilling. In 2003, 'Yokoyama' pollen was pollinated with the flower of Japanese pear strain 'TH3' just after castration. 'TH3' is a S1 of 'Osa-Nijisseiki', which has homozygous of S4sm as the gene of self-compatibility haplotype. Using the seedlings obtained, the budbreak percentage in the parents, 'Yokoyama' and 'TH3', and their F1 was investigated for 3 years.

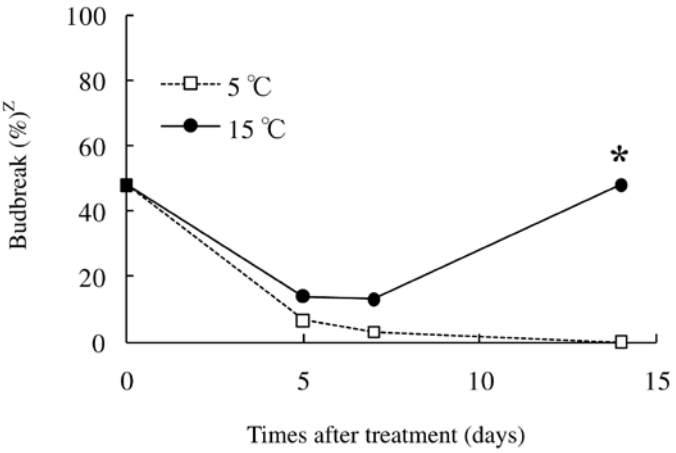

Fig. 2 - Effect of temperature on endodormancy induction of leaf bud in 'Gorld-Nijisseiki' pear (Takemura *et al*., 2011).
(z) 28 days after forcing at 23°C.

Table 3 - Chilling requirement for breaking leaf bud endodormancy in *Pyrus* plants evaluated by the seasonal changes in percent leaf budbreak (Takemura *et al*., 2013)

Chilling requirement (CU)	Registered name
-400	'Yokoyama'
400-800	*P. fauriei*
800-1000	*P. calleryana*, *P. dimorphophylla*, 'Ci Li', 'Akibae', 'Hosui', 'Ninomiya'
1000-1200	*P. betulaefolia*, 'Kosui', 'Wasekouzo'
1200-1400	*P. pyrifolia*, 'Qui Bai Li', 'Ya Li', 'Chojuro', 'Hakko', 'Niitaka', 'Nijisseiki', 'Taihaku', 'Yakumo'
1400-1600	*P. longipes*, 'Choju', 'Hattastu', 'Imamuraaki', 'Kikusui', 'Kumoi',
1600-1800	*P. aromatica*, *P. communis*, 'Bai Li', 'Beijing Bai Li', 'Akaho', 'Amanogawa', 'Doitsu', 'Kimitsukawase', 'Okusankichi', 'Shinsetsu', 'Shinsui'

Table 2 - Effect of temperature and photoperiod on endodormancy induction of leaf bud in 'Gorld-Nijisseiki' pear (areas in Japan) (Takemura *et al*., 2011)

Temperature conditions	Photoperiod conditions	Bud break (%)					
		Sampling date/Days of forcing					
		24 Oct.			10 Dec.		
		14 days	21 days	28 days	14 days	21 days	28 days
Greenhouse (18°C)	16 hr.	18.0 a[z]	40.0 a	60.0 a	36.0 a	50.0 a	60.0 a
	Natural	18.0 a	34.0 a	44.0 b	22.0 a	50.0 a	64.0 a
Open field	16 hr.	0.0 b	14.0 b	16.0 c	0.0 b	8.0 b	8.0 b
	Natural	0.0 b	18.0 b	18.0 c	0.0 b	6.0 b	16.0 b

[z] Different letters within the same column show a significant difference at $P < 0.05$ by *t*-test.

The data obtained showed that the percentage of budbreak in 'Yokoyama' was higher than 60% on all observational days. The percentage of budbreak in 'TH3' was lower than in 'Yokoyama' on all observational days, and it gradually increased from early December to early January. The percentage of budbreak in F1 plants was widely distributed between that of 'Yokoyama' and 'TH3'. On 8 of all observational days, the average percentage of budbreak in F_1 plants was near to that of 'TH3' rather than 'Yokoyama' (Fig. 3) (Takemura *et al*., 2012). Thus, we formulated the hypothesis that 'TH3' is homozygous for a dominant gene involved in the depth of endodormancy, but the hypothesis was rejected based on a chi-square test. Therefore, it was suggested that pear plant had quantitative trait loci (QTL) to decide CR as the genetic factor. It has recently been reported that the fifth chromosome of apricots or ninth chromosome of apples possesses QTL that influence the CR for breaking endodormancy (van Dyk *et al*., 2010; Campoy *et al*., 2011).

