Development of a Molecular Test to Ensure Good Flowering of *Viburnum opulus* var. *roseum*

A. Kromwijk and F. van Noort
Wageningen UR Greenhouse Horticulture
P.O. Box 20, 2665 ZG Bleiswijk
The Netherlands

N. Verhoef, P. Balk and M. van Wordragen
NSure
Binnenhaven 5, 6700 AA Wageningen
The Netherlands

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Abstract

During wintertime *Viburnum opulus* var. *roseum* (snowball) shrubs are forced in warm greenhouses to harvest early cut flowers. Early forcing is occasionally unsuccessful. This is probably due to a lack of hours with low temperatures that is needed to break bud dormancy. To gain more insight about the effect of temperature on breaking dormancy and early forcing results, shrubs were transferred into cold storage in week 41, 43 and 45 (2009) for 4, 6 or 8 weeks at a temperature of 2, 5 or 8°C. After storage, the shrubs were forced at a day-/night temperature of 28/23°C. Shrubs stored for at least 8 weeks from week 41, 6 weeks from week 43 and 4 weeks from week 45 gave good forcing results, regardless of storage temperature. Since growing conditions outside vary every year and differ between nurseries, it is difficult to determine when forcing can be started. To prevent poor forcing results, we aimed to develop a molecular diagnostic assay to determine the moment at which winter dormancy of snowball flower buds is sufficiently broken. In order to identify genes involved in bud breaking dormancy, an experiment was performed on a commercial nursery from October 2010 to January 2011. Flower buds of snowball shrubs outside were sampled weekly and in December and January batches of shrubs were placed every week in a greenhouse to observe forcing results. Genes involved in bud breaking dormancy were identified by performing next generation Illumina RNA sequencing on bud samples with poor and good forcing results. A set of candidate genes was validated during a second trial at two different commercial nurseries in 2011-2012.

INTRODUCTION

Under natural conditions *Viburnum opulus* var. *roseum* (snowball) flowers in spring. To harvest early cut flowers in the Netherlands, shrubs are forced in greenhouses at high temperatures during the winter. In 2011, 11 529 782 snowball cut flower stems were sold at the auctions in the Netherlands with a turnover of € 5 670 641 euro. Early forcing in a greenhouse is occasionally unsuccessful; only a few or sometimes even none of the flower buds develop into racemes leading to financial loss. Unsuccessful bud break and uneven flowering can occur in woody perennials, such as temperate-zone deciduous fruit trees (Faust et al., 1997), *Rubus idaeus* L. (raspberry; Mazzitelli et al., 2007), *Hydrangea macrophylla* pot plants (Hanke, 1996) and *Rhododendron simsii* (azalea) pot plants (Kromwijk and Van Leeuwen, 2003). To gain more insight about the effect of temperature on breaking dormancy and early forcing results, snowball plants were transferred into cold storage in week 41, 43 and 45 for 4, 6 or 8 weeks at a temperature of 2, 5 or 8°C and forcing results were determined at a day/night temperature of 28/23°C.

Furthermore, we investigated whether it is possible to monitor the moment of bud break dormancy release in snowball at molecular level. The last decade several studies have been performed to investigate the molecular pathways underlying dormancy release in various plant species such as raspberry (Mazzitelli et al., 2007), *Prunus mume* Sieb. et Zucc. (Japanese apricot; Yamane et al., 2008), *Ribus nigrum* L. (Hedley et al., 2010), *Vitis riparia* (Mathiason et al., 2009) and *Betula pubescens* (birch; Welling et al., 2004). In order to find genes that can be used to determine the dormancy transition, RNA sequencing (RNA-Seq), a version of Next Generation Sequencing, was performed on

flower bud samples collected at a nursery in 2010-2011. RNA-Seq has proven to be a powerful technique and during the last couple of years it has been broadly used in plant science to compare transcriptomes of different kind of samples (Zenoni et al., 2010; Severin et al., 2010; Stattin et al., 2012). The strength of this technology is that no prior sequence information of the plant is needed, which is a solution for poor researched species such as snowball. A subset of candidate genes was verified by real-time RT PCR and validated during a second trial at two different commercial nurseries in 2011-2012.

