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Noninfectious Bud-failure As a Model For Studying Age Related Genetic Disorders in Long-Lived Perennial Plants

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Abstract

Plants disseminated through vegetative propagation avoid the meiotic recombination and associated rejuvenation found during sexual seed propagation. The resulting natural and human selected clones allow accumulation of genetic as well as non-genetic (epigenetic) interactions as long as the studied trait remain true-to-type following vegetative propagation. The consequent 'immortalization' of the clone also allows large clonal population sizes and long-term plant lifespans required for the later accumulation of budsport mutations in such long-lived genotypes. While both natural and human selection result in clones with desirable changes, the long-term preservation of these clones also facilitate the occurance of genetic disorders. In almond (*Prunus dulcis*, DA Webb) Noninfectious Bud-failure (NBF) is an economically important disorder of California cultivars because it severely affects two major commercial cultivars, 'Nonpareil' and 'Carmel', and has led to the abandonment of many otherwise productive cultivars and breeding selections over the last half century. NBF expression shows some epigenetic characteristics including an increase in expression with tree as well as clone age, so that new cultivars may possess the NBF factor but it remains latent for years to decades. The resulting tree and clone aging 'time-bomb' makes this disorder particularly devastating since extensive commercial plantings may have been established before the disorder is first identified. Because of this economic significance, clone lineage or clone-source selection strategies have been developed to identify this disorder within breeding lineages as well as within individual nursery propagation sources. Proven though somewhat tedious phenotype-based methods for identifying NBF-associated factors, both among and within genotypes have proven effective in 30 years of field testing. Results from preliminary characterizations of DNA-(de)methylation profiling using methylation-sensitive amplified fragment length polymorphisms (MS-AFLP) targeting NBF and the related aging-process within individual plants as well as within individual clones has provided promising insights. Challenges remain in identifying both the mechanism as well as governing tissue for clonal age-memory in plants.

Aberrant performance in vegetative and/or reproductive growth of clonal tree cultivars is the consequence of either outside (environmental) or internal (genetic and epigenetic) factors. Environmental disorders, such as those resulting from temperature, nutrient or pest/disease stresses, can often be remedied by modification of the environment, as through cultural management (i.e. pruning) and the application of appropriate agrochemicals. Genetic disorders refer to those problems that, once established, are usually permanent and are not rectified by standard cultural practices, presumably because the problem arose from deleterious changes at the genetic level of plant development. Until effective molecular diagnostics become available, the standard indicator of genetic disorders was that all attempts to remediate and/or identify the disorder using standard practices had so far failed. Although the genetic mechanisms remain unknown, the disorders often display characteristic patterns indicating losses in required gene function. Within an individual plant, the disorder can be expressed either

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Fig. 1. Diagram showing the ramified or branching pattern typical for tree growth. Disorders are typically expressed as a spontaneously occurring bud-sport where all subsequent growth of a shoot is affected (red) or as a successive age-linked pattern where new growth of roughly similar age is expressed throughout the tree canopy (yellow).

spontaneously, affecting distinct branches, or progressively, becoming more pronounced with general plant age (Fig. 1). Because these novel phenotypes are often brought about by discrete genetic or epigenetic changes, clonal plants propagated from buds from affected limbs would very likely be genetically indistinguishable from those propated from unaffected limbs by most molecular diagnostics. Trees propagated from the budsport branch often continue to show the aberrant symptoms in progeny trees. Such genetic or epigenetic-like disorders are relatively common in long propagated Prunus clones including 'Rusty-Blotch' and 'False Shot-Hole' disease in plums, 'Russet-Scab' in prune, 'Gumboil' in apricot, and 'Crinkle-Leaf ' disease in cherry and plum (Ogawa and English 1991, Hadidi et al. 2011). More recently, a propagation disorder of the UCB1 pistachio rootstock referred to as 'bushy-top' syndrome has resulted in multi-million-dollar losses in California though a definitive cause has yet to be determined using genomic and epidemiological methods (Randall et al., 2018). The careful selection of propagation source-material remains the only available control option though selection strategies remain poorly defined. The identification of reliable true-to-type propagation-sources for horticultural clones (i.e. the source-clone) is thus of paramount importance to plant propagators and breeders. Source-clone quality is evaluated by careful examination of the source tree as well as by careful and long-term evaluation of clonal pedigrees, including vegetative progeny previously propagated from that source. Noninfectious Bud-failure (NBF) is a disorder expressed as a nonpathogenic failure of vegetative bud development leading to tree decline and decreased reproductive growth that directly affects commercial yield. NBF was shown to be inherited in both vegetative and sexual progeny with predictable levels of expression that was related to the age and propagation history of a specific clone-source (Kester, 1978, 1974; Kester et al., 2004; Kester et al.,

