**Crop Science Society of America**
5585 Guilford Road • Madison, WI 53711-5801 • Tel. 608-273-8080 • Fax 608-273-2021
www.crops.org • email: headquarters@crops.org

# *The Plant Genome* Proofing Instructions

Following are the galley proofs for your *Plant Genome* manuscript. Please review the galley proofs carefully; they will not be read by editorial staff. Review of the word-processing file at the query letter stage should not be considered a substitute for galley proof review.

Note that you are being asked to correct errors, not to revise the paper. You will not be charged for our editing mistakes or typographical errors, but you will be charged for any alterations from the original text that you make on the galley proofs. Extensive alteration may require Editorial Board approval, possibly delaying publication. Alterations to the galley proof carry a $5 per line charge.

***Reviewing the proofs***. Please follow these guidelines for reviewing the proofs:

- Print a copy of this file; do not review the galley proofs on-screen. The exception is figures, which generally have a truer representation on-screen than in the printed copy.
- Check all type on the galley proofs, including the footers that appear at the bottom of each page, the title and byline, the abbreviations list, and the author–paper documentation paragraph.
- If your galley proofs have tables, check the table data against that in your original tables.
- If your galley proofs have equations, check them against those in your original manuscript.
- If your galley proofs have figures, check to be sure that all type is legible, including any small-print text. If a figure requires alteration, *you must provide a PDF file of the revised figure via email attachment*.

***Returning the proofs***. If the galley proofs require alterations, return the marked galley proofs via express delivery to the address below.

Alternatively, you may return the marked galley proofs by fax. If you choose this option, you must also send an email message that includes a description of each alteration. You may also return the marked galleys as an email attachment.

If the galley proofs do not require alterations, you may send an email message indicating that no changes are needed.

(more)

COLOR REPRINT COVERS AVAILABLE! For reprint covers, we are now offering high-quality color copies of the journal cover. Your covers will be taken from the issue in which your manuscript is published. Covers include manuscript title, byline, and page numbers.

Thank you for your contribution to *The Plant Genome*.

Sincerely,

Elizabeth Gebhardt, Managing Editor
*Crop Science, The Plant Genome*
5585 Guilford Road
Madison, Wisconsin 53711 USA

608-268-4950 (phone), 608-273-2021 (fax), egebhardt@sciencesocieties.org (email)

Revised 2/10

# Abiotic Stress-Related Expressed Sequence Tags from the Diploid Strawberry *Fragaria vesca* f. *semperflorens*

Maximo Rivarola, Agnes P. Chan, David E. Liebke, Admasu Melake-Berhan, Hui Quan, Foo Cheung, Shu Ouyang, Kevin M. Folta, Janet P. Slovin,* and Pablo D. Rabinowicz*


## Abstract

Strawberry (*Fragaria* spp.) is a eudicotyledonous plant that belongs to the Rosaceae family, which includes other agronomically important plants such as raspberry (*Rubus idaeus* L.) and several tree-fruit species. Despite the vital role played by cultivated strawberry in agriculture, few stress-related gene expression characterizations of this crop are available. To increase the diversity of available *Fragaria* transcriptome sequence, we produced 41,430 *Fragaria vesca* L. expressed sequence tags (ESTs) from plants growing under water-, temperature-, and osmotic-stress conditions as well as a combination of heat and osmotic stresses that is often found in irrigated fields. Clustering and assembling of the ESTs resulted in a total of 11,836 contigs and singletons that were annotated using Gene Ontology (GO) terms. Furthermore, over 1200 sequences with no match to available Rosaceae ESTs were found, including six that were assigned the "response to stress" GO category. Analysis of EST frequency provided an estimate of steady state transcript levels, with 91 sequences exhibiting at least a 20-fold difference between treatments. This EST collection represents a useful resource to advance our understanding of the abiotic stress-response mechanisms in strawberry. The sequence information may be translated to valuable tree crops in the Rosaceae family, where whole-plant treatments are not as simple or practical.


Published in The Plant Genome 4:1–12. Published xx Jan. 2011.
doi: 10.3835/plantgenome2010.08.0018
© Crop Science Society of America
5585 Guilford Rd., Madison, WI 53711 USA
An open-access publication

All rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Permission for printing and for reprinting the material contained herein has been obtained by the publisher.

The cultivated strawberry (*Fragaria* ×*ananassa* Duchesne ex Rozier) is a valuable crop, recognized for its sweet and nutritious berries. This hybrid species is an octoploid with at least two subgenomes that share a common ancestor with extant diploid species, *Fragaria vesca* L. and *F. iinumae* Makino (Folta and Davis, 2006; Rousseau-Gueutin et al., 2009). Over the last decade, the *F. vesca* system has gained attention as an attractive model for genomics in the *Fragaria* genus, the Rosoidiae subfamily, and the Rosaceae family as a whole. *Fragaria vesca* has one of the smallest genomes among crop plants. It can be grown in a relatively small space, cycles rapidly from seed to seed, is readily transformed and regenerated, and, like the octoploid, is amenable to both sexual and vegetative propagation (Battey et al., 1998; Davis and Pollard, 1991; Slovin and Rabinowicz, 2007; Uratsu et al., 1991). All of these attributes make *F. vesca* an outstanding system to functionally characterize how specific

M. Rivarola, D.E. Liebke, and P.D. Rabinowicz, Institute for Genome Sciences, Dep. of Biochemistry and Molecular Biology, Univ. of Maryland, School of Medicine, BioPark Building II, 801 West Baltimore St., Baltimore, MD 21201; A.P. Chan, A. Melake-Berhan, H. Quan, F. Cheung, S. Ouyang, and P.D. Rabinowicz, The J. Craig Venter Institute, 9704 Medical Center Dr., Rockville, MD 20850; K.M. Folta, Horticultural Sciences Dep. and the Graduate Program in Plant Molecular and Cellular Biology, 1301 Fifield Hall, Univ. of Florida, Gainesville, FL 32611; J.P. Slovin, USDA-ARS, Genetic Improvement of Fruits and Vegetables Lab. Henry A. Wallace Beltsville Agricultural Research Center, 10300 Baltimore Ave., Beltsville, MD 20705. Received 1 Aug. 2010. M. Rivarola and A.P. Chan contributed equally to this work. *Corresponding authors (janet.slovin@ars.usda.gov), (prabinowicz@som.umaryland.edu).

Abbreviations: cDNA, complementary DNA; EST, expressed sequence tag; FOM, figure of merit; GO, Gene Ontology; HSP, heat shock protein; LMW, low molecular weight; mRNA, messenger ribonucleic acid; NCBI, National Center for Biotechnology Information; RNA, ribonucleic acid; RUBISCO SSU, ribulose 1,5 bisphosphate carboxylase small subunit; TGICL, the Institute for Genomic Research gene indices clustering tool.

genes contribute to traits of interest in basic science or horticultural production.

Despite its utility, the *Fragaria* genus has an incomplete representation of expressed sequences in public databases. While such resources for some members of the Rosaceae family have grown considerably in recent years (Gasic et al., 2009; Vizoso et al., 2009), there is substantial need for improvement of these resources for *Fragaria*. Especially lacking had been transcript sequences from abiotically stressed plants. Such sequence information would provide a valuable foundation from which to probe the mechanisms that underlie how strawberry plants adjust or acclimate to unfavorable changes in their ambient environment.

Development of resources to study stress physiology in strawberry is pertinent to current trends in strawberry production. Agriculture throughout the world is facing increases in abiotic stresses such as dramatic fluctuations in temperature, water shortages, and soil salinization as a result of irrigation practices. Drought, high salinity, and even mild but chronic temperature variations result in reduced cell division in main meristematic tissues and a decrease in cell expansion (Potters et al., 2009), resulting in major crop loss worldwide (Boyer, 1982; Bray et al., 2000; Ort and Boyer, 1985). While plants are unable to physically escape an environmental stressor, they have adapted to cope with stresses in a variety of ways.

A substantial body of work has uncovered many of the underlying processes that contribute to a plant's ability to tolerate some level of abiotic stress, either separately (Ito et al., 2006; Kotak et al., 2007; Larkindale et al., 2005; Ma et al., 2006; Seki et al., 2003; Shinozaki et al., 2003; Tattersall et al., 2007) or in combination (Kant et al., 2008; Mittler, 2006; Potters et al., 2009; Rizhsky et al., 2004). Such processes can result in morphological changes that involve redistribution of plant growth sites with concomitant changes in plant architecture (Potters et al., 2009) and consequent effects in crop yield. Plant defense responses to abiotic stresses also lead to changes in the chemical components of cells, including the production of solutes such as glycine, betaine, and proline in response to drought (Caramelo and Iusem, 2009; Ma et al., 2006; Seki et al., 2003; Shinozaki et al., 2003; Wang et al., 2003) or the production of new lipids or proteins that change the fluidity or permeability of membranes in response to temperature or ionic environment changes (Heinen et al., 2009; Kotak et al., 2007; Larkindale et al., 2005; Luu and Maurel, 2005; Ma et al., 2006; Munns, 2002; Ueda et al., 2004). Another plant adaptive response involves the production of protective or restorative proteins such as the late embryogenesis abundant (LEA) proteins (Battaglia et al., 2008), the large class of molecular chaperones known as heat shock proteins (HSPs) (Swindell et al., 2007; Tonsor et al., 2008; Waters et al., 2008), and enzymes that remove damaging reactive oxygen species (ROS) (Apel and Hirt, 2004; Conklin and Barth, 2004).