MATERIAL AND METHODS

Cold Storage Experiment

In 2009, snowball plants grown outside in container pots with soil and watered with droppers were transferred at week 41 (no leaves fallen), 43 (20-50% leaves fallen) and 45 (almost all leaves fallen) into cold storage rooms (dark) with a mean temperature of 2.3, 5.1 and 8.1°C and a relative humidity of 89, 80 and 93% (week 41-53) for 4, 6 and 8 weeks, resulting in 27 treatments (5 plants each). Shrubs were watered before and when needed, during storage. After storage, plants were watered and forced in a greenhouse at a temperature set point of 28°C (7 am to 7 pm) and 23°C (7 pm to 7am) resulting in a mean temperature of 25.4°C in week 45-1. Transition from 28°C to 23°C and vice versa was gradually in 3 hours. Plants were watered when needed and the floor was sprayed with water regularly. A second greenhouse with the same set points (mean temperature 24.7°C week 49-1) was used for plants forced from week 49, 51 and 53. When flower buds sprouted 4-5 cm, shrubs were moved to a third greenhouse set at 21°C (mean temperature 20.7°C week 49-1). Furthermore, outdoor grown shrubs were directly transferred into a forcing environment in week 53. Data were analysed using a Generalized Linear Model based on a Poisson distribution using the statistical package GenStat Release 14 (VSN International Ltd).

Plant Material Used to Identify and Validate Genes Involved in Bud Break Dormancy

Flower buds of 10 shrubs grown outside at a commercial nursery (nr. 1) from October 2010 to January 2011 were weekly frozen in liquid nitrogen. From week 48, shrubs were forced every week in the greenhouse under nursery conditions to determine the forcing results. A similar experiment was performed from October 2011 to February 2012 at three nurseries (plants grown in pots with soil and watered with droppers at nursery 1 and 3 and plants grown in pots with rockwool on an ebb and flood system at nursery 2). In addition a batch covered with reed from week 42 was followed at one nursery. Instead of freezing the buds, bud tissue was processed according to the sampling procedure of NSure. The top flower buds from 10 shrubs were collected, halved and crushed into a vial containing extraction fluid. Subsequently, one drop of extract was transferred to an FTA card (Whatman). From week 48/49 shrubs were weekly placed into the greenhouse to determine the forcing results.

Selection of Genes Involved in Bud Break Dormancy

From the experiment performed on a commercial nursery in 2010-2011, total RNA was isolated out of frozen top buds sampled from 10 shrubs in week 41 and 49 and sent to ServiceXS (The Netherlands) for next generation Illumina RNA sequencing and analysis. Clustering and DNA sequencing using the Genome Analyzer IIx (Solexa) was performed according manufacturer's protocols. Two sequencing reads of 100 and 48 cycles using the Read 1 sequencing and Read 2 sequencing primers were performed with the flow cell. Image analysis, basecalling and quality check was performed with the Illumina Genome Analyzer data analysis pipeline RTAv1.8.70.0 and/or OLB v1.8 and CASAVA v1.7.0. The reads were assembled into contigs using the de novo assembler PEassembly in the NextGENe v.1.95 (SoftGenetics) package. Assembled contigs were blasted against the Genbank nucleotide (nt) database for annotation (http://www.ncbi.nlm.nih.gov/genbank/). The reads were aligned onto the assembled contigs. Quantification of a gene was

performed by counting the number per read per gene of each sample. For differential analysis between the samples DESeq (http://www.huber.embl.de/users/anders/-DESeq/) was used with a Pvalue cutoff of <0.1. Subsequently, a selection of potential indicators was selected and specific primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/). Expression levels of all collected frozen bud samples from the first trial in 2010 and FTA cards (Whatman) from the second trial in 2011 were measured using real-time RT PCR (iQ5 real-time PCR detection system, Bio-Rad) using standard protocols.