2003). NBF remains a major threat to almond (*Prunus dulcis*, DA Webb) production in California as it severly affects two major cultivars, 'Nonpareil' and 'Carmel' that together make up almost 50% of the 540,000 ha of commercial plantings. Recent research has proposed genetic ageing mediated by DNA-(de)methylation as a mechanism involved in NBF exhibition (Fresnedo-Ramírez et al., 2017). Consequently, the involvement of ageing for both the selection of propagation-wood as well as selection of parents for breeding needs to be understood.

This review uses NBF in almond as a model system for the study of aging related disorders in perennial crop plants, a neglected topic in horticulture considering its high commercial importance. Strategies for effective source-clone selection, management, and maintenance are presented that have proven effective in long-term, field-based trials. These strategies have allowed continued commercial production of vulnerable cultivars as well as the rehabilitation of strongly affected cultivars. In addition to providing rational approaches for the selection of propagation and breeding source material, the resulting model-based performance offers insights into the genetic/epigenetic basis of this disorder. The approach should provide similar basic and applied information for identifying and managing genetic disorders of other clonal crops.

Spontaneous vs. gradually progressing genetic disorders

Changes or mutations in gene expression result from nucleic acid changes in the genes themselves or through epigenetic changes in the genetic organization including chemical/structural components (Fig. 2) which act to regulate gene activity (Campisi and Vijg, 2009; D'Aquila et al., 2013; Garinis et al., 2008; Thomas, 2004). Known epigenetic-like mechanisms that can modify the level of gene

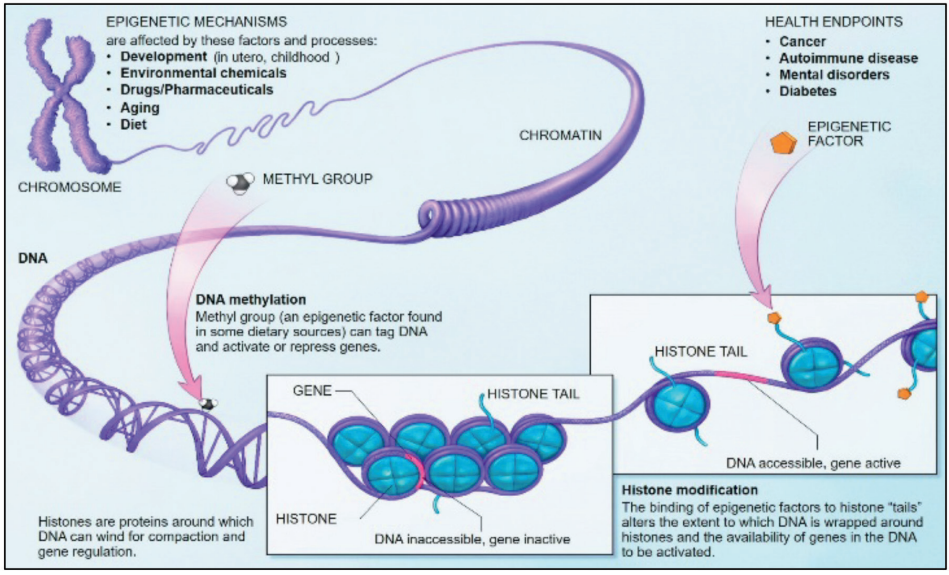

Fig. 2. Proposed epigenetic mechanisms from human research where control of a trait is determined not just by the presence or absence of a gene but also by epigenetic mechanisms which act to enhance or suppress its expression. (National Institutes of Health ttp://commonfund.nih.gov/epigenomics/figure.aspx, Public Domain, https://commons.wikimedia.org/w/index.pp?curid=9789221)

Fig. 3. A spontaneous bud-sport in almond showing distorted narrow leaves, sterility and low vigor (left) compared to normal growth at right. The causal mutation was the presence of an extra chromosome in the nucleus (Martínez-Gómez and Gradziel, 2001).