The responses of fruit crops such as strawberry that rely on sexual reproductive development to produce fruit have been less well studied than those of the major crops such as wheat (*Triticum aestivum* L.), soybean [*Glycine max* (L.) Merr.], or the model plant *Arabidopsis thaliana* (L.) Heynh. In rosaceous fruits, many abiotic factors have been identified by growers as having an impact on yield and fruit quality. Drought, heat, cold, and soil salinity are widely recognized as having the largest effects, although flooding, sunburn, and air pollution also affect fruit yield and quality. Fruit trees or perennial plants such as strawberry, raspberry (*Rubus idaeus* L.), and blackberry (*Rubus* spp.) may be more or less susceptible to drought, temperature, and soil salinity, depending on the stage in their developmental cycles.

We report here the generation and analysis of a substantial set of *F. vesca* expressed sequence tags (ESTs) from seedlings and various tissues of mature strawberry plants subjected to stress. These sequence data provide the research community with a robust gene discovery tool as the ESTs represent a snapshot of the transcriptome corresponding to discrete genetic programs that were otherwise unaccounted for. This comprehensive stress EST set considerably expands previous efforts to characterize strawberry transcriptomes and is also an important resource for development of simple sequence repeat (SSR) or EST-polymerase chain reaction (PCR) markers for mapping, as well as for genomic annotation and transcriptome analysis.

## Materials and Methods

### Plant Material

*Fragaria vesca* L. subsp. *vesca* forma *semperflorens* (Duchesne) Staudt Hawaii-4 [Germplasm Resources Information Network (GRIN) Accession number: PI551572, GRIN Local ID: CFRA 197, also known as "FV10" (Oosumi et al., 2006)] was obtained from V. Shulaev (Virginia Biotechnology Institute, Virginia Tech, Blacksburg, VA). Plants were grown in three different systems: aseptically on agar plates, in nonsoil substrate in growth chambers, or hydroponically. For tissues from mature plants, seed from the original plant were sown in Metro Mix 510 (Scotts-Sierra Horticultural Products, Marysville, OH) mixed 2:1 vermiculite and supplemented with 50.0 g. of dolomitic lime per 10.0 L of substrate. Plants were kept in growth chambers (E15, Conviron, Winnipeg, MB, Canada) under a 12/12 h light/dark cycle of ~200 mM photon $m^{-2}$ $sec^{-1}$ and fertilized once per week with Miracle-Gro Tomato Plant Food (Scotts Miracle-Gro Products, Inc. Marysville, OH) as directed by the supplier.

Seeds for seedling treatments were surface sterilized by treating them for 5 min with 95% ethanol followed by several rinses with sterile $H_2O$ and incubation overnight at 5°C in sterile water with gentle shaking. Seeds were then treated with 30% commercial bleach containing 5 mL $L^{-1}$ of Tween-20 for 15 min, rinsed in sterile $H_2O$ as before, and then sown on 0.5x Murashige and Skoog media (Murashige and Skoog, 1962) containing 3% sucrose

solidified with 0.8% Phytagar (Invitrogen, Carlsbad, CA) in square Petri dishes. The appropriate reagents for each treatment were added as needed to the medium before autoclaving. Plates were sealed with Micropore tape (3M, St. Paul, MN) and one edge further sealed with parafilm before the plates were placed vertically in racks in a temperature controlled (25°C) tissue culture room. Seedlings were grown under a 12/12 h light/dark cycle at ~20 mM photon $m^{-2}$ $sec^{-1}$ supplied by cool white fluorescent bulbs.

Runner plants attached to the mother plant were grown hydroponically in growth chambers on 1:100 diluted Miracle-Gro Tomato Plant Food (Scotts Miracle-Gro Products, Inc., Marysville, OH) by suspending the runners on Nylon window screening over the solution. The diluted fertilizer was maintained in the dark to inhibit algal growth. Root initials elongated within a few days and roots were harvested between 1 and 2 wk later.

## Plant Treatments and Complementary DNA Library Construction

Plants or seedlings were collected after the experimental treatments, rapidly frozen in liquid N, and stored at −80°C until ribonucleic acid (RNA) extraction.

Cold treatment (Cold H4 library): Three- to four-week-old seedlings growing aseptically on vertical agar plates in a growth room at 25°C were transferred to 5°C ±2°C in either complete dark or 12/12 h light/dark cycle (~20 mM photon $m^{-2}$ sec−1) for various periods (from 15 min to 4 d) before being removed from the plates, gently rinsed with distilled water, patted dry, weighed and frozen in liquid N.

Drought treatment (Drought H4 library): Leaves, buds, flowers, roots, petioles, runner tips (terminal 1.0 cm from runners), young fruit, and crown tissues were harvested from 12- to 15-wk-old plants maintained in growth chambers in Metro Mix, vermiculite, and dolomitic lime substrate as described above. Plants were chronically wilted by reducing watering to 10% relative to normal growth conditions for 6 to 8 d before harvest. Hydroponically growing roots were incubated in 10% polyethylene glycol 6000 for 3 h before harvest, as this treatment is known to reduce the pressure and osmotic potentials (Kaufmann and Eckard, 1971).

Heat treatment (Heat H4 library): Leaves, roots, buds, flowers, and crown tissues were harvested from moderately heat-stressed plants growing for more than 3 wk at 32/25°C day/night in growth chambers under saturating humidity. Young leaves, buds, flowers, petioles, and runner tips were harvested from these same plants heat shocked at 45°C for 30 min or 1 h in the dark, with pots set in pans with 2.0 cm of water at 45°C. Entire 3- to 4-wk-old seedlings growing aseptically on the surface of agar on vertical plates were harvested after treatment in the dark under saturating humidity for 30 min at 37°C followed by 1 h at 25°C and subsequent exposure to either 1 h at 37°C or 30 min at 45°C.

High salt treatment (Salt H4 library): Leaves, buds, open flowers, roots, petioles, runner tips, young fruit, and crown tissues were harvested from plants watered daily for 5 d with 50 mM NaCl. Entire seedlings were grown aseptically for 3 to 6 wk on the surface of agar containing 25, 50, or 75 mM NaCl in square Petri dishes vertically positioned under a 12/12 h light/dark cycle (~20 mM photon $m^{-2}$ $sec^{-1}$). Both 50 and 75 mM NaCl had previously been shown in preliminary experiments to greatly inhibit seedling root elongation but it did not result in death of 6-wk-old seedlings (data not shown).

Combined high salt and heat treatment (Salt/Heat H4 library): Leaves, buds and open flowers, roots, petioles, runner tips, fertilized achenes from very young fruit, and crown tissues were harvested from plants watered daily for 1 wk with 50 mM NaCl and then treated in the dark for 30, 60, or 90 min at 37°C. Entire seedlings were aseptically grown for 3 to 6 wk on the surface of agar containing 25 mM NaCl in vertically positioned Petri dishes under a 12/12 h light/dark cycle (~20 μM photon $m^{-2}$ $sec^{-1}$) and then treated in the dark for 30 min or 2 h at 37°C before harvesting tissues.

For all libraries, total RNA was extracted, separately from each tissue type and treatment, using a modified hot borate method (Wan and Wilkins, 1994). For the Cold H4 library, equal amounts of RNA from the various samples were pooled before messenger ribonucleic acid (mRNA) isolation using the Oligotex Direct mRNA Mini Kit (Qiagen Inc., Valencia, CA). The complementary DNA (cDNA) library was constructed in the Gateway pDONR222 vector using the CloneMiner kit (Invitrogen, Carlsbad, CA). The remaining libraries were commercially constructed using mRNA from pooled RNA samples in a Gateway pENTR vector (Virotech International, Inc., Gaithersburg, MD).