RESULTS

Cold Storage Experiment

Forcing results improved with starting date and duration of the storage (Table 1). No significant differences were found between 2, 5 and 8°C. Plants stored for at least 8 weeks from week 41, 6 weeks from week 43, 4 weeks from week 45 and outdoor grown plants forced from week 53 gave good forcing results. Outside temperatures (5-minute values) registered by www.LetsGrow.com at a location near the production site outdoors and temperatures during cold storage were used to calculate the accumulated chilling units (CU) before and during storage for each treatment. Calculations were based on Richardson et al. (1974) with some adjustments regarding the low temperature as no significant differences were found between 2, 5 and 8°C. One CU was calculated for each hour at a temperature between 2 and 9.1°C, 0.5 CU for temperatures between 1 and 2°C and 0 CU for temperatures below 1°C. CU were calculated from week 43 (20 to 50% leaves fallen), based on Walser et al. (1981). Bud break accelerated and flowering results improved with increasing number of CU and no further improvement was found above 800 CU (Fig. 1).

Identification of Genes Involved in Bud Breaking Dormancy

To investigate changes in gene expression associated with dormancy breaking next generation Illumina RNA-seq was performed on buds collected in week 41 and week 49 from the time course experiment performed in 2010-2011 at nursery 1. Differential expression analysis on the raw counts was performed by DESeq, a model based on negative binomial distribution. From the 62764 contigs, 120 contigs were up-regulated in time and 73 contigs down-regulated. Genes that were differentially expressed included genes involved in metabolism, cell defense (antifreeze genes such as dehydrins and pathogenesis related genes), cell wall, transcription and (hormonal) signal transduction pathways. To validate the expression profiles obtained by RNA-Seq, real time RT-PCR was performed on a subset of respectively 27 and 21 genes that were positively and negatively correlated to bud break dormancy. They were chosen based on their putative function and high difference in expression. The expression levels of *VI1*-*VI5*, genes categorized in different plant processes, showed a clear seasonal pattern (Fig. 2). At the beginning *VI1*-*VI5* were expressed at low levels and gradually increased till week 48/49 were they reached a stable level of expression and subsequently declined (Fig. 2). Genes *VI6*, *VI7* and *VI8*, respectively involved in cell defense, the cell wall and a hormonal signal transduction pathway, showed an opposed trend in time (Fig. 2). For the real time RT-PCR expression profiles of the remaining genes of the subset similar seasonal gene expression patterns were found (data not shown). From week 48 shrubs were placed in a greenhouse under forcing conditions to determine the moment of bud break dormancy. Forcing results were satisfactory from week 50 (Table 2). Due to capacity problems, the forcing results might be negatively influenced as the forcing day temperature of 28-29°C was shorter than needed.

To confirm that these genes are involved in bud break and dormancy release, a similar experiment was performed in 2011-2012 at three different nurseries. In comparison to the previous experiment, the indicators showed a similar expression pattern (Fig. 3). The gene activity profiles of the shrubs at nursery 3 were ahead in comparison to the shrubs grown outside at nursery 2 (Fig. 3). This corresponds with the forcing results,

as forcing at nursery 2 was satisfactory from week 52, while the shrubs of nursery 3 were already successfully forced in week 49 (Table 2). The experiment was also repeated at nursery 1 and similar expression patters were observed (data not shown). Some nurseries cover their shrubs to enhance bud break dormancy. Starting from week 42, nursery 3 also covered a part of the batch with reed. The gene activity profiles of the covered batch were slightly behind the uncovered shrubs (Fig. 3). However, the forcing time of the uncovered shrubs moved to a forcing environment in week 49, took 4 days longer than the covered shrubs (Table 2).

