Molecular Marker Based Diagnostics for Almond Budfailure

Project No.: **14-HORT7-Gradziel**

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Project Objectives:

A. Consolidate historical and recent data from almond x almond as well as almond x peach breeding populations for evaluation of possible inheritance patterns of budfailure (BF) in progeny.
B. Develop genetic/epigenetic model(s) based on compiled progeny segregation and development patterns and current research on similar genetic/epigenetic afflictions.
C. Initiate a preliminary assessment currently available molecular-based diagnostics for discriminating between high and low-BF expression.
D. Publish results from BF-heritability studies as a basis for a subsequent USDA proposal targeting molecular-based BF predictors.

2014 Objectives:

1) Examine profiles of individual DNA methylation markers identified in 2013 using our clonal sources of different ages and BF-potentials to test for level of association of each individual marker with clone age and, separately, BF-potential within the same clones as well as with BF-potential among varieties.
3) If markers with good associations with either an ageing or BF-expression are identified, use them to detect genomic regions associated with these traits as a basis for future development of more precise markers.
4) Continue to develop almond BF as a model system for epigenetic disorders in plants, as a basis for more extensive outside research funding targeting the more basic mechanisms involved as well as applied predictors and possible genetic manipulations (including the possibility of BF remission).

Interpretive Summary:

True noninfectious budfailure is characterized by the death of terminal or sub-terminal shoot buds during the development of dormancy in the previous fall, which can be verified by a brown necrosis of the internal bud tissue at that time as well as failure of subsequent bud swelling and development during the subsequent winter and spring. The disorder becomes evident with the failure of the vegetative buds to grow the following spring resulting in sections of blind or bare shoot-wood and the subsequent pushing of the still-viable basal vegetative buds. Flower buds are not affected and can often develop into fully formed nuts despite the lack of any nearby vegetative leaf growth. A third distinct BF characteristic is that once budfailure symptoms develop, normal growth is not restored in subsequent seasons but rather the disorder gets worse with each following season. This recurring sequence of terminal shoot-budfailure and pushing of a viable basal buds results in a punctuated and erratic shoot development pattern commonly termed "crazy top" which with time can degrade orchard productivity for commercially important cultivars such as Carmel and Nonpareil.

Several lines of evidence suggest that almond BF is not controlled by the presence or absence of a controlling gene but rather the normal expression of key development gene(s) are suppressed by chemical or physical modification of these controlling genes which corrupts their normal expression. Since the gene DNA would remain unchanged, standard DNA-based molecular markers would not be effective in identifying the cause. This study explores the use of methylation-based (i.e. chemically altered) markers for predicting level of BF expression in different nursery clonal sources, as well as the clone age (i.e. number propagations from original seedling tree) which is known to be related to BF expression level.

Over 12,000 markers were evaluated for clones of different BF-potential as well as clonal age. Promising markers showing clear differences among the tested samples were identified using the Methylation-Sensitive Representational Difference Analysis (MS-RDA). This technique utilizes the methylation-sensitive restriction endonuclease HpaII to recognize the 5'-CCGG-3' 4-bp motif and thereby isolates DNA fragments differentially methylated within clone variants. We have now identified a number of methylation-markers associated with the level of BF expression, as well as with the age of the clone. Because of the large number of potential markers and the inherent difficulties in accurately scoring both BF-potential and clone age, we are now analyzing the data through both large-scale statistical analysis and individual assessment of putative candidate gene function to identify epigenetic markers associated with BF expression. A strong association might then be used as predictor of the ultimate level of BF expression in vegetative progeny from different nursery source trees. If highly correlated, these markers may help identify the gene(s) controlling this disorder, which in turn might lead to a better understanding of BF development as well as its control. Ongoing animal studies also suggest opportunities for rehabilitation of important Carmel and Nonpareil clonal sources where the level of BF expression has curtailed nursery use.

Introduction:

This research is a joint project funded by the Almond Board of California (ABC) and the California nursery industry (IAB). It consolidates and advances previous UC Davis (UCD) studies which have led to an understanding of the pattern of Non-infectious Budfailure (BF) development within propagation sources (clones) of commercially important almond cultivars including Nonpareil and Carmel, which allow effective selection of clonal sources with lower probabilities of expressing BF during the crucial early years of orchard growth. Attempts to develop molecular markers as indicators of BF-potential have proven unsuccessful, leading to the hypothesis that BF genetic deterioration is not associated with changes in the marker-targeted DNA sequence of the gene(s) involved, but rather involves suppression of gene activity through still poorly understood epigenetic mechanisms. Clonal analyses, as well as tree development studies have also supported an epigenetic/environmental control. This project is thus pursuing epigenetic markers based on the methylation patterns for individual genes from clones of Nonpareil and other important almond cultivars which differ in the level of BF expression and/or the clone age (since it is known that the potential for BF-expression increases with age of susceptible cultivars).

Non-infectious Budfailure (BF) remains a major threat to almond production in California, particularly with the recent rapid expansion of acreage into more inherently water and heat stressed regions. It is a particularly serious problem for the commercially important cultivars Nonpareil and Carmel, which together make up approximately 50% of total plantings. Clonal selection of low BF sources has allowed continued plantings of both Nonpareil and Carmel after BF first became a problem in these cultivars. However, BF-potential (which is related to the age and propagation history of the cultivar) in even the best clonal sources of Carmel may not be sufficiently low to ensure continued commercial use. Careful selection of low-BF Nonpareil clones in the 1970s, 80s and 90s has allowed continued plantings of this dominant variety (**Figure 1**), though recent BF expression in some Nonpareil sources caution that they may also be progressing towards a new round of BF expression. High BF expression was also a major contributor to the early abandonment of otherwise very promising cultivars such as Merced, and will likely be found in some of the recently released California varieties, particularly those which have the BF-susceptible cultivar Nonpareil as a parent (which includes virtually all currently commercially important cultivars).

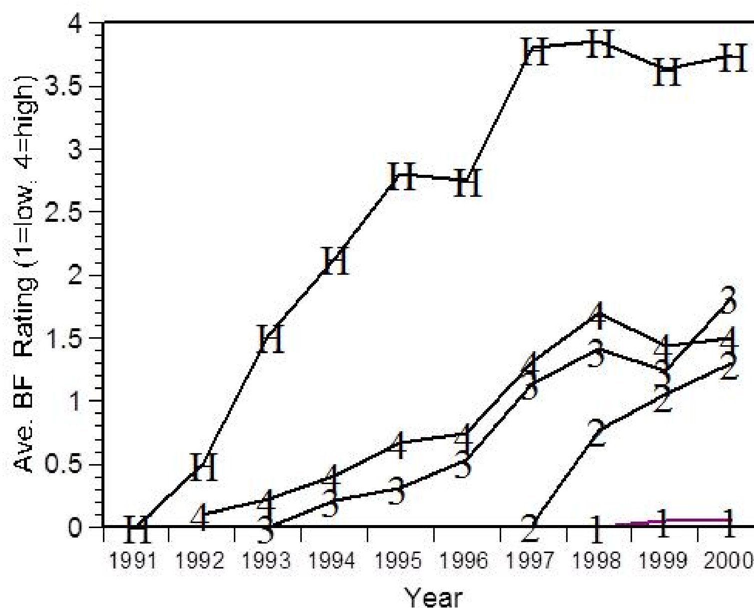


Figure 1. Development of BF- expression in vegetative progeny of different clonal sources of Carmel. (1-original Carmel seedling tree, 2-standard low-BF *FPS#1* source, 3-medium-BF *FPS#2* source, H-high expression commercial source).

BF-like symptoms have been observed in isolated trees of some recent releases including the cultivar Winters. Molecular marker analysis has verified the Winters identity but the source of the budwood was not virus-free FPS foundation stock but was probably propagated from virus infected wood gathered from the early Delta research block trials. Similarly, BF-like 'crazy-top' shoot growth was also observed in Marcona trees recently planted in the southern San Joaquin Valley. ELISA analysis however showed the symptoms to be the result of Prunus Necrotic Ringspot virus infection. While BF has been shown to be inherited in progeny, the genetic control of BF remains elusive.

Figure 2. Characteristic shoot development pattern of noninfectious budfailure resulting from a seasonal pattern of die-back and regrowth. Lower inset shows the characteristic die back of buds the previous fall with no further development of buds through the winter and following spring (upper inset).

Populations which should segregate for BF-expression have been developed from crosses of almond selections to high-BF Nonpareil clones (to assess BF-potential among clones of the same variety), and recently from crosses of almond varieties to early-flowering peach genetic-tester lines (to assess latent BF-potential among different varieties). Resultant inheritance data has been used to establish and test different genetic and molecular models for BF.

Results and Discussion:

Budfailure characterization.
Farm calls over the course of this project have typically identified multiple and distinct causes of shoot budfailure in almond:

- Nutrient deficiencies/toxicities
- Variety growth habit
- Low winter chilling
- Wind rubbing
- Virus/viroids
- Bacterial Bud-drop
- Noninfectious Budfailure (BF) (also known as Crazy Top)

Figure 3. 'Rough-bark' trait sometimes observed in severe noninfectious bud failure.

True noninfectious budfailure is characterized by the death of terminal or sub-terminal shoot buds during the previous Fall, which can be verified by a brown necrosis of the internal bud tissue at that time (see insets in **Figure 2**) as well as failure of subsequent bud swelling and development during the subsequent winter and spring. The disorder becomes evident with the failure of the vegetative buds to grow the following spring resulting in sections of blind or bare shoot-wood and the subsequent pushing of the still-viable basal vegetative buds. Flower buds are not affected and can often develop into fully formed nuts despite the lack of any nearby vegetative leaf growth. A third distinct BF characteristic is that once budfailure symptoms develop, normal growth is not restored in subsequent seasons but rather the disorder gets worse with each following season (though the extent and rate of symptom development may vary in subsequent years depending upon growth rate, heat stress from the previous summer, etc.). This recurring sequence of terminal shoot-budfailure and pushing of a viable basal buds results in a punctuated and erratic shoot development pattern commonly termed "crazy top" (**Figure 2**). In some severe cases of BF, the bark on young shoots can develop a characteristic splitting or cracking often called 'rough bark' (**Figure 3**). BF is 'noninfectious' i.e. it cannot be transmitted to other trees by budding, grafting or transferred by feeding insects. BF is often confused with other aberrations in bud development, the most common of which are summarized in **Figure 4** and below.