## Sequencing and Analysis

Clones were picked using a Q-Pix robot (Genetix, San Jose, CA) and templates were prepared using alkaline lysis and ethanol precipitation in a 5-prime DNA purification workstation (Eppendorf, Hamburg, Germany). Sequencing was done using a 5′-end sequencing primer and Big Dye Terminators (Applied Biosystems, Foster City, CA). Sequencing reactions were run in 3730XL genetic analyzers (Applied Biosystems). Following electrophoresis and fluorescence detection, quality values were assigned to each base by Tracetuner software (Paracel, Inc., Pasadena, CA), and vector and low quality sequences were removed. Sequences are available in the dbEST division of GenBank under the accession numbers listed in Table 1.

To generate unique sequences, ESTs were grouped into clusters based on shared sequence similarity using the TGICL (the Institute for Genomic Research gene indices clustering tool) clustering utilities with default parameters (Pertea et al., 2003) that produces "clustered" sequences and "singletons." The TGICL clustering is performed by a slightly modified version of the National Center for Biotechnology Information's (NCBI) Megablast (Zhang et al., 2000) and the resulting

clusters are then assembled using CAP3 (Huang and Madan, 1999). Alignments containing gaps (or inserts) longer than nine nucleotides were discarded. Expressed sequence tags that did not assemble into a contig were kept as "singletons."

Gene Ontologies (GO; http://www.geneontology.org [verified 30 Dec. 2010])) were used to provide a controlled vocabulary to describe putative genes (Ashburner et al., 2000). Gene Ontology annotations were assigned using the program Blast2Go (Conesa et al., 2005) and all plant proteins available in GenBank were used for the initial BLASTX ($E < 10^{-10}$) searches. Blast2Go combines sequence similarity searches with statistical analyses to transfer GO annotations to query sequences from homologous sequences in a selected database. After assigning GO annotations, one unique sequence (CL2Contig1) similar to bacterial transposases was removed from all further analysis.

Unique sequences with similar expression profiles were identified using *k*-means clustering analysis (The *k*-means algorithm is a partitional method that by iterative reallocation of cluster members minimizes the overall within-cluster dispersion. See Sturn et al., 2002, for more details.) with Pearson's correlation coefficient and the Genesis software package (Sturn et al., 2002). Expressed sequence tag abundances for all 1471 unique sequences composed of five or more ESTs were normalized relative to the Cold H4 library and adjusted by applying Log base(2). *K*-means clustering was run for a maximum of 50 iterations and resulted in a converged output. Using the figure of merit (FOM) analysis (Yeung et al., 2001) in the Genesis software package, the optimal number of clusters was determined to be 14, which resulted in good quality clusters with distinct profiles.

Estimates of differential gene expression were derived following the methodology of Audic and Claverie (Audic and Claverie, 1997) implemented with the IDEG6 software package (Romualdi et al., 2003). IDEG6 allows identification of differentially expressed genes and assigns a *p*-value by statistical analysis of large matrices of multiconditional EST expression data. Results were confirmed using a permutation test with the same set of 1471 sequences composed of five or more ESTs. Briefly, the mean difference was estimated between randomly drawn EST counts from a pool created by combining the counts from each of two stress conditions. The pool consisted of EST counts from each condition, in which at least one of the two conditions had a count greater than 0. From this pool, two counts were drawn at random (uniform 0.5 probability) and their difference taken and recorded. This process was repeated using the same pool (sampling with replacement) until the number of recorded differences was the same as the original sample size ($n = 1471$). The mean of the differences was then calculated and recorded and the whole process was repeated until there were 1000 mean-difference values, which represent the distribution of mean difference values expected if there was no difference between the two experimental conditions (null hypothesis). The significance of the differences was determined for each value by calculating the 0.005 and 0.995 quantiles of the permutated mean-difference values and determining if the actual difference lies outside of those quantiles.

## Results and Discussion

### Expressed Sequence Tag Sequencing and Annotation

Five cDNA libraries labeled Cold H4, Drought H4, Heat H4, Salt H4, and Salt/Heat H4 were produced with mRNA extracted from *F. vesca* seedlings and plants that were exposed to one of four different abiotic stresses or to a combination of these stresses that often occurs in irrigated fields. Treatments and tissue selections were designed to maximize the number of different transcripts present. The Cold H4 library was constructed using in vitro-grown seedlings treated from 15 min to 4 d at 5°C, and samples were collected at different intervals. Plants for the Drought H4 library were subjected to chronic wilting for 6 to 8 d or treated with polyethylene glycol (PEG) solution. The Heat H4 library was constructed from mRNA extracted from five tissues of plants subjected to long-term, moderately elevated temperatures and from these same plants further subjected to short-term heat shock at 37 or 42°C. The osmotic stress treatment (Salt H4) library was constructed with RNA from eight different tissues from mature plants and seedlings treated for extended periods with moderately elevated levels of salt that had previously been shown to reduce growth but not result in plant death (Slovin and Rabinowicz, 2007). Finally, the combined stress (Salt/Heat H4) library was constructed using mRNA from eight different tissues or from seedlings exposed for extended periods to moderately elevated salt concentration followed by heat shock at 37°C.

From these five libraries, 50,496 sequence attempts resulted in 41,430 EST sequences. These ESTs were clustered and assembled using the TGICL pipeline (Pertea et al., 2003), which uses a set of stringent criteria to discard low-quality sequences. A total of 41,074 high-quality EST sequences were assembled including 9639 from the Cold H4 library, 6846 from the Drought H4 library, 8885 from the Heat H4 library, 8577 from the Salt H4 library, and 7483 ESTs from the Salt/Heat H4 library (Table 1). The average read length was 675 bp. A total of 11,836 unique sequences were generated from all the ESTs, including 4433 consensus sequences from assemblies (contigs) and 7403 ESTs that could not be assembled (singletons). The length of these unique sequences ranged from 100 to 4731 bp with an average of 818 bp.

Abiotic stresses can broadly affect gene expression as well as biochemical reactions and properties of cellular components, including membrane fluidity and protein folding. To obtain information about their potential functions in these processes, the unique sequences were annotated using Blast2Go, which assigns GO annotation using a combination of similarity searches and statistical analysis (Conesa et al., 2005). In this way, nearly 13,000 GO terms for a particular biological process, cellular component, or molecular function were assigned

**Table 1. Library and sequence specifications.**

| Library | Accession numbers | Sequence attempts | High quality ESTs[†] | Avg. read length (bp) | Cloned cDNA[‡] fraction |
|---|---|---|---|---|---|
| Heat H4 | EX665399–EX674283 | 9984 | 8885 | 649 | 0.8–4 kb |
| Cold H4 | DY666641–DY676279 | 10,368 | 9639 | 733 | 1.5 kb (average) |
| Drought H4 | EX674284–EX681129 | 9984 | 6846 | 695 | 0.8–4 kb |
| Salt H4 | EX656822–EX665398 | 9984 | 8577 | 665 | 0.8–4 kb |
| Salt/Heat H4 | EX681130–EX688612 | 10,176 | 7483 | 625 | 0.8–4 kb |
| Total | | 50,496 | 41,430 | 675 | |

[†]EST, expressed sequence tag.

[‡]cDNA, complementary DNA.

to 6040 of the unique sequences. Sequences obtained by EST assembly may not contain an entire gene sequence, so domain motifs necessary for making GO assignments may not be present on a given unique sequence. Of the original 11,836 unique sequences, nearly 81% had a BLASTX match ($E < 10^{-10}$) in the NCBI plant protein database and 85% had a match in the nonredundant protein database; however, GO terms could not be assigned to almost one half (5918) of the unique sequences.

A "biological process" GO term could be assigned to 2982 sequences, and most of these sequences could be assigned to one or more of 21 categories (Fig. 1A; Supplemental Table S1; Supplemental Table S2). Many of these sequences appear to be associated with metabolic processes, and, as expected, a large number of the unique sequences was associated with "response to stress" GO terms. Further scrutiny of the 433 unique sequences that were assigned the "response to stress" category (Supplemental Table S3) showed that 88 corresponded to "response to cold," 86 to "response to heat," 34 to "response to water deprivation," and 31 to "response to osmotic stress" (Fig. 1B).

Several of the assembled unique sequences contained large numbers of ESTs. Among the 20 assembled from the highest numbers of ESTs (Table 2) are 17 that show similarity to well-known stress response proteins, including metallothioneins, a dehydrin, a secretory peroxidase, and low molecular weight heat shock proteins (LMW HSP), providing evidence that many of the expected stress-related genes are expressed in *F. vesca*. Heat shock protein and metallothionein gene families are involved in heat and oxidative stress responses, respectively (Cobbett and Goldsbrough, 2002). The metallothioneins are low molecular weight cysteine-rich proteins that regulate the intracellular supply of zinc and copper ions and protect cells from the effects of elevated levels of these and other metal ions, as well as of reactive oxidant species. Abundant sequences also included those typically highly expressed in leaf tissue, such as ribulose 1,5 bisphosphate carboxylase small subunit (RUBISCO SSU) and chlorophyll a/b binding proteins, which are expected in non-normalized cDNA libraries.

