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Rudell, Jr. et al.

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(54) **GENE EXPRESSION MONITORING FOR RISK ASSESSMENT OF APPLE AND PEAR FRUIT STORAGE STRESS AND PHYSIOLOGICAL DISORDERS**

G06N 5/048 (2013.01); *C12Q 2600/118* (2013.01); *C12Q 2600/13* (2013.01); *C12Q 2600/158* (2013.01)

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(58) **Field of Classification Search**
None
See application file for complete search history.

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C12Q 1/6883 (2018.01)
G06F 19/20 (2011.01)
G06F 19/22 (2011.01)
G06N 5/04 (2006.01)
C12Q 1/6895 (2018.01)
G06F 19/18 (2011.01)

(52) **U.S. Cl.**
CPC *C12Q 1/6883* (2013.01); *C12Q 1/6895* (2013.01); *G06F 19/18* (2013.01); *G06F 19/20* (2013.01); *G06F 19/22* (2013.01);

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(57) **ABSTRACT**

The present invention is a tool for diagnosis and prediction using biomarker-based risk assessment for chilling-related disorders of Rosaceous fruit crops including apple and pear. Provided are methodology and genes whose relative and absolute expression can accurately indicate disorder risk throughout the production and supply chain of these crops. This technology describes a necessary and novel management tool for stakeholders producing, servicing, or retailing these crops.

1 Claim, 6 Drawing Sheets
(6 of 6 Drawing Sheet(s) Filed in Color)

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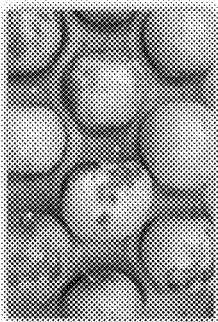


Fig. 1A

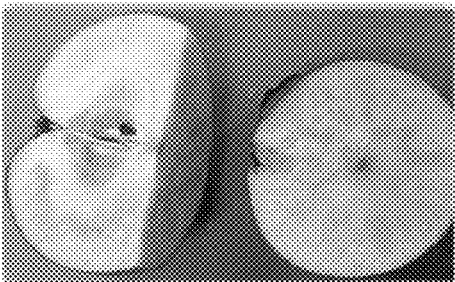


Fig. 1B



Fig. 1C

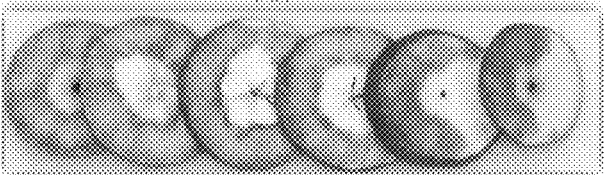


Fig. 1D

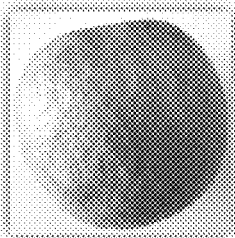


Fig. 1E

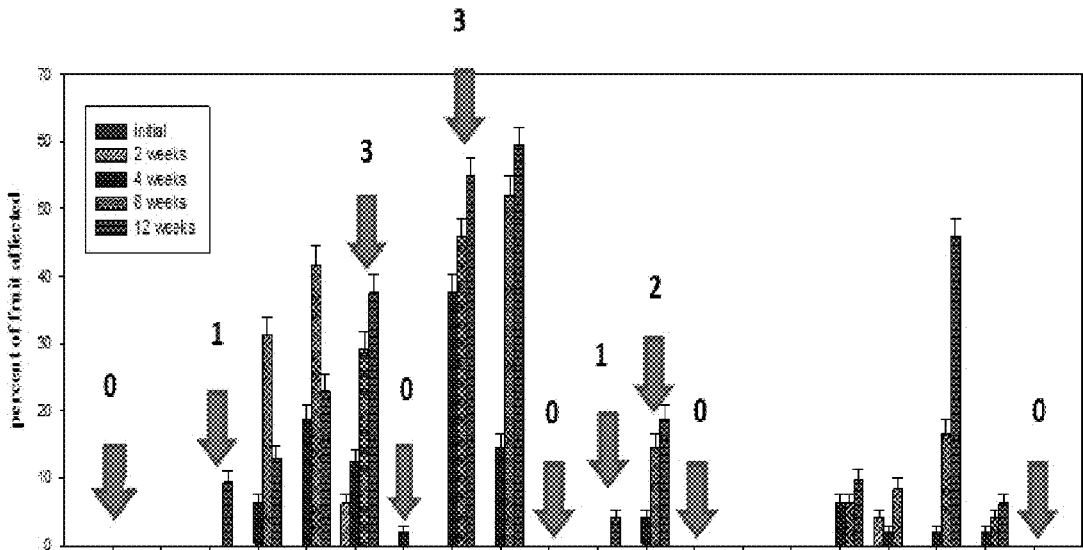


Fig. 2

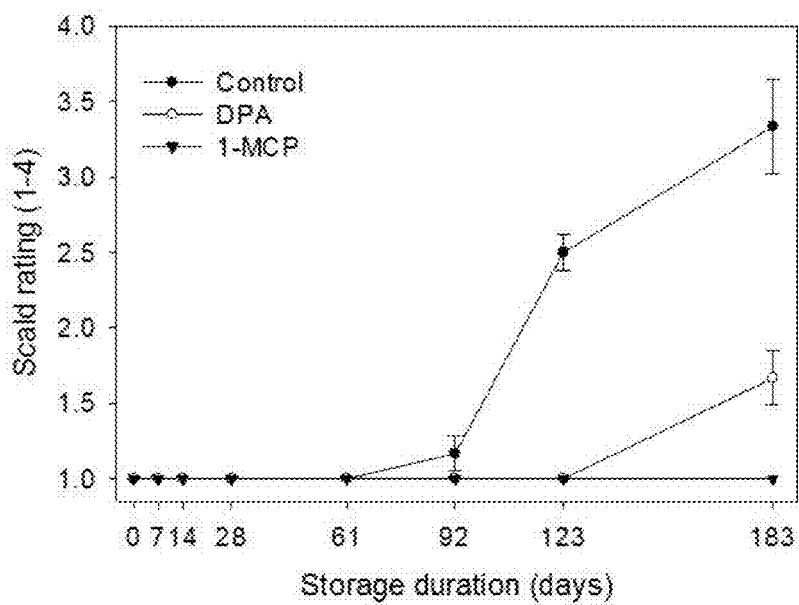
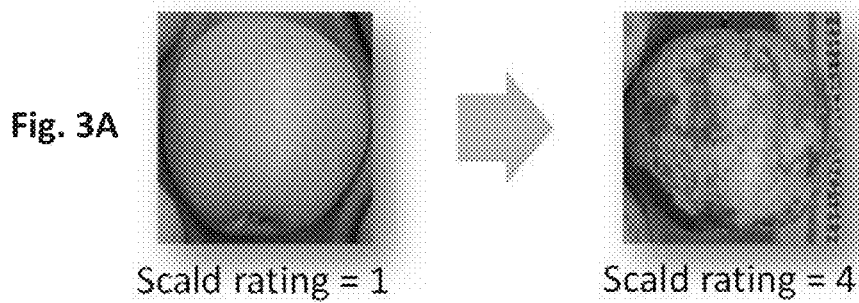