DISCUSSION

Regulation of dormancy has become of significant importance to horticultural industries. Dormancy is released by a certain amount of hours with low temperatures varying between different crops and varieties within a crop. This study demonstrates that cold storage of snowball plants for at least 8 weeks from week 41, 6 weeks from week 43 or 4 weeks from week 45 in 2009 at 2 to 8°C, released dormancy and gave good early forcing results. This corresponds to Hydrangea pot plants, who can be forced successfully after 40 days storage (960 hours) at a temperature of 2-5°C (Hanke, 1996) and azalea pot plants who need a cold storage period of 6 to 8 weeks at 8°C for even flowering (Kromwijk and van Leeuwen, 2003). Calculating chilling units (CU) is used to determine when dormancy is released for peach trees (Richardson et al., 1974; Walser et al., 1981) and several other fruit trees. This study indicates that snowball plants need 800 CU from 20-50% leaf abscission in the fall to be forced successfully. However, additional research is needed for further development of the model and to test predictions of the model in different years and at nurseries that differ in early forcing results (for instance the closely situated nurseries 2 and 3).

To gain insight into the genetic control of dormancy transition in snowball, RNA-Seq was performed. RNA-Seq revealed 193 candidate genes that may be involved in the release of dormancy. Expression of 62% of these genes raised upon dormancy release, 38% declined. The functional classification of a large portion of the genes into cell defense, metabolism, (hormonal) signaling pathways, transcription and cell wall were also previously identified by other researchers that studied gene expression in relation to dormancy release in various other plant species (Mazzitelli et al., 2007; Mathiason et al., 2009; Hedley et al., 2010). A subset of 48 genes was further analyzed by real-time RT-PCR. Interestingly, a similar seasonal expression profile was observed for all up-regulated genes irrespectively of their function as well as for the down-regulated genes. Similar seasonal expression patterns have been observed in *Vaccinium* spp., raspberry and several trees such as birch and Japanese apricot (Dhanaraj et al., 2004; Welling et al., 2004; Mazzitelli et al., 2007; Yamane et al., 2008). Validation at three nurseries in 2011-2012 showed that the observed gene expression profiles were consistent in time and among different nurseries. A similar expression profile was observed for the shrubs that were covered. Comparison of the forcing results to the gene expression profiles suggest that the moment of gene expression stabilization, might be the moment that snowball shrubs go from an endodormant to an ecodormant stage. This transition is a little earlier than the moment that forcing results were satisfactory for the growers. Since forcing results are not only determined on full budburst, but also on the equality of budburst, it is imaginable that just at the moment of transition equal flowering is not optimal as certain other physiological processes after the release of bud dormancy might play role. Furthermore, it does not seem likely that the moment of bud break varies with the position of the buds along the branch as the flower buds just below the top flower buds collected at nursery 1 in 2010-2011 showed similar gene expression patterns (data not shown). At last, cultivation techniques and bud set conditions might also influence the equality of flowering.

This study has successfully identified several genes that may be used in the future to develop a molecular test to determine when dormancy is sufficiently released in snowball.

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Tables

Table 1. Number of days for flower buds to sprout 4-5 cm and number of racemes per branch at harvest of snowball plants stored from week 41, 43 and 45 (2009) for 4, 6 and 8 weeks. Means of 3 storage temperatures (2, 5 an 8°C) with 5 plants each.

	Forcing time (days)			Nr. racemes/branch		
Start\Duration	4 weeks	6 weeks	8 weeks	4 weeks	6 weeks	8 weeks
Week 41	54 c[1]	31 b	22 a	0.5 a	2.4 c	3.4 ef
Week 43	33 b	24 a	21 a	2.1 b	2.9 d	3.2 de
Week 45	24 a	21 a	19 a	3.2 de	3.5 ef	3.6 f

[1] Different letters indicate significant differences between treatments ($p<0.05$).