## Diversity and Abundance of Unique Sequences

To have a sense of the overall diversity of transcribed sequences and their relative expression level under each treatment, the number of ESTs that compose each unique sequence was used as an indication of the level of expression of the corresponding putative genes (assuming that each unique sequence represents one gene). To approximate relative transcript abundance between libraries, the number of ESTs in each library was normalized to the Cold H4 library, from which the largest number of ESTs has been generated. The number of unique sequences was then plotted against bins representing the number of EST components (Fig. 2). The Cold H4 library had the largest amount of diversity of expressed sequences, while the other libraries had from 35 to 48% less diversity. Drought, heat, salt, and combined salt and heat treatments each resulted in a few transcripts being present in high abundance. On the other hand, no such highly abundant transcripts were found in the Cold H4 library. Putative genes having the highest expression level in the Cold H4 library include the RUBISCO SSU and light harvesting complex protein genes described in Table 2 (Supplemental Table S1).

A similar analysis of the 433 unique sequences assigned the GO category "stress response" resulted in a comparable profile to that seen in Fig. 2 for all the sequences: the largest diversity of "stress response" sequences was found in the Cold H4 library, and few genes appear to be highly expressed as a result of cold treatment, compared to the other stresses (Fig. 3). The Heat H4 and Salt/Heat H4 libraries showed the largest numbers of highly expressed, stress-related unique sequences (18 and 19, respectively) while the Cold H4 and Heat H4 showed the largest diversity of stress-related unique sequences (195 and 194, respectively) (Supplemental Table S4).

## Differential Expression of Unique Sequences among Stresses

The 1471 unique sequences assembled from five or more ESTs were subjected to pair-wise comparisons between libraries using the Audic and Claverie test (Audic and Claverie, 1997) (Supplemental Table S5). Only significant p values (<0.01) were considered. An additional permutation test (see Methods) confirmed the results of the Audic and Claverie test, but we report here the results of only the more stringent Audic and Claverie test. From these analyses it was found that 787 unique sequences (53%) were found at significantly different levels between at least two treatments (Supplemental Table S5). Of these, 91 showed at least a 20-fold difference between any two treatments, indicating stress-specific expression (Supplemental Table S1).

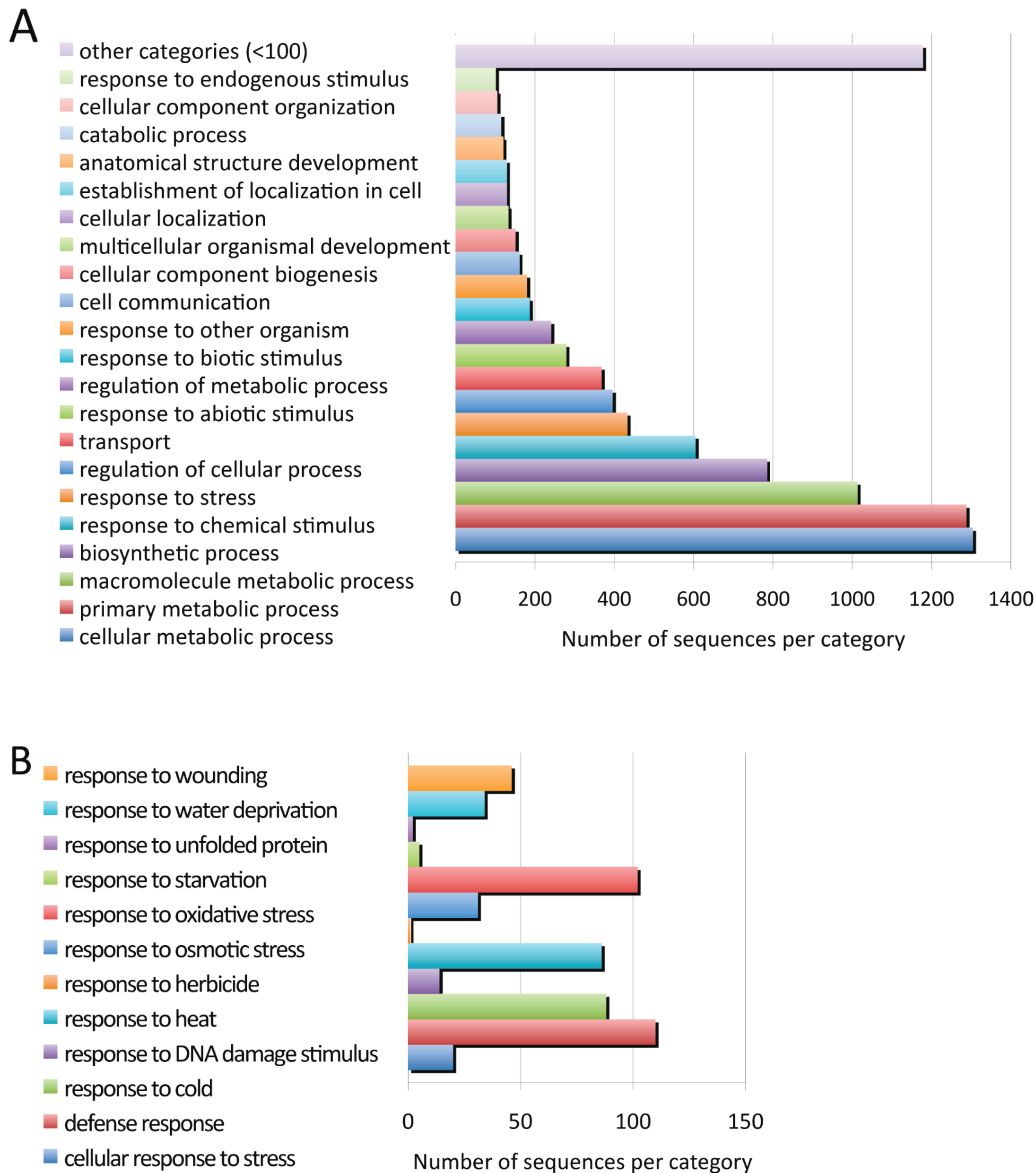


Figure 1. Distribution of "Biological Processes" Gene Ontology (GO) terms in the *F. vesca* expressed sequence tags (ESTs). A) Distribution of GO "Biological Processes" categories for all contigs and singletons. Gene Ontology annotation categories assigned to fewer than 100 sequences are combined in "other categories." B) Distribution of level four GO categories assigned to sequences within the level 3 "response to stress" category.

The heat, high salt, and combined salt and heat treatments provided an opportunity to discover genes whose expression is affected by only a combination of stress conditions that has not been examined before at the molecular level yet is becoming more common in the field as a result of irrigation. A pair-wise analysis of sequences from these three libraries identified a set of 423 unique sequences that have differential expression in at least one comparison. The comparison between the high salt and heat treatments revealed 118 differentially expressed unique sequences, while each of these two treatments resulted in an increased number of differentially expressed unique sequences compared to the combined stress treatment (309 and 310, respectively; Supplemental Table S5). The Salt/Heat H4 library contained 189 differentially expressed unique sequences that showed no significant difference in abundance between Heat H4 and Salt H4 libraries. Therefore, the combined stress treatment highlighted sequences that would have not been identified using individual stress treatments. Furthermore, under this combined treatment the largest number of unique sequences (34) showing a peak in expression level was found while only four were found under cold treatment (Supplemental Table S6). These results suggest that fewer genes are induced specifically by cold stress than other stresses.

