Plant Breeding and Evaluation

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Inheritance of Pink Flower Color in *Styrax japonicus*

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Significance to Industry: Combining pink flower color and other ornamental traits, such as weeping plant form, in *Styrax japonicus* Sieb. et. Zucc. could result in superior cultivars for the market. Because efforts to transfer pink flower color from 'Pink Chimes' via controlled pollinations have not been successful, it has been speculated that the deep pink flower color of this cultivar is chimeral in nature. By producing and examining 'Pink Chimes' selfed progeny, this study demonstrated that the deep pink color of 'Pink Chimes' flowers is heritable and that a breeding effort to combine flower color with other desirable traits is feasible.

Nature of Work: *Styrax japonicus* (Japanese snowbell) is a small deciduous tree that is cultivated as an ornamental. Native to Japan, China, Korea, Taiwan and the Philippines, it was introduced into the U.S. in 1862 (1). The species grows from 6 to 9 m (20 to 30 ft) in height with a similar spread, making it a valuable plant for use in small residential landscapes or under utility lines. Flowers are bell-shaped, approximately 2 cm (0.8 in) in diameter, and very fragrant. They are produced in mid-spring and hang beneath the foliage in three- to six-flowered racemes.

While most *S. japonicus* cultivars produce white flowers, a few pink-flowered forms have been reported. 'Pink Chimes' is the most widely grown pink-flowered form and the only *S. japonicus* cultivar with deep pink flowers that hold their color even under hot growing conditions (1, 3). Over the past 12 years, we have made numerous crosses for the purpose of combining the deep pink flower color of 'Pink Chimes' with other ornamental traits, especially weeping plant habit. Although we have recovered plants with pale pink flowers, we have never found any plant with flowers similar in intensity to those of 'Pink Chimes'. In addition, two new introductions that have been described as pink-flowered, weeping forms have been disappointing. The flowers of 'Rubra Pendula' and 'Pink Cascade' develop only a pale pink color when grown in the heat of the southeastern U.S. (2, personal observation). Because of the rarity of deep pink flower color in *S. japonicus*, it has been speculated that 'Pink Chimes' is a periclinal chimera. The objective of this study was to determine if the deep pink flower color of 'Pink Chimes' is heritable or chimeral in nature.

Four white-flowered selections (G258-20, G258-90, G258-98 and G259-36) were hybridized with 'Pink Chimes'. When F_1s flowered, pink-flowered plants from two

groups of progeny (G258-98 × 'Pink Chimes' and G259-36 × 'Pink Chimes') were used for making full-sib crosses. 'Pink Chimes' was also self-pollinated. Controlled cross- and self-pollinations were performed as previously described (4).

Styrax seeds exhibit double dormancy and thus require both a heat and cold treatment for germination (1). Seeds from pollinations were collected in early fall, sown immediately in a pine-bark based medium and kept damp in a warm greenhouse for 5 months. Seeds were then placed in a refrigerated chamber for 3 months, after which they were moved back to the greenhouse. Seedlings were initially grown in containers, but were transplanted to the field in Fall 2008.

Flower color was rated in spring 2008 and 2009 on a scale of 1 to 5, where 1= white, 2 = very pale pink, 3 = pale pink, 4 = medium pink, and 5 = deep pink. A rating of 5 was considered to be equal to the flower color of 'Pink Chimes'. Mean flower color rating was determined for each F_1 and F_2 plant.

Results and Discussion: Flower color among F_1 plants ranged from white to pale pink, with 67% having white, 21% very pale pink and 12% pale pink flowers. Mean flower color ratings and flower color distribution were similar between F_1 and full-sib F_2 populations (Table 1; Fig. 1). Flower color among the full-sib F_2s ranged from white to medium pink, with the majority of plants (68%) having white flowers. Distribution of flower color was considerably different among full-sib and selfed F_2 populations (Fig. 1). Flower color among 'Pink Chimes' self-progeny ranged from white to deep pink, but almost half of the plants in this population had flowers rated as medium pink or darker. Five 'Pink Chimes' selfed plants (18%) had flowers comparable in color intensity to those of 'Pink Chimes'.