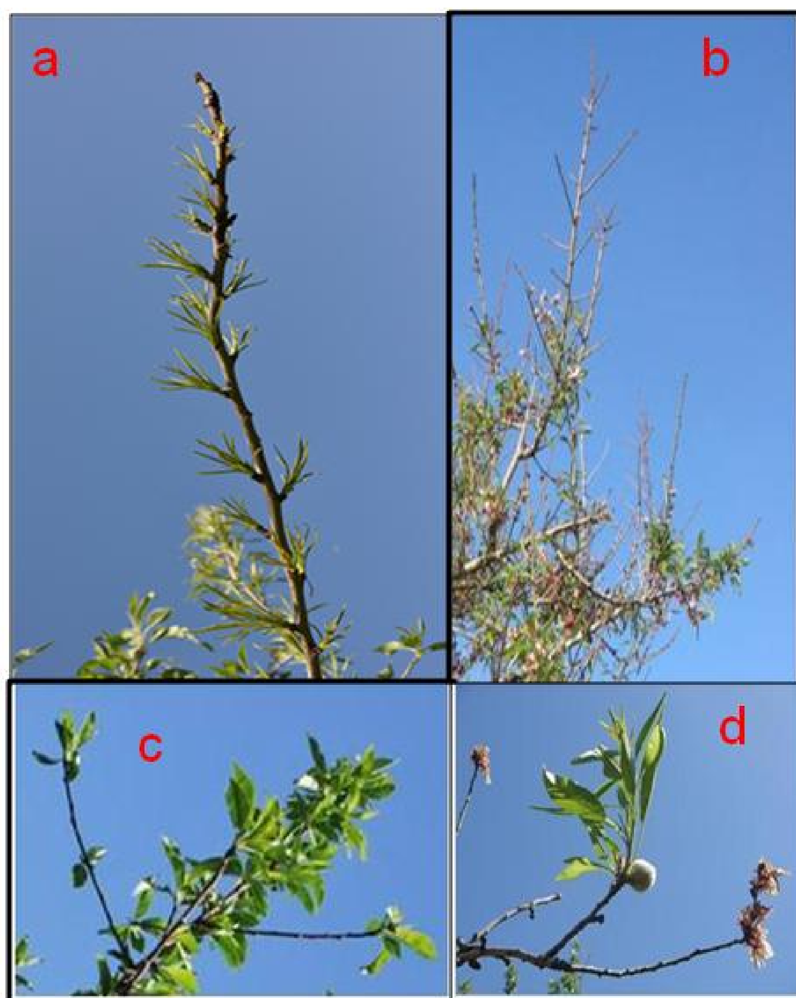


Figure 4. Expression of budfailure from different biotic and abiotic agents.

[a] Budfailure from nutrient deficiencies/toxicities (including some herbicide toxicities) often show some bud development during the winter chilling period and subsequent spring growth, as is the case with zinc-deficiency in **Figure 4a**. Leaf and shoot appearance is often characteristic of the specific toxicity/deficiency. Normal growth can also be restored with the proper nutrient treatment.

[b] Similarly, some varieties such as the late-blooming variety Savanna (**Figure 4b**) show a late leafing-out on terminal shoots that give an early impression of BF. Close examination

of shoots, however, typically shows that buds are developing although at a delayed rate. This can also be confirmed by revisiting the orchard one to two weeks later when normal shoot growth should be observed.

[c] In years with low winter chilling, some varieties, including Carmel, may also show a delay in terminal or subterminal lateral bud development (**Figure 4c**). Again, a close examination of the buds will show some degree of swelling or development from the previous fall, ruling out noninfectious bud failure. As with late blooming varieties, buds may continue development at a later date though in some cases they appeared to become dormant or even desiccated. Serious BF-like symptoms were observed in the spring of 2012 and again in 2013-15 for the cultivar Monterey. While initial symptoms, including normal floral bud development with terminal and sub-terminal axillary vegetative budfailure, appear similar to Noninfectious Budfailure, later observations showed that many vegetative buds were still viable and pushed normal looking spring growth, although at a much delayed time (**Figure 5**). A similar appearance is sometimes caused when shoots or branches rubbed together in the wind causing the sloughing of buds. Close examination of the shoots can often identify the physical damage

Figure 5. BF-like symptoms in the cultivar Monterey observed in the spring. While initial symptoms, including normal floral bud development with terminal and sub-terminal axillary vegetative budfailure, appear similar to Noninfectious Budfailure, later observations showed that many vegetative buds were still viable and pushed normal looking spring growth, although at a much delayed time.

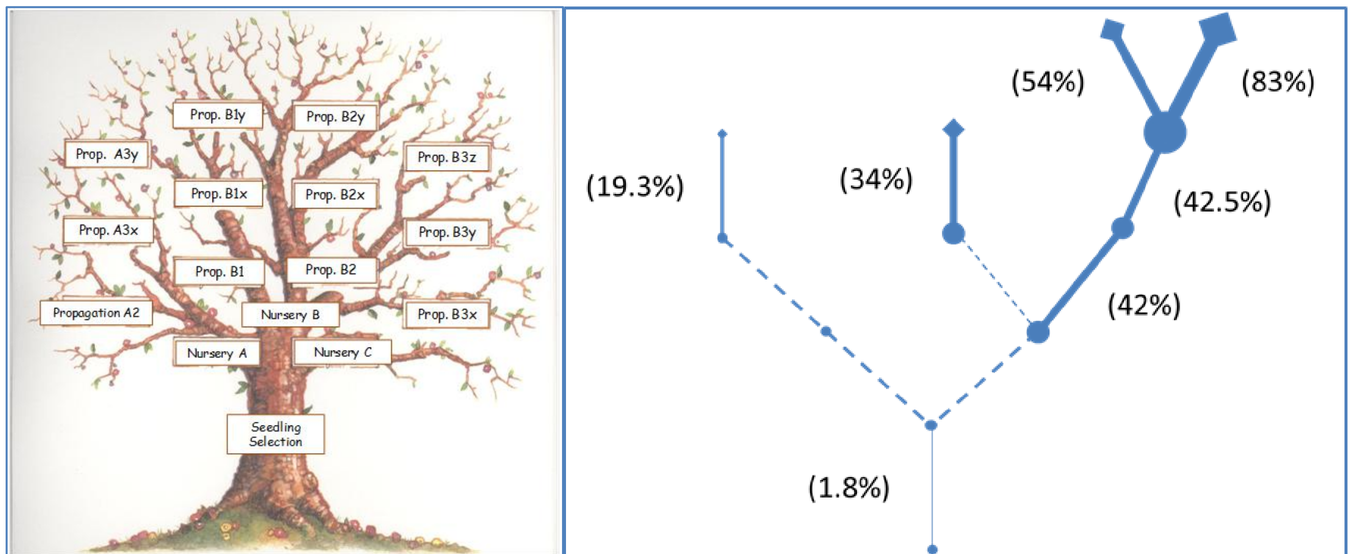


Figure 6. [Left] Tree model for the increase in potential for BF appearance either in an orchard tree or (analogously) nursery propagation sources. [Right] Actual BF expression in vegetative progeny populations from Carmel clone sources of known relation to the original tree (basal point).

from rubbing as well as the responsible branch.

[d] A form of budfailure often observed on old to very old trees is infectious budfailure, or budfailure caused by virus infection (typically Prunus Necrotic Ringspot Virus or Prunus Dwarf Virus). Where noninfectious budfailure will typically first appear in the rapidly growing shoots at the tops of trees, infectious budfailure tends to be more prevalent at the slower growing shoots on the trees lower branches. New shoot growth tends to show shortened internodes and be willowy giving a 'mules-tail' appearance (**Figure 4d**). Flowers may or may not be affected depending upon the virus and variety. Diagnosis of infectious budfailure is by graft or bud transmission to a susceptible host, or by ELISA or molecular analysis (see **Appendix A** and **B**).

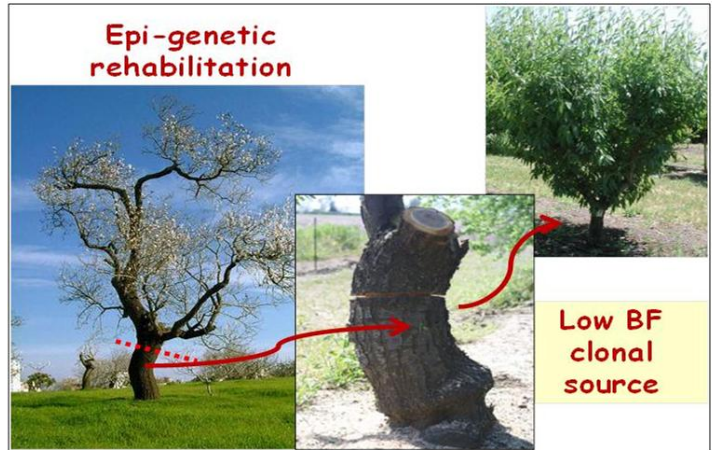


Figure 7. Rehabilitating Nonpareil almond to a lowered BF status by propagating new nursery foundation blocks from BF-dormant basal epicormic buds pushed from 100-year-old trees.

Models for Noninfectious Budfailure development.

In our evolving model of BF, the critical fall bud degeneration results from the deterioration in function of gene(s) vital to vegetative bud transition to winter dormancy. This deterioration results from a gradual genetic 'ageing' of a crucial gene complex as a consequence of repeated phase cycling in controlling cells. Such cycling occurs during the yearly growth phases of almond shoots and appears to also occur, and may even be amplified, by vegetative propagation. The typically ramified propagation history of most vegetatively propagated tree crops is thus analogous to the growth and development of a mature tree (**Figure 6**). Since BF appears to be determined by an 'internal aging' process, the appearance of BF symptoms at the terminus of one branch (clonal propagation source) is a good predictor of imminent BF appearance on other branches (propagation sources) all of which arose from a common source. Genetic deterioration also appears to be correlated with environmental stresses, particularly heat, during early-season bud development of

Table 1. Proportion of trees showing BF.-expression in FPS foundation stock and grower trees derived from that stock (but 2 to 3 generations more advanced through nursery propagations).