**Table 2. Twenty unique sequences assembled from the highest numbers of expressed sequence tags (ESTs).**

| Unique sequence name | Protein sequence match[†] | No. of EST components | Sequence length (bp) | Best match accession No. | % Identity | % Similarity | % Coverage | Taxon |
|---|---|---|---|---|---|---|---|---|
| CL1Contig 62 | Metallothionein-like protein 2 | 497 | 1294 | P93134 | 96.25 | 97.50 | 100 | *Fragaria ×ananassa* Duchesne ex Rozier |
| CL1Contig 88 | Class I LMW HSP[‡] | 432 | 804 | AAR25848 | 83.57 | 94.29 | 90.91 | *Carica papaya* L. |
| CL1Contig 129 | Nonspecific lipid transfer protein | 310 | 2443 | AAY83341 | 100 | 100 | 100 | *F. ×ananassa* |
| CL1Contig 68 | Nonspecific lipid transfer protein | 261 | 959 | AAY83346 | 93.97 | 96.55 | 99.15 | *F. ×ananassa* |
| CL1Contig 25 | RUBISCO SSU[§] | 255 | 1319 | AAA33866 | 86.90 | 95.24 | 93.85 | *Malus domestica* Borkh × *Pyrus communis* L. |
| CL1Contig 47 | Class I LMW HSP | 252 | 850 | AAC39360 | 97.42 | 98.71 | 99.36 | *F. ×ananassa* |
| CL1Contig 1 | Metallothionein-like protein | 224 | 881 | CAA04766 | 100 | 100 | 100 | *Fragaria vesca* L. |
| CL3Contig 1 | Dehydrin | 215 | 1387 | ABG56268 | 78.65 | 80.90 | 38.86 | *Malus domestica* |
| CL1Contig 60 | Hypothetical Protein | 183 | 1505 | BAD46202 | 75.36 | 79.71 | 27.82 | *Oryza sativa* L. |
| CL1Contig 49 | Class III Heme Dep. Peroxidase | 176 | 1341 | AAA99868 | 87.50 | 95.31 | 96.39 | *Gossypium hirsutum* L. |
| CL1Contig 15 | Peptidylprolyl isomerase | 168 | 2061 | CAC84116 | 92.98 | 95.32 | 98.84 | *Betula pendula* Roth |
| CL1Contig 56 | LMW HSP | 165 | 862 | ABK92179 | 81.41 | 91.03 | 100 | *Nelumbo nucifera* Gaertn. |
| CL1Contig 131 | Glycine Rich Protein-like 2 | 158 | 4731 | ABG76000 | 93.60 | 96.80 | 95.82 | *Gossypium hirsutum* |
| CL1Contig 14 | Fragaria a 1 allergen | 156 | 867 | CAJ29538 | 100 | 100 | 100 | *F. ×ananassa* |
| CL1Contig 132 | Light-harvesting chl a/b binding protein | 153 | 1120 | AAC34983 | 95.47 | 98.11 | 100 | *Prunus persica* (L.) Batsch |
| CL1Contig 93 | Putative Glycine-rich RNA[¶]-binding protein | 136 | 1042 | AAL13082 | 86.23 | 88.02 | 93.82 | *Prunus avium* (L.) L. |
| CL4Contig1 | LMW HSP20 | 136 | 684 | ABD32352 | 84.92 | 96.03 | 79.25 | *Medicago truncatula* Gaertn. |
| CL1Contig64 | Class I LMW HSP | 129 | 1086 | AAC39360 | 97.44 | 98.72 | 100 | *F. ×ananassa* |
| CL1Contig98 | Light-harvesting chl a/b binding protein LHCII type I | 127 | 996 | AAB87573 | 94.59 | 97.68 | 97.37 | *Panax ginseng* C. A. Mey. |
| CL6Contig1 | Auxin-repressed dormancy associated 12.5 KDa protein | 126 | 1061 | Q05349 | 100 | 100 | 100 | *F. ×ananassa* |

[†]Defined by the best BLASTX match with $E < 10^{-10}$ against all plant proteins.

[‡]LMW HSP, low molecular weight heat shock protein.

[§]RUBISCO SSU, ribulose 1,5 bisphosphate carboxylase small subunit.

[¶]RNA, ribonucleic acid.

## Coincident Expression of Unique Sequences

To further identify groups of unique sequences that share similar expression patterns we performed *k*-means clustering analysis of the 1471 unique sequences composed of five or more ESTs using the Genesis software package (http://genome.tugraz.at/genesisclient/genesisclient_description.shtml [verified 4 Jan. 2011]) (Supplemental Fig. S1A; Supplemental Table S5) (Sturn et al., 2002). A FOM analysis (Supplemental Fig. S1B) determined that the optimal number of 14 clusters yielded distinct expression patterns. Clusters 1, 3, 5, 8, and 9 depict sequences that are predominantly expressed in response to two of the treatments, while Clusters 6, 12, 13, and 14 each depict patterns reflecting treatment specific expression. Cluster 14 contains 155 unique sequences that show a peak in expression under low temperature conditions. This cluster includes sequences to which "response to cadmium" and "light harvesting complex proteins" GO terms were assigned. These results are consistent with previous studies in Rosaceae and other dicots (Wisniewski et al., 2008; Fowler and Thomashow, 2002), except that under cold stress we observed induction of some photosynthetic genes induced, which seem to be typically downregulated under cold stress in apple (*Malus domestica* Borkh.) (Wisniewski et al., 2008). Nevertheless, even the most highly expressed genes in the Cold H4 library are transcribed at a 2- to 11-fold lower level than the highest expressed sequences in other libraries. Furthermore, only 29 unique sequences expressed in response to cold were represented by 16 or more ESTs, whereas several unique sequences in the other libraries were represented by 65 or more ESTs (Supplemental Table S6; Fig. 2).

Cluster 7 depicts the differential expression of putative genes in the high salt, heat, and combined stress conditions described above, and groups 174 unique sequences that show higher expression under the combined stresses than under either high salt or heat alone. Among these unique sequences are 88 of the 189 differentially expressed under a combination of high salt and heat stress. The expression pattern for CL10Contig1, which shows similarity to 11S globulin seed storage proteins, is depicted in cluster 7.

Cluster 1 represents sequences expressed in response to both cold and drought, and Cluster 5 represents sequences expressed in response to both to salt and drought. Cluster 8 shows a well-known response of plants to heat stress in

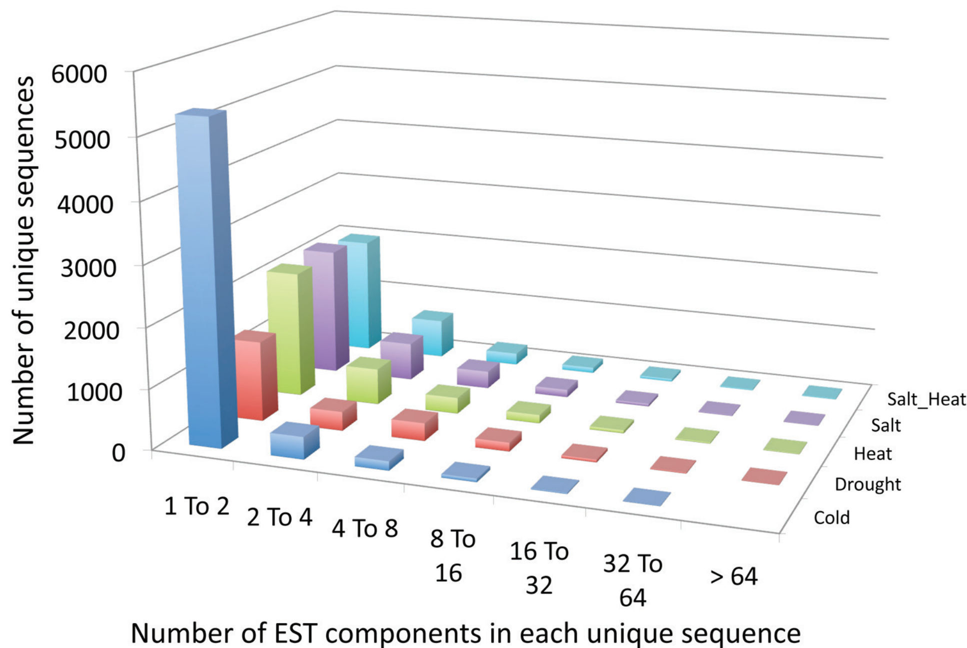


Figure 2. Diversity of unique sequences among the different expressed sequence tag (EST) libraries. The histogram shows the normalized number of ESTs in all unique sequences, classified by library and number of EST components. Diversity of expressed sequences is higher under cold than under other stresses.

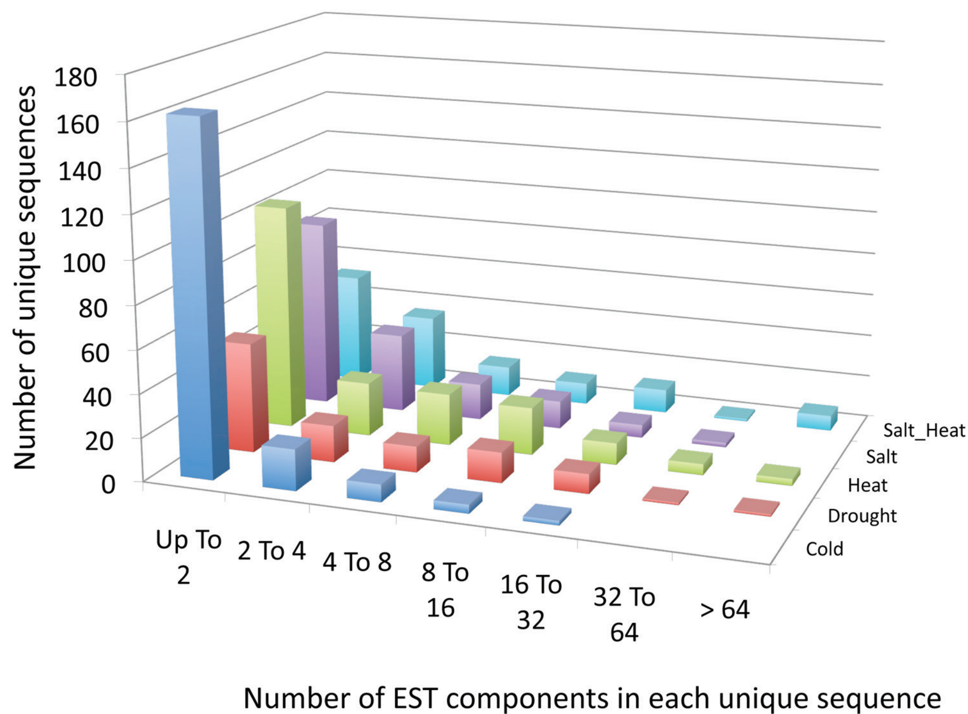


Figure 3. Diversity of stress-related unique sequences among the different expressed sequence tag (EST) libraries. The histogram shows the normalized number of ESTs in unique sequences that were assigned to the Gene Ontology (GO) category "response to stress." Cold stress induced the expression of the highest number of different sequences, while the other stress conditions showed high levels of expression of some sequences.