In addition to these researches, it has been investigated about biochemical or molecular biological changes during induction or breaking periods of endodormancy in the buds of deciduous fruit trees. Previously researches about cold-hardening during winter in many higher plants shown the change of the lipid composition within cellular membrane (Uemura and Steponkus, 1994; Uemura *et al*., 1995) or the accumulation of compatible solute within the cell (Koster and Lynch, 1992; Wanner and Junttila, 1999; Kamata and Uemura, 2004). In the peach buds, the accumulating period of total soluble sugar contents and water contents during endodormancy seasons were different between two cultivars having difference of CRs, even though plants were of the same specie (Yooyongwech *et al*., 2009). Moreover, Yooyongwech *et al*. (2009) have shown that expression levels of Pp-PIP1 and Pp-γTIP1 genes encoding aquaporin regulating water transport in tonoplast and plasma membrane increased in the peach buds of high-chilling cultivars than that of low-chilling cultivars before endodormancy breaking. In addi-

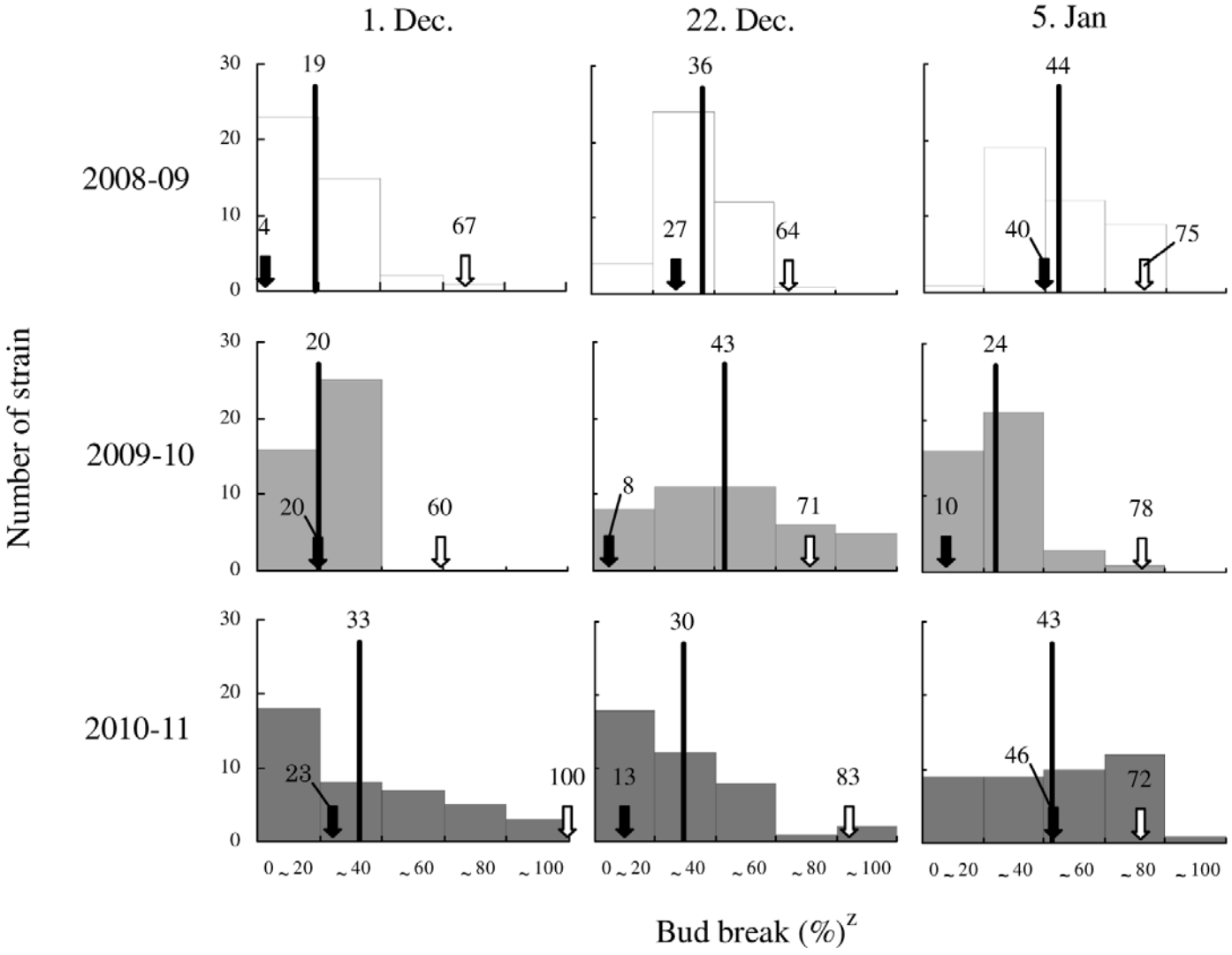

Fig. 3 - Distribution of percentage budbreak on F1 seedlings of 'TH3' × 'Yokoyama' (Takemura *et al*., 2012).
(z) 28 days after forcing at 23°C.
Vertical bars indicate mean of percentage budbreak on F1 seedlings.
: Budbreak of 'TH3' and 'Yokoyama, respectively.

tion, it has been reported that the change of dehydrin levels has been associated with cold hardiness, endodormancy and the content/state of water in the tissue of tree (Arora *et al*., 1997, 2003; Erez *et al*., 1998; Karlson *et al*., 2003). Yamane *et al*. (2006) reported that the role of dehydrin in the bark tissue during the dormant season is common to all *Prunus* species from research compared dehydrin expression level between evergreen and deciduous peach genotypes. However, Yakovlev *et al*. (2008) found that the expression of some dehydrin genes in Norway spruce (*Betula