expression include changes in chromosome number and organization, changes in histone, chromatin and other proteins, which make up the structure of the chromosome, as well as modifications in the chemistry of the DNA nucleotides, such methylation, and ubiquitination or phosphorylation in the case of proteins (Messerschmidt et al., 2014). While most epigenetic changes are "reset" during epigenetic reprograming in the germline and subsequent seed development, clonal propagation often maintains and so can accumulate both genetic and epigenetic changes. This allows greater opportunities for both natural and breeding selection but also greater chances for genetic disorders. Most research on plant mutation rates have focused on the simpler annual, seed-propagated plant species where mutation rates to detrimental forms during lifetime mutation-accumulation experiments have been reported as high as 0.1% (Garinis et al., 2008). Mutation rates in woody perennial plants have been estimated to be up to 20 to 40 times greater because of their larger plant size (i.e. more total cells) and longer life expectancies (Munne-Bosch 2008). Because clonally propagated plants accumulate both genetic and epigenetic mutations, the probabilities for deleterious changes in gene expression would be expected to be even higher. Many mutations appear to have negligible impact on plant fitness and yield. Some mutations may add value to the cultivar. For example, 'Tardy-'Nonpareil' is a cultivar resulting from a mutation of 'Nonpareil' that delays bloom by approximately 10 days and so can improve frost avoidance. Most genetic or epigenetic change to critical genes will likely result in reduced fitness since the original genetic forms are the product of extensive natural selection for optimal function. Consequently, mutations of crucial genes are often lethal, resulting in rapid elimination of mutated cells from further growth. Deleterious though not lethal changes may survive which, if present in meristematic tissue, could affect future shoot growth from that point on, resulting in the appearance of a spontaneous 'budsport' (see Fig. 1 and 3). Since active meristems represent only a small fraction of the total cells in a plant, the majority of deleterious mutations will have little to no long-term effect. Because plant mutations are fixed in their source tissue (i.e. they cannot be transmitted to other parts of the organism as with animal cancers), bud-sport mutations will only be observable if present in actively growing meristematic cells and thus will affect only growth following the initial mutation event. In addition, because most mutations will be deleterious, the resulting growth often shows lower vigor and is usually outcompeted by neighboring unaffected shoots, and so consequently

rarely noted by observers (as in Fig. 3). Spontaneous budsport mutations suppressing fruiting, however, may consequently redirect energy to vegetative growth resulting in much larger but generally fruit-sterile 'bull' trees which often get attention because of the larger plant size and the reduced and often distorted crop.

Noninfectious Bud-failure.

In contrast to typical budsports, NBF is not limited to a specific branch or vegetative lineage. It can occur throughout the tree canopy once a certain shoot age has been achieved and occurs only in certain clonal lineages which themselves are at an advanced clonal age relative to propagation from the original seedling tree (Kester, 1978, 1974, 2004; Kester and Asay, R. N. 1978a, 1978b). NBF is characterized by the failure to transition to winter dormancy with subsequent death of terminal or subterminal vegetative buds during the previous fall. Vegetative bud death can usually be identified by a brown necrosis of the internal bud tissue (see lower inset in Fig. 4). Bud-necrosis can range from a small dark spot to the blackening and shriveling of the entire axillary bud and its budscales. The necrotic areas may also extend outwardly from

Fig. 4. Characteristic 'crazy-top' shoot development pattern of NBF resulting from a multi-seasonal pattern of die-back and regrowth. Lower inset shows the characteristic necrosis of bud meristems the previous fall with no further development of buds through the winter and following spring (upper inset).

Fig. 5. 'Rough-bark' trait sometimes observed in severe NBF.

the base of the leaf petiole and sometimes discolored internal tissue can be detected near the external lesions. Vegetative buds on affected shoots are often abnormally small. The diameter of the affected shoots may also be smaller than normal. Necrotic buds are essentially dead, and no swelling or development occurs during the ensuing winter months. The disorder becomes most evident with the failure of the vegetative buds to grow-out the following spring, resulting in sections of blind or bare shoots with the subsequent pushing of the more basal vegetative buds that often remain viable. When terminal buds fail, the shoots die-back (Fig. 4). On other shoots, particularly those that are most vigorous, the terminal sections remain relatively unaffected while the middle portion of the shoot shows signs of failure, giving a 'mule-tail' type of appearance.