Fig. 3B

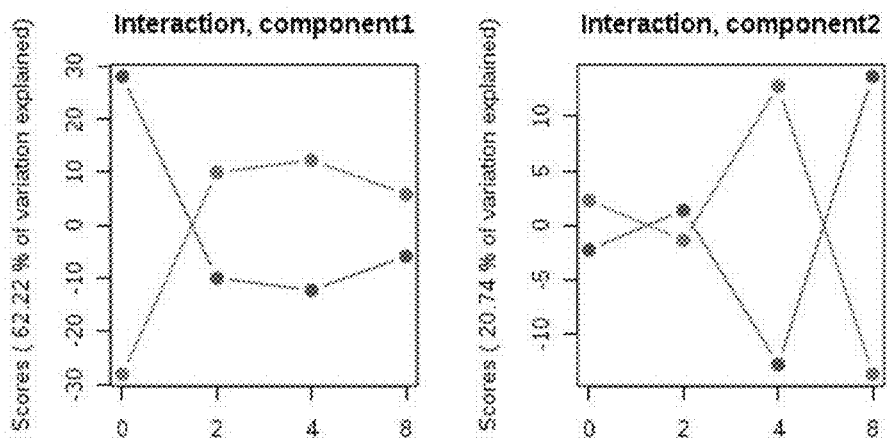


Fig. 4

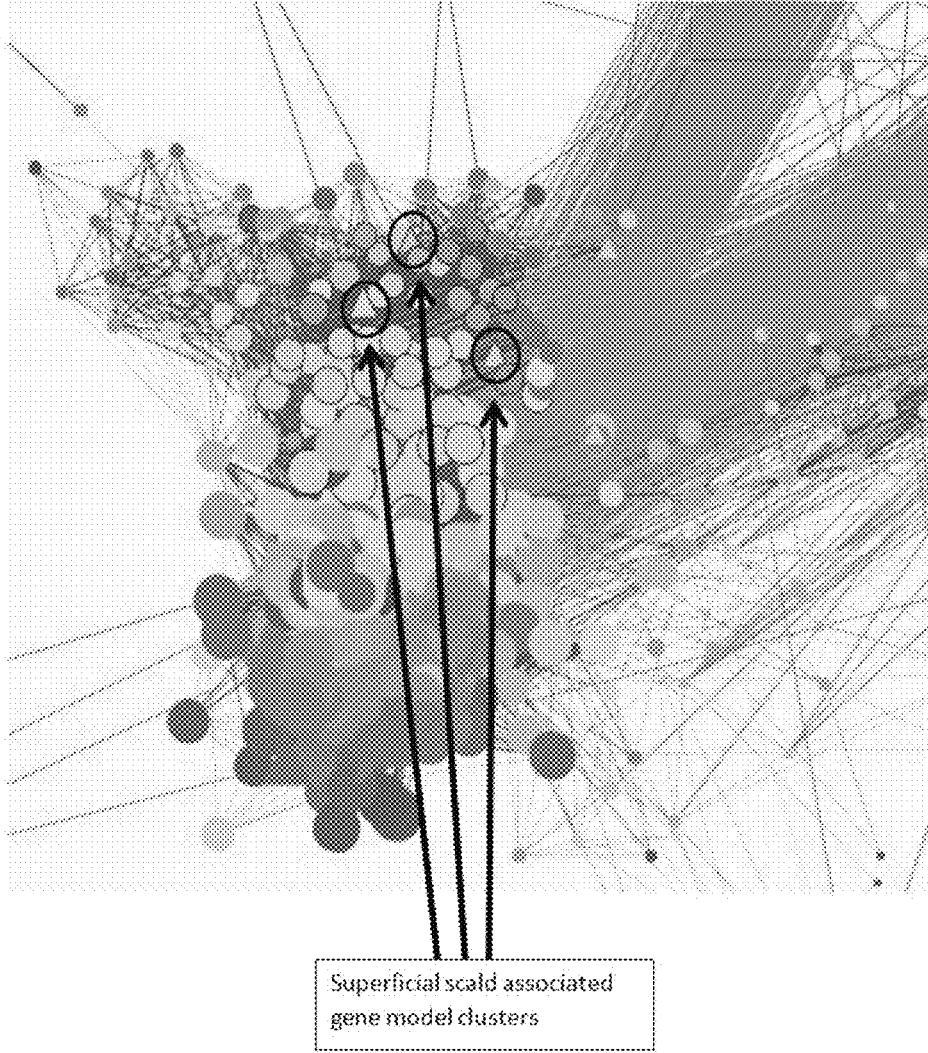


Fig. 5

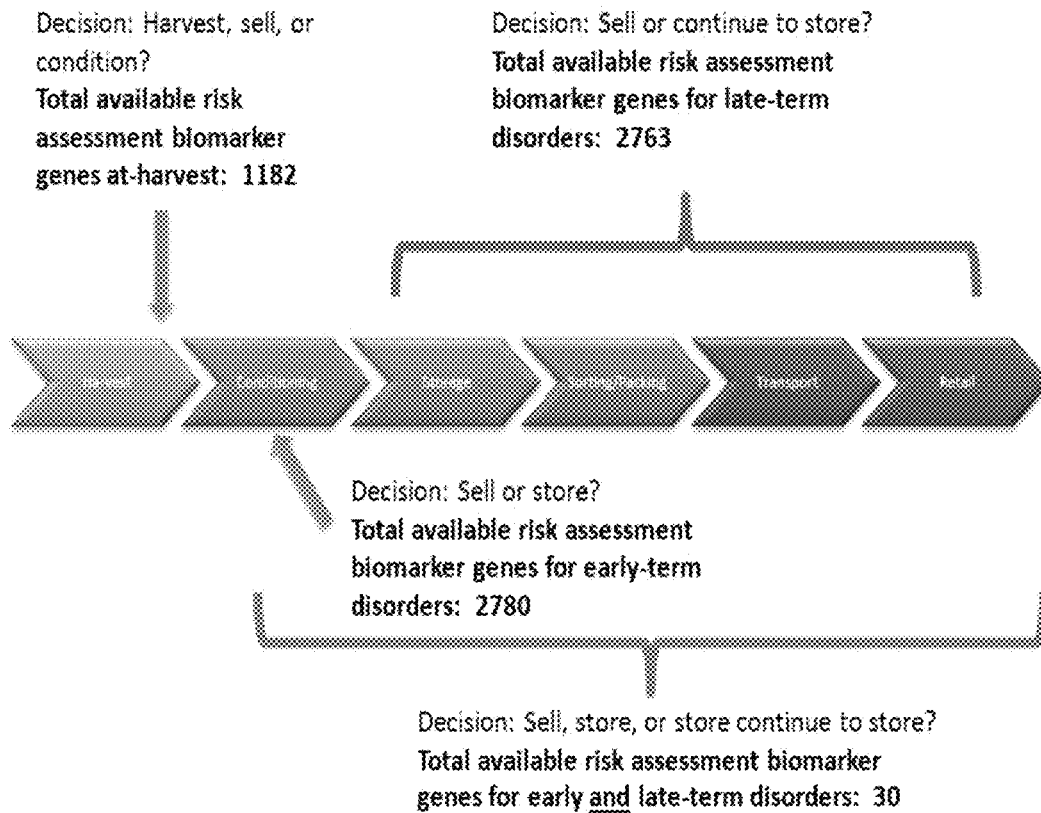


Fig. 6

**GENE EXPRESSION MONITORING FOR
RISK ASSESSMENT OF APPLE AND PEAR
FRUIT STORAGE STRESS AND
PHYSIOLOGICAL DISORDERS**

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention is a biomarker-based risk assessment tool for predicting, diagnosing and distinguishing postharvest chilling-related physiological disorders of Rosaceous fruit crops, including apple and pear.

Background of the Invention

In the fruit industry, multiple browning disorders result in significant annual losses. A major obstacle to developing strategies that reduce such losses is the lack of methods for evaluating the risk of any of the multiple browning disorders materializing at any time during the twelve month storage and distribution period due to chilling stress imposed by the required cold storage. There have been no biomarkers identified that are useful to assess the effect of cold storage stress where the disorder is multifactorial and where the outcome will not become evident for many months. The risk is difficult to determine as existing tests aimed at estimating fruit quality are not linked with conditions associated with browning disorder risk. Thus, there is currently a need to develop more effective techniques for identification of endpoints for monitoring disorder progression during the storage and the distribution period and for evaluating the effectiveness of changes instituted in efforts to treat and control such disorders. To date, no consistently effective risk assessment exists targeting postharvest disorders and control measures are even lacking for many prevalent browning disorders.

SUMMARY OF THE INVENTION

We have identified biomarkers that predict, diagnose, and distinguish multiple postharvest browning disorders during the cold storage period, thereby enabling a strategy for assessing risk for the occurrence of multiple browning disorders throughout the cold storage and distribution periods and for adjusting controls and marketing strategies to reduce product loss.

In accordance with this discovery, it is an object of the invention to provide a method of using biomarkers to identify stages of the progression of the multiple browning disorders soft scald, soggy breakdown, firm flesh browning, external CO₂ injury and superficial scald during the cold storage period as part of a strategy of risk assessment in order to facilitate storage and supply chain management decisions.

It is an object of the invention to provide measurable metabolites and identified biomarkers (expressed gene sequences reflecting mRNA changes) that can be monitored to assess risk of storage disorder development as changes in levels of multiple expressed genes precede browning disorder development by weeks or months.

It is a further object of the invention to provide biomarker profiles whose relative and absolute expression can accurately predict and diagnose disorder risk throughout the production and supply chain of these crops.

It is another object of the invention to monitor biomarker levels in apple and pear tissues throughout the entire growing and supply chain as a means to predict and diagnose and assess risk for disorder development, to check effectiveness of control strategies, and to adjust disorder control or

marketing strategies in order to avoid losses and thereby provide a more consistent, high quality product to consumers.

It is an additional object of the invention to provide a biomarker-based diagnostic tool as a necessary and novel management tool for stakeholders producing, servicing, or retailing these crops.

It is an additional object of the invention to provide such monitoring strategies in order to provide effective treatment and control practices that can be monitored by reliance on the same biomarkers as indicators of their effect on maintenance of fruit quality with the result that previously employed crop protectant applications or energy input into the storage environment are no longer necessary.

Other objects and advantages of this invention will become readily apparent from the ensuing description.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the U.S. Patent and Trademark Office upon request and payment of the necessary fee.

FIGS. 1A-1E depict examples of chilling-related postharvest physiological disorders of apple. These disorders are physiologically and etiologically distinct. Superficial scald (FIG. 1A) and firm flesh browning (FIG. 1B) were chosen to discover risk assessment biomarkers for late-term disorders; soft scald (FIG. 1C), soggy breakdown (FIG. 1D), and CO₂ injury (FIG. 1E) for early term disorders.

FIG. 2 depicts usage of the combination of harvest maturity and orchard incidence variability to discover risk assessment biomarkers for soft scald and soggy breakdown (shown in FIGS. 1C and 1D) and other early-term physiological storage disorders of apple and other Rosaceous fruit crops. Soft scald was categorized for statistical analysis on a scale of 0-3 (0=no incidence, 1=0-10%; 2=10-25% and 3=over 25%).

FIG. 3A shows superficial scald development of 'Granny Smith' apples. FIG. 3B depicts the usage of this combination to discover risk assessment biomarkers for this and other late-term physiological storage disorders of apple and other Rosaceous fruit crops. Control apples (no crop protectants applied) developed the highest incidence of superficial scald (FIG. 3B). This "pharmaceutical" contrast was used to discover risk assessment biomarkers for this and other late-term postharvest physiological disorders of Rosaceous fruit crops. Abbreviations (DPA, diphenylamine; 1-MCP, 1-methylcyclo-propene).

FIG. 4 depicts a summary of overall changes of gene expression in 'Honeycrisp' apple from high (green) and low (red) risk orchards. The left graph indicates that the largest impact on the transcriptome occurred between harvest and 2 weeks when cold storage was imposed.

FIG. 5 shows a metabolic-gene expression correlations network illustrating closely related metabolites (circles, representing a single metabolite) and gene model k-means clusters (triangles, representing many highly correlated genes). Nodes closely positioned and linked with red edges (lines) are highly, positively correlated. Nodes outlined in black are those highly correlated with methanol (R>0.700), a metabolite linked with superficial scald symptom development in 'Granny Smith'. Risk assessment biomarker genes for superficial scald and other late-term disorders reside in the three highlighted gene model clusters.

FIG. 6 depicts a testing scheme for a disorder risk management system. If risk assessment biomarker levels are higher (or lower, see Table), decisions on how long a particular lot, alone or compared to other lots, should proceed through the supply chain can be made at key decision points based on the assessed risk for developing various browning disorders.

DETAILED DESCRIPTION OF THE INVENTION

Multiple browning disorders that lead to severe damage of the peel and cortex (FIG. 1) are a major problem in the fruit industry, causing low quality, inconsistent fruit products for the consumer and severe annual losses. Costs for protectants and additional energy costs also add to these losses. We have applied metabolic and gene expression profiling to discover biomarkers that can be used to predict, diagnose, and distinguish economically significant apple postharvest physiological disorders. Changes in levels of multiple expressed genes precede disorder development by weeks or months. The invention reduces product loss caused by multiple browning disorders by indicating which fruit have a high risk for developing the disorders and therefore can serve as a basis for storage and supply chain management decisions. We have identified metabolites and biomarkers (gene sequences reflecting mRNA changes) that can be monitored and used to assess risk of storage disorder development. These identified genes have not been previously associated with fruit chilling stress in Rosaceous crops. Further, these identified genes are very similar among Rosaceous fruit crops. Fruit response to stress events that lead to these collective disorders are detected and monitored using this approach. Unlike previous technologies, this invention targets storage risk assessment and only incorporates differences in fruit quality factors or fruit ripeness or maturity when these conditions impact disorder development. Monitoring biomarker levels in apple and pear tissues throughout the entire growing and supply chain provides a means to check effectiveness of control strategies, diagnose and assess risk for disorder development and, then, adjust disorder control or marketing strategies to avoid losses and provide a more consistent, high quality, disorder-free product to consumers. Integrated production approaches using this new tool potentially reduce unnecessary crop protectant application or energy input into the storage environment by reducing uncertainty of effectiveness of technologies presently in use.