2015 Data	DELTA	KERN	FPMS
CARMEL#1			
3-56-1-90	7%	28%	-
NONPAREIL			
3-8-2-70		11%	-
3-8-6-72		8%	-
3-8-5-72	-		
3-8-8-72	-		-
3-8-16-90			-
3-8-12-72			-
3-8-18-92			-

summer dormancy (4, 20). Low BF-potential propagation sources have been selected from among clonal lines in which gene ageing is limited owing to their lineage (recent position in line of descent from original cultivar seedling tree; see **Figure 6**) and previous growth environment (including low heat stress and propagation method) (12). Such vegetative progeny based clonal studies, however, typically require 10 or more years to accurately characterize clonal-source BF-potentials. A well-characterized example of this approach was the selection in the 1990s of Carmel clonal nursery sources which showed lower potential for developing BF symptoms when used as propagation material (**Figure 1**). Significantly, even the best sources showed symptoms within the first 10 years of tree growth showing that while the BF potential could be reduced dramatically, it would still be a concern even in the most promising propagation sources (particularly since an additional 2 vegetative generations of ageing {i.e. mother block and grower trees plantings and growth} are required prior to commercialization). This clonal-source selection as applied to Carmel was originally applied to Nonpareil when BF symptoms became particularly problematic in the 60s, 70s and 80s. To, in a sense, turn back the internal-aging clock, epicormic buds from the base of old Nonpareil trees initially planted in the early 1900s were pushed to develop shoot growth from which clonal source material was propagated (**Figure 7**). Because the Nonpareil cultivar originated in the 1880s, these basal epicormic (i.e. poorly differentiated) buds from old trees would represent relatively low BF potentials (because they were laid down early in tree growth and remained largely dormant in the intervening years). As such, they would serve as good foundation material for continued Nonpareil propagations. That it took approximately 50 years for Nonpareil to initially show BF-symptoms indicates that the original seedling selection had relatively low initial BF potential. However, while low BF-potential was recovered from trees planted in the early 1900s, their BF-potential would be expected to gradually age (decay) in the ensuing 50 years to the point that BF-expression again became a problem by the 1960s. Nonpareil clone rehabilitation through appropriate epicormic bud selection at that time has allowed Nonpareil to remain relatively free from budfailure, though the passage of an additional 50 years since those initial epicormic selections suggest that BF may again become a problem in this cultivar.

Variety	DELTA	KERN	FPMS	Grower
Aldrich	-	-	-	
Butte	-	-	-	
Chip's	-	?		
Donna	-	-		
Fritz	-	-	-	
Jenette	-	X		
Jiml	-	-		
Johlyn	-	?		
Kahl	-	?		?
Kaperiel	-	-	-	
Livingston	-	-		
Milow	-	-	-	
Mission	-	-	-	
Monterey	-	-	-	
Morley	?	-		
NPU	-	-	-	
Padre	-	-	-	
Peerless	-	-	-	
Plateau	-	-		
Price	-	-	-	
Rosetta	-	-	-	
Ruby	-	-	-	
Sano	?	?		
Savana	?	-		
Sonora	-	-	-	
Wood Colony	-	-		
Yokut	?	X		
Winters	-	-		X
Kester	-	-	-	

Figure 8. Results from 2012-15 BF surveys from the Delta and Kern Regional Variety Trials as well as local grower trials and FPS foundation plots sources.

Evidence of such low BF-potential erosion has recently been observed in a Nonpareil-clonal source originally identified for low BF-expression/BF-potential (**Table 1**). While increasing levels of BF-expression are expected in relatively young (20 years) clonal sources of Carmel because of its higher (initial seedling tree) BF potential, it has not been previously observed in the generally more durable low BF-potential Nonpareil clonal sources selected in the 60s, 70s and 80s. The commercially important IR2 Nonpareil selection (3-8-2-70) was selected at a similar time and from similar material as the other industry important sources, Jeffries and McEnespy. BF expression in Nonpareil trees from this and related lineages has recently been documented with a slight increase in recent years (**Table 1**). [Data in **Table 1** was developed from 20 plus year-old orchards of these initial clonal sources which are still present in some Sacramento and San Joaquin Valley locations]. Consequently, the BF expression levels serve as an indication of the BF-durability of these different sources. Southern San Joaquin Valley locations (Kern County in **Table 1**) consistently give some of the best assessments of long-term BF-durability (see Reference 12) because of the generally greater heat stress. [Interestingly, the IR2 (3-8-2-70) Nonpareil clone also shows some of the highest levels of cumulative production in recent San Joaquin regional trial studies by Bruce Lampinen et al. (**Appendix C**).]

Figure 9. BF-like symptoms on Winters trees in Fresno County in 2010-11**.**

While careful selection in the 60s, 70s and 80s source material based on BF-expression (as determined using both vegetative progeny tests and the more rapid test-crosses method described below), allowed continued production of low BF Nonpareil trees, even these more elite lines are beginning to again show BF. Reduced BF-expression may be maintained by carefully selection of those propagation lineages remaining free from BF-expression or returning to the original mid-1900s selections (where available). As part of this project, new FPS parent clonal stock were established via such basal epicormic buds rehabilitation (**Figure 7**) for the Nonpareil sessions (3-8-5-72), (3-8-2-70), (3-8-8-72) and (3-8-16-91) and Carmel accession 3-56-1-90.

Figure 10. Budfailure in the Marcona almond variety resulting from Prunus Necrotic Ringspot Virus infection.

Several recent varieties such as Yokut, Kochi and Jenette continued to show evidence of early BF expression in recent years (**Figure 8**). However, since plantings of these varieties are not expected to be commercially significant, the evaluation/selection of low BF potential sources may not be warranted. A single case of potential BF in the more commercially important cultivar *Winters* has been identified in eastern Fresno County (**Figures 8 & 9**). The low number of trees showing symptoms also showed growth habits somewhat inconsistent with the Winters variety. Molecular analysis of leaf samples collected from these trees, however, has verified that they are the cultivar Winters (**Appendix B**). Winters has been known to be vulnerable to BF based on both lineage (it has the BF-affected cultivars Nonpareil, Harriet, and Jordanolo as parents) and from BF test-crosses [in an earlier Winters x high BF Nonpareil cross, progeny showed a low proportion of budfailure trees indicating a low BF potential]. The low potential for Winters was comparable to Sonora, which gave similar progeny test results and despite its extensive plantings has only shown the occasional BF tree). A more recent and more accurate test of BF potential involves the control crossing with an early flowering peach tester stock (UCD 40A-17) as described below in Genetic/Epigenetic Models. Results (described below) support an existing but low BF potential for the Winters cultivar. In addition, the bud-wood source used to propagate the early Fresno County test block trees where BF was observed was not from the established FPS foundation source, but was traced back to very early test plantings in the Stockton area which were later found to be virus-infected.

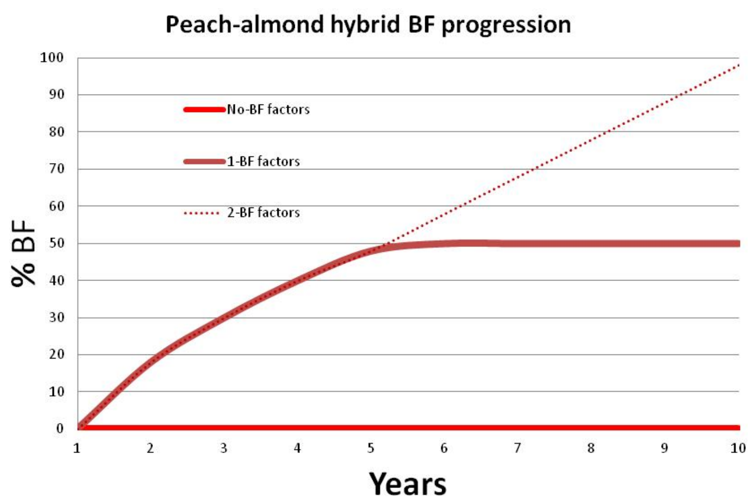


Figure 11. Expected peach by almond progeny performance when the almond parent contains one or two high BF-genes forms.

BF-like symptoms have also been observed in Southern San Joaquin Valley *Marcona* plantings (**Figure 10**). Molecular (ELISA) analysis however showed the symptoms to be the result of virus induced budfailure, in this case due to infection of Prunus Necrotic Ringspot Virus (PNRSV in **Appendix A**). The virus was also verified through graft-transmission (work done in cooperation with FPS labs). Extensive source selection/virus testing of different Marcona clones has identified a single tree source which has been shown to be negative for both Prunus Necrotic Ringspot Virus and Prunus Dwarf Virus (**Appendix A**). In 2013, this clonal source material has been transferred to FPS foundation stock orchards after and undergoing final trueness-to-type testing in 2015 has been included for long-term evaluation in the new RVT trials.

Genetic/epigenetic models and associated molecular-based diagnostics.
Different genetic control models, including control by 1 to 3 Mendelian-type genes, as well as various epigenetic mechanisms are consistent with observed segregation patterns (**Figure1 & 11**). In diploid almond by almond crosses, the possible interaction between functional and non-functional forms of the BF gene(s) is possible because each parent will contribute a genetic

factor and the presence of a functional factor may act to mask the presence of a nonfunctional BF-factor.