Typically, multiple, flushes of vegetative growth during the summer affect the subsequent expression of NBF in susceptible clone-sources. Spur and shoot-flushes of growth, which are completed early in the spring, usually do not show bud-failure the following year. Shoots that have second flushes of growth, either terminally or laterally in late spring or early summer, generally show the most failure. Shoots that grow in the relatively cooler temperatures later in the summer have fewer failed buds. If the bud-failure potential of a given clone-source is sufficiently high, all buds on all shoots may fail. Growth patterns showing multiple flushes of vegetative growth, particularly during the heat of mid-summer, are unique to the high fertilizer and water input and subsequent high-yield almond cultural practices of California's Central Valley. In contrast, vegetative growth of wild almond as well as almonds grown under the more traditional dryland conditions of Mediterranean culture where NBF occurance is rare, typically show only a short flush of vegetative growth early in the spring with a second, smaller flush possible in late summer if sufficient moisture becomes available.

Flower buds are not affected even when developing from the same node as a NBF necrotic vegetative bud, and can often develop into fully formed nuts even in the absence of any nearby leaf growth (Fig. 4). Eventually, the number of flower buds become severely reduced, partly from the suppression of new fruit-wood and tree vigor. Bloom is also often delayed in NBF affected branches.

A distinct NBF characteristic is that once bud-failure symptoms develop, normal growth is not restored in subsequent seasons but rather the disorder gets progressively worse (though the extent and rate of symptom development may vary depending on growth rates, heat during the previous summer, and other stresses). This recurring sequence of terminal shoot-bud-failure followed by the pushing of viable basal buds, results in a punctuated and erratic shoot development pattern commonly termed 'crazy top' (Fig. 4). In some severe cases of NBF, the bark on young shoots can develop a characteristic splitting or cracking called 'rough bark' (Fig.

5). Rough-bark sometimes encircles the stem within distinct bands and locations on branches two or more years old. Necrosis and death of meristematic tissue also appear to be involved, but in this case, it affects the more general meristem responsible for growth and expansion of the cambium or bark.

Productivity losses from NBF have been sufficiently large to result in the abandonment of otherwise important cultivars and advanced breeding selections. In the mid-1900s, these included the 'Jordanolo' and 'Harpareil' and later 'Merced'. NBF remains an important concern for the two dominant California cultivars 'Nonpareil' and 'Carmel' and is expected to eventually occur in some of the newly released cultivars, making NBF one of the most economically important genetic disorders in cultivated plants (Kester et al., 1998).

Variability within genotypes (clones)

To assess variability within a genotype or clone, four propagation sources (source-clones) of the almond cultivar 'Carmel' with a known history of variable NBF expression were selected for long-term assessment. Approximately 200 vegetative progeny trees representing different source-clones were propagated onto Nemaguard rootstock and then planted in test plots in the western San Joaquin Valley where the high summer temperatures, that enhance NBF expression, are common. Trees were evaluated yearly for level of NBF expression using standardized criteria (Kester et al., 2004). Results plotted over time (Fig. 6) show differences in individual source-clone variability both for the time of initial NBF expression as well as the rate of increase within vegetative progeny populations once expression had occurred. The lowest levels of NBF expression were observed in trees propagated directly from the original 'Carmel' seedling tree. Similar though slightly higher expression levels were observed in trees one generation removed from the original seedling tree (i.e. from a budwood increase tree propagated

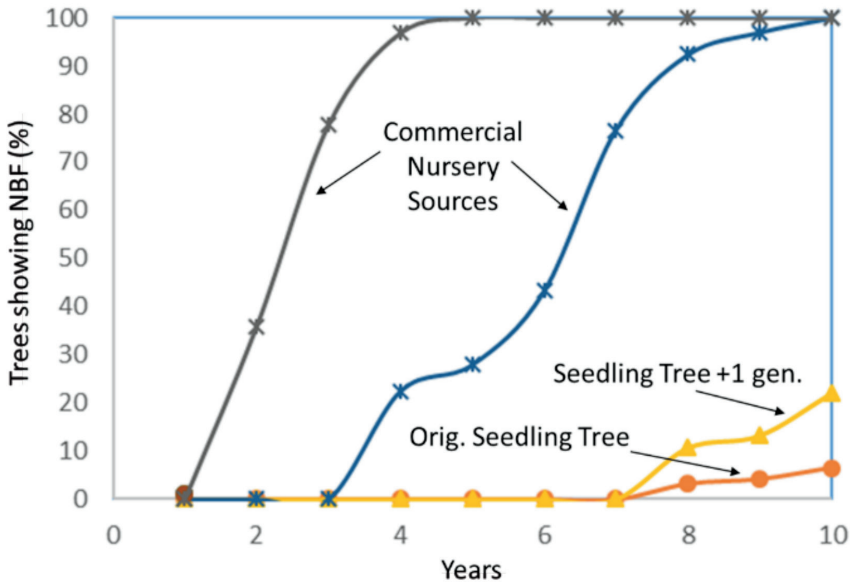

Fig. 6. Patterns of increase in NBF expression over time for vegetative progeny from different 'Carmel' propagation-sources (source-clones), allowing the identification of the most promising sources for future commercial propagation of low NBF trees.