Expression of risk assessment genes and metabolites levels are evaluated at key decision points from before harvest until the end of the supply chain. mRNA samples are extracted from peel or cortex tissue and the extract prepared depending upon the evaluation method to be employed. Expression levels of the given gene sequences can be monitored using RNAseq Solexa protocols (Solexa, Illumina, Hayward, Calif.). Expression levels are considered absolutely, depending upon the platform employed, and/or relatively, regardless of the platform and may be negatively or positively associated with disorder risk. Expression levels are considered in the context of mitigating conditions and stresses applied during the production chain and alongside levels of metabolites that less accurately indicate elevated risk for certain disorders.

Softscald and soggy breakdown (FIGS. 1C and 1D) are related but distinct disorders associated with low temperature storage. Soft scald symptoms are sharply defined brown lesions on the apple skin which can extend into the flesh

(Snowdon, A. L. 1990. A Color Atlas of Post-harvest Diseases and Disorders of Fruits and Vegetables, Vol. 1. CRC Press, Boca Raton, Fla., 213 pp.; Watkins and Rosenberger. 2002. Cornell Fruit Handling and Storage Newsletter, 14pp. [Retrieved from the internet: hort.comell.edu/department/faculty/Watkins/extpubs.] while soggy breakdown is an internal disorder with soft, brown, sponge-like tissue, sometimes including most of the flesh (Watkins and Rosenberger, supra). Softscald and soggy breakdown are early-term severe chilling-provoked browning disorders of apple and pear peel and cortical tissue impacting multiple economically significant cultivars including 'Honeycrisp' and Jazz™. Typically symptoms appear following 0-2 months following cold storage imposition. Tests and validation of the biomarkers considered seasonal variation and multiple climatic, orchard, developmental, crop protectant, and storage factors employed using common industry practices. Risk assessment was performed at harvest and within 1-2 weeks following cold storage imposition. Evaluating risk assessment biomarker gene expression levels (Tables 1 and 2) provided an early, accurate assessment of disorder risk in these cultivars up to 4 weeks prior to symptom appearance. Comparing relative levels before and after cold storage imposition and/or among expressed biomarkers provided additional confidence in the assessment.

CO₂ injury is exhibited externally and/or internally depending on the cultivar and growing conditions (Colgan et al. 1999. *Postharvest Biol. Technol.* 16:223-231; Elgar et al. 1998. *Hortsci.* 33:719-722; Elgar et al. 1999. *Hortsci.* 34:305-309; Fernandez-Trujillo et al. 2001. *J. Amer. Soc. Hort. Sci.* 126:235-241; Watkins et al. 1997. *HortScience* 32:1242-1246). Susceptible cultivars include 'Braeburn', 'Cortland', 'Empire', 'Fuji', 'Golden Delicious', 'Honeycrisp', and 'McIntosh'. Diphenylamine (DPA) reduces or prevents development of both external and internal CO₂ injury and, when DPA is not used, significant losses result (Argenta et al. 2002. *Postharvest Biol. Technol.* 24:13-24; Burmeister and Dilley. 1995. *Postharvest Biol. Technol.* 6:1-7; Colgan et al. 1999, supra; Fernandez-Trujillo et al., supra). Unlike superficial scald, 1-methylcyclopropene (1-MCP) treatment exacerbates CO₂ injury (Fawbush et al. 2008. *Postharvest Biol. Technol.* 48:92-98). CO₂ injury has been associated with accumulation of succinate in the tissue of fruit exposed to high CO₂ concentrations (Hulme, A. C. 1956. *Nature* 178:218-219), but Fernandez-Trujillo et al. (supra) found similar levels of succinate in injured controls and non-injured fruit treated with DPA. Risk assessment biomarker gene expression levels (Table 3) change with disorder risk following cold storage inception caused by chilling and elevated CO₂ and mitigated by crop protectant and other orchard and storage factors.

Superficial scald is a late-term chilling-related peel browning disorder of multiple apple and pear cultivars; typically symptoms appear after at least 2 months cold storage (FIG. 3A) (Bain and Mercer. 1963. *Aust. J. Biol. Sci.* 16:442-449). Superficial scald is associated with oxidative stress ostensibly linked to the build-up of oxidation products of the sesquiterpene (E,E)- α -farnesene (Huelin and Coggiola. 1970. *J. Sci. Food Agric.* 21:584-589; Whitaker et al. 2000. *Postharvest Biol. Technol.* 20:231-241; Rowan et al. 2001. *J. Agri. Food Chem.* 49:2780-2787). Pre-storage treatment of apples with DPA inhibits oxidation of α -farnesene and largely prevents scald development (Huelin and Coggiola, supra). Moreover, exposure of apple fruit to the ethylene action inhibitor 1-MCP greatly curtails α -farnesene production and markedly reduces scald incidence and severity (Fan et al. 1999. *J. Agri. Food Chem.* 47:3063-3068;

Rupasinghe et al. 2000. *J. Hort. Sci. Biotech.* 75:271-276; Watkins et al. 2000. *Postharvest Biol. Technol.* 19:17-32; Shaham et al. 2003. *J. Am. Soc. Hort. Sci.* 128:761-766). Controlled atmosphere storage (low oxygen) can reduce scald incidence and severity (Fidler et al. 1973. In: *Commonwealth Agricultural Bureaux*, England. Pp. 113-116; Lau, O. L. 1990. *J. Am. Soc. Hort. Sci.* 115:959-961), but not consistently if risk is high. Symptoms of this disorder typically appear from 4 to 6 months after cold storage imposition and can be controlled during conventional production using multiple approaches and during organic production using controlled atmosphere storage. However, reduced acceptance of antioxidant crop protectants used to control superficial scald and inconsistent efficacy of control using controlled atmosphere storage assures that this disorder remains a significant annual economic world-wide consideration for producers of susceptible apple and pear cultivars, including 'Granny Smith' and 'Delicious'. Risk assessment biomarker gene expression levels were different at harvest (Table 4) depending upon the risk of apples from individual orchards to develop the disorder. Other biomarkers (Table 5) increased dramatically during storage starting at 3 months prior to first symptom appearance in 'Granny Smith' apples, providing an early and accurate risk assessment of conditions evoked by production, crop protectant and storage conditions. Monitoring relative biomarker gene expression levels alongside oxidation of superficial scald-associated metabolites, such as α -farnesene and methanol, can improve assessment accuracy using this technology.

Firm flesh browning is considered a form of chilling injury that results in patterned darkening of the flesh. It is a long term problem of the 'Empire' cultivar. 'Empire' is considered highly desirable by the fresh cut apple industry and flesh browning is unacceptable. Increasing storage temperatures from 0° C. to 2° C. reduces the symptoms although unacceptable softening occurs at 3° C. 1-MCP treatment is now the industry norm to meet market requirements, but 1-MCP-treated fruit develop flesh browning at both low and high storage temperatures (Jung et al. 2011. *Postharvest Biol. Technol.* 59:219-226). DPA and other treatments that control flesh browning are ineffective in 1-MCP-treated fruit. These flesh browning symptoms are visually indistinguishable from those previously considered to be chilling injury. Risk assessment biomarker gene expression levels (Table 6) change with the risk of occurrence of the disorder following cold storage inception caused by chilling and mitigated by crop protectant and other orchard and storage factors.

Experimental evidence supports that expression levels of these risk assessment biomarkers change similarly with stresses that can lead to multiple early and late storage term disorders. Biomarker expression does not correlate with any traditional quality-associated phenotype such as firmness or flavor loss. Instead, biomarker expression specifically represents changes with stress and storage factors that lead to browning disorder development.

Apple [*Malus sylvestris* (L.) Mill var. *domestica* (Borkh.) Mansf.] was chosen as the model for discovering biomarkers for Rosaceous fruit postharvest browning disorders as different apple cultivars are susceptible to developing many distinct postharvest browning disorders of the peel and flesh, and our understanding of the controls and etiology of these disorders is best in apples. There is a high degree of gene homology among Rosaceous fruit crops, including pear.

This and ripening and postharvest physiological similarities make risk assessment biomarkers behave similarly under high or low risk conditions.

The present invention provides improved systems and strategies for predicting the progression of multiple browning disorders. According to the present invention, soft scald, soggy breakdown, superficial scald, firm flesh browning and CO₂ injury of Rosaceous fruit may be predicted or diagnosed by obtaining a profile of biomarkers from a sample obtained from Rosaceous fruit tissue. The present invention is particularly useful for predicting and diagnosing soft scald, soggy breakdown, superficial scald, firm flesh browning and CO₂ injury during storage and distribution.

Biomarker profiles may be a ratio of two or more measurable aspects of a biomarker. A biomarker profile comprises at least one measurement, where the measurements can correspond to the same or different biomarkers. A biomarker profile may also comprise at least three, four, five, 10, 20, 30 or more measurements. The profile of biomarkers obtained from an individual apple tissue specimen, namely the candidate biomarker profile, is compared to a reference biomarker profile. The reference biomarker profile can be generated from one individual or a population of individuals at different time points during storage.

The reference biomarker profile and the candidate biomarker profiles that are compared in the methods of the present invention may be generated from the same population for the purpose of monitoring disorder progression. In this instance it would be expected that the candidate and reference profiles are generated from biological samples taken at different time points and compared to one another, i.e., the reference profile will be expression of the biomarker at the earlier time point and compared to the candidate's biomarker expression at the later time point. Such a comparison may be used, for example, to determine the risk status of developing a browning disorder in the individual tissue by repeated measurements over time. The reference biomarker profiles may be chosen from tissue of fruit that has a risk of a browning disorder or the reference biomarker profile may be generated from a healthy individual or population that is not at risk, i.e., those that are in a non-affected environment, in a controlled storage population, or in an environmental crop protected situation. In addition, it would be expected that the candidate and reference profiles are generated from biological samples taken from different orchard locations, reflecting environmental and disorder development differences, and compared to one another. i.e., the reference profile will be expression of the biomarker from a sample with known disorder development and compared to the candidate's biomarker expression from another population.