This study, to some extent, utilized plants developed for breeding purposes and was not designed to elucidate mode of inheritance of pink flower color in *S. japonicus*. However, the production of plants with flowers similar in color intensity to those of 'Pink Chimes' following self-pollination indicates that the deep pink flower color of this cultivar is inheritable. While the data indicates that flower color is not a simply inherited trait, additional generations and larger numbers of progeny are necessary to determine numbers of genes involved in determining *S. japonicus* flower color. Large F_2 populations will likely be needed for recovering progeny with both deep pink flower color and other desired traits; these may best be produced using bee-mediated pollinations.

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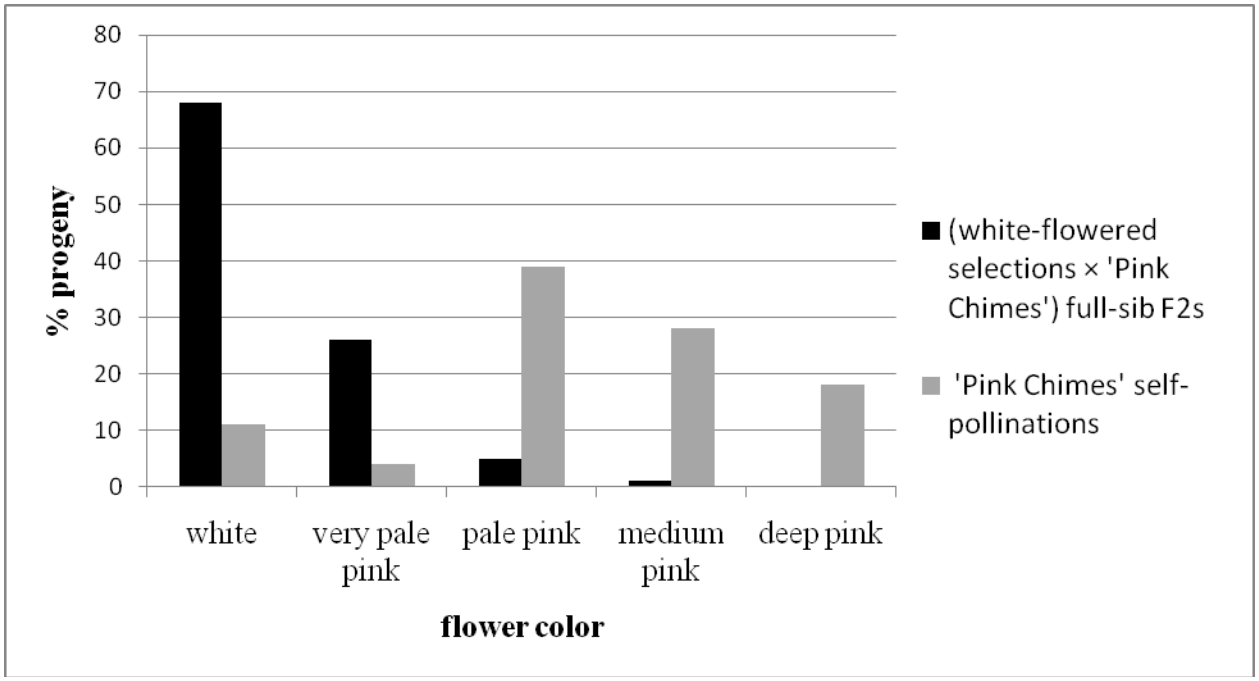
Table 1. Flower color in F_1 and F_2 populations of *Styrax japonicus*.

Generation[z]	No. plants	Color rating, mean ± s.e.[y]
F_1		
G258-22 × 'Pink Chimes'	3	1.7 ± 0.67
G258-90 × 'Pink Chimes'	10	1.4 ± 0.27
G258-98 × 'Pink Chimes'	6	1.8 ± 0.33
G259-36 × 'Pink Chimes'	5	1.4 ± 0.25
F_2		
(G258-98 × 'Pink Chimes') full-sib crosses	58	1.4 ± 0.08
(G259-36 × 'Pink Chimes') full-sib crosses	20	1.6 ± 0.18
'Pink Chimes' self-pollinations	28	3.5 ± 0.23

[z]G258-22, G258-90, G258-98 and G259-36 are white-flowered selections of *S. japonicus*.

[y]Flower color is a mean of 2008 and 2009 data. Flower color was rated on a scale of 1 to 5, where: 1 = white; 2 = very pale pink; 3 = pale pink; 4 = medium pink; and, 5 = deep pink. A rating of 5 was considered equivalent to the color of 'Pink Chimes'.