Table 2. Forcing results at commercial nurseries in 2010-2011 and 2011-2012.

Nursery nr. and year	Week number	Forcing result	Remark
1: 2010-2011	48	-	Longer forcing of ± one week was required
	49	-	They flower, but unequal
	50	+	
	51	+	
	52	+	
	1	+	
	3	+	
2: 2011-2012	48[1]	-	More than 60% branches do not flower
	49[1]	-	Almost all branches flower, but flowering is not equal
	50[1]	-	About 20% of all branches do not flower, but flowering is not equal
	51[1]	-/+	All branches flower, but still a bit unequal
	52	+	
	1	+	
	2	+	
	3	+	
3: 2011-2012	49 (covered)	+	
	49 (outside)	+	Forcing time 4 days longer than covered batch
	50 and later	+	

[1] Day temperature of 24°C not guaranteed.

Figures

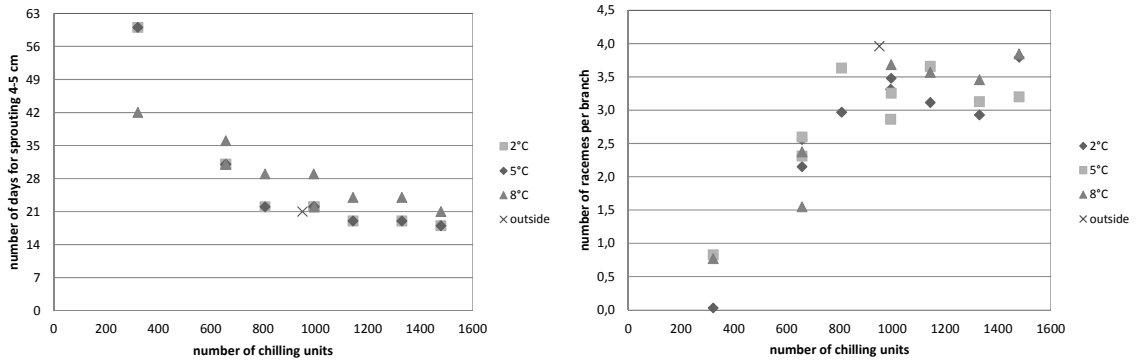

Fig. 1. Days at 28/23°C to sprouting 4-5 cm (left) and number of racemes per branch at harvest (right) in relation to the accumulated chilling units from week 43 (20-50% leaves fallen) of snowball plants stored from week 41, 43 and 45 (2009) for 4, 6 and 8 weeks at 2, 5 and 8°C and for snowball plants grown outside until forcing from week 53.