The methods of the present invention comprise comparing a candidate biomarker profile with a reference biomarker profile. The present invention is based on the identification of new biomarkers of multiple browning disorders. A biomarker is useful if it is specific for a browning disorder and measurable. In particular, of the 63,541 genes screened for assessing risk for soft scald and soggy breakdown and CO₂ injury, the present invention provides the identity of 82 candidates found useful for assessing risk at-harvest (Table 1) and 494 for 2 week risk assessment of peel and/or cortex tissue (Table 2). Accordingly, in one aspect, the present invention provides for the identification, generation, and use of expression profiles of sets of genes selected from the genes disclosed herein.

TABLE 2-continued

Accession numbers (Velasco et al., 2010; Retrieved from the Internet: Rosaceae.org) of biomarker genes for soft scald assessment during cold storage following cold storage imposition. Higher or lower expression levels can result from cold stress resulting from storage imposition. Where lower expression is associated with risk, accession numbers are italicized.

MDP0000225867	MDP0000310803	MDP0000284374	MDP0000303924	MDP0000724726	MDP0000228064
MDP0000646125	MDP0000709073	MDP0000122895	MDP0000392540	MDP0000128265	MDP0000315323
MDP0000279149	MDP0000243428	MDP0000319512	MDP0000524677	MDP0000287199	MDP0000157932
MDP0000163088	MDP0000686661	MDP0000637142	MDP0000136933	MDP0000642530	MDP0000467241
MDP0000204212	MDP0000231619	MDP0000207937	MDP0000065186	MDP0000120398	MDP0000873235
MDP0000271479	MDP0000138275	MDP0000236176	MDP0000360077	MDP0000161521	MDP0000168076
MDP0000891353	MDP0000314606	MDP0000232589	MDP0000277370	MDP0000239442	MDP0000124881
MDP0000262367	MDP0000249502	MDP0000520097	MDP0000870230	MDP00000917844	
MDP0000165325	MDP0000156727	MDP0000216267	MDP0000506697	MDP0000212628	
MDP0000215235	MDP0000139194	MDP0000194823	MDP0000264406	MDP0000298280	
MDP0000561738	MDP0000274685	MDP0000940086	MDP0000889787	MDP0000368098	
MDP0000273126	MDP0000244593	MDP0000342041	MDP0000916486	MDP0000563592	
MDP0000879217	MDP0000170568	MDP0000196064	MDP0000898951	MDP0000234503	
MDP0000249248	MDP0000219876	MDP0000169170	MDP0000873427	MDP0000130994	
MDP0000226838	MDP0000249858	MDP0000321157	MDP0000294840	MDP0000775468	
MDP0000188052	MDP0000211229	MDP0000131731	MDP0000216027	MDP0000153185	
MDP0000156246	MDP0000319315	MDP0000150429	MDP0000150710	MDP0000263844	
MDP0000842137	MDP0000260116	MDP0000260377	MDP0000214906	MDP0000121783	
MDP0000174971	MDP0000898232	MDP0000447975	MDP0000120330	MDP0000252488	
MDP0000151767	MDP0000453114	MDP0000320239	MDP0000127732	MDP0000547788	
MDP0000279576	MDP0000725984	MDP0000253102	MDP0000418187	MDP0000220129	
MDP0000651801	MDP0000277459	MDP0000787701	MDP0000147201	MDP0000222196	
MDP0000188054	MDP0000307795	MDP0000234782	MDP0000146449	MDP0000719275	
MDP0000757641	MDP0000193241	MDP0000131486	MDP0000903417	MDP0000143473	
MDP0000165187	MDP0000160077	MDP0000272522	MDP0000273225	MDP0000197624	
MDP0000590974	MDP0000297123	MDP0000268258	MDP0000121830	MDP0000564318	
MDP0000014856	MDP0000791166	MDP0000197330	MDP0000151362	MDP0000280632	
MDP0000198015	MDP0000710467	MDP0000250386	MDP0000151457	MDP0000166159	
MDP0000356821	MDP0000222944	MDP0000363287	MDP0000421679	MDP00000418062	
MDP0000762756	MDP0000321469	MDP0000242205	MDP0000279018	MDP0000641544	
MDP0000311618	MDP0000681634	MDP0000272640	MDP0000152774	MDP0000719559	
MDP0000178043	MDP0000500159	MDP0000920394	MDP0000294531	MDP0000139291	
MDP0000312998	MDP0000154049	MDP0000230727	MDP0000941000	MDP0000804427	

35

Because superficial scald can be adequately controlled using appropriate storage conditions, indicating whether storage conditions are actually working or monitoring risk during storage is also a useful tool for this disorder.

External CO₂ injury browning disorder can be controlled using application of commercially used postharvest chemicals. We were able to use these methods to discover putative biomarkers, as different treatments following harvest induce contrasting disorder development. Controlled atmosphere combined with treatment of apples with 1-MCP (Smart-Fresh) following harvest enhances external CO₂ injury. In contrast postharvest treatment with the antioxidant diphe-

nylamine (DPA) almost eliminates external CO₂ injury completely. Candidate biomarker genes from an RNAseq experiment were selected by pairwise comparison using the differential gene expression program edgeR (R, Bioconductor) with a p-value cut off of 0.05. Genes were selected and peel tissue collected from New York state grown ‘Empire’ apples, provoked by storage treatments that affect external CO₂ injury incidence. From a total of 63541 gene models, 2330 that expression changed at least 4-fold and that had an average expression RPKM value of at least 1 per sample were selected as potential predictive or diagnostic biomarkers for external CO₂ injury (Table 3).

TABLE 3

Accession numbers (Velasco et al., 2010; Retrieved from the Internet: Rosaceae.org) of biomarker genes for CO₂ injury assessment during cold storage following cold storage imposition. Higher or lower expression levels can result from cold stress resulting from storage imposition.

MDP0000840536	MDP0000708692	MDP0000349942	MDP0000261740	MDP0000863792	MDP0000140151	MDP0000252752
MDP0000661951	MDP0000357718	MDP0000590966	MDP0000565270	MDP0000148686	MDP0000435842	MDP0000400084
MDP0000745805	MDP0000716794	MDP0000126361	MDP0000233319	MDP0000573540	MDP0000135191	MDP0000414314
MDP0000940411	MDP0000152673	MDP0000809337	MDP0000432471	MDP0000127630	MDP0000310700	MDP0000321031
MDP0000794258	MDP0000251180	MDP0000697676	MDP0000461015	MDP0000130797	MDP0000310582	MDP0000184143
MDP0000769492	MDP0000642253	MDP0000245702	MDP0000300161	MDP0000160197	MDP0000167683	MDP0000251957
MDP0000769493	MDP0000202184	MDP0000844682	MDP0000283288	MDP0000330474	MDP0000298689	MDP000032865
MDP0000200896	MDP0000649783	MDP0000496027	MDP0000168735	MDP0000295392	MDP0000126481	MDP0000228366
MDP0000755567	MDP0000260404	MDP0000192733	MDP0000131763	MDP0000501957	MDP0000172296	MDP0000180012
MDP0000564079	MDP0000120347	MDP0000769764	MDP0000321302	MDP0000238081	MDP0000607920	MDP0000273201
MDP0000348107	MDP0000231748	MDP0000350778	MDP0000835211	MDP0000926304	MDP0000202781	MDP0000164134
MDP0000864747	MDP0000309314	MDP0000274714	MDP0000374881	MDP0000140878	MDP0000695737	MDP0000531811
MDP0000637737	MDP0000585462	MDP0000897962	MDP0000294677	MDP0000205306	MDP0000232535	MDP0000616079
MDP0000575740	MDP0000334047	MDP0000158089	MDP0000316698	MDP0000122792	MDP0000225088	MDP0000262337
MDP0000899351	MDP0000568045	MDP0000814899	MDP0000297123	MDP0000162529	MDP0000145449	MDP0000121243

TABLE 3-continued

Accession numbers (Velasco et al., 2010; Retrieved from the Internet: Rosaceae.org) of biomarker genes for CO2 injury assessment during cold storage following cold storage imposition. Higher or lower expression levels can result from cold stress resulting from storage imposition.

Table with 7 columns of accession numbers. The first column contains numbers from MDP0000523205 to MDP0000119071. The remaining six columns contain corresponding accession numbers ranging from MDP000040539 to MDP0000293886.

TABLE 3-continued

Accession numbers (Velasco et al., 2010; Retrieved from the Internet: Rosaceae.org) of biomarker genes for CO₂ injury assessment during cold storage following cold storage imposition. Higher or lower expression levels can result from cold stress resulting from storage imposition.

MDP0000127858	MDP0000126669	MDP0000196404	MDP0000466345	MDP0000282415	MDP0000150868	MDP0000467552
MDP0000261492	MDP0000242979	MDP0000902434	MDP0000635582	MDP0000215958	MDP0000266089	MDP0000161881
MDP0000145045	MDP0000230141	MDP0000133811	MDP0000597773	MDP0000154506	MDP0000287459	MDP0000260154
MDP0000787216	MDP0000123747	MDP0000568825	MDP0000383450	MDP0000227405	MDP0000394944	MDP0000215382
MDP0000564318	MDP0000435315	MDP0000910895	MDP0000654783	MDP0000376469	MDP0000273795	MDP0000130884
MDP0000241592	MDP0000158047	MDP0000232344	MDP0000256389	MDP0000124866	MDP0000161424	MDP0000157408
MDP0000191304	MDP0000154734	MDP0000676744	MDP0000257242	MDP0000184300	MDP0000563788	MDP0000165719
MDP0000280001	MDP0000426496	MDP0000128790	MDP0000138447	MDP0000302538	MDP0000458350	MDP0000301119
MDP0000548790	MDP0000190504	MDP0000157404	MDP0000314763	MDP0000295029	MDP0000173508	MDP0000157771
MDP0000552120	MDP0000368720	MDP0000864660	MDP0000668252	MDP0000430546	MDP0000883367	MDP0000147902
MDP0000566057	MDP0000442260	MDP0000884047	MDP0000190181	MDP0000896307	MDP0000221867	MDP0000155603
MDP0000842877	MDP0000135898	MDP0000279395	MDP0000197292	MDP0000249932	MDP0000856686	MDP0000237150
MDP0000306121	MDP0000281041	MDP0000889931	MDP0000134341	MDP0000145764	MDP0000128058	MDP0000284488
MDP0000854541	MDP0000251656	MDP0000181188	MDP0000251025	MDP0000266683	MDP0000869501	MDP0000170101
MDP0000635152	MDP0000281816	MDP0000343280	MDP0000766072	MDP0000860226	MDP0000188388	MDP0000270603
MDP0000188054	MDP0000172320	MDP0000127930	MDP0000312731	MDP0000897242	MDP0000165286	MDP0000190016
MDP0000253075	MDP0000192074	MDP0000787808	MDP0000309587	MDP0000650358	MDP0000136653	MDP0000155060
MDP0000239443	MDP0000825373	MDP0000275026	MDP0000181521	MDP0000237733	MDP0000037814	

Susceptibility to develop superficial scald is often quite different depending on environmental location of the apples. To discover putative biomarkers, genes were selected from ‘Red Delicious’ apple peel tissue collected from 6 different orchard locations with observed differences in superficial scald injury incidence. Candidate biomarker genes were selected using Pearson correlation analysis (R, Bioconductor) with a p-value cut off of 0.05. Genes that expression changes at harvest correlated with superficial scald injury incidence, with a correlation coefficient of at least 0.6 were selected for further filtering. From a total of 63541 gene models, 1106 that changed at least 4-fold in tissues showing the lowest and highest development of superficial scald and

with an average expression value of at least 1 RPKM per sample were selected as potential at harvest predictive biomarkers of superficial scald (Table 4). Because superficial scald can be adequately controlled using appropriate storage conditions, indicating whether storage conditions are actually working or monitoring risk during storage is also a useful tool for this disorder. Increases in superficial scald risk-associated gene expression (FIG. 5) began at 1 month for a few candidates, finally resulting in the selection of 690 candidates for early indication of superficial scald risk (Table 5). Monitoring multiple risk assessment biomarkers can better ensure that changes are based on more biochemical systems associated with risk.