Fig. 2. Distribution of flower color among full-sib and selfed F_2 *Styrax japonicus* progeny.



Seed Stratification, Germination and Greenhouse Performance of Diverse *Rosa* Species

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Significance to Industry:
There are more than 130 recognized species in the genus *Rosa* (1). However, only about 7-10 species of *Rosa* are found in the background of most modern rose cultivars (3). Research into breeding systems (sexual reproduction) and pollination relationships with only a few exceptions has concentrated mostly on cultivar development (2). To expand the genetic background for modern roses, breeders should more extensively explore wild rose resources. A better knowledge of wild rose resources will make it possible to incorporate many valuable traits into garden rose breeding programs and to develop improved garden rose cultivars that are more broadly adapted. The unexplored wild rose species included in these experiments may have many potential horticultural traits and resistance or tolerance to stresses. They have potentially not been evaluated under the environmental extremes present in Texas along with the Southeast and Southern plains. *Rose* spp. that we are currently evaluating is likely to have potential as breeding materials as well as for direct commercial marketability.
Therefore, this research was conducted with two primary objectives: 1) investigate the influence of stratification on seed germination and the performance of seedlings in growing media or substrate, and 2) evaluate aphid and powdery mildew susceptibility of seedlings growing under greenhouse conditions.

Nature of Work: Seeds of wild rose species (provided by Dr. Kevin Conrad, Curator, U.S. National Arboretum, Washington DC) were placed in plastic bags containing moistened, clean, washed quartz sand and moss (1:1 ratio). The plastic bags were sealed and stratified in a refrigerator at approximately 4° C (40° F) until seeds sprouted (about 6 months for most species). For best germination, care was taken that the seeds never dried-out once they were placed in bags and were never held in standing water. If not enough seeds germinated after 4 months, cold stratification (same plastic bag returns to refrigerator) was continued until seeds sprouted. After stratification, seeds were carefully removed from the sand-moss mixture by hand or by sieving through a screen. Sprouted seeds were sown immediately in 18 holed sheet pots (3.5″-18 count 3″ deep) using a soilless substrate (for example, peat-based mixture or pine bark and moss based substrate). Pots were kept at room temperature (ca. 21°C, or 70°F) for two days and then transferred to the greenhouse. Fresh seeds without stratification were also sown in the same two substrate/media.

Results and Discussion: The purpose of this study was to develop methods to increase germination percentage, shorten germination time, provide more synchronous germination, and to result in more efficient seed propagation techniques for rose. Data indicated that fresh (without stratification) seeds of wild rose have high physiological dormancy and did not germinate in either of the two medias. We treated 99 Rosa species for cold stratification. So far, however, only 26 species sprouted during the stratification process (4-6 months) (Table 1). Rose can be produced in one week following sowing of stratified seeds in peat based media. Higher percent of seedlings emergence and better growing performance was observed in a peat based substrate than in the pine bark based substrate (Fig. 1). Peat-based media are useful for seed germination because they are relatively sterile, light in texture and weight, and are uniform. The light texture enables seed to readily germinate and emerge, allows tender roots to elongate, and makes transplanting seedlings easier. We developed 235 plants from 11 species of roses (Table 1) among the 40 wild rose species. Genotypes for these 11 species vary in leaves, plant types, and thorns, indicating significant genotypic diversity (Fig. 2).

Gardeners may choose to grow thornless roses for a variety of reasons. Four thornless (code as '0') rose species (Table 1, Fig. 3), produced from this collection along with additional plants that may germinate later, will be our potential breeding parents for thornless rose breeding. Roses are host to a wide range of insect and disease pests. For most rose genotypes, aphids and black spot leaf fungus are the main pest and disease concerns. Until now, no aphid and black spot infestation have been observed in the genotypes resulting from the hybridizations in this study, but it is still very early in this set of experiments. Infections from powdery mildew, however, have been observed in some species. This disease covers new leaves and flower buds with a distinctive white, powder-like growth (Fig. 3). Eight wild roses exhibit resistance to this disease (Fig. 3). Plants will be evaluated for alkalinity tolerance and aphid resistance as more plant material is available for each of the test plants.

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Table 1: Rose species, country of origin/ collector's notes, no. of plants and their some features.