pubescens* Ehrh.) gradually decreased when approaching bud burst, and suggested that the observed changes cannot be related to winter dormancy. From results in our research using bud in Japanese pear, it was not clear that dehydrin genes is involved in development of endodormancy because the results cannot be find the common pattern in two cultivars having difference CRs (Fig.4; A13F). On the other hands, expression levels of gene encoding GAST-like gibberellin (GA) regulated protein increased with development of endodormancy in both two Japanese pear cultivars (Fig.4; B9C).

In previous research, there is a lot of reports focused on association between endodormancy breaking and phytohormone, including GA (Erez *et al*., 1979), Ethylene (Wang *et al*., 1985), Auxin (Nakano *et al*., 1980), Cytokinin (Broome and Zimmerman, 1976; Sterrett and Hipkins, 1980) and Abscisic acid (ABA) (Corgan and Peyton, 1970; Seeley and Powell, 1981; Tamura *et al*., 1992, 1993).

Among them, ABA is termed 'dormin' or 'dormancy inductor' (Addicott, 1983), and considered the most important growth inhibitor. On endodormancy induction period, it has been thought that the accumulation of ABA in shoots of grape controlled the induction or development of endodormancy (Düring and Alleweldt, 1973). In addition, the contents of ABA within leaf bud in Japanese pear on mid-November increased in only the open field condition which confirmed inducing endodormancy (Fig. 5) (Takemura *et al*., 2011). Moreover, Tamura *et al*. (1992) reported the decrease of ABA contents and the increase of GAs contents on endodormancy breaking period in leaf bud of Japanese pear.

The vernalisation has been controlled by GAs interacting closely with ABA and assessed similarities with dor-

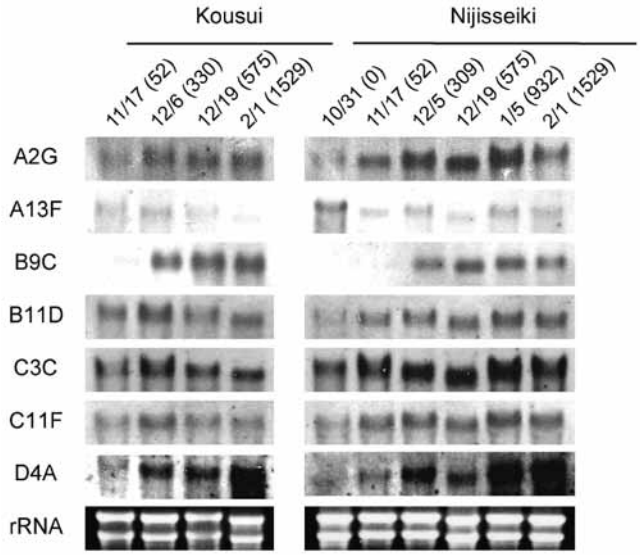

Fig. 4 - Northern blot analysis of the candidate genes is shown in Table 4 in Japanese pear buds of 'Kousui' and 'Nijisseiki'. CU is shown as number in parentheses on the right side of calendar day.