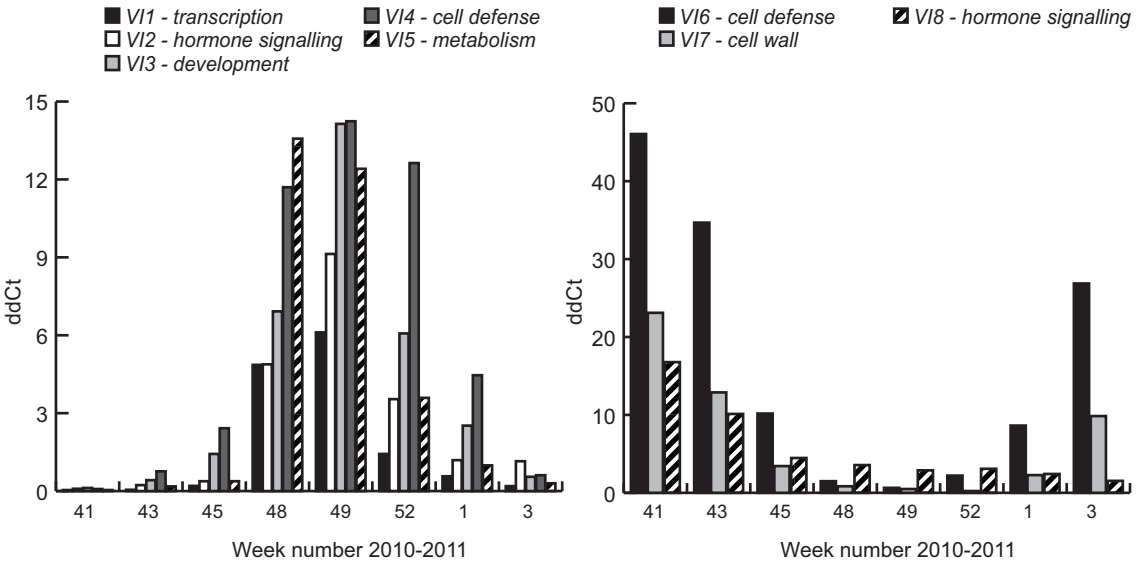

Fig. 2. Gene expression profiles that were positively (*VI1*-*VI5*) or negatively (*VI6*-*VI8*) associated with bud break dormancy in snowball grown outside. Expression was given as ddCt (delta-delta-tresholdcycle) algorithm, which is dCt difference between the gene of interest and an internal standard.

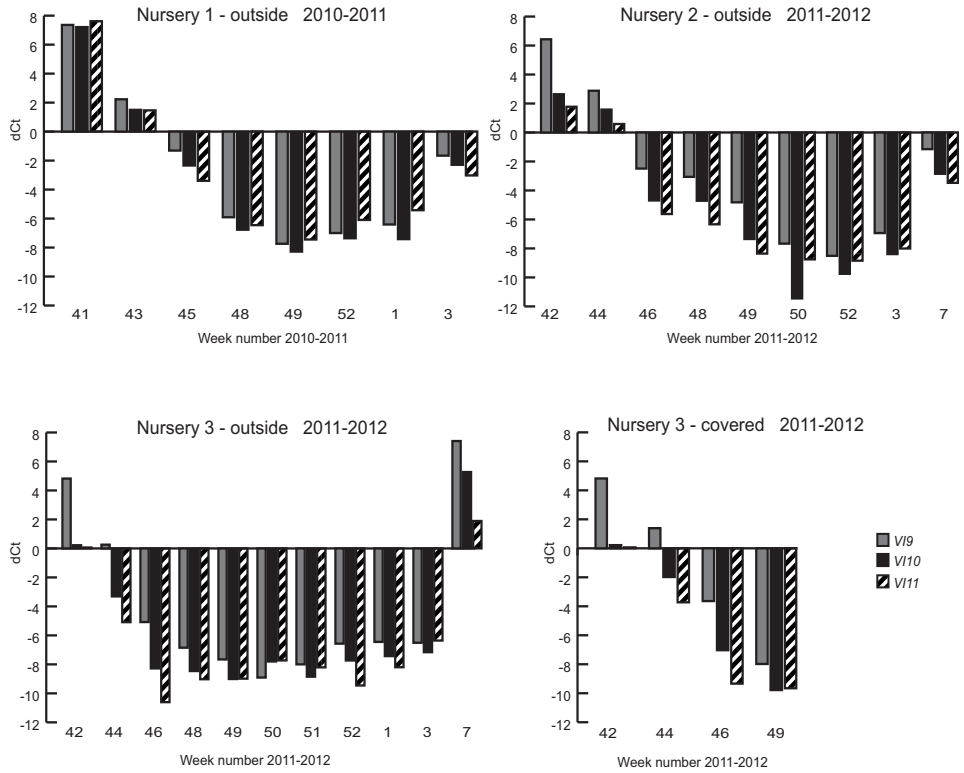

Fig. 3. Indicator profiles that are correlated to bud break dormancy in snowball grown outside. Expression was given as dCt, the dCt difference between an up- and down-regulated gene.