TABLE 4

Accession numbers (Velasco et al., 2010; Retrieved from the Internet: Rosaceae.org) of biomarker genes for superficial scald risk assessment during at-harvest. Higher or lower expression levels can result from cold stress resulting from storage imposition.

MDP0000289575	MDP0000213320	MDP0000177522	MDP0000717184	MDP0000631171	MDP0000655623	MDP0000731637
MDP00000313905	MDP0000638953	MDP0000144115	MDP0000320382	MDP0000262190	MDP0000202740	MDP0000180378
MDP0000433093	MDP0000232968	MDP0000283613	MDP0000215302	MDP0000319726	MDP0000176025	MDP0000361876
MDP0000516194	MDP0000125476	MDP0000163474	MDP0000135287	MDP0000588196	MDP0000871409	MDP0000275553
MDP0000156132	MDP0000124687	MDP0000235002	MDP0000507270	MDP0000153860	MDP0000132044	MDP0000267615
MDP0000318040	MDP0000172972	MDP0000304954	MDP0000120272	MDP0000121120	MDP0000820453	MDP0000197521
MDP0000415930	MDP0000138035	MDP0000215722	MDP0000124509	MDP0000156068	MDP0000183189	MDP0000261492
MDP0000261079	MDP0000291259	MDP0000344650	MDP0000868649	MDP0000474856	MDP0000882683	MDP0000120700
MDP0000212886	MDP0000120956	MDP0000313254	MDP0000256881	MDP0000207314	MDP0000319079	MDP0000214384
MDP0000140157	MDP0000193646	MDP0000279740	MDP0000601017	MDP0000653408	MDP0000853568	MDP0000179389
MDP0000522839	MDP0000347935	MDP0000347863	MDP0000279176	MDP0000142739	MDP0000387483	MDP0000169344
MDP0000686132	MDP0000369898	MDP0000904058	MDP0000141949	MDP0000209761	MDP0000243224	MDP0000230950
MDP0000277425	MDP0000737713	MDP0000320910	MDP0000291399	MDP0000312108	MDP0000215578	MDP0000411018
MDP0000182628	MDP0000250672	MDP0000237931	MDP0000349941	MDP0000399965	MDP0000165443	MDP0000943790
MDP0000159869	MDP0000176753	MDP0000776092	MDP0000255275	MDP0000310682	MDP0000946443	MDP0000293407
MDP0000361139	MDP0000242656	MDP0000133066	MDP0000305689	MDP0000309382	MDP0000167951	MDP0000197294
MDP0000378591	MDP0000851390	MDP0000288632	MDP0000401045	MDP0000933747	MDP0000318862	MDP0000169201
MDP0000224489	MDP0000202040	MDP0000230821	MDP0000214259	MDP0000314365	MDP0000334306	MDP0000941890
MDP0000224176	MDP0000194377	MDP0000496646	MDP0000281427	MDP0000661747	MDP0000529463	MDP0000880462
MDP0000165543	MDP0000704323	MDP0000175408	MDP0000574556	MDP0000647735	MDP0000310288	MDP0000259615
MDP0000294123	MDP0000137325	MDP0000072936	MDP0000432499	MDP0000188386	MDP0000164814	MDP0000249932
MDP0000203358	MDP0000898595	MDP0000293236	MDP0000295321	MDP0000215049	MDP0000185616	MDP0000192825
MDP0000443024	MDP0000223864	MDP0000303280	MDP0000224372	MDP0000268711	MDP0000342064	MDP0000259615
MDP0000258335	MDP0000149963	MDP0000799890	MDP0000358823	MDP0000417755	MDP0000282435	MDP0000170551
MDP0000830611	MDP0000274608	MDP0000722481	MDP0000169335	MDP0000135721	MDP0000262784	MDP0000197833
MDP0000239443	MDP0000254595	MDP0000415109	MDP0000796588	MDP0000368412	MDP0000256575	MDP0000161846
MDP0000854541	MDP0000116595	MDP0000520902	MDP0000293072	MDP0000712524	MDP0000949494	MDP0000287512
MDP0000351376	MDP0000722373	MDP0000320289	MDP0000242946	MDP0000876051	MDP0000793261	MDP0000842724
MDP0000568871	MDP0000164181	MDP0000140641	MDP0000458965	MDP0000306215	MDP0000310582	MDP0000634462
MDP0000156263	MDP0000119092	MDP0000119066	MDP0000678128	MDP0000417786	MDP0000585315	MDP0000149647

TABLE 5-continued

Accession numbers (Velasco et al., 2010; Retrieved from the Internet: Rosaceae.org) of biomarker genes for superficial scald risk assessment during cold storage. Biomarker expression levels increase as risk of developing superficial scald increases.

MDP0000126873	MDP0000157408	MDP0000363760	MDP0000157879	MDP0000318500	MDP0000304378	MDP0000184157
MDP0000292176	MDP0000119600	MDP0000129506	MDP0000421759	MDP0000167088	MDP0000831840	MDP0000189570
MDP0000230316	MDP0000204626	MDP0000873295	MDP0000320322	MDP0000311389	MDP0000599427	MDP0000722934
MDP0000218952	MDP0000601893	MDP0000009080	MDP0000279360	MDP0000776146	MDP0000144640	MDP0000279785
MDP0000192816	MDP0000196627	MDP0000806350	MDP0000321558	MDP0000163006	MDP0000182333	MDP0000241928
MDP0000300351	MDP0000679687	MDP0000648218	MDP0000131377	MDP0000775613	MDP0000164161	MDP0000440918
MDP0000812560	MDP0000184480	MDP0000175141	MDP0000531811	MDP0000228304	MDP0000190430	MDP0000274901
MDP000012534	MDP0000195070	MDP0000184238	MDP0000609114	MDP0000891965	MDP0000175839	MDP0000298769
MDP0000268320	MDP0000757070	MDP0000135041	MDP0000205725	MDP0000131763	MDP0000138284	MDP0000215026
MDP0000276264	MDP0000832469	MDP0000262811	MDP0000202900	MDP0000242266	MDP0000318293	MDP0000328419
MDP0000711374	MDP0000788707	MDP0000163669				MDP0000134278
MDP0000131004	MDP0000130822	MDP0000897807				
MDP0000227886	MDP0000122413	MDP0000269136				
MDP0000882268	MDP0000130200	MDP0000734649				
MDP0000199661	MDP0000318900	MDP0000677352				
MDP0000155809	MDP0000296600	MDP0000241462				
MDP0000689033	MDP0000720974	MDP0000238976				
MDP0000213383	MDP0000306888	MDP0000125850				

Firm flesh browning disorder can be controlled using appropriate storage conditions. We were able to use these methods to discover putative biomarkers, since different treatments following harvest, induce contrasting disorder development. Controlled atmosphere combined with lower temperature (0.5° C.) storage causes increased injury over that observed at warmer storage temperature (3.0° C.). In addition, treatment of apples with 1-MCP (SmartFresh) following harvest also enhances firm flesh browning injury. Candidate biomarker genes were selected by pairwise com-

parison using the differential gene expression program edgeR (R, Bioconductor) with a p-value cut off of 0.05. Genes were selected from cortex/flesh tissue collected from New York state grown ‘Empire’ apples, provoked by storage treatments that affect firm flesh browning injury incidence. From a total of 63541 gene models, 2581 had an expression change of at least 4-fold, and had an average expression RPKM value of at least 1 per sample, and were selected as potential predictive or diagnostic biomarkers for firm flesh browning (Table 6).

TABLE 6

Accession numbers (Velasco et al., 2010; Retrieved from the Internet: Rosaceae.org) of biomarker genes for firm flesh browning risk assessment during cold storage. Higher or lower expression levels can result from cold stress resulting from storage imposition.