However, ongoing research with almond by peach interspecies hybrids, (**Figure 11**), has demonstrated that the very early flowering peach tester (UCD40A-17) appears to lack a BF-type gene and so would not act to mask any aberrant BF-gene expression of the almond parent tested. With no homologous BF-functional gene to mask the expression of BF-expressing genes, progeny should show BF-symptoms when BF-forms of the gene are present and normal symptoms when normal BF-forms are present. Because BF-factors would be inherited entirely from the almond parent, the performance of the peach by almond progeny could be used to precisely determine the almond parent genotype as shown in **Figure 11**. If the almond parent contained no BF-inducing factors/genes then no progeny would show BF (solid basal red line in **Figure 11**). If the almond parent had one BF factor and one normal factor than only half the progeny would be expected to eventually show BF (curved rust line in **Figure 11**). If both factors/genes in the tested almond parent were BF then all progeny would be expected to eventually show BF (dotted line in **Figure 11**). Thus progeny performance can identify the BF-potential of almond parents even when no BF has previously been observed in those parents, though the test requires several years for completion. In addition, data from earlier studies suggest that the strength of BF-potential in the almond source will be correlated with the rate of BF expression in the seedlings and the final level of BF expression in individual seedlings.

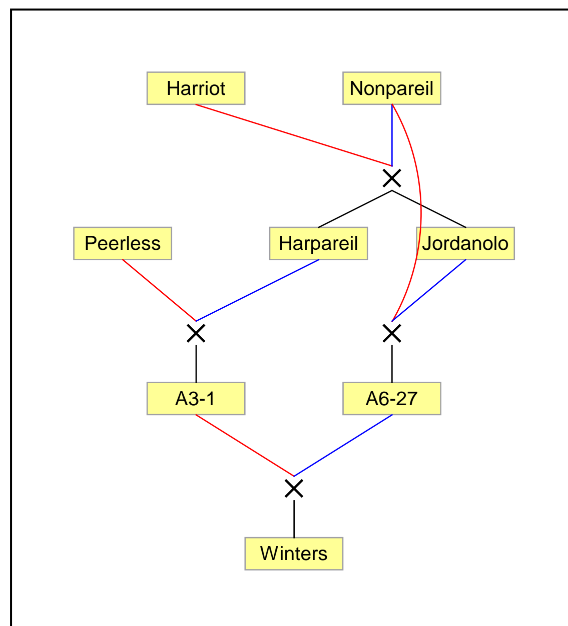


Figure 12. Lineage analysis of the Winter's variety as developed using PediMap software which may allow the correlation of BF-expression with specific molecular markers (if determined by genetic differences) and possibly epigenetic markers (if determined by a change or alteration in gene function/activity).

Thus, while test-cross progeny from an almond x high-BF almond cross are useful in identifying low-BF sources within the same clone, test-cross progeny from almond x early-flowering peach testers are useful in the early identification of general BF-potential of new breeding selections and varieties such as Winters, Sweetheart and Kester. We are currently in the 7th year of progeny testing from a Winters by UCD40A-17 test cross. Of 25 individuals in the population, none has shown budfailure to date though according to the peach-almond gene model, approximately 30% of the individual should be showing budfailure if Winters was a strong carrier. Similar results have also been obtained with Sonora and other well-established almond cultivars such as Peerless, which have occasionally shown budfailure symptoms, but only in isolated instances. Because of Winters unique and well-established lineage (**Figure 12**) including having both the BF vulnerable Nonpareil and Jordanolo as parents, this variety as well as high-and low-BF Nonpareil clones and breeding selections are being further analyzed using high-resolution genetic mapping. Association mapping procedures could then be used to identify certain marker combinations in progeny which are always associated with

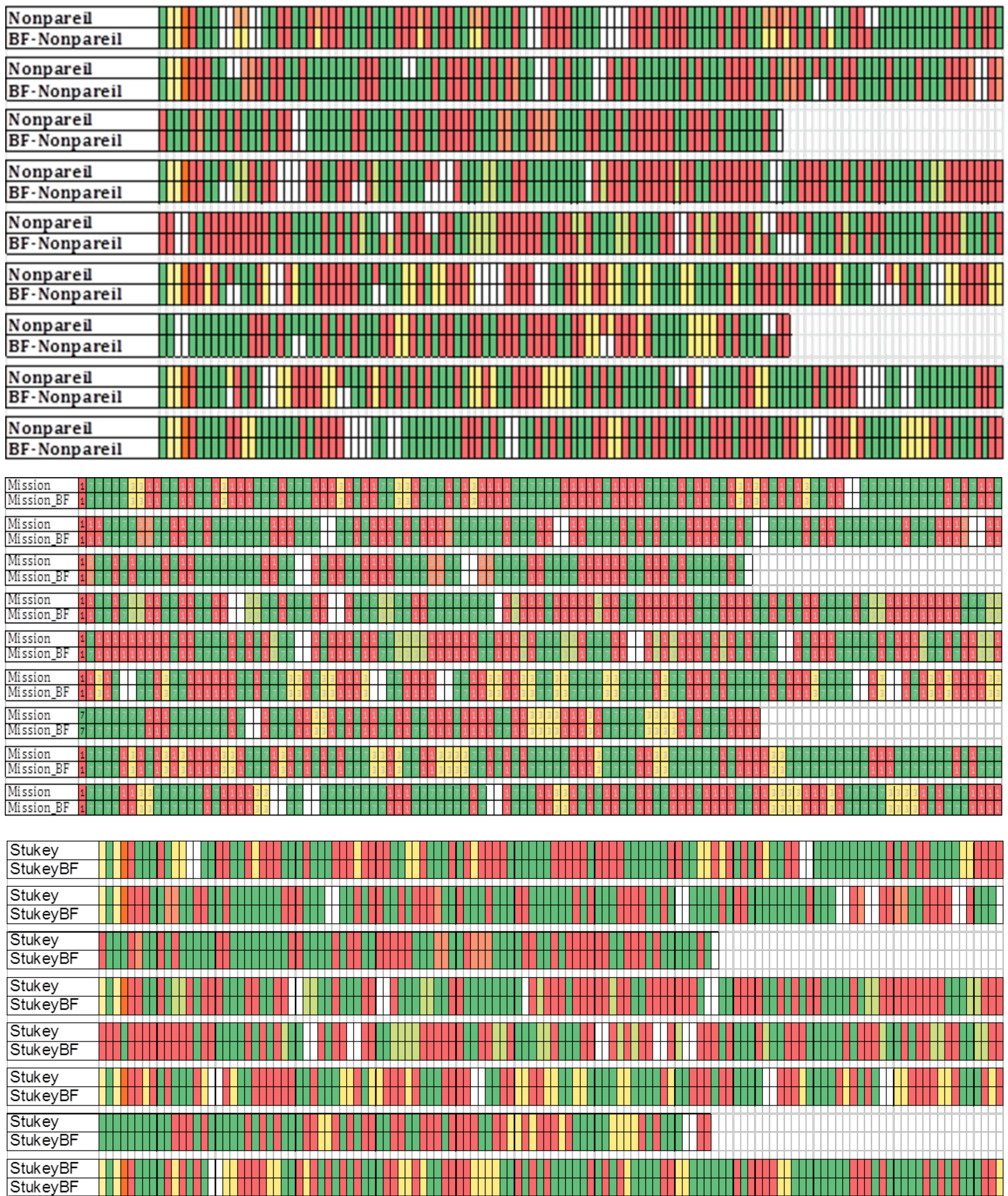


Figure 13. Sampling from final RosBreed analysis of high-BF versus low-BF Clones of *Nonpareil*, *Mission* and Stuckey (a synthetic clone derived from embryo budding) showing no genetic differences in the over 500 markers saturating all eight Prunus chromosomes.

BF expression even if that DNA not causative (i.e. the BF-gene). These genes might then be used as markers (since their association with that trait indicates that they are closely linked to the causative gene) as well as a starting point to identify the specific causative gene. Final results from the 2010-2014 RosBreed-1 project, however, have shown no marker differences between high-BF and low-BF clones of key varieties (**Figure 13**). Results support, but do not prove, the hypothesis that BF is due to a change in function (epigenetic change) of the BF-gene rather than a change to genetic nucleotide structure (mutation) which is required for standard marker assisted selections.

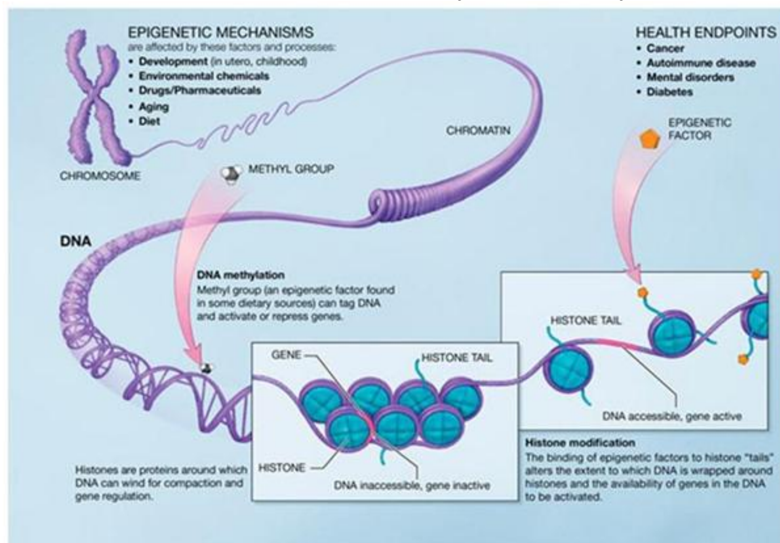


Figure 14. A summary of possible epigenetic mechanisms from more advanced human studies where control of a trait is determined not just by the simple presence or absence of a gene but rather by epigenetic mechanisms which act to enhance or suppress expression of genes.

Because, as previously described, genetic differences may be discernible in certain intraspecific peach by almond hybrids as presence/absence rather than variability in the expression level, several hundred progeny from a high-BF Carmel by UCD40A-17 test cross, in which progeny are expected to strongly segregate for BF (based on previous performance), have been generated in 2011-14 and planted in 2014 and 2015. The presence and extent of BF in individual progeny trees will be rated based on criteria developed in literature (see reference # 12). Information on the time that BF was first observed in individual progeny trees will also be included in the database. The rate of BF progression in both individual trees as well as in the combined progeny population will be evaluated as a possible predictor of BF-potential of the almond parent variety. Inheritance models supported by this preliminary data will then be evaluated. Previously established genetic relationships (see citations 3, 4, 8) among almond varieties tested will also be considered when evaluating inheritance models.

Epigenetic model.