which drought responsive genes are turned on together with heat response genes (Rizhsky et al., 2004).

Among the 433 unique sequences assigned the "stress response" GO category, several were expressed at very high levels in the plants subjected to drought, heat, or a combination of salt and heat (Fig. 3). Among these is a sequence similar to a metallothionein (CL1Contig62) in cluster 10 that appears to be highly expressed under drought and to a lesser extent under heat and high salt but hardly expressed in the combined stress. Also very

highly expressed under combined stress but not under cold or drought treatments is a putative cytosolic LMW HSP (CL1Contig88), depicted in cluster 4. Sequences with similarity to HSP family members and metallothioneins (Supplemental Table S1) were included among the most abundant transcripts in our *F. vesca* abiotic stress EST set. Further examination of the abundance of these sequences in the various libraries revealed that accumulation of several of these transcripts was not uniform among the libraries. For example, the two metallothionein-like sequences (CL1Contig1 and CL1Contig62 in clusters 11 and 10, respectively) were highly abundant in the heat- or high salt-treated plants but were barely detected in plants subjected to a combination of these stresses. Another LMW HSP-like sequence (CL4Contig2), found in cluster 7, was highly abundant in the Salt/Heat H4 library; however, somewhat surprisingly, its expression appeared to be low or not detected in either the Heat H4 or Salt H4 libraries. A different LMW HSP sequence (CL1Contig47 in cluster 8) was highly abundant in heat- or drought-treated plants and was also found in the Cold H4 library, albeit at very low level. Most of the HSP-like sequences found in our EST set belong to the small HSP family. These small protein chaperones are abundantly expressed in most plants in response to heat, sometimes at different levels during development (Almoguera et al., 1993, 2002), and can also be induced by transcriptional regulators that respond to drought and salt stresses (Sakuma et al., 2006; Lim et al., 2006; Sun et al., 2001). Moreover, a small HSP has also been found to protect against chilling injury (Sabehat et al., 1996). Cold and other nonthermal stress treatments such as wounding and osmotic and salt stress can induce expression of HSPs as well as affect expression of the heat shock transcription factors that regulate HSP expression.

Potential overlap in function and cross-talk among stress response pathways is graphically illustrated in Fig. 4A, which shows that a core of 411 unique sequences that include putative genes similar to metallothioneins, LMW HSPs, and other well known stress-related protein coding sequences such as a dehydrin, a poorly understood auxin repressed, dormancy-related protein, and lipid transfer proteins are expressed in all stress treatments, although not at the same level. As became evident from the clustering analysis, a large number of sequences were expressed in plants experiencing different types of stress, which is consistent with the observation that one stress can induce protection against another (Lurie and Klein, 1991; McKersie and Leshem, 1994). Many genes that are turned on in response to drought are also turned on in response to cold (Fowler and Thomashow, 2002; Seki et al., 2003) or to heat stress (Larkindale et al., 2005). At low temperatures, water is removed from cells by freezing, triggering a drought response. At high temperatures, stomata are opened to facilitate evaporative cooling on the leaf surface, causing the plants lose a large amount of water. If water is not available from the soil, this can also trigger a drought response. Our results show that a large number of sequences (993) are expressed both in drought and osmotic stress (Fig. 4A), which has not been studied before. Interestingly, our data show that about 1% of our *Fragaria* unique sequences show expression in cold, drought, and salt stresses (Fig. 4A). This overlap in the expression patterns of certain genes under these stresses has been observed in similar studies conducted in other dicot and monocot plants (Seki et al., 2002; Rabbani et al., 2003; Wong et al., 2006), although the proportions of genes showing such expression profiles were larger.

Figure 4A also shows that there are treatment-specific unique sequences, and some of them appear to encode proteins with known stress-related functions. For example, several members of the LMW HSP family are found only in the Heat H4 library, and a specific member of the S-adenosyl methionine decarboxylase gene family (CL247Contig1) was detected only in the Cold H4 library. Moreover, the Cold H4 library showed the largest proportion (73%) of unique sequences expressed specifically under the corresponding stress (Fig. 4A),

Closer examination of the sequences in the individual Salt H4, Heat H4, and Salt/Heat H4 libraries showed that there are 590 sequences found in all three conditions (Fig. 4B). Many of these are abundant sequences that were assigned a "stress response" GO term. Among this group is the previously mentioned CL1Contig88 sequence (class I LMW HSP), which was not detected in the Cold H4 and Drought H4 libraries. Although in general library-specific sequences are found at low levels, some of those detected only in the Salt/Heat H4 library were found in fairly high abundance, including a hydroxyproline-rich glycoprotein (HRGP), an RNA binding domain protein, and a translationally controlled tumor protein homolog (TCTP) that is a component of the target of rapamycin (TOR) signaling pathway (Berkowitz et al., 2008) and has been implicated in osmotic stress resistance (Deprost et al., 2007).

## Enhancing *Fragaria* Expressed Sequence Tag Resources

As the goal of this work was to increase the diversity of sequences available for rosaceous species we looked for expressed sequences not present in other Rosaceae EST sets. Analysis of the *F. vesca* unique sequences using stringent TBLASTX ($E < 10^{-10}$) against nearly half a million publicly available Rosaceae ESTs revealed 1286 unique sequences that had no matches. These novel strawberry sequences potentially encode proteins that differentiate *Fragaria* from its closest relatives and may include proteins responsible for fruit development and plant growth habit. Among these novel sequences were six singletons assigned to "stress-related" GO categories (Table 3). Four of these sequences were found in the Cold H4 library, one in the Heat H4 library, and one in the Salt/Heat H4 library. Two of these sequences share similarity with proteins of unknown function, one is similar to BiP (a well studied HSP 70 chaperone protein involved in the unfolded protein response in the endoplasmic reticulum), and two others are related to DNA repair proteins.

Interrelationships and cross-talk among the various stress responses are the subjects of several recent

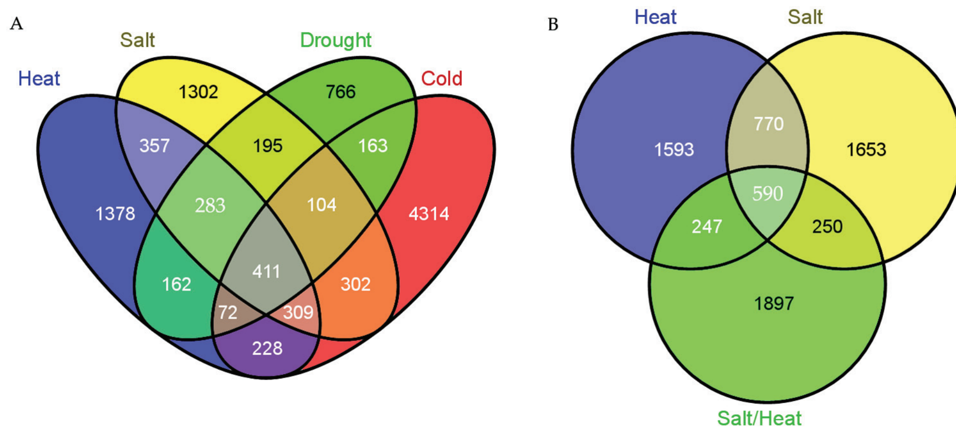


Figure 4. Distribution of all unique sequences among the treatments. A) Venn diagram showing the distribution of the unique sequences from the Cold H4, Heat H4, Drought H4, and Salt H4 libraries. Four hundred eleven unique sequences were found under all conditions. B) Venn diagram showing the distribution of all unique sequences from the Heat H4, Salt H4, and Salt/Heat H4 libraries. A substantial number of unique sequences were expressed under the combined stresses and not under each stress condition individually.