Acc No	Species	Country of origin/Collector's Notes	No. of Plants	Powder mildew		Thorn	
R13	*Rosa canina*	Ukraine, Sumy	2	R	1	No	1
R15	*Rosa canina*	Ukraine, Sumy	29	R	0	No	0
R22	*Rosa gymnosperm*	Wash. Pk. Arb. U. of Wash. Seattle WA	2				
R24	*Rosa henryi*	unknown	1	R	2	No	1
R27	*Rosa majalis*	Uppsala, Sweden	1	R	2	Yes	5
R31	*Rosa moschata*	Local name: 'Ching'	4				
R40	*Rosa pimpinellifolia*	Wild Collected USSR	22	R	0	Yes	5
R41	*Rosa pisocarpa*	unknown	3				
R47	*Rosa rugosa*	unknown	2	R	1	Yes	5
R49	*Rosa rugosa*	unknown	19	R	2	No	0
R55	*Rosa rugosa*	Wild Collected Russia/ dark pink flower	23				
R57	*Rosa rugosa*	unknown	3	S	4	Yes	5
R58	*Rosa rugosa*	Matthare B.G. Anne Arbor, Michigan	4	S	3	Yes	5
R59	*Rosa rugosa*	Chollipo Arb. Seoul, Korea	1				
R69	*Rosa woodsii*	G-13105 mont. 1965--1964 seed	4	R	0	Yes	4
R71	*Rosa woodsii*	G-13106 Mont.	4				
R72	*Rosa woodsii*	unknown	2				
R73	*Rosa woodsii*	unknown	7	R	0	Yes	4
R74	*Rosa woodsii*	72NC323895ai01	5	S	3	No	1
R75	*Rosa woodsii*	Colorado	14	S	3	Yes	3
R78	*Rosa sp.*	unknown	7	S	4	Yes	5
R83	*Rosa sp.*	Albania / Ned Garvey	17	S	3	Yes	3
R87	*Rosa sp.*	Ames 25538	40	S	4	No	0
R90	*Rosa sp.*	unknown	12	R	0	Yes	4
R96	*Rosa sp.*	1965 ERJensen NMSU Hort.G14990 sd. strat:65	5	S	5	No	0
R99	*Rosa sp.*	1964-1965 seed G13107 Montan.	2				
26	11		235				

Note: plants with most powdery mildew susceptible (5) and most resistant (0); most thorned (5) and thornless (0)

Fig. 1: Rose seedlings in peat based (a), pine based (b) media; seedlings susceptible (c) and resistant (d) to powdery mildew disease.

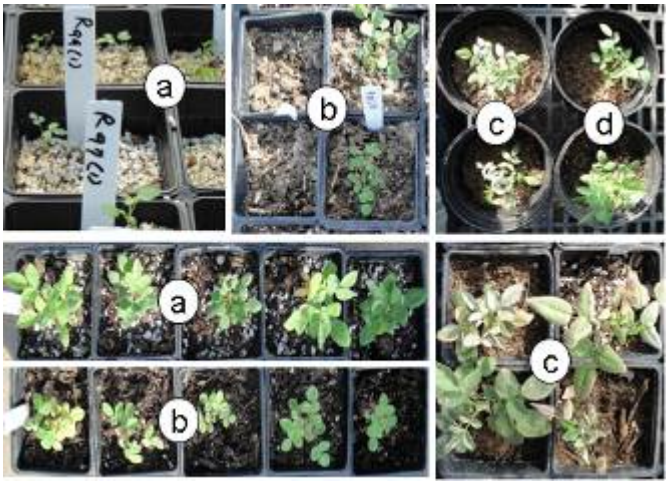


Fig. 2. Thorn characteristics (A- thornless code “0”; B- thornless code “1”; C- thornless code “2”; D- thorn code “3”; E- thorn code “4” and F- thorn code “5” in Table 1).