Standard genetic dogma states that a trait such as BF results from the action of a specific protein controlling a specific plant developmental process. Since the specific protein structure is coded for by a unique sequence of DNA (gene), the definitive marker for that trait is the DNA sequence coding for the controlling protein. This model has proven successful in describing and genetically manipulating numerous processes in plant development and has led to a proliferation of accurate DNA-based molecular markers for many traits. Previous findings suggest that BF is a genetic disorder in almond which is expressed as a failure of vegetative bud growth leading ultimately to tree decline. Current data indicates that BF does not fully follow the standard genetic model but rather is due to the failed expression of a gene/gene complex required for normal growth and development. In this case the DNA sequence (gene) is identical in both the normal and BF condition, obviating the value of traditional molecular markers as predictors of this disorder. The aberrant nature of such 'epigenetic' conditions

have discouraged their research in mainstream genetics with most early studies limited to genetic disorders with dramatic economic consequences, such as almond BF and cherry crinkle and also some disorders and diseases in humans (**Figure 14**). Recent advances in our understanding of organismal genomics has shown that a diversity of epigenetic mechanisms exists which can play important roles in development. This realization has led to a research surge on epigenetic mechanisms, including the development of more accurate molecular-based diagnostics and possible treatments. For models based on standard Mendelian-gene control, a diverse array of molecular-based diagnostics is available (as summarized in literature citations 3, 4, 8, 14, 16, 21). In this case, the choice of molecular diagnostic would be made using standard marker assisted selection approaches such as PediMAP/Flex QTL software (see **Figure 13** and citation 8). Initial field data, however, shows non-Mendelian segregation patterns, again supporting epigenetic control. Unlike Mendelian genetic control, where genes/traits are either present/absent, epigenetic mechanisms can vary in their degree of trait suppression resulting in varying levels of BF-phenotype.

Figure 15. 104-year-old Mission (top-left) and Nonpareil (top-right) trees use for clonal age sampling. (Bottom left). Sample of the fruiting spur from the top of the trees from which 'Top' leaf samples were collected for testing. (Bottom-right, arrow) epicormic shoots from the base of the trees from which 'Base' leaf samples were collected.

Epigenetic analysis.
Thirty-seven different selections showing either differences in BF, differences in clonal age, or both, were selected for testing (**Table 2**). BF- level was determined by vegetative progeny testing as described in Kester et al. (Ref. 12). High BF rating indicated that the sample was taken from a tree showing BF expression. Medium-to low-BF ratings were given to trees that showed no BF expression but were known by from previous vegetative progeny testing to

Table 2. Thirty-seven different selections showing either differences in BF, differences in clonal age or both.

C#	Item	Source	BF potential	Age
1	Carmel	WEO	High	
2	Carmel-Mod-BF	Arb-Marine Rootstock	Medium-Hi	Medium
3	Carmel-OK	FPS19-9	Medium	Medium
4	Drake-Base	Esparto	No-BF	Low
5	Drake-Middle	Esparto	No-BF	Medium
6	Drake-Top	Esparto	No-BF	High
7	Mission-BF	WEO-BF	High	Medium
8	Mission-OK	FPS19-13	No-BF	Medium
9	Nonpareil-Base-OK	Esparto	Low	Low
10	Nonpareil-Top-BF	Esparto	High	High
11	Nonpareil-BF	Arb Nonp Lane	High	Medium
12	Nonpareil-Mod-BF	Arb-Marine Rootstock	Medium-Low	Medium
13	Nonpareil-OK1	FPS21-17	Medium-Low	Medium
14	Nonpareil-OK2	FP21-25	Medium-Low	Medium
15	Nonpareil-OK3	FPSxx-x	Medium-Low	Medium
16	TurkmenTopBF	Repo	High	Medium
17	TurkmenBaseOK	Repo	Low	Medium
18	Peerless-Base	Esparto	No-BF	Low
19	Peerless-Middle	Esparto	No-BF	Medium
20	Peerless-Top	Esparto	No-BF	High
21	Primal 161	2007,12-161	No-BF	Low
22	Primal 164	2007,12-164	No-BF	Low
23	Primal 192	2005,20-192	No-BF	Low
24	Primal 209	2007,12-209	No-BF	Low
25	STU 5-1-OK	F5C, 5-1	No-BF/Lo	Low
26	STU 5-2BF	F5C, 5-2	High	Low
27	STU 6-BF	F5C, 6-7or9	High	Low
28	STU 6-OK	F5C, 6-8	No-BF/Low	Low
29	Winters-OK	FPS2137	No-BF	Medium
30	Winters-BF	DELTA-BF-BROWNE	High	Medium
31	BF Winters-Upper	R11 fr N east	High	Medium
32	BF Winters-Lower	R11 fr N east	High	Medium
33	"Healthy" Winters	Greg Browne	No-BF	Medium
34	Carmel A	Billings-Kern	Mid	Medium
35	Carmel B	Billings-Kern	Mid	Medium
36	MONTEREY-OK	FPS21-13	No-BF	Medium
37	MONTEREY-PeudoBF	Arb-Marine Rootstock	No-BF	Medium

show medium-to low-(respectively) levels of BF expression in vegetative progeny trees (i.e. clonal trees propagated from that individual). 'No-BF' ratings were applied to selections showing no BF expression in the selected tree as well as vegetative progeny from that tree or clone. STU 5 and STU 6 (samples 25-28) were uniquely paired trees derived from the same initial embryo which divided very early in development to produce twin-seed (two embryos within the same seedcoat). As such, they represent identical genotypes (see **Figure 13**) that differ in phenotype (including, in this case, BF expression) presumably because of epigenetic differences possibly associated with different methylation patterns. Primal selections (samples 21-24) represented unique and rare peach genotypes which appear to spontaneously revert to a wilder almond-type tree leaves and fruit.

Samples differing in clonal age were also collected from 104 year old almond trees from a dryland almond orchard in the Capay Valley near Esparto, California (**Figure 15**). Leaf samples were collected from the most recent top growth, from mid-level growth, and from epicormic shoots at the base of the tree.

The Methylation-sensitive representational difference analysis (MS-RDA) procedure was followed as recommended by Ushijima and Yamashita (Ref. 17) and adapted as needed for almond samples (which usually contain high levels of phenolic compounds). MS-RDA is a genome subtraction method that isolates DNA fragments differentially methylated between two genomes.

MS-RDA Results and discussion.
Over 12,000 markers were evaluated for clones of different BF-potential as well as clonal age. Promising markers showing clear differences among the tested samples were identified using the Methylation-Sensitive Representational Difference Analysis (MS-RDA). This technique utilizes the methylation-sensitive restriction endonuclease HpaII to recognize the 5'-CCGG-3' 4-bp motif and thereby isolates DNA fragments differentially methylated within clone variants {orange boxes} (**Figure 16**). This test represents one of the first successful applications to Prunus and the first in almond, of epigenetic differentiation via methylation patterns. Because of the huge amount of data generated, the major challenge is accurate interpretation of the data. This is typically achieved through powerful

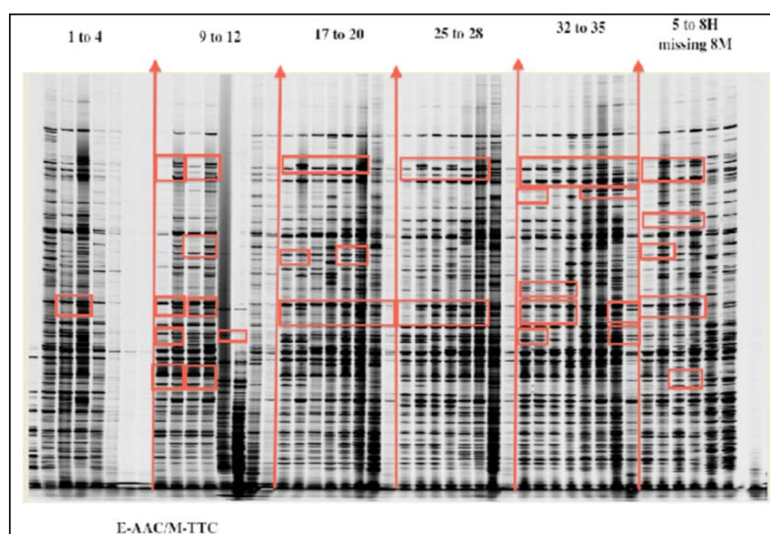


Figure 16. Results from 2013 showing a large number of variable and so potentially useful markers from MS-RDA, which utilizes the methylation-sensitive restriction endonuclease HpaII to recognize the 5'-CCGG-3' 4-bp motif and thereby isolates DNA fragments differentially methylated within clone variants {orange boxes}.

statistical packages which can identify significant associations in the data (in this case between certain methylation patterns in the degree of BF and, separately, the clonal age of the cultivar