MDP0000034165	MDP0000312902	MDP0000133552	MDP0000218946	MDP0000310711	MDP0000716315	MDP0000234244
MDP0000060869	MDP0000313281	MDP0000134105	MDP0000219155	MDP0000311039	MDP0000720656	MDP0000234570
MDP0000074782	MDP0000313454	MDP0000134162	MDP0000220129	MDP0000311884	MDP0000720974	MDP0000234689
MDP0000085889	MDP0000314088	MDP0000134341	MDP0000220168	MDP0000312044	MDP0000722139	MDP0000235112
MDP0000085893	MDP0000314777	MDP0000134389	MDP0000220633	MDP0000312397	MDP0000722229	MDP0000235661
MDP0000086296	MDP0000317237	MDP0000134603	MDP0000221075	MDP0000312434	MDP0000727570	MDP0000235822
MDP0000088659	MDP0000317475	MDP0000135374	MDP0000221400	MDP0000313382	MDP0000729533	MDP0000237637
MDP0000092478	MDP0000317502	MDP0000135529	MDP0000221435	MDP0000313664	MDP0000731537	MDP0000238100
MDP0000119926	MDP0000318069	MDP0000135540	MDP0000221742	MDP0000313949	MDP0000731637	MDP0000239011
MDP0000120188	MDP0000318184	MDP0000135831	MDP0000221867	MDP0000314286	MDP0000732666	MDP0000239029
MDP0000120975	MDP0000318347	MDP0000135974	MDP0000222184	MDP0000314435	MDP0000732713	MDP0000240138
MDP0000121374	MDP0000318613	MDP0000136892	MDP0000222403	MDP0000314478	MDP0000736385	MDP0000241650
MDP0000121657	MDP0000319048	MDP0000137225	MDP0000222430	MDP0000314505	MDP0000736490	MDP0000241840
MDP0000121897	MDP0000320017	MDP0000137705	MDP0000223153	MDP0000315227	MDP0000742957	MDP0000242052
MDP0000122086	MDP0000320496	MDP0000137792	MDP0000223422	MDP0000315498	MDP0000744636	MDP0000242083
MDP0000122235	MDP0000320534	MDP0000138035	MDP0000223749	MDP0000315959	MDP0000745371	MDP0000243721
MDP0000122297	MDP0000320763	MDP0000138500	MDP0000223871	MDP0000315998	MDP0000746166	MDP0000244067
MDP0000122540	MDP0000321910	MDP0000138538	MDP0000223878	MDP0000316002	MDP0000746652	MDP0000244851
MDP0000124008	MDP0000322034	MDP0000138727	MDP0000224040	MDP0000316095	MDP0000747845	MDP0000245067
MDP0000124585	MDP0000322229	MDP0000138729	MDP0000224773	MDP0000316207	MDP0000748035	MDP0000245173
MDP0000125807	MDP0000322543	MDP0000138789	MDP0000225212	MDP0000316310	MDP0000749755	MDP0000245245
MDP0000126274	MDP0000322563	MDP0000139058	MDP0000225313	MDP0000316379	MDP0000750374	MDP0000245702
MDP0000126479	MDP0000323033	MDP0000139525	MDP0000225502	MDP0000316397	MDP0000753547	MDP0000245760
MDP0000126481	MDP0000324254	MDP0000139721	MDP0000225524	MDP0000316490	MDP0000754524	MDP0000245817
MDP0000126601	MDP0000324681	MDP0000140046	MDP0000225569	MDP0000316497	MDP0000755474	MDP0000246198
MDP0000127732	MDP0000324831	MDP0000140259	MDP0000226247	MDP0000317227	MDP0000755567	MDP0000247211
MDP0000128678	MDP0000325376	MDP0000141463	MDP0000226279	MDP0000317247	MDP0000756536	MDP0000248168
MDP0000128887	MDP0000325652	MDP0000141686	MDP0000227119	MDP0000317975	MDP0000757613	MDP0000249105
MDP0000129164	MDP0000326734	MDP0000142080	MDP0000227640	MDP0000318256	MDP0000758050	MDP0000249183
MDP0000129874	MDP0000330372	MDP0000142814	MDP0000227657	MDP0000318702	MDP0000761113	MDP0000249561
MDP0000130244	MDP0000331536	MDP0000143458	MDP0000228070	MDP0000318866	MDP0000762600	MDP0000250254
MDP0000130449	MDP0000337873	MDP0000143462	MDP0000228247	MDP0000318901	MDP0000766223	MDP0000250737
MDP0000130822	MDP0000343219	MDP0000143463	MDP0000228302	MDP0000319037	MDP0000766240	MDP0000250951

TABLE 6-continued

Accession numbers (Velasco et al., 2010; Retrieved from the Internet: Rosaceae.org) of biomarker genes for firm flesh browning risk assessment during cold storage. Higher or lower expression levels can result from cold stress resulting from storage imposition.

Table with 7 columns of accession numbers. The first column contains 100 entries from MDP0000211459 to MDP0000251570. The second column contains 100 entries from MDP0000742278 to MDP0000900422. The third column contains 100 entries from MDP0000174168 to MDP0000193050. The fourth column contains 100 entries from MDP0000266003 to MDP0000287029. The fifth column contains 100 entries from MDP0000495793 to MDP0000590966. The sixth column contains 100 entries from MDP0000123893 to MDP0000168543. The seventh column contains 100 entries from MDP0000366291 to MDP0000617956.

TABLE 6-continued

Accession numbers (Velasco et al., 2010; Retrieved from the Internet: Rosaceae.org) of biomarker genes for firm flesh browning risk assessment during cold storage. Higher or lower expression levels can result from cold stress resulting from storage imposition.

MDP0000293842	MDP0000125709	MDP0000209713	MDP0000302905	MDP0000676693	MDP0000216376	MDP0000848515
MDP0000293974	MDP0000126455	MDP0000209897	MDP0000303101	MDP0000679280	MDP0000216945	MDP0000853531
MDP0000294379	MDP0000126718	MDP0000210022	MDP0000303198	MDP0000680042	MDP0000217142	MDP0000858452
MDP0000295039	MDP0000126850	MDP0000210238	MDP0000303384	MDP0000681106	MDP0000217310	MDP0000859733
MDP0000295223	MDP0000127834	MDP0000210254	MDP0000303430	MDP0000682471	MDP0000217362	MDP0000863049
MDP0000295540	MDP0000127930	MDP0000210260	MDP0000303496	MDP0000684216	MDP0000217499	MDP0000863404
MDP0000295543	MDP0000128265	MDP0000211252	MDP0000303872	MDP0000685420	MDP0000217803	MDP0000864442
MDP0000295562	MDP0000128267	MDP0000211643	MDP0000304254	MDP0000685425	MDP0000217850	MDP0000866840
MDP0000295794	MDP0000128423	MDP0000211758	MDP0000304881	MDP0000685724	MDP0000218078	MDP0000868044
MDP0000298232	MDP0000128463	MDP0000211874	MDP0000305029	MDP0000686466	MDP0000219430	MDP0000868659
MDP0000298527	MDP0000128531	MDP0000211955	MDP0000305338	MDP0000688188	MDP0000219802	MDP0000873893
MDP0000299181	MDP0000128560	MDP0000212045	MDP0000305695	MDP0000688643	MDP0000219838	MDP0000874667
MDP0000300808	MDP0000128786	MDP0000212077	MDP0000305886	MDP0000689162	MDP0000220167	MDP0000883367
MDP0000301666	MDP0000128790	MDP0000212327	MDP0000305934	MDP0000689889	MDP0000220328	MDP0000891353
MDP0000302024	MDP0000128937	MDP0000212372	MDP0000306224	MDP0000691413	MDP0000221160	MDP0000892526
MDP0000302440	MDP0000129051	MDP0000212760	MDP0000306907	MDP0000692523	MDP0000222089	MDP0000895380
MDP0000302671	MDP0000129445	MDP0000212975	MDP0000306990	MDP0000694318	MDP0000222724	MDP0000899413
MDP0000303449	MDP0000129648	MDP0000213508	MDP0000307150	MDP0000694562	MDP0000223057	MDP0000901379
MDP0000304620	MDP0000129681	MDP0000213863	MDP0000307340	MDP0000696497	MDP0000223568	MDP0000903481
MDP0000304719	MDP0000130630	MDP0000213948	MDP0000307432	MDP0000696624	MDP0000223905	MDP0000905321
MDP0000305094	MDP0000130716	MDP0000214579	MDP0000307685	MDP0000697378	MDP0000224209	MDP0000905924
MDP0000306738	MDP0000130769	MDP0000214797	MDP0000307853	MDP0000697676	MDP0000224389	MDP0000906703
MDP0000306888	MDP0000131142	MDP0000215239	MDP0000308181	MDP0000698024	MDP0000224417	MDP0000910353
MDP0000307717	MDP0000131267	MDP0000215270	MDP0000308285	MDP0000698038	MDP0000224930	MDP0000911731
MDP0000308205	MDP0000131368	MDP0000215276	MDP0000308395	MDP0000700189	MDP0000225340	MDP0000921067
MDP0000309160	MDP0000131377	MDP0000215777	MDP0000308491	MDP0000702868	MDP0000226276	MDP0000921871
MDP0000309314	MDP0000131386	MDP0000216638	MDP0000308907	MDP0000703059	MDP0000227692	MDP0000928898
MDP0000309732	MDP0000131731	MDP0000216907	MDP0000308938	MDP0000705359	MDP0000227742	MDP0000937996
MDP0000309741	MDP0000132207	MDP0000216952	MDP0000309169	MDP0000706828	MDP0000228529	MDP0000942516
MDP0000310093	MDP0000132209	MDP0000217406	MDP0000309382	MDP0000708299	MDP0000228670	MDP0000942873
MDP0000310940	MDP0000132381	MDP0000217451	MDP0000309530	MDP0000708692	MDP0000229338	MDP0000944409
MDP0000311556	MDP0000132436	MDP0000217745	MDP0000309676	MDP0000709073	MDP0000231369	MDP0000947607
MDP0000312449	MDP0000132623	MDP0000218252	MDP0000309805	MDP0000709523	MDP0000231477	MDP0000949486
MDP0000312701	MDP0000132726	MDP0000218451	MDP0000310374	MDP0000711379	MDP0000232264	MDP0000950422
MDP0000312765	MDP0000132855	MDP0000218748	MDP0000310430	MDP0000711832	MDP0000232789	
MDP0000312878	MDP0000132952	MDP0000218810	MDP0000310641	MDP0000711891	MDP0000233356	

The methods of the present invention may be practiced using any set of genes selected from the candidate genes disclosed herein, as long as the expression profiles of the genes within a given set discriminate between browning disorder progression outcomes.

The identification of such sets of genes may be performed by any suitable selection method, including, but not limited to, cluster analysis, supported vector machines, neural networks or other algorithms. A set of genes identified by such selection methods is generally capable of predicting the classification of an unknown sample based on the expression levels of genes used for the discrimination. "Leave one out" cross-validation may be used to test the performance of various models and to help identify weights (genes) that are uninformative (e.g., redundant) or detrimental to the predictive ability of the gene model.

As will be appreciated by those of ordinary skill in the art, sets of genes whose expression profiles correlate with browning disorder progression, and which can discriminate between browning disorder progression outcomes, may be used to identify/study unknown Rosaceous tissue samples. Accordingly, the present invention provides methods for characterizing Rosaceous tissue in Rosaceous fruit crops suspected of having the risk of developing multiple browning disorders.

The diagnostic/prognostic methods of the present invention generally involve the determination of expression levels of a set of genes in a Rosaceous tissue sample. Determination of gene expression levels in the practice of the inventive methods may be performed by any suitable method. For

example, determination of gene expression levels may be performed by detecting the expression of mRNA expressed from the genes of interest and/or by detecting the expression of a polypeptide encoded by the genes. Here, we have exemplified the determination of gene expression by the RNAseq method.