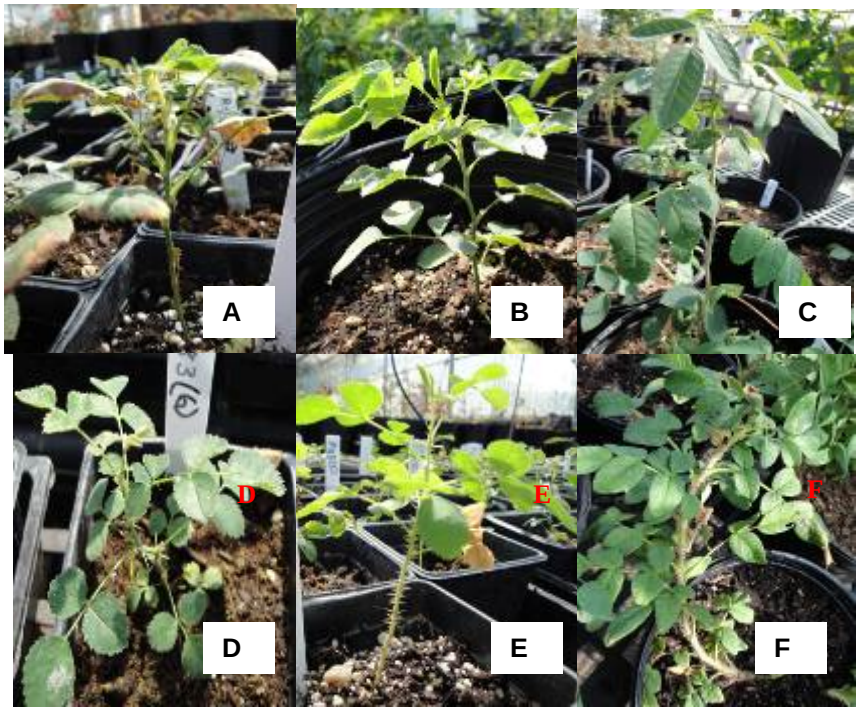
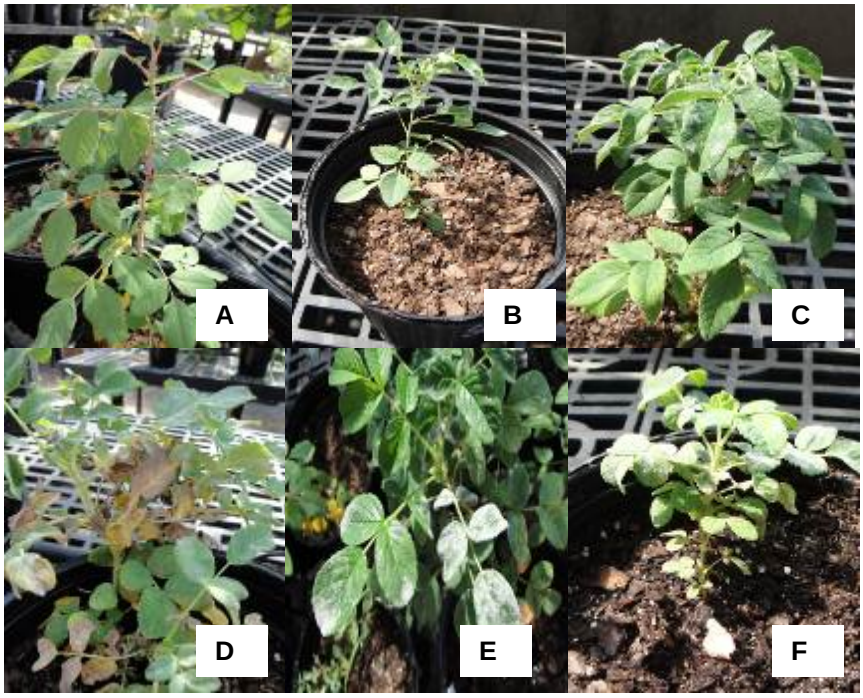


Fig. 3. Powdery mildew infestation (A- resistant code “0”; B-resistant code “1”; C-resistant code “2”; D- susceptible code “3”; E- susceptible code “4” and F-susceptible code “5” in Table 1).



Identification of Mechanisms for Cold Tolerance in *Helleborus orientalis* Lam.

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Significant to the Industry: The purpose of this research was to isolate genes in *H. orientalis* that allow its flower buds to survive extreme cold temperature fluctuations. Cold induced genes were successfully isolated and identified from flower buds and molecular models for cold tolerance were formulated based on the putative function of identified genes. The results of this work will be useful in the development of mechanisms that illustrate how *H. orientalis* is able to survive extreme cold fluctuations. Mechanisms that confer cold tolerance in plants are needed for the development of new ornamental plants that are able to survive such cold stresses.

