21st Century Approach to Improving Burbank's 'Stoneless' Plum

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Abstract. The theme running through many of Luther Burbank's breeding programs was to make plants more tailored to human uses. Mr. Burbank thought that the stone in plum fruits was unessential to a tree that was propagated vegetatively, so he chose stoneless plums as a breeding goal. He made two releases, 'Miracle' in 1903 and his final and almost perfect 'Conquest' in 1916, which he considered one of his best accomplishments in plum breeding. 'Conquest' had only a grain of stone and flavor and size comparable to the best French types of the time but was not commercially successful. In view of the current desire for convenience food such as seedless fruit (citrus, grapes, watermelon) and advanced knowledge of genetics and breeding technologies, we have taken up where Mr. Burbank left off in the production of a better than "almost perfect" stoneless plum. We began by locating what were most likely remnants from Mr. Burbank's breeding program and we are now using 21st century technology to achieve a completely stoneless, high-quality plum fruit. These technologies include molecular markers, genetic engineering, and accelerated breeding cycles (FasTrack). Initial experiments had characterized the stoneless trait as a decrease in the number of endocarp cells that form the stone. We defined the time critical to the formation of endocarp by analyzing gene expression of a number of transcription factors involved with determining endocarp cells. We identified genes that were expressed differently during this period between normal stone cultivars and one of the stoneless cultivars. In addition, we targeted genes for genetic engineering to reduce the lignification in endocarp and to reduce or convert endocarp cells to non-lignifying cells. A system, FasTrack, using a flowering gene from poplar, has been incorporated to reduce the juvenility period and eliminate the seasonal aspect of fruiting to see the results of the breeding as well as the genetic engineering approach much faster. The combination of these approaches is now in place to attempt to improve on Mr. Burbank's stoneless plum.

HISTORY OF THE STONELESS PLUM

Luther Burbank was heralded in his day as a genius in breeding (New York Times, 30 Sept. 1906), especially in the area of practical breeding (Jones, 1928). One theme that ran through his breeding approach was to select and improve plants that had lost certain characteristics that were of no use to people. To this end, Luther Burbank thought that the plum stone that surrounds the seed was not

necessary. The plum was not seed propagated, hence protecting the seed was not important. "But a moment's reflection makes it clear that the plum stone serves man no useful purpose, while the inconvenience it gives us is obvious" (Burbank, 1914a). So he began a breeding program to obtain plums without stone through his project "An Experiment in Teaching a Plant Economy" (Fig. 1).

Part of Mr. Burbank's success as a breeder was his choice of germplasm. He believed in

incorporating germplasm from wide-ranging sources. There existed a so-called stoneless plum, 'Sans Noyau', in France that Luther Burbank used as the source of his stoneless trait. "There has been known for several hundred years, a wild plum, an unproductive, thorny bush, which bore insignificant, acid, bitter, wild berry-like fruits with only half or two-thirds of a stone" (Burbank 1914c). He imported this plum from Transom Freres Nurseries in France and began to cross it beginning in 1890 to his high-quality 'Agen' or French-type germplasm (Fig. 2). From these seedlings he was able to select better quality and nearly stoneless germplasm, from which he could backcross to the 'Agen' plum. He made his first release of 'Miracle' (Fig. 3A) (Burbank, 1903) as a beta-version

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Fig. 1. Cover of Luther Burbank's re-publication of the stoneless plum bulletin: "The Stoneless Plum: An Experiment in Teaching a Plant Economy." <<http://www.amazon.com/The-Stoneless-Plum-Experiment-Teaching/dp/141470125X>>.

Fig. 2. A picture taken from *Burbank Methods and Discoveries* (1914a) entitled "Three Stages of Development." At the left is the original wild French plum, called the 'San Noyau'—of insignificant size and practically inedible. It is almost stoneless. Mr. Burbank improved the plum by hybridizing it with cultivated varieties, retaining the stoneless condition and introducing the qualities that make a commercial fruit. The central figure shows the plum at an intermediate stage of development; at the right the improved stoneless descendant a generation or two later.

Fig. 3. A composite of pictures taken from *Burbank Methods and Discoveries* (1914a): (A) 'Miracle', the first stoneless release; (B) typical stoneless seedlings showing a sliver of stone. (C) 'Conquest' and (D) typical stoneless plum showing the complete lack of stone surrounding the seed.

stoneless plum, because it still had a crescent of stone and not quite the flavor and size of the French-type plums. "A representative of the Oregon Nursery Company, on a visit to my Sebastopol grounds in 1903, was greatly pleased with this variety, and at once purchased it.....At the time it was the best stoneless plum in existence. But its chief merit was that it was the forerunner of a race of stoneless plums and prunes which will in time be grown wherever these fruits are raised" (Burbank, 1914c). Mr. Burbank had grand plans for this program. He released a second stoneless plum, 'Conquest', in 1916 (Fig. 3B) (Burbank, 1914a, 1914b, 1914c). He has included this plum in his four favorite plums. "The stone has been eliminated wholly with the exception of a tiny speck. The fruit is so very valuable and the tree so very productive that I have consented to introduce it this season" (Burbank, 2014c). He goes on to say that the quality of the fruit and size are similar to the French prune.