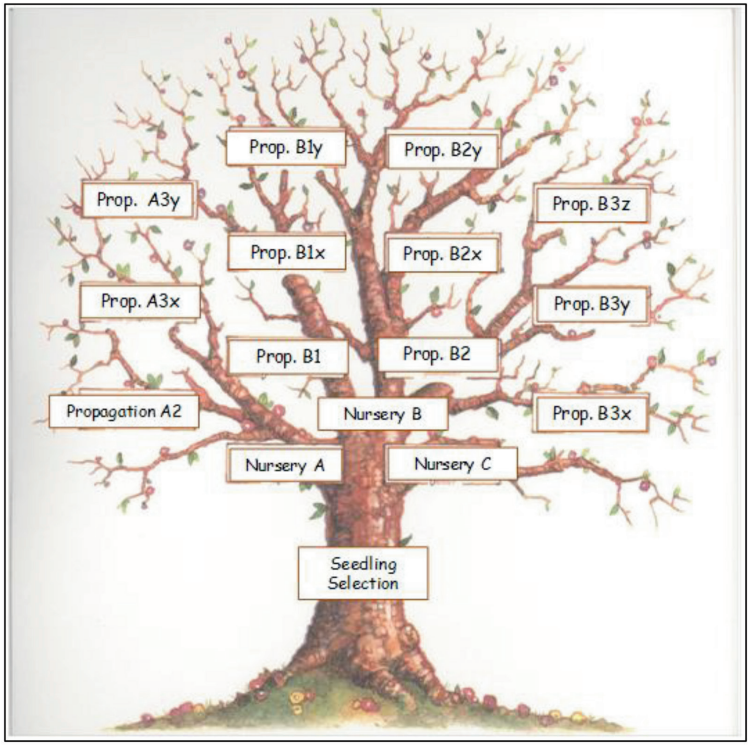

Fig. 7. Tree model for the increase in potential for NBF appearance either in an orchard tree or (analogously) nursery propagation sources.

from the original seedling). High levels of NBF expression were observed in standard commercial nursery sources. Vegetative progeny testing can thus determine the risk of different propagation sources for NBF even when the propagation lineage of that source is unknown.

In NBF, the critical fall vegetative degeneration appears to result from the deterioration in function of gene(s) vital to vegetative bud transition to winter dormancy. This deterioration appears to result from a gradual genetic 'aging' of a crucial dormancy factor, probably as a consequence of repeated growth-phase cycling or oscillations in affected cells (Fenton et al., 1988; Kester et al., 2003). Such cycling occurs during the yearly growth phases of almond shoots and appears to also occur, and may even be amplified, by cycles of vegetative propagation. The typically ramified propagation history of most vegetatively propagated clonal cultivars is thus analogous to the growth and development of a mature tree where each ramification or subsequent branching (either through orchard growth or through a series of vegetative propagations) reflects an incremental increase in clone-aging for that cultivar (Fig. 7). For example, the cultivar *'*Carmel*'* was first selected as a seedling tree in the 1960s. Initially, propagation mother-block trees were propagated using buds from that first seedling tree (represented as the trunk in Fig. 7). As *'*Carmel' became commercially important, the increased need for propagation-wood necessitated bud collection from young 'Carmel' production orchards which possessed the abundant

and vigorous shoot-growth needed for harvesting the required tens of thousands of vegetative buds (represented by the scaffolds in Fig. 7). As these plantings aged, new propagation wood was subsequently collected from more recent or younger clonal orchards because the more rapid vegetative growth in these younger trees produced better quality budwood. In this way a large number of successive sources for 'Carmel' propagation wood (clone-sources or source-clones) were developed, with each having a unique developmental lineage or branch-pattern but with all tracing back to the initial seedling selection. Since NBF appears to be determined by an internal 'ontogenetic clock' or 'aging' process, the increasing branch ramifications also represent increases in the potential for NBF expression. Thus, the appearance of NBF symptoms at the terminus of one branch (either actual tree or nursery clonal propagation source) is a good predictor of imminent NBF appearance at similar locations on the other branches (including nursery propagation sources of the same lineage or similar genetic age) even though all arose from a common low potential source (Kester et al, 2004). The rate of clone-source deterioration with clonal-aging also appears to be accelerated with environmental stresses, particularly high temperatures during early to mid summer.