Our method can be readily adapted to currently available, existing platforms for measuring biomarkers. The expected end users of our products include apple producers, storage operators, shippers, retailers, agricultural laboratories, and agrichemical service providers. The same or similar equipment is already regularly employed world-wide by agricultural/agrchemical service providers to measure biomarkers and for other chemical analysis (plant nutrient levels, plant growth regulators, chemical residue analysis). Other existing easy to use, field-based platforms, many of which are already employed in apple production and apple packing plants can be adapted for biomarker measurement in diverse settings including hand-held electronic devices, dip-stick tests, and bench-top, hand-held, and packing-line mounted non-destructive sorting sensors. Examples of agricultural applications using gene expression biomarkers include an easy-to-use, mail-in platform that measures biomarkers that determine optimum apple and pear fruit harvest date.

As used herein, the term "gene" refers to a polynucleotide that encodes a discrete macromolecular product, be it RNA or a protein, and may include regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. As more than one polynucleotide may encode a discrete product, the term also

includes alleles and polymorphisms of a gene that encode the same product, or a functionally associated (including gain, loss, or modulation of function) analog thereof.

The term “gene expression” refers to the process by which RNA and proteins are made from the instructions encoded in genes. Gene expression include transcription and/or translation of nucleic acid material.

The terms “gene expression pattern” and “gene expression profile” are used herein interchangeably. They refer to the expression of an individual gene or of a set of genes. A gene expression pattern may include information regarding the presence of target transcripts in a sample, and the relative or absolute abundance levels of target transcripts.

The term “differentially expressed gene” refers to a gene whose level of expression is different in a subject (or a population of subjects) afflicted with a disorder relative to its level of expression in a healthy or control subject (or a population of healthy or control subjects). The term also includes a gene whose level of expression is different at different stages of, as described here, browning disorder. As will be appreciated by those skilled in the art, a gene may be differentially expressed at the nucleic acid level and/or protein level, may undergo alternative splicing resulting in a different polypeptide product, or the protein could be a target of posttranslational modification and degradation. Differential expression includes quantitative, as well as qualitative, differences in the temporal or cellular expression pattern in a gene or its expression products. As described in greater details below, a differentially expressed gene, alone or in combination with other differentially expressed genes, is useful in a variety of different applications in diagnostic, therapeutic, prognosis and related areas. The expression patterns of the differentially expressed genes disclosed herein can be described as a fingerprint or a signature of browning disorder progression. They can be used as a point of reference to compare and characterize unknown samples and samples for which further information is sought.

The term “RNA transcript” refers to the product resulting from transcription of a DNA sequence. When the RNA transcript is the original, unmodified product of a RNA polymerase catalyzed transcription, it is referred to as the primary transcript. An RNA transcript that has been processed (e.g., spliced, etc.) will differ in sequence from the primary transcript. A processed RNA transcript that is translated into protein is often called a messenger RNA (mRNA). The term “messenger RNA or mRNA” refers to a form of RNA that serves as a template to direct protein biosynthesis. Typically, but not always, the amount of any particular type of mRNA (i.e., having the same sequence, and originating from the same gene) reflects the extent to which a gene has been expressed.

The term “complementary DNA or cDNA” refers to a DNA molecule that is complementary to mRNA. cDNA can be made by DNA polymerase (e.g., reverse transcriptase) or by directed chemical synthesis.

The term “complementary” refers to nucleic acid sequences that base-pair according to the standard Watson-Crick complementary rules, or that are capable of hybridizing to a particular nucleic acid segment under relatively stringent conditions. Nucleic acid polymers are optionally complementary across only portions of their entire sequences.

The term “hybridizing” refers to the binding of two single stranded nucleic acids via complementary base pairing. The terms “specific hybridization” and “specific binding” are used herein interchangeably. They refer to a process in which a nucleic acid molecule preferentially binds,

duplexes, or hybridizes to a particular nucleic acid sequence under stringent conditions (e.g., in the presence of competitor nucleic acids with a lower degree of complementarity to the hybridizing strand). In certain embodiments of the present invention, these terms more specifically refer to a process in which a nucleic acid fragment (or segment) from a test sample preferentially binds to a particular genetic probe and to a lesser extent or not at all, to other genetic probes, for example, when these genetic probes are immobilized on an array.

The term “gene expression array” refers to an array comprising a plurality of genetic probes immobilized on a substrate surface that can be used for quantitation of mRNA expression levels. The term “genetic probe”, as used herein, refers to a nucleic acid molecule of known sequence, which has its origin in a defined region of the genome and can be a short DNA sequence (i.e., an oligonucleotide), a PCR product, or mRNA isolate. Genetic probes are gene-specific DNA sequences to which nucleic acid fragments from a test sample are hybridized. Genetic probes specifically bind to nucleic acid of complementary or substantially complementary sequence through one or more types of chemical bonds, usually through hydrogen bond formation. Here, we have used RNAseq profiling.

As used herein, the term “a reagent that specifically detects expression levels” refers to one or more reagents used to detect the expression of one or more genes (e.g., genes selected from the groups of 82 (Table 1), 494 (Table 2) and 2330 (Table 3) genes provided herein. Examples of suitable reagents include, but are not limited to, nucleic acid probes capable of specifically hybridizing to the gene of interest, PCR primers capable of specifically amplifying the gene of interest, and antibodies capable of specifically binding to proteins expressed by the gene of interest. The term “amplify” is used in the broad sense to mean creating an amplification product. “Amplification”, as used herein, generally refers to the process of producing multiple copies of a desired sequence, particularly those of a sample. A “copy” does not necessarily mean perfect sequence complementarity or identity to the template sequence.

The term “browning disorder profile” refers to a presentation of expression levels of a set of genes in Rosaceous fruit tissue (e.g., tissue at time of harvest, tissue after cold storage imposition). In preferred embodiments, profiles are generated from pooled samples comprising tissue samples from a plurality of fruits at the same stage.

As used herein, the term “modulation of browning disorder progression” refers to the ability of a treatment or management strategy to increase or decrease the likelihood that browning disorder will occur. Generally, useful strategies are those that decrease the likelihood of multiple browning disorder progression.

EXAMPLES

Having now generally described this invention, the same will be better understood by reference to certain specific examples, which are included herein only to further illustrate the invention and are not intended to limit the scope of the invention as defined by the claims.

Example 1

Biomarker Discovery: Early Term Physiological Disorder Risk Assessment

For initial candidate selection, ‘Honeycrisp’ apples were obtained from 15 orchards distributed among the Lake

Chelan/Brewster, Columbia Basin, and Yakima Valley growing regions in Washington State and the Hood River growing region of Oregon between Sep. 2 and Oct. 21, 2011. No pre-harvest treatments were applied, with the exception of Retain™, an ethylene biosynthesis inhibitor, (Valent Bio-Science Corporation, Libertyville, Ill., USA) to Orchard M. To determine the effects of harvest-timing on postharvest soft scald and soggy breakdown, fruit from one site was harvested at three different times, to represent an early, mid-, and late harvest (Orchard A). On the day of harvest, quality was assessed. Fruit were stored at 1° C. with no atmospheric modification and after 12 weeks, the final incidence of soft scald and soggy breakdown was assessed. For the selection process, the intent was to account for orchard to orchard variation of soft scald/soggy breakdown incidence given the impacts of pre-harvest environment and cultural controls as well as harvest maturity.

Example 2

Biomarker Discovery: Late Term Physiological Disorder Risk Assessment

‘Granny Smith’ apples were harvested 140 days after full bloom (approximately 1 month prior to commercial harvest) at a research orchard near Orondo, Wash. After transport to the laboratory, analysis of fruit maturity and application of DPA and 1-MCP were performed. Apples were stored in air at 1° C. for up to 6 months. Six replications of 3 fruit per treatment were removed from storage at 1, 2, and 4 weeks and 2, 3, 4, and 6 months. Upon removal from storage, scald development was rated on a 0-4 scale and peel sampled and stored from each treatment for subsequent analysis. In a parallel experiment, additional apples were treated with 2000 mg L⁻¹ DPA after 1-4 weeks, and 2 months after storage inception to determine the length of the transitional period during which scald can be suppressed. Scald development on these fruit was evaluated after 2, 3, 4, and 6 months storage.

Example 3

Gene Expression Evaluation: RNA Extraction and RNAseq Profiling

Harvested apple tissue was immediately snap frozen in liquid nitrogen and stored at -80° C. until required. Tissue was ground into a fine powder in liquid N₂ and total RNA was extracted from 500 mg of tissue in 0.8 mL of extraction buffer (4M guanidine isothiocyanate, 25 mM EDTA, 2.5% polyvinylpyrrolidone (MW 40,000), 2% sarkosyl, 1% β-mercaptoethanol, 0.2M sodium acetate, pH 5.0) at 70° C. for 10 min. Following incubation, chloroform (0.8 mL) was added, tubes vortexed and then spun in a benchtop centrifuge at top speed for 15 minutes. The resulting upper aqueous phase was collected, and a half volume of ethanol added and mixed by inversion. Total RNA was then purified through columns as per the manufacturer’s instructions (Qiagen, RNeasy), and eluted in nuclease free water. The resulting total RNA was checked for integrity via gel electrophoresis then quantified and diluted appropriately.

Libraries for RNAseq were made using 2 μg of total RNA following the method of Zhong et al. (2011). *Cold Spring Harbor Protocols* 8:940-949) and Gapper et al. (2013). *AoB Plants* 5:plt021) with slight modification. In short, mRNA was isolated from total RNA, mRNA was fragmented and used as a template to produce cDNA by reverse transcription

using Superscript III (Invitrogen). Following first strand cDNA synthesis, the second strand was synthesized with a dNTP mix incorporating dUTP instead of dTTP by DNA polymerase (Enzymatics). The ends of the double stranded cDNAs were then repaired (Enzymatics), dA tailed by the Klenow enzyme (Enzymatics) and universal adapters ligated to the double stranded cDNA fragments. Following ligation of adapters, the second strand was digested by Uracil DNA Glycosylase (UDG), to enable strand specific enrichment of the library. The UDG digested cDNA was then used as a template to enrich the libraries by PCR with Illumina Tru-seq primers using the high fidelity enzyme Phusion (New England Biolabs) with the following conditions: 95° C. 2 min; 15 cycles of 98° C. 11 s, 65° C. 30 s, 72° C. 25 s; 72° C. 2 min; 4° C. soak.