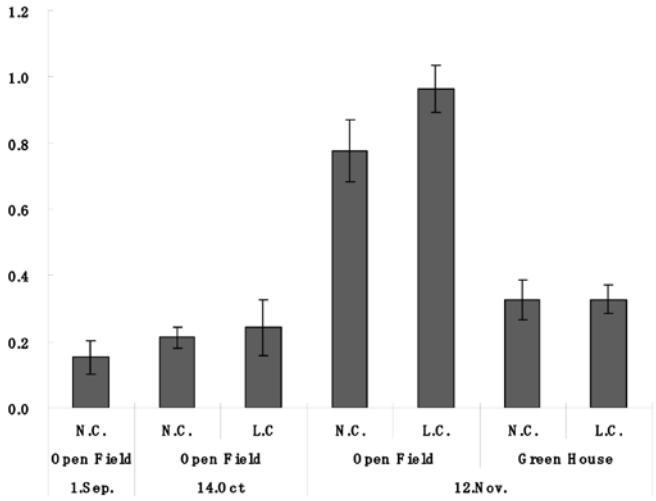

Fig. 5 - The change of ABA contents on induction period of endodormancy in the leaf bud of Japanese pear. N.C. and L.C. are shown as natural day-length condition and long-day condition, respectively (Takemura *et al*., 2011).

Table 4 - Results of sequence homology search for the candidate genes related to endodormancy

Reference clone	cDNA size (bp)	Best database match	E-value
A2G	596	Proline-rich protein 1 (*Vitis vinifera*; AAL02329.1)	7.00E-41
A13F	826	Dehydrin 1 (*Eriobotrya japonica* ; ACL01288.2)	2.00E-16
B9C	468	Putative GAST-like gibberellin regulated protein (*Prunus dulcis*; ABR13302.1)	1.00E-23
B11D	794	PREDICTED: early light-induced protein, chloroplastic (*Vitis vinifera*; XP_002283398.1)	2.00E-59
C3C	927	Cysteine protease inhibitor cystain (*Malus x domestica*; AAO19652.1)	2.00E-137
C11F	503	PREDICTED: uncharacterized RNA-binding protein C23E6.01c-like isoform 1 (*Vitis vinifera*; XP_002285479.1)	3.00E-25
D4A	1049	Unnamed protein product (*Vitis vinifera*; CBI31586.3)	3.00E-18

mancy release (Chouard, 1960). In Arabidopsis, the vernalisation after prolonged exposure to low temperatures was regulated by FLOWERING LOCUS C (FLC) which is MADS-box gene (Sung and Amasino, 2005). In evergrowing peach, Bielenberg *et al*. (2008) reported a cluster of six MADS-box transcription factors (DORMANCY-ASSOCIATED MADS-BOX: DAM) as candidate genes for the regulation of terminal bud formation. The expression of two of these genes, DAM5 and DAM6, is suppressed by chilling temperatures and inversely correlated with bud break rate in peach (Jimenez *et al*., 2010 b), whereas DAM4 and DAM6 expression is promoted by short photoperiods (Li *et al*., 2009). Recent studies have also shown that DAM genes are differentially expressed in response to seasonal dormancy transitions in other plant, inclucing raspberry (*Rubus idaeus* L.) (Mazzitelli *et al*., 2007), Japan apricot (*Prunus mume*) (Yamane *et al*., 2008), peach (Jimenez *et al*., 2010 a; Leida *et al*., 2010) and pear (Ubi *et al*., 2010).

4. Conclusions

Endodormancy in temperate-zone deciduous fruit trees is an essential mechanism to defend buds from unfavourable conditions during winter. To predict the day of breaking bud endodormancy is very important for cultivating. Especially on the forcing culture, a lack of CR for breaking endodormancy caused growth inhibitor as non-germination during spring. Therefore, molecular markers which can estimate whether plants are broken to the endodormancy or not should be developed. Until now, detail of mechanism regulating the induction and the breaking of endodormancy is still unknown. In the future, protein analysis related endodormancy in addition to these genetic approaches will lead us the final conclusion of the mechanisms.

On the other hands, it is suggested that pear and other plants had QTL to decide CR as the genetic factor. To provide for global warming in the future, deciduous fruit tree having lower CR should be bread in the future.

The data of the present paper showed that endodormancy in Japanese pear was induced by low temperature, without effect of day length. Japanese pear had quantitative trait loci (QTL) as genetic factor to decide chilling requirement (CR) for breaking endodormancy. The increase of abscisic acid (ABA) and gibberellin (GA) concentrations in the bud of Japanese pear is related with induction and breaking of endodormancy.

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