These two releases appear to be the only commercial cultivars from Mr. Burbank's very large stoneless program (Fig. 3C and D). He had several thousand stoneless seedlings planted and had generated hundreds of thousands of seed, but he had a hard time preserving them as a result of higher levels of rot and consumption by pests resulting from the lack of stone. Mr. Burbank states that in one experiment to test the preservation of seeds, he began with 100,000 seeds stored in several manners. He was able, however, to plant out several thousand seedlings to evaluate but none of those seedlings had the required combination of stoneless and commercial fruit quality. "Every color of plum now appears in these stoneless hybrids—white, pale yellow, orange, scarlet, crimson, violet, deep blue, almost black, striped, spotted, variegated, and mottled in every way imaginable" (Burbank, 1914c). Neither 'Miracle' nor

'Conquest' appear to have been a success in the marketplace although they had great publicity (New York Times, 1906, 1912; Burbank, 1903). It could be because the growers were paid by the pound and these stoneless plums weighed less without the stone (L.J. Rombough, personal communication).

In teaching plums to be stoneless, Mr. Burbank did not feel he had actually achieved his goal.

"So my ideal of an eatable plum having no stone about its seed was almost achieved. I say almost achieved because there still remained, in the case of the plums of best quality, a fragment of shell which varied from a small crescent about on side of the kernel to an almost invisible granule. There were some individual plants among the numberless seedlings that bore fruit in which the stone was absolutely eliminated and, in some cases, the seed also" (Burbank, 1914a).

Burbank felt he did all the pioneering work, "Even though the fruit should not be of better quality than that which it supplants, the fact that the elimination of the stone permits an increased abundance of fruit, to say nothing of the value of the stoneless fruit itself, will offer an inducement that the progressive fruit raiser will find conclusive" (Burbank, 1914a). He did believe that he had not reached the plants limit, but that with more crosses and screening of seeds that he already had, he would find the perfect stoneless and seedless, high-quality plum (Burbank, 1914a). Mr. Burbank found that it was much easier to combine stoneless with poor-quality fruit than it was to obtain a high-quality fruit with a stone (Burbank, 1914b). We look at his results as a proof of concept in that he was able to obtain fruit that were completely stoneless and even seedless. This accomplishment suggests that it is possible to accomplish his goal but that it just needed more combinations to combine the required fruit quality traits with stoneless and even seedless. We have picked up Luther

Burbank's goal of stoneless plums 100 years later. We believe the time is now ripe for accomplishing this because of the gain in knowledge and technology over the last century. When 'Miracle' and 'Conquest' were being released, Gregor Mendel's work was just being rediscovered and was not accepted by everyone, including Mr. Burbank, who still believed that acquired traits could be inherited (Stansfield, 2006). Today there is a better understanding of genetics and what influences traits. There are also the techniques of molecular biology, which allow us to introduce traits not in the germplasm as well as manipulate specific genes rather than recombine two genomes from the two different parents. For plum, there is now a FasTrack breeding system, which is able to shorten the generation cycle from 4 to 7 years to 1 year and the ability to breed year-round in the greenhouse rather than seasonally (Srinivasan et al., 2012). Using this new knowledge and techniques, we have begun a program to produce a high-quality fruit that has no stone (and in the future no seed). We are using breeding in much the manner of Mr. Burbank but with not only a goal of stoneless combined with quality fruit, but with understanding the genetics of the trait. We are using molecular biology to also understand the trait and identify genes that could be manipulated to obtain the stoneless phenotype. Lastly we are using our FasTrack system of breeding that reduces the generation time in plum to 1 year from seed to fruiting plant for both the breeding and the genetic engineering approaches.

21st CENTURY BREEDING

We chose as a goal for modern breeding a completely stoneless plum not only for the reason of no useful purpose that Mr. Burbank used (Burbank, 1914a), but for several additional reasons. First, the removal and detection of stone and stone fragments is a

large expense for the processed food industry; next, that the production of the energy-dense stone could be limiting to fruit production; and lastly, that the creation of a novel fruit could stimulate the public to increase consumption of a healthy food. The idea that a plum or any stone fruit could be consumed without having to carefully eat around the stone is very appealing. It also could allow for smaller fruit, even grape-sized, to be desired. Ideally the fruit would be completely pitless—no stone and no seed—but our focus is initially on the stone because that is a major focus of all *Prunus* processing industries.