While the relationship of different vegetative clone-sources to the initial seedling source provides useful information concerning the probability of NBF expression in vegetative progeny of a susceptible cultivar propagation-source, the specific relationships often were not recorded in early nursery records. However, the tree model for the increase in NBF expression provides a protocol for the assessment of the different nursery propagation sources (source clones) for susceptible cultivars. For example, clone-sources showing the lowest proportion of their vegetatively propagated progeny trees expressing bud-failure over time could thus be identified as the best budwood sources for future propagations. Significantly, even the best sources of 'Carmel' showed symptoms within the first 10 years of tree growth (Fig. 6), demonstrating that while NBF risk could be dramatically reduced, it would still be a threat even for the most promising propagation sources for this very susceptible cultivar. This clone-source selection approach as applied to 'Carmel', was originally applied to 'Nonpareil' when NBF symptoms became particularly problematic in the 1960s, 70s and 80s (Kester 1978, 1974). To recover propagation wood of reduced NBF age, epicormic buds from the base of very old 'Nonpareil' trees, initially planted in the late 1800's to early 1900s, were pushed to develop shoot growth from which clone-source material was propagated (Fig. 8). Because the 'Nonpareil' cultivar originated in the 1880s (Wickson 1889), these basal epicormic (a poorly differentiated type of meristem) buds from old trees would represent relatively low NBF potentials because they were laid down early in tree growth and remained largely dormant in the intervening years. Long-term commercial test-plantings of 'Nonpareil' from these sources developed no NBF over the typical 20 year commercial orchard-life even when planted in NBF inducing climates (Gradziel et al., 2019). As such, they provided good propagation wood sources or foundation source-clones with virtually all of the 162,000 ha of current 'Nonpareil' commercial plantings being derived from these or closely related sources (Gradziel et al., 2019). That it took approximately 50 years for 'Nonpareil' to initially show NBF-symptoms indicates that the original seedling selection had relatively low initial NBF risk. Thus, while 'Nonpareil' clone rehabilitation through appropriate epicormic bud selection in the mid- to late 1900s has allowed 'Nonpareil' to remain relatively free from NBF in commercial plantings, the passage of an additional 50 years since those initial epicormic selections suggest that NBF may again become a problem in this cultivar.

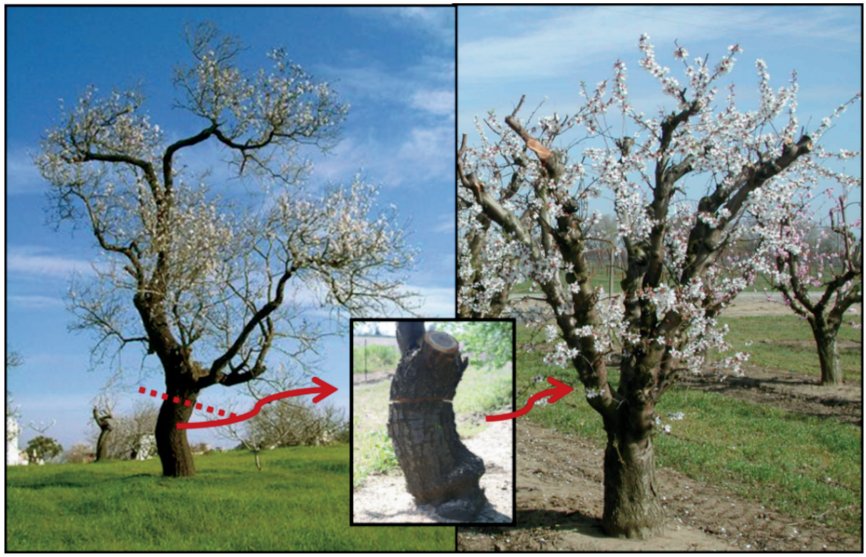

Fig. 8. Pushing basal, 100+ year old dormant epicormic buds in order to develop low NBF nursery foundation-stocks. Because NBF increases with tree age, this recovery of long-dormant epicormic bud-derived sources allows the epigenetic 'rejuvenation' of nursery propagation stock (modified from Gradziel et al, 2019).