**Table 3. *Fragaria vesca* stress-response sequences not found in other Rosaceae expressed sequence tag (EST) sets.**

| GenBank accession No. | Description | Library | Biological process |
|---|---|---|---|
| EX673244 | Unknown protein | Heat H4 | Response to oxidative stress |
| EX686969 | BiP luminal binding protein | Salt/Heat H4 | Protein folding<br>Response to bacterium<br>Response to heat |
| DY673869 | ATP dependent DNA<br>Ligase family protein | Cold H4 | DNA repair<br>DNA replication<br>DNA recombination |
| DY672681 | Mre11 (meiotic recombination protein 11)<br>Serine/threonine phosphatase | Cold H4 | Double-strand break repair |
| DY672970 | Zinc-binding dehydrogenase family protein | Cold H4 | Response to cold |
| DY670217 | Unknown protein | Cold H4 | Heat acclimation |

studies (Kant et al., 2008; Rizhsky et al., 2004; Swindell et al., 2007). However, to our knowledge, none have specifically addressed the plant response to the combined stresses of a salinized soil coupled with the elevated temperatures anticipated to accompany global climate change. The sequences found in our Salt/Heat H4 *F. vesca* cDNA library will be useful for studying specific responses to these stresses to ameliorate their effect on the large strawberry industry worldwide. Like many herbaceous perennials, strawberries can cold acclimate and acquire the ability to tolerate extracellular ice formation in vegetative tissues. However, open flowers are highly susceptible to late spring cold spells with freezing temperatures. Strawberry production is also highly susceptible to elevated temperatures and day-neutral strawberry plants produce few and misshapen fruit during the hot days of summer. The *F. vesca* abiotic stress EST collection reported here represents a useful tool for continuing studies toward understanding the molecular responses of strawberries to the abiotic environment. The stress related sequences obtained from this small-statured crop may translate to valuable tree crops, in which whole-plant treatments are not as simple or practical.

## Supplemental Information Available

Supplemental material is available free of charge at http://www.crops.org/publications/tpg.

### Acknowledgments

This research was supported by a USDA-CSREES grant (USDA-59-1275-5-364) to JPS (PI) and PDR (CoPI). We thank the J. Craig Venter Institute's Joint Technology Center and the Informatics group for carrying out sequencing and bioinformatics support, respectively.

### References

Almoguera, C., M.A. Coca, and J. Jordano. 1993. Tissue-specific expression of sunflower heat shock proteins in response to water stress. Plant J. 4:947–958.

Almoguera, C., A. Rojas, J. Diaz-Martin, P. Prieto-Dapena, R. Carranco, and J. Jordano. 2002. A seed-specific heat-shock transcription factor involved in developmental regulation during embryogenesis in sunflower. J. Biol. Chem. 46:43866–43872.

Apel, K., and H. Hirt. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55:373–399.

Ashburner, M., C.A. Ball, J.A. Blake, D. Botstein, H. Butler, J.M. Cherry, A.P. Davis, K. Dolinski, S.S. Dwight, J.T. Eppig, M.A. Harris, D.P. Hill, L. Issel-Tarver, A. Kasarskis, S. Lewis, J.C. Matese, J.E. Richardson, M. Ringwald, G.M. Rubin, and G. Sherlock. 2000. Gene ontology: Tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25:25–29 10.1038/75556.

Audic, S., and J.M. Claverie. 1997. The significance of digital gene expression profiles. Genome Res. 7:986–995.

Battaglia, M., Y. Olvera-Carrillo, A. Garciarrubio, F. Campos, and A.A. Covarrubias. 2008. The enigmatic LEA proteins and other hydrophilins. Plant Physiol. 148:6–24 10.1104/pp.108.120725.

Battey, N.H., P. LeMiere, A. Tehranifar, C. Cekic, S. Taylor, K.J. Shrives, P. Hadley, A.J. Greenland, J. Darby, and M.J. Wilkinson. 1998. Genetic and environmental control of flowering in strawberry. p. 111–131. *In* K.E. Cockshull et al. (ed.) Genetic and environmental manipulation of horticultural crops. CABI Publishing, New York, NY.

Berkowitz, O., R. Jost, S. Pollmann, and J. Maslea. 2008. Characterization of TCTP, the translationally controlled tumor protein, from *Arabidopsis thaliana*. Plant Cell 20:3430–3447 10.1105/tpc.108.061010.

Boyer, J.S. 1982. Plant productivity and environment. Science 218:443–448.

Bray, E., J. Bailey-Serres, and E. Weretilnyk. 2000. Responses to abiotic stresses. p. 1158–1203. *In* B.B. Buchanan et al. (ed.) Biochemistry and molecular biology of plants. American Society of Plant Biologists, Rockville, MD.

Caramelo, J.J., and N.D. Iusem. 2009. When cells lose water: Lessons from biophysics and molecular biology. Prog. Biophys. Mol. Biol. 99:1–6.

Cobbett, C., and P. Goldsbrough. 2002. Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. Annu. Rev. Plant Biol. 53:159–182 doi:10.1146/annurev.arplant.53.100301.135154.

Conesa, A., S. Gotz, J. Garcia-Gomez, J. Terol, M. Talon, and M. Robles. 2005. National Centre for Biotechnology Information Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21:3674–3676.

Conklin, P., and C. Barth. 2004. Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. Plant Cell Environ. 27:959–970.

Davis, T.M., and J.E. Pollard. 1991. *Fragaria vesca* chlorophyll mutants. HortScience 26:311.

Deprost, D., L. Yao, R. Sormani, M. Moreau, G. Leterreux, M. Nicolai, M. Bedu, C. Robaglia, and C. Meyer. 2007. The Arabidopsis TOR kinase links plant growth, yield, stress resistance and mRNA translation. EMBO Rep. 8:864–870 doi:10.1038/sj.embor.7401043.

Folta, K.M., and T.M. Davis. 2006. Strawberry genes and genomics. Curr. Rev. Plant Sci. 25:399–415.

Fowler, S., and M.F. Thomashow. 2002. Arabidopsis transcriptome profiling Indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14:1675–1690.

Gasic, K., D.O. Gonzalez, J. Thimmapuram, L. Liu, M. Malnoy, G. Gong, Y. Han, L.O. Vodkin, H.S. Aldwinckle, N.J. Carroll, K.S. Orvis, P. Goldsbrough, S. Clifton, D. Pape, L. Fulton, J. Martin, B. Theising, M.E. Wisniewski, G. Fazio, F.A. Feltus, and S.S. Korban. 2009. Comparative analysis and functional annotation of a large expressed sequence tag collection of Apple. Plant Gen. 2:23–38 doi:10.3835/plantgenome2008.11.0014.

Heinen, R.B., Q. Ye, and F. Chaumont. 2009. Role of aquaporins in leaf physiology. J. Exp. Bot. 60:2971–2985 doi:10.1093/jxb/erp171.

Huang, X., and A. Madan. 1999. CAP3: A DNA sequence assembly program. Genome Res. 9:868–877.

Ito, Y., K. Katsura, K. Maruyama, T. Taji, M. Kobayashi, M. Seki, K. Shinozaki, and K. Yamaguchi-Shinozaki. 2006. Functional analysis of Rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant Cell Physiol. 47:141–153.

Kant, P., M. Gordon, S. Kant, G. Zolla, O. Davydov, Y.M. Heimer, V. Chalifa-caspi, R. Shaked, and S. Barak. 2008. Functional-genomics-based identification of genes that regulate *Arabidopsis* responses to multiple abiotic stresses. Plant Cell Environ. 31:697–714.

Kaufmann, M.R., and A.N. Eckard. 1971. Evaluation of water stress control with polyethylene glycols by analysis of guttation. Plant Physiol. 47:453–456.

Kotak, S., J. Larkindale, U. Lee, P. v. Koskull-Döring, E. Vierling, and K.-D. Scharf. 2007. Complexity of the heat stress response in plants. Curr. Opin. Plant Biol. 10:310–316.

Larkindale, J., M. Mishkind, and E. Vierling. 2005. Plant responses to high temperature. *In* M. Jenks and P. Hasegawa (ed.) Plant abiotic stress. Blackwell Publishing Professional, Ames, IA.

Lim, C.J., K.A. Yang, J.K. Hong, J.S. Choi, D.J. Yun, J.C. Hong, W.S. Chung, S.Y. Lee, M.J. Cho, and C.O. Lim. 2006. Gene expression profiles during heat acclimation in Arabidopsis thaliana suspension-culture cells. J. Plant Res. 119:373–383.