Libraries were quantified, and 20 ng of each pooled for sequencing. Up to 48 libraries were multiplexed per sequencing reaction using an Illumina HiSeq 2500 next generation sequencer at the Weill Medicine School Sequencing Facility (Cornell University, New York City, N.Y.). Three biological replicates were sequenced for each sample. Short (40 bp) single-end, strand-specific RNAseq reads were filtered by aligning to adapter, ribosomal RNA and tRNA sequences using Bowtie (allowing two mismatches). The resulting high quality reads were aligned to the apple genome (Velasco et al. 2010. *Nat. Genet.* 42(10):833-839) using Tophat (allowing 1 segment mismatch) (Benjamini and Hochberg. 1995. *J. Royal Stat. Soc. Series B (Method.)* 57(1):289-300). Following alignments, raw counts were normalized to reads per kilobase of exon model per million mapped reads (RPKM).

Example 4

In Vitro Transcription and Chilling Stress

Soft scald and soggy breakdown. Only genes with expression levels over 2 RPKM for any replication were considered for candidate selection. Only genes whose expression was variable across the whole data set were considered for subsequent correlation analysis. Peel and cortex expression levels (RPKM) at harvest and following 2 weeks cold storage were compared with soft scald and soggy breakdown incidence (coded as 0=no incidence, 1=0-10%; 2=10-25%; 3=over 25%) taken at 12 weeks using Pearson’s correlation analysis. Gene expression with a correlation coefficient above R²=10.5001 or R²=10.7001 for at-harvest and 2 week comparisons (respectively) were considered candidate biomarkers for risk assessment monitoring.

Soft scald and soggy breakdown began to develop beginning at 4 weeks in some high risk orchards eventually developing to maximum incidence and severity by 12 weeks, as expected from earlier work using this cultivar (FIG. 2). The RNAseq protocol provided one of many sensitive, accurate, and precise platform for candidate expression evaluation in both apple peel and flesh. Of the 63,541 genes screened in this experiment, 82 candidates were found useful for assessing risk at-harvest (Table 1) and 494 for 2 week risk assessment of peel and/or cortex tissue (Table 2). Biomarker gene expression primarily increased although some decreased with increasing risk. Accordingly, a summary of overall gene expression levels indicated that more models were linked with eventual injury at 2 weeks, or following cold storage imposition, rather than at-harvest (FIG. 4) indicating that the cold stress that provokes the injury followed by candidate expression evaluation will provide the most accurate risk assessment. Given the dra-

matic change in gene expression provoked by cold storage imposition, measuring candidate expression levels both before and at one or more points following cold storage may yield even more accurate information by documenting upward or downward trends. Similarly, monitoring multiple risk assessment biomarkers can better ensure that changes are based on more biochemical systems associated with risk.

Superficial scald. Only genes with peel expression levels over 2 RPKM for any time point were considered for modeling and candidate selection yielding 35,644 gene models for subsequent screening. Gene models alongside superficial scald incidence were clustered across all 3 treatments over the 6 month storage period using k-means correlation clustering algorithm (Matlab) yielding 86 clusters. Gene models with increasing expression in control fruit, but not antioxidant or 1-MCP-treated fruit 2 months or more prior to superficial scald incidence, were considered candidates. K-means correlations clustering was used to group genes similarly expressed over the entire experiment in all treatments. Clusters comprised of superficial scald risk storage monitoring biomarker candidates were those that correlated $R^2=0.700$ with conjugated trienol (CTOL) levels or injury incidence.

The first symptoms of superficial scald began to develop on control fruit between 2 and 3 months and continued to increase in severity until 6 months as is typical for this cultivar when stored in air without any control steps taken (FIG. 3B). Both crop protectants either eliminated or markedly reduced symptom development while providing two physiologically different means of control to select the most accurate risk assessment candidates from. Because superficial scald can be adequately controlled using appropriate storage conditions, indicating whether storage conditions are actually working or monitoring risk during storage is the most useful tool for this disorder. Increases in superficial scald risk-associated gene expression began at 1 month for a few candidates (FIG. 5) 690 candidates for early indication of scald risk (Table 3). Monitoring multiple risk assessment biomarkers can better ensure that changes are based on more biochemical systems associated with risk.

Example 5

Risk Assessment

Multiple candidates are part of previously unidentified metabolic responses to stress and, monitoring these candidates appear to be effective for assessing risk across disorders and cultivars following, or during, cold stress events. Changes in expression levels before and following cold storage imposition as well as fold difference between high risk and low risk orchards for developing soft scald/soggy breakdown (early-term disorders) are included as examples of broadly effective risk assessment biomarkers (Table 7). For soft scald/soggy breakdown risk assessment, the fold difference from the highest incidence orchard to lowest incidence orchard is indicated as well as the % change in expression following cold storage imposition. For superficial scald, a late-term disorder, the time from first detection of risk using a specific biomarker until symptom appearance and the % change in expression during that period are indicated. Expression of many of these genes change dramatically indicating repeated measurement around the point of cold storage imposition may be used to improve confidence. Monitoring the same genes during storage of cultivars at risk for developing superficial scald or other late-term apple and pear physiological disorders leads to

expression changes that ultimately culminate in symptom development 1 month or more later. Increased expression of up to 98% transpired up to 6 weeks prior to disorder development. Biomarker genes are from multiple stress-related biochemical processes not related to stress in fruit prior to this discovery. Monitoring biomarkers from multiple processes can be used for a more confident evaluation. For instance, candidates putatively involved in phenolic metabolism and indole acetic acid (IAA) metabolism could be monitored in tandem.

TABLE 7

Apple protein Accession Numbers (Velasco et al., 2010; Retrieved from the Internet: Rosaceae.org) of biomarker genes for early and late term apple peel and flesh disorders.

Gene accession number	Early-term disorders		Late-term disorders	
	Fold difference ¹	% change ²	Time before symptoms (weeks) ³	% change ⁴
MDP0000591260	7.8*, nd	75.0	8	52.8
MDP0000272351	6.4, 11	89.0	8	41.9
MDP0000145813	11.5, 13.8	43.9	8	82.0
MDP0000551974	16.9, 56.6	86.4	8	46.2
MDP0000424447	21.9, 54.4	85.6	8	36.6
MDP0000215525	20.2, 29.0	46.3	8	83.9
MDP0000715898	12.7, 15.5	81.9	6	60.1
MDP0000306121	18.2, 30.0	46.6	8	84.3
MDP0000290801	19.1, 41.0	56.2	8	83.4
MDP0000307237	17.9, 27.4	40.4	6	81.1
MDP0000769764	20.1, 224	59.6	8	77.7
MDP0000545122	13.6, 27.0	88.2	6	44.7
MDP0000733506	22.0, 31.3	82.4	8	48.0
MDP0000617956	47.6, nd	82.3	8	46.9
MDP0000755567	11.6, 25.2	96.6	6	17.9
MDP0000334047	11.0, 8.09	64.4	6	36.7
MDP0000688645	34.2, 16.4	98.1	8	43.9
MDP0000576682	4.8, nd	-10.6	6	66.2
MDP0000191939	4.0, 3.1	-6.3	8	46.7
MDP0000195213	15.3, 19.4	63.3	8	41.8
MDP0000211643	19.5, 8.2	82.2	8	20.3
MDP0000292164	9.0, 16.4	64.7	6	50.2
MDP0000130244	51.0, 20.5	96.8	6	75.2
MDP0000190809	4.9, 10.0	62.8	6	-22.1
MDP0000748916	10.0, 4.2	48.8	6	21.6
MDP0000199009	8.4, 5.3	62.8	6	21.5
MDP0000143611	1.8, 2.8	17.9	8	46.7
MDP0000166302	1.6, 2.1	18.9	8	24.5
MDP0000278475	3.3, 4.3	34.8	8	21.8
MDP0000640906	4.1, 8.1	85.9	6	28.0

¹Fold difference of biomarker levels at 2 weeks between GH (highest risk) and GLE1 (lowest risk).

²Percent gene expression change in GH harvest to 2 weeks.

³The time (in weeks) between elevated biomarker levels in high risk fruit and symptom development at 3 months.

⁴Percent change in gene expression in high risk fruit from 2 to 8 weeks.

As apple and pear fruit transition through the supply chain, they undergo many stress events. Our invention allows for an easy means for interrogating fruit undergoing these transitions to assess risk for developing these disorders. FIG. 6 provides a scheme for some of the ways which our biomarker-based risk assessment scheme can be used to direct storage and marketing decisions that can mitigate or avoid disorder development.

All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

The foregoing description and certain representative embodiments and details of the invention have been presented for purposes of illustration and description of the invention. It is not intended to be exhaustive or to limit the

invention to the precise forms disclosed. It will be apparent to practitioners skilled in this art that modifications and variations may be made therein without departing from the scope of the invention.

We claim:

1. A method comprising:

- a) isolating mRNA from peel tissue or cortex tissue of at least one apple or at least one pear;
- b) performing reverse transcription PCR on said isolated mRNA; and
- c) assaying the level of expression of a set of biomarkers, wherein the set of biomarkers consists of:

MDP0000848515,	MDP0000238780,
MDP0000260007,	MDP0000153149,
MDP0000585315,	MDP0000273646,
MDP0000216786,	MDP0000180580,
MDP0000737425,	MDP0000298967,
MDP0000808492,	MDP0000273866,
MDP0000353053,	MDP0000910032,
MDP0000145050,	MDP0000263844,
MDP0000158999,	MDP0000547254,
MDP0000196325,	MDP0000224653,
MDP0000321382,	MDP0000270602,
MDP0000233661,	MDP0000665342,
MDP0000246831,	MDP0000823528,
MDP0000408705,	MDP0000518327,
MDP0000291249,	MDP0000154589,

5
10
15
20
25

- MDP0000202817,
- MDP0000312397,
- MDP0000529726,
- MDP0000639894,
- MDP0000183676,
- MDP0000797616,
- MDP0000782908,
- MDP0000562305,
- MDP0000361351,
- MDP0000412192,
- MDP0000149492,
- MDP0000125882,
- MDP0000163006,
- MDP0000329063,
- MDP0000665685,
- MDP0000200783,
- MDP0000862371,
- MDP0000441757,
- MDP0000599531,
- MDP0000272980,
- MDP0000268175,
- MDP0000637194,
- MDP0000287262,
- MDP0000164966,
- MDP0000318068,
- MDP0000225326, and MDP0000125411.

- MDP0000590954,
- MDP0000213383,
- MDP0000312071,
- MDP0000307665,
- MDP0000754521,
- MDP0000818877,
- MDP0000266443,
- MDP0000228366,
- MDP0000737001,
- MDP0000223032,
- MDP0000264361,
- MDP0000498460,
- MDP0000225132,
- MDP0000170865,
- MDP0000196079,
- MDP0000182956,
- MDP0000321792,
- MDP0000313657,
- MDP0000297583,
- MDP0000317502,
- MDP0000722139,
- MDP0000125700,
- MDP0000745534,
- MDP0000389794,
- MDP0000322237,

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