Variability among genotypes

To assess variability among different genotypes, crosses were made between *'*Carmel*'* and the very low-chill and so very early blooming peach genotype 40A-17 using almond as the seed parent. Trees were evaluated yearly for NBF expression using standard identifiers (Kester et al., 2004) with the results plotted over time (Fig. 9). Hybrid progeny showed characteristic population development patterns indicative of the presence of epigenetic NBF in the parent almond genotype (Kester, 1978). The characteristic pattern develops as a rapid increase in the proportion of sexual progeny showing NBF expression, particularly roughbark, with a subsequent population leveling at approximately 50% of the progeny showing NBF expression and approximately 50% continueing to remain free of the disorder (Fig. 9). It is hypothesized that while peach is genetically similar and so readily cross-hybridized with almond, it has evolved different developmental pathways and, as a consequence, lacks the equivalent of the almond NBF genetic component (which appears to be involved in a type of summer dormancy typical for almond but absent in peach). Consequently, peach has been utilized as an effective test-parent for NBF detection in new breeding selections and cultivars. Interestingly, a recent DNA methylation analysis by Prudencio et al. (2018) identified possible candidates controlling dormancy release in almond flower buds using epi-genotyping by sequencing.

DNA-(de)methylation epigenetic profiling as a potential biomarker to approach NBF

DNA-(de)methylation is associated with gene silencing during development as well as aging in plants (Fraga, 2002), and has been associated with vernalization in crucifers and cereals, as well as flower induction in sugar beet. Thus, it may offer the opportunity to develop efficient molecular markers for age-related epigenetic changes (Campisi and Vijg, 2009; D'Aquila et al.,

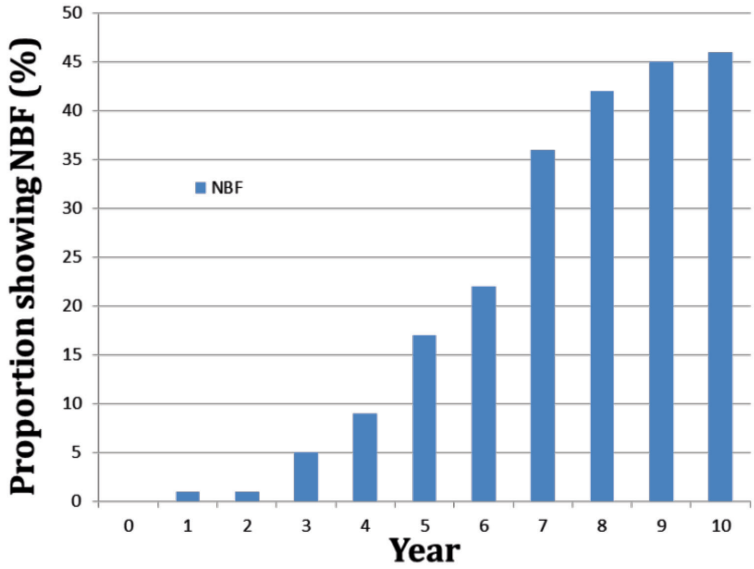

Fig. 9. The proportion of 'Carmel' almond by 40A-17 peach hybrid progeny showing NBF over time, demonstrating a characteristic single-factor inheritance pattern of a rapid increase in expression with a subsequent leveling near 50%.

2013; Garinis et al., 2008; Thomas, 2004). Almond leaf genomic DNA was isolated from multiple accessions of genetically diverse almond cultivars including 'Carmel' and 'Nonpareil' following standard methods with modifications (Fresnedo-Ramírez et al., 2017). Methylation-sensitive amplified fragment length polymorphisms (MS-AFLP), a modification of the standard AFLP protocol, were utilized. The use of methylation-sensitive restriction enzymes is incorporated into this technique. The protocol uses the isoschizomers *HpaII* and *MspI* instead of *MseI* as 'frequent cutter' enzymes as described by Fresnedo-Ramírez et al. (2017).

A total of 1251 fragments were scored in nine primer combinations per accession. In total 24,794 bands were scored for 22 accessions. Results showed a remarkably high level of genome-wide DNA-(de)methylation polymorphisms. Every primer combination provided polymorphic bands. We observed 1129 polymorphic fragments representing the largest amount of polymorphic MS-AFLP bands reported to date for a plant species (Fresnedo-Ramírez et al 2017) despite the relatively small genome size of almond at approximately 240 Mb. Typically, the number of polymorphic bands was not more than 30 sites genome-wide in other research reports (Ashikawa 2001). The degree of DNA-(de)methylation was associated with clone age, almond cultivar, and the presence of NBF. Results indicated that epigenetic mechanisms may be involved with aging as well as differential NBF exhibition both within and among almond clones. Two bands, e22 and i19 (corresponding to the primer combinations: E-AGT + HM-TAA and E-ACG + HM-TAA, respectively), demonstrated total correspondence with BF-expression (presence/absence) in all 22 propagation-sources of the seven cultivars evaluated. The bands were recovered and sequenced, but the results were inconclusive since no significant alignment was found in a GeneBank search at that time.