Lurie, S., and J.D. Klein. 1991. Acquisition of low-temperature tolerance in tomatoes by exposure to high-temperature stress. J. Am. Soc. Hortic. Sci. 116:1007–1012.

Luu, D.-T., and C. Maurel. 2005. Aquaporins in a challenging environment: Molecular gears for adjusting plant water status. Plant Cell Environ. 28:1–85.

Ma, S., Q. Gong, and H.J. Bohnert. 2006. Dissecting salt stress pathways. J. Exp. Bot. 57:1097–1107 doi:10.1093/jxb/erj098.

McKersie, B.D., and Y.a.Y. Leshem. 1994. Stress and stress coping in cultivated plants. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Mittler, R. 2006. Abiotic stress, the field environment and stress combination. Trends Plant Sci. 11:15–19.

Munns, R. 2002. Comparative physiology of salt and water stress. Plant Cell Environ. 25:239–250.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 72:473–497.

Oosumi, T., H.A. Gruszewski, L.A. Blischak, A.J. Baxter, P.A. Wadl, J.L. Shuman, R.E. Veilleux, and V. Shulaev. 2006. High-efficiency transformation of the diploid strawberry (*Fragaria vesca*) for functional genomics. Planta 223:1219–1230.

Ort, D.R., and J.S. Boyer. 1985. Plant productivity, photosynthesis and environmental stress. p. 279–313. *In* B.G. Atkinson and D.B. Walden (ed.) Changes in eukaryotic gene expression in response to environmental stress. Academic Press, New York, NY.

Pertea, G., X. Huang, F. Liang, V. Antonescu, R. Sultana, S. Karamycheva, Y. Lee, J. White, F. Cheung, B. Parvizi, J. Tsai, and J. Quackenbush. 2003. TIGR gene indices clustering tools (TGICL): A software system for fast clustering of large EST datasets. Bioinformatics 19:651–652.

Potters, G., T.P. Pasternak, Y. Giuisez, and M.A.K. Jansen. 2009. Different stresses, similar morphogenic responses: Integrating a plethora of pathways. Plant Cell Environ. 32:158–169.

Rabbani, M.A., K. Maruyama, H. Abe, M.A. Khan, K. Katsura, Y. Ito, K. Yoshiwara, M. Seki, K. Shinozaki, and K. Yamaguchi-Shinozaki. 2003. Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol. 4:1755–1767.

Rizhsky, L., H. Liang, J. Shuman, V. Shulaev, S. Davletova, and R. Mittler. 2004. When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. Plant Physiol. 134:1683–1696.

Romualdi, C., S. Bortoluzzi, F. D'Alessi, and G.A. Danieli. 2003. IDEG6: A web tool for detection of differentially expressed genes in multiple tag sampling experiments. Physiol. Genomics 12:159–162 doi:10.1152/physiolgenomics.00096.2002 00096.2002 [pii].

Rousseau-Gueutin, M., A. Gaston, A. Aïnouche, M.L. Aïnouche, K. Olbricht, G. Staudt, L. Richard, and B. Denoyes-Rothan. 2009. Tracking the evolutionary history of polyploidy in *Fragaria* L. (strawberry): New insights from phylogenetic analyses of low-copy nuclear genes. Mol. Phylogenet. Evol. 51:515–530.

Sabehat, A., D. Weiss, and S. Lurie. 1996. The correlation between heat-shock protein accumulation and persistence and chilling tolerance in tomato fruit. Plant Physiol. 110:531–537.

Sakuma, Y., K. Maruyama, F. Qin, Y. Osakabe, K. Shinozaki, and K. Yamaguchi-Shinozaki. 2006. Dual function of an Arabidopsis transcription factor DREB2A in water-stress-responsive and heat-stress-responsive gene expression. Proc. Natl. Acad. Sci. USA 103:18822–18827.

Seki, M., A. Kamei, K. Yamaguchi-Shinozaki, and K. Shinozaki. 2003. Molecular responses to drought, salinity and frost: Common and different paths for plant protection. Curr. Opin. Biotechnol. 14:194–199.

Seki, M., M. Narusaka, J. Ishida, T. Nanjo, M. Fujita, Y. Oono, A. Kamiya, M. Nakajima, A. Enju, T. Sakurai, M. Satou, K. Akiyama, T. Taji, K. Yamaguchi-Shinozaki, P. Carninci, J. Kawai, Y. Hayashizaki, and K. Shinozaki. 2002. Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J. 3:279–292.

Shinozaki, K., K. Yamaguchi-Shinozaki, and M. Seki. 2003. Regulatory network of gene expression in the drought and cold stress responses. Curr. Opin. Plant Biol. 6:410–417.

Slovin, J., and P.D. Rabinowicz. 2007. *Fragaria vesca*, a useful tool for Rosaceae genomics. p. 112–117. *In* F. Takeda (ed.) North American Strawberry Symp., Ventura, CA. 9–12 Feb. 2007. American Society for Horticultural Science, Alexandria, VA.

Sturn, A., J. Quackenbush, and Z. Trajanoski. 2002. Genesis: Cluster analysis of microarray data. Bioinformatics 18:207–208.

Sun, W., C. Bernard, B. Van de Cotte, M. Van Montagu, and N. Verbruggen. 2001. At-HSP17.6A, encoding a small heat-shock protein in Arabidopsis, can enhance osmotolerance upon overexpression. Plant J. 27:407–415.

Swindell, W.R., M. Huebner, and A.P. Weber. 2007. Transcriptional profiling of *Arabidopsis* heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. BMC Genomics 8:125.

Tattersall, E.A.R., J. Grimplet, L. De Luc, M.D. Wheatley, D. Vincent, C. Osborne, A. Ergül, E. Lomen, R.R. Blank, K.A. Schlauch, J.C. Cushman, and G.R. Cramer. 2007. Transcript abundance profiles reveal larger and more complex responses of grapevine to chilling compared to osmotic and salinity stress. Funct. Integr. Genomics 7:317–333.

Tonsor, S.J., C. Scott, I. Boumaza, T.R. Liss, J.L. Brodsky, and E. Vierling. 2008. Heat shock protein 101 effects in *A. thaliana*: Genetic variation, fitness and pleiotropy in controlled temperature conditions. Mol. Ecol. 17:1614–1626. doi:10.1111/j.1365–294X.2008.03690.x.

Ueda, A., A. Kathiresan, M. Inada, Y. Narita, T. Nakamura, W. Shi, T. Takabe, and J. Bennett. 2004. Osmotic stress in barley regulates expression of a different set of genes than salt stress does. J. Exp. Bot. 55:2213–2218.

Uratsu, S., H. Ahmadi, R.S. Bringhurst, and A. Dandekar. 1991. Relative virulence of *Agrobacterium* strains on strawberry. HortScience 26:196–199.

Vizoso, P., L.A. Meisel, A. Tittarelli, M. Latorre, J. Saba, R. Caroca, J. Maldonado, V. Cambiazo, R. Campos-Vargas, M. Gonzalez, A. Orellana, and H. Silva. 2009. Comparative EST transcript profiling of peach fruits under different post-harvest conditions reveals candidate genes associated with peach fruit quality. BMC Genomics 10:423. doi:1471-2164-10-423 [pii] 10.1186/1471–2164–10–423.

Wan, C.-Y., and T.A. Wilkins. 1994. A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). Anal. Biochem. 223:7–12.

Wang, W., B. Vinocur, and A. Altman. 2003. Plant responses to drought, salinity and extreme temperatures: Toward genetic engineering for stress tolerance. Planta 218:1–14.

Waters, E.R., B.D. Aevermann, and Z. Sanders-Reed. 2008. Comparative analysis of the small heat shock proteins in three angiosperm genomes identifies new subfamilies and reveals diverse evolutionary patterns. Cell Stress Chaperones 13:127–142.

Wisniewski, M., C. Bassett, J. Norelli, D. Macarisin, T. Artlip, K. Gasic, and S. Korban. 2008. Expressed sequence tag analysis of the response of apple (Malus × domestica 'Royal Gala') to low temperature and water deficit. Physiol. Plant. 133:298–317.

Wong, C.E., Y. Li, A. Labbe, D. Guevara, P. Nuin, B. Whitty, C. Diaz, G.B. Golding, G.R. Gray, E.A. Weretilnyk, M. Griffith, and B.A. Moffatt. 2006. Transcriptional profiling implicates novel interactions between abiotic stress and hormonal responses in Thellungiella, a close relative of Arabidopsis. Plant Physiol. 4:1437–1450.

Yeung, K.Y., D.R. Haynor, and W.L. Ruzzo. 2001. Validating clustering for gene expression data. Bioinformatics 17:309–318.

Zhang, Z., S. Schwartz, L. Wagner, and M. Miller. 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 1–2:203–214.