The first thing we set out to do was to obtain some of Mr. Burbank's germplasm with which to study and proceed. It would be a lot quicker starting with the fruit quality of 'Conquest' and only a speck of a stone. Because Mr. Burbank's two plums were not successful, they appeared to have been lost from nursery catalogs and we were unable to locate them. Plums with the name of 'Stoneless' and 'Sans Noyau', one of which was a bush like the originally described 'Sans Noyau', were available from a number of sources including the USDA National Clonal Germplasm Repository, Davis, CA, the University of California, Davis breeding program, the USDA-ARS program at Parlier, CA, and from a private owner, Lon Rombough, who had a stoneless breeding program as a hobby (Fig. 4). These sources were budded to rootstocks and planted in the fields of USDA-ARS–Appalachian Fruit Research Station, Kearneysville, WV. The first trees that bloomed and fruited were the 'Stoneless' from ARS. After several seasons of fruiting, it was noted that in some years it had a nearly complete, albeit soft stone (Fig. 4E), and in others, it had almost no stone at all, only a speck at the funiculus (Fig. 4C). The degree of stone formed appeared to correlate with the temperature around pollination and early fruit development in that when it was warm after pollination, there was more stone tissue. This plum, in its best years, resembled the description of 'Conquest' because its size was similar if not larger than the French types and fruit quality was good (Fig. 4).

Next, we wanted to know something about the genetics of the trait so that we could potentially mark the gene(s) with

molecular markers to aid in its incorporation into high-quality germplasm.

Mr. Burbank, unfortunately, did not keep good breeding records for the stoneless plum project so little has been known about the genetics of the stoneless trait—whether it is a single gene and whether it is a dominant gene. First, we found out that the parental 'Stoneless' was self-incompatible, that is, it could only be pollinated with pollen from other plums. Mr. Burbank had mentioned that 'Miracle' is an uneven fruit bearer and that may be because it needed an appropriate pollenizer (Burbank, 1914c). Our 'Stoneless' flowers were pollinated by a mixed collection of pollen from forced flowers from other plums as well as any plum trees that flowered in overlapping times. Fruit was harvested and seeds germinated. The first-generation seedlings were planted and began to flower and fruit 4 years later. There was considerable diversity in terms of leaf size and shape, tree size and shape, and fruit size and shape that may reflect the recent introduction of the small fruited, bush-like 'Sans Noyau' in the heritage of 'Stoneless'. After 6 or 7 years, less than half of the seedlings had fruited. Of those that had, $\approx 50\%$ had a stone defect, either a partial stone or a stone thin enough to cut through with a knife (Fig. 5).

That 50% of the fruiting seedlings in the F_1 generation had a stone defect suggests that at least part of the trait of stonelessness is dominant and segregates as a single gene. It also suggests that the parent 'Stoneless' is not homozygous for the stoneless trait because half the F_1 fruiting seedlings had what appeared to have been a normal stone.

The future goal for breeding is to generate molecular markers for the putative single-dominant stoneless gene. To this end, the whole genome of 'Stoneless' and a number of normal stone cultivars have been sequenced. There are over 1,000,000 differences in single bases, small insertions and small deletions between 'Stoneless' and 'Improved French', a normal stone cultivar also resulting from Mr. Burbank's breeding program. These will be analyzed further to pick out appropriate polymorphisms to use as molecular markers to map the F_1 population, looking for linkages to the stoneless trait that can be used in furthering the breeding effort.

MOLECULAR STUDIES

Armed with information about segregation and expression of genes, we wanted to further understand the stoneless trait. This knowledge could give us targets for looking at the molecular control of that process. A careful study of the growth and size of the different tissue layers, exocarp (skin), mesocarp (flesh), endocarp (stone), and seed tissue was undertaken to determine if the same amount of endocarp was formed in 'Stoneless' (Callahan et al., 2009). Using dry weight measurements and lignin stains with phloroglucinol, we determined that there were fewer endocarp cells in 'Stoneless' than in a normal stone cultivar. RNA levels for enzymes involved in the hardening of the stone were also measured and found to be similar in endocarp (the little that was made) for both 'Stoneless' and for the normal stone cultivar (Callahan et al., 2009). A finer level examination of the cells that form the mesocarp and the endocarp of 'Stoneless' and of a normal stone cultivar, *Cacanska lepotica*, showed that there are many fewer layers of endocarp cells being formed in the 'Stoneless' confirming the conclusion that 'Stoneless' has little or no stone because it has far fewer endocarp cells that differentiate into the stone (Fig. 6).

This study then gave us a target process to evaluate that of endocarp formation. RNA accumulation levels were measured in peach to look at gene expression associated with endocarp formation. From these studies it was found that there were specific genes involved with lignification that were expressed in endocarp and then only at specific times in development (Dardick et al., 2010). Genes responsible for formation of endocarp and the resulting lignification had been previously identified in *Arabidopsis* (Ferrandiz, 2002; Irish, 2010). These were also tested to see if a similar process took place in peach. Results showed that for a number of transcription factors, *SHATTERPROOF (SHP)*, *SEED-STICK (STK)*, *FRUITFULL (FUL)*, *ALCATRAZ (ALK)*, and *INDEHISCENT (IND)*