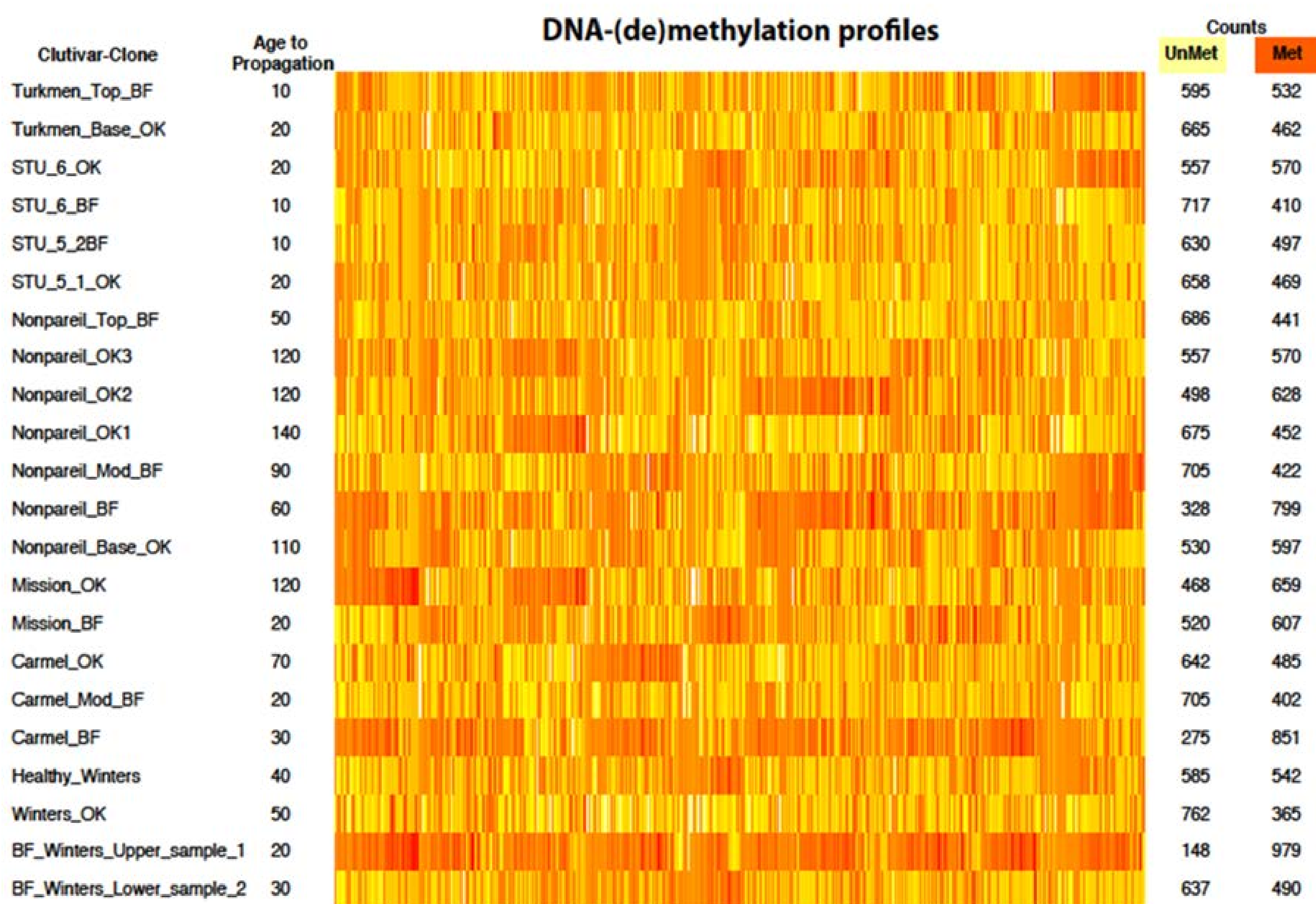
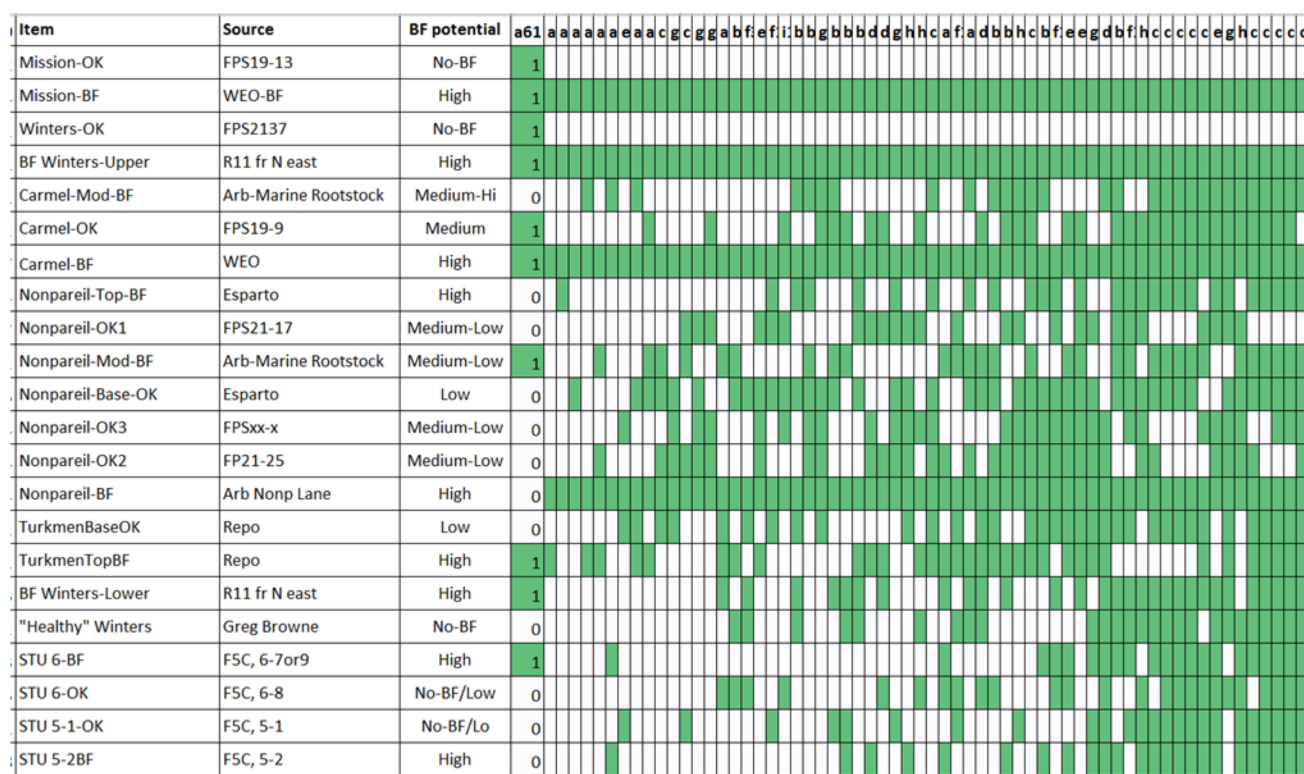


Figure 17. Graphical summary of DNA methylation/demethylation profiles for the evaluated clones. Propagation age is given in estimated years from clone divergence from non-BF to BF types. Counts (right columns) for the number of unmethylated to methylated markers.

being tested. Within any individual clone, we would also expect to see a correlation between clonal age and potential for showing BF. The difficulty with such statistical analysis is that the results are only as good as the data. While a certain amount of error is to be expected in the development of the marker profiles (for example, see discussion of RosBreed marker errors in the 2012/13 Almond Variety Development report), the bigger challenge here is an accurate characterization of both clonal age and BF potential as both involve a large degree of estimation. (Differences in clonal age are given in estimated years from clone divergence from non-BF to BF types). DNA methylation /demethylation profiles for the pooled data are summarized in **Figure 17**. The graphical display strongly hints at differences between BF and non-BF types but it is difficult to determine whether these represent associations within certain clones or broader correlations between specific markers and BF-phenotype. **Figure 18** displays results when markers are selected based on Mission BF phenotype and then

compared to a more limited number of other genotypes were BF potential is more clearly defined. Interestingly, almost identical patterns are observed between non-BF and BF Mission and non-BF and BF Winters with some additional agreement with other genotypes. Because BF is particularly rare in both Mission and Winters, they were felt to be good initial candidates for inspection. However the extensive nature of these differences as well as their apparent general diffusion throughout the eight chromosomes (data still under evaluation) suggests that the association may be primarily with clonal age and only secondarily with BF (since BF is expected to be correlated with clonal age based on the epigenetic model).

A pooled analysis of all the methylation-sensitive markers with BF expression for different clonal sources using more powerful, but obtuse statistical methods shows greater promise for differentiating between clones of differing levels of BF as well as different 'age' status (**Figure 19**). Several, though not all, profiles suggest the ability to differentiate BF from non-BF clones. The ability to differentiate clones with low BF potential (no BF expression in original tree and vegetative progeny from that tree) versus medium BF-potential (no BF expression in original tree but some levels of BF expression in vegetative and/or sexual progeny) also appears promising for some clones are not others. [This ability would be crucial to the application of such markers since the real need by industry is to be able to select among non-symptomatic clones for those sources that will remain nonsymptomatic in vegetative progeny and against those sources which would show BF symptoms (within the first 5 to 10



Item	Source	BF potential	a61
Mission-OK	FPS19-13	No-BF	1
Mission-BF	WEO-BF	High	1
Winters-OK	FPS2137	No-BF	1
BF Winters-Upper	R11 fr N east	High	1
Carmel-Mod-BF	Arb-Marine Rootstock	Medium-Hi	0
Carmel-OK	FPS19-9	Medium	1
Carmel-BF	WEO	High	1
Nonpareil-Top-BF	Esparto	High	0
Nonpareil-OK1	FPS21-17	Medium-Low	0
Nonpareil-Mod-BF	Arb-Marine Rootstock	Medium-Low	1
Nonpareil-Base-OK	Esparto	Low	0
Nonpareil-OK3	FPSxx-x	Medium-Low	0
Nonpareil-OK2	FP21-25	Medium-Low	0
Nonpareil-BF	Arb Nonp Lane	High	0
TurkmenBaseOK	Repo	Low	0
TurkmenTopBF	Repo	High	1
BF Winters-Lower	R11 fr N east	High	1
"Healthy" Winters	Greg Browne	No-BF	0
STU 6-BF	F5C, 6-7or9	High	1
STU 6-OK	F5C, 6-8	No-BF/Low	0
STU 5-1-OK	F5C, 5-1	No-BF/Lo	0
STU 5-2BF	F5C, 5-2	High	0

Figure 18. Association patterns for markers selected for differential expression between BF and non-BF expression in the cultivar Mission for BF occurs but is very rare. An almost identical pattern is observed in Winters were BF expression is also very rare, suggesting markers may be more for clonal aging residents specific BF phenotype.

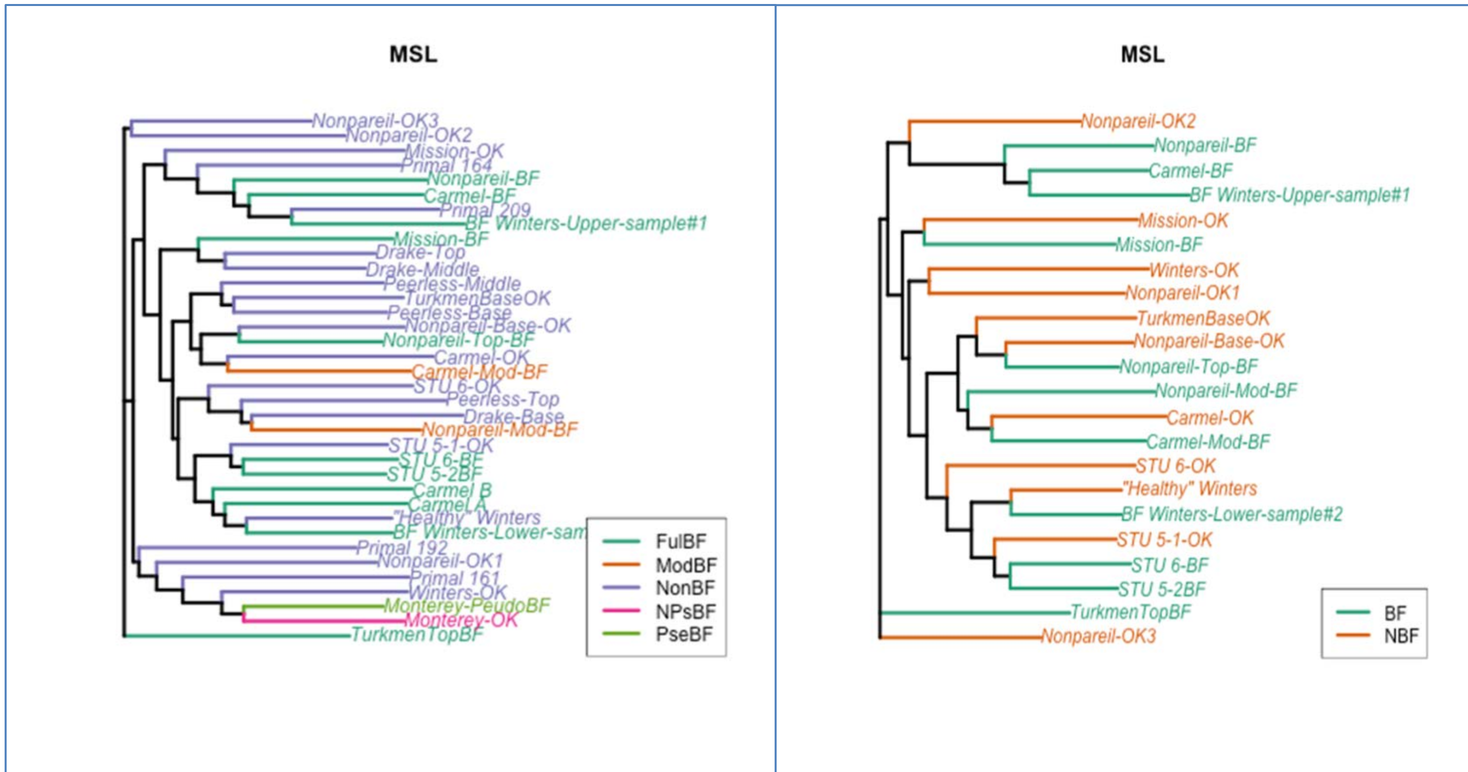


Figure 19. Pooled statistical analysis of all the methylation-sensitive markers with clone age and/or BF expression for different clonal sources shows differing differentiation profiles depending on initial assumptions (estimations) for clone age and specific level of BF-potential. Several, though not all, profiles suggest the ability to differentiate BF from non-BF clones. The ability to differentiate clones with low BF potential (no BF expression in original tree and vegetative progeny from that tree) versus medium BF-potential (no BF expression in original tree but some levels of BF expression in vegetative and/or sexual progeny) also appears promising for some clones are not others.

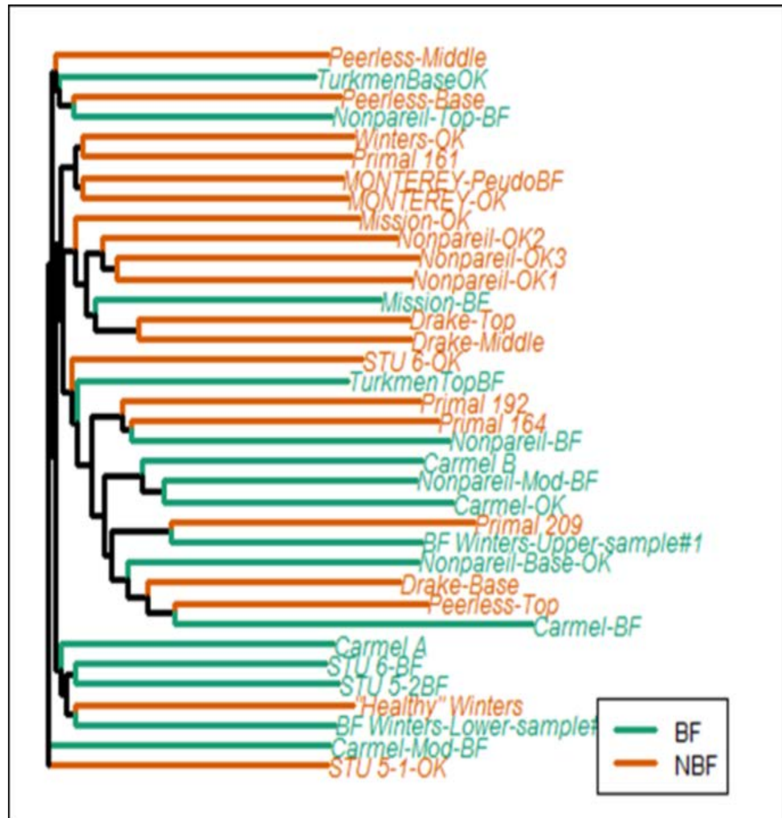


Figure 20. Markers selected for their strong association with BF expression in a subgroup of clones whose BF-potential scores were based on more extensive field testing of BF expression in vegetative progeny, resulting in more accurate BF scoring. Two markers (blue arrows) show good correlation between marker expression (level of methylation) and BF expression, and so represent candidates for more detail genetic analysis.

years) in vegetative progeny]. Because of the value of such a marker, even utility within a single clone such as Nonpareil or Carmel, would be particularly useful, though they may inherently be more error-prone if the marker is for closely associated but not causative epigenetic event. Thus while statistically analyzed data is very promising, the verification of utility will require further analysis both in focusing in on markers most strongly associated with clonal aging and/or BF expression, and concurrently, more accurately defining what is meant by clonal age in BF expression. Consequently, success will depend on large-scale statistical analysis anchored by an accurate characterization of the developmental processes involved. Thus we are pursuing the analysis in a convergent process where we are using the statistical analysis to identify promising marker candidates and then testing whether those candidates facilitate the development of a specific developmental model is a specific genetic targets for further analysis. For example, in **Figure 20**, two markers have been identified which show a very high association with BF expression in a select group of clones were competence in the level of BF-potential is based on an extensive assessment of vegetative progeny populations (see **Figure 6**) and so more dependable. This approached has refined the number of promising markers. For example, **Figure 21**, two different markers a29 and i92 show good (though not perfect) agreement with BF Potential level as calculated in one of several specific models currently under consideration. In this analysis, the initial association was tested for samples from the 104 year-old Nonpareil tree (**Figure 15**) since both sample age and BF level were better defined and external variables were minimized. Because over 1000 makers were analyzed, the possibility exists that the observed association are by chance only and not indicative of a causal relationship. (In this analysis, over 200 markers showed positive association of increasing methylation levels with the 3 categories of increasing tissue age (early, intermediate and late), while over 100 showed a negative association, demonstrating the opportunities for random associations or associations with clone age, independent of BF potential (though this would still be a useful indicator of BF potential within clones).

a29	i92	BF Potential	Item
1	0	5	Nonpareil-Top-BF
0	0	3	Nonpareil-Mod-BF
0	1	1	Nonpareil-Base-OK
1	0	5	Carmel-BF
0	1	4	Carmel-Mod-BF
0	1	2	Carmel-OK
1	0	5	Mission-BF
0	1	0	Mission-OK
0	1	5	TurkmenTopBF
0	0	3	TurkmenBaseOK
1	1	5	Nonpareil-BF
0	1	1	Nonpareil-OK3
0	1	1	Nonpareil-OK1
0	1	1	Nonpareil-OK2
1	1	4	Winters-BF Upper
0	0	3	Winters-BF Lower
0	1	0	Winters-Healthy
0	0	0	Winters-OK
0	1	5	STU 5-2BF
0	1	3	STU 5-1-OK
0	0	5	STU 6-BF
0	1	3	STU 6-OK

Figure 21. Methylation markers a29 and i92 positive association with BF Potential level as calculated in one of several specific models currently under consideration. In this analysis, the initial association was tested for samples from the 104 year-old Nonpareil tree (**Figure 15**) since both sample age and BF level were better defined and external variables were minimized. Results were then compared against other clone samples where origin and BF potential were more clearly defined either because samples were from a single tree (top Nonpareil rows, Winters, Turkmen) or related clonal sources (Carmel and Mission). STU samples are unique in they are derived from early budding of the developing seed embryo and so are identical in genotype and environmental history, differing in epigenetic status. (Markers a29 shows a positive association with BF while i92 shows a negative association. They are also located on different chromosomes).

We are currently working on a more ambitious Bayesian analysis of the data which incorporates more stringent definitions of BF potential and clone age (though most involve considerable speculation and so are primarily useful for initial modeling. While any markers (such as a29 and i92 in **Figure 21**) would thus be tenuous, our plan is to test the genes they are closely linked with against the growing database of genes involved in this type of development. A prime target would be genes involved in dormancy or endodormancy. Endodormancy in almonds refers to the summer growth suppression which occurs under normal dryland conditions. This summer dormancy can be characterized by suppression of continued shoot growth in early summer (typically June in California) even when terminal buds are pinched or removed and cultured in controlled growth chambers (**Table 3**). Normal endodormancy is suppressed under commercial high water/fertilizer conditions and this suppression is believed associated with later failures to achieve (winter) dormancy in BF susceptible clones (i.e. The characteristic bud decline during fall when dormancy should be occurring). [This is why little BF is observed under traditional Mediterranean culture but does show up, even with local cultivars, when high input farming is introduced]. **Table 3** summarizes

some early work which shows a clear association with summer endodormancy in almond and level of BF expression in different Nonpareil and Carmel clones. The identification of candidate dormancy/BF genes would then allow a much more thorough analysis of controlling DNA for genetic/epigenetic changes that would have values as predictors of BF potential in putative nursery source clones. In human epigenetic studies, which are much more advanced (i.e. better funded) than plants, opportunities to restore normal epigenetic states are now being advanced to clinical trials, suggesting opportunities for clone rehabilitation in varieties such as Carmel as well.

Table 3. Level of summer endodormancy (expressed as level of continued bud growth when removed and placed in culture) in clones differing BF expression level, showing lower BF when normal endodormancy is allowed to occur.

Material	***Treatment***	**% of shoots sprouted**	**% of bud sprouted**	**% of green buds**	**% of death buds**
Low BF	Control	33	3	52	45
	42ºC/1h	83	22	40	38
	42ºC/2h	83	19	51	30
Means		*66*	*15*	*48*	*37*
Medium BF	Control	33	5	29	66
	42ºC/1h	100	29	48	23
	42ºC/2h	66	21	53	26
Means		*66*	*18*	*43*	*39*
High BF	Control	0	0	26	74
	42ºC/1h	0	0	8	92
	42ºC/2h	16	1	6	93
Means		*5*	*0.3*	*13*	*87*

Appendix A. ELISA confirmation that BF-symptoms in Marcona are the result of infection by Prunus Necrotic Ringspot Virus (PNRSV; PDV – Prunus Dwarf Virus).

ELISA Testing for Marcona trees

	PNRSV		PDV
Marcona, tree BL7	positive	4/30/2010	negative
Marcona, tree DRT3	positive	4/30/2010	negative
Marcona, tree DRT4	positive	4/30/2010	negative
Marcona, tree DRT7	positive	4/30/2010	negative
Marcona, tree DRT11	negative	4/30/2010	negative
Marcona, tree DRT14	positive	4/30/2010	negative
Marcona, tree DRT18	positive	4/30/2010	negative

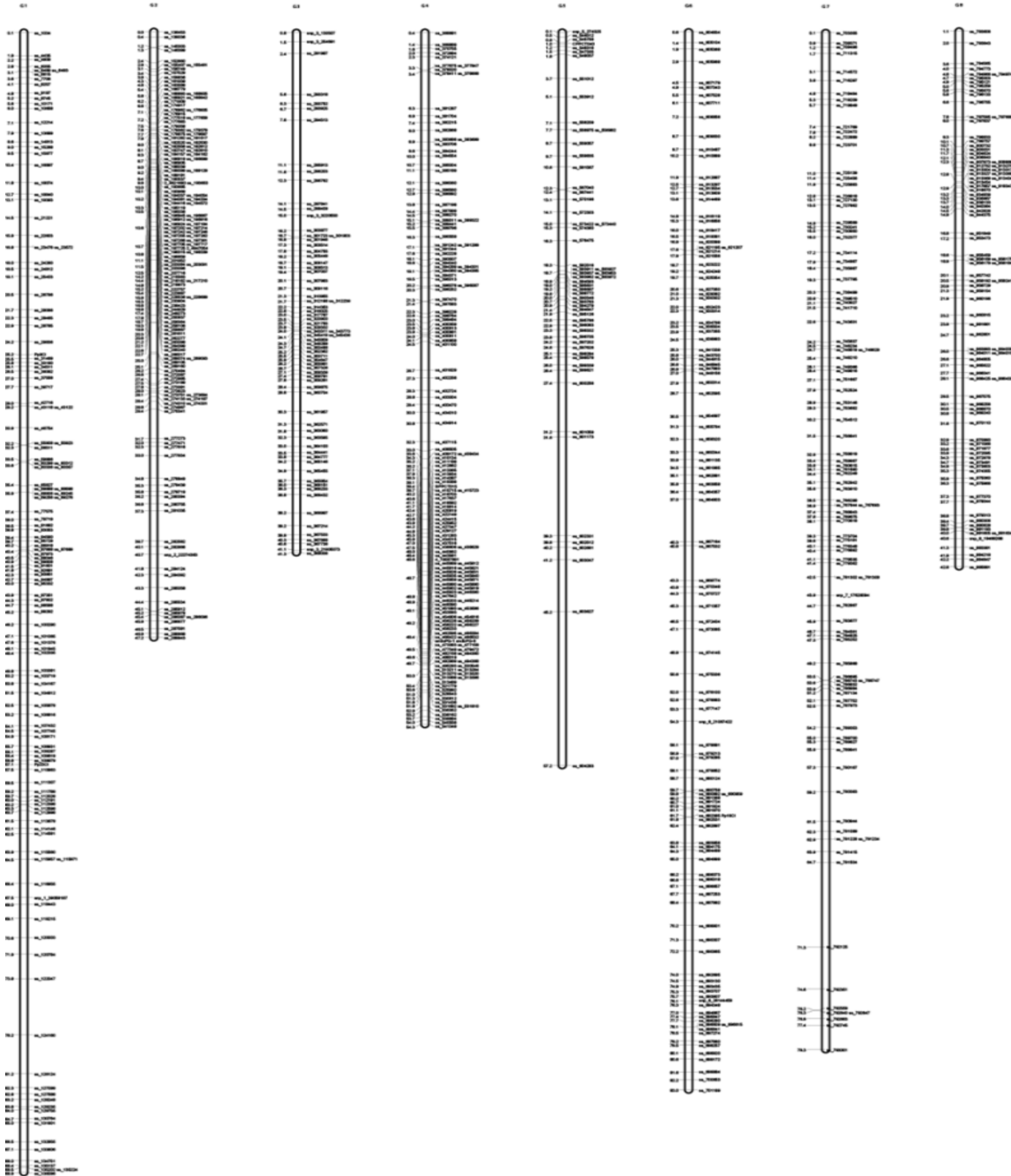
Appendix B. Molecular marker analysis verifying that the affected trees (Brown-Winters) are the Winters variety and not a propagation error.

NONPAREIL	182 194	130 148	142 146 211 233	99 110	212 259 224 236	155 155	148 158 151 167	115 121	134 152
PADRE	182 196	122 180	136 142 199 209	99 108	227 227 236 244	129 147	148 148 143 149	141 145	146 168
PRICE	194 196	146 148	130 148 199 211	110 114	212 227 224 236	129 155	148 148 167 167	121 141	134 166
RUBY	194 196	122 142	136 146 199 211	108 110	227 227 224 236	147 155	148 156 143 167	141 145	134 168
SOLANO	182 194	130 130	130 142 211 233	99 99	212 255 224 236	155 155	138 158		
SONORA	182 194	148 148	130 142 211 233	99 99	255 259 224 236	145 155	138 158 149 151	115 121	134 152
THOMPSON	194 216	122 130	130 142 203 211	99 114	212 227 224 236	147 155	136 148		
WINTERS	182 200	130 136	132 132 233 233	116 116	227 229 236 242	155 155	146 158 143 167	121 145	134 134
Brown-Winters	182 200	130 136	132 132 233 233	116 116	227 229 236 242	155 155	146 158 143 167	121 145	134 134

Appendix C. Yield performance of selections at the Billings Regional Variety Trials showing particularly high yields of Nonpareil clonal source (3-8-2-70) (from Bruce Lampinen RVT Annual Report)

				Kernel pounds per			
2011			Shelling	unit PAR int.	Tree	Acre	Cumulative kernel
Variety	No. of nuts/tree	Average kernel wt (g)	percentage				yield (lbs/acre)
Nonpareil-Nico	18776.9 a	0.99 bcde	68.0 abc	86.7 a	41.0 a	4964.2 a	19522.7 a
Nonpareil-3-8-2-70	17744.2 abc	1.05 bc	70.7 a	87.9 a	41.0 a	4962.3 a	18878.1 ab
Nonpareil-Newell	17790.9 abc	1.00 bcd	70.1 ab	81.0 ab	39.2 a	4744.7 a	18746.5 ab
Nonpareil-Driver	17943.0 ab	0.98 bcde	66.0 abcd	84.3 a	38.7 ab	4682.6 ab	18593.4 abc
Nonpareil-5	15744.6 de	1.03 bc	70.4 ab	78.0 ab	35.9 abc	4341.9 abc	17886.9 bcd
Nonpareil-6	16630.0 bcde	1.04 bc	70.0 ab	81.6 ab	38.1 ab	4618.5 ab	17838.3 bcd
2-19e	18253.3 ab	0.91 bcde	64.8 abcd	73.6 ab	36.8 ab	4459.7 ab	17560.0 bcd
Nonpareil-7	17078.8 abcd	0.83 e	69.2 abc	76.1 ab	31.4 bcd	3804.0 bcd	17235.0 cd
Nonpareil-Jones	16992.6 abcd	0.96 bcde	70.0 ab	81.6 ab	36.0 abc	4359.4 abc	17050.7 d
Winters	15979.0 cde	0.83 e	58.7 ef	76.3 bc	29.3 cde	3553.5 cde	14757.0 e
Chips	11900.6 f	0.94 bcde	60.3 de	51.4 de	24.6 de	2984.7 de	13917.8 e
Sweetheart	14969.2 e	0.86 de	64.1 bcde	52.5 de	28.2 de	3411.8 de	13712.5 e
Kahl	12420.0 f	0.89 cde	53.5 f	59.1 cd	24.4 de	2953.2 de	13514.3 e
Marcona	9633.4 g	1.07 b	30.8 g	51.8 de	22.7 e	2746.0 e	12053.7 f
Kochi	8701.4 g	1.22 a	63.5 cde	43.4 e	23.3 e	2825.2 e	11246.5 f

Appendix D. Improved almond by peach molecular marker map developed by our program (Ref. 22 and in review) using 864 markers (860 SNPs and 4 SSRs). Molecular markers in almond by peach test progeny which may be found to be highly correlated with BF expression can then be used both as a marker or predictor of BF as well as a starting point to identify the specific gene(s) controlling this trait.



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