Considerable developmental plasticity was also observed by methylation profiling, even within individual trees. For example, in comparing methylation patterns in a 108-year-old 'Nonpareil' tree, leaf samples from epicormic shoots at the base as well as the middle section (both non-expressing) and from the top (expressing NBF) showed no difference in methylation status (methylated/demethylated) from bottom to top in over half the samples (Fig. 10). In approximately 20% of the samples, the patterns flip-flopped from base to middle to top. Only one-quarter of the samples showed a consistent transition with tree aging (demethylated to methylated or methylated to demethylated). We concluded that leaves may not be the best tissue for detecting clone-age related changes since differential gene methylation is a well-established process controlling tissue specific ontogeny (Fraga, 2002). Consequently, leaf tissue methylation status may not represent the ideal biomarker to study NBF, and additional studies are being undertaken to remedy this. The identity of the plant tissue that determines the memory of clone-age remains elusive (Munne-Bosch, 2008), as does the mechanism. Because pith and/or cambium derrived dormant epicormic meristems do not appear to age but retain the NBF potential existing at their initial formation, these tissues represent promising targets for future research for identifying the basis of such age-related changes.

While little is known concerning the mechanism of aging even in humans, it is known that the process can involve complex interactions at the cellular level including stem cell exhaustion, altered intercellular communication, genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, and cellular senescence (López-Otín et al. 2013). All these phenomena have been neglected in the development of the plant model systems such as *Arabidopsis thaliana*. Thus, almond provides a unique opportunity to identify epigenetic models and biomarkers of aging with both applied and basic implications. The current availability of new genomic resources for almond such as genomic and transcriptomic data, combined with the availability of efficient high-throughput genome sequencing technologies represent ongoing attempts to produce new data,

Epi-type	Proportion	Group Sum
{From base}		
(+++)	0.27	
(- - -)	0.29	0.56
(+ - +)	0.10	
(- + -)	0.09	0.19
(+ - -)	0.15	
(- ++)	0.10	0.25

Fig. 10. Changes in methylation status between methylated (+) and demethylated (-) for leaf samples taken from basal, mid-level and top sections, respectively, of a 108 year old 'Nonpareil' tree.

contrast hypotheses, and develop improved models for this disorder.

Summary

Utilizing the NBF disorder as a relatively well-studied example of epigenetic ageing in almond, strategies for epigenetic selection both within and among genotypes have been successfully developed and deployed but the phenotype-based assessment remains tedious and time consuming. These strategies also show promise for characterizing the nature and inheritance of other genetic disorders such as 'cherry-crinkle' (Ogawa and English, 1991) and pistachio 'bushy-top' (Randall et al., 2018) where molecular-based diagnostics have proven inconclusive. While these field-based clone-source-integrity assessments are more time-consuming than molecular-based diagnostics, they directly measure actual field performance and so are readily justified when propagation errors can result in substantial monetary loss and propagator liabilities. Results also demonstrated that epigenetic variability exists within and among almond genotypes/cultivars that is available for cultivar maintenance and improvement. Until more accurate diagnostics become available, long-term vegetative progeny-based statistical assessment remains the only proven epigenetic selection strategy.

MS-AFLP documented extensive changes in epigenetic methylation patterns within individual trees as well as individual clones. Both NBF as well as a broad range of MS-AFLP patterns changed with plant as well as clone age, which, while consistent with epigenetic control, confounded the development of definitive MS-AFLP diagnostics for NBF status.

Future research is needed to identify the appropriate tissue to be sampled for detecting markers of clone age-related changes. In the customarily sampled leaf tissue, the differential gene methylation involved in the inherently complex leaf ontogeny would act to dilute more subtle age-associated changes. The cellular location and biological mechanism for such age-memory in long-lived perennials also remains elusive. However, the increasing availability of efficient technologies and genetic/epigenetic lineages for interrogating genomes and cellular mechanism promise new opportunities to advance this basic model to eventually allow the characterization of other, more complicated clone-based disorders.

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