Nature of Work: Damage caused by late winter and early spring freezing temperatures affects the commercial values of most plants. *Helleborus orientalis* (Lenten-rose) is an evergreen ornamental that can withstand multiple freezing-thawing cycles without encountering significant damage. The goal of this research project was to identify molecular mechanisms and genes in flower buds of Lenten-rose that provide protection from tissue damage caused by low temperature fluctuations.

Lenten-rose flower buds used in this study were collected on three days in March 2008. On March 9th, there was a freezing event; maximum temperature remained at or below -5˚C. On March 10th, the temperature was above 0˚C and by the 14th, it was above 10˚C.

Collected flower buds were flash frozen in liquid nitrogen and kept frozen until analyzed. Total RNA was isolated from each flower bud sample and reverse-transcribed into single strand cDNAs. Transcript populations from samples collected at different dates were compared using fluorescent differential display RNAspectra Red Kits from Genhunter Company (Nashville, TN) following the protocol described previously (1,2). Differentially expressed cDNA fragments were identified, cloned, sequenced and their putative functions were determined using bioinformatics analysis. The expression of each clone was validated using quantitative RT-PCR analysis.

Through these analyses, it was discovered that the gene expression patterns in *H. orientalis* flower tissue changed after being subjected to freezing and thawing cycle. Response and tolerance mechanisms observed included changes in sugar and secondary protection mechanisms, protease activity, photosynthetic machinery, signal transduction and cell growth. When gene sequences were searched in both the nucleotide and the protein databases (NCBI (National Center for Biotechnology

Information, http://www.ncbi.nlm.nih.gov/), few clones had match sequences in the databases. The lack of database matches indicated that the flower buds of Lenten-rose contained cold-responsive genes that have not been reported. In general, expression levels were lower for most genes isolated from flower tissues that had been frozen prior to collection. Some genes had no expression or reduced expression when the ambient temperature increased from -5˚C to 10˚C. Results obtained through these analyses are providing basic information on molecular survival mechanisms in Lenten-rose that allow flower bud tissues to survive freezing. For the development of cold resistant plants, these genes could potentially be introduced into susceptible species to improve their tolerance or resistance to freezing and thawing cycles.

Results and Discussion: Gene IDs for some clones were identified by blasting against NCBI nucleotide database. Peptide sequences were then searched in the protein database and also for conserved domain at (PROSITE profiles) [prf], Pfam HMMs (local models) [pfam_fs], Pfam HMMs (global models) [pfam_ls] (3).

A total of 103 gene fragments were sequenced. Among these, 82 could not be identified due to low homology with reported sequences in the database(s). The 21 identified genes were putatively involved in the following cellular processes:

1. Aquaporin for preserving cellular water balance;
2. Chloroplast component proteins;
3. Maintaining integrity of photosystem I and II;
4. Transcriptional factor for modulating gene expression;
5. Peptidases for removal of toxic peptides;
6. Ascorbate oxidase for increasing antioxidant activity;
7. Other cellular processes;

The relative expression levels of genes were further confirmed using real-time quantitative PCR (qPCR) method following the protocol for Two-Step-RT-PCR with SYBR Green chemistry (4). One gene (clone 428, primer 76), which expressed at stable level under all the temperature regimes, was chosen as the housekeeping gene, or endogenous reference gene. All the genes were corrected by normalization to the housekeeping gene before comparing between different temperature treatments. The fold change in target gene expression was calculated using the $2^{-\Delta\Delta C}$T method (5). According to the fold difference between cold treatments (-5 and 0˚C) and control (10˚C), cloned genes were divided into seven groups.

Group one consisted of ten genes that had stable expressions as the temperature warmed from below freezing to 0˚C and 10˚C. Group two had low gene expression at temperature below freezing, and increased significantly at warmer temperature (10˚C). Group three consisted of nine genes that had low expression during freezing. However, the gene expressions of this group were activated when the temperature increased to 0˚C, and remained at that same expression level as temperature increased to 10˚C. Group four consisted of one protein-protein interaction motif PCI domain containing protein, one myrcene synthase gene, and one unidentified gene. Group five consisted of four genes; with the exception of two unidentified genes, the ammonia transporter

gene was 1.5-2.0 folds higher at -5 and 0˚C compared to 10˚C. Group six consisted of fifteen genes that increased their expression as the temperature increased from below freezing to 0˚C and to 10˚C. Group seven consisted of nine genes. These genes were activated as temperature decreased to 0˚C, and were down-regulated as the temperature increased to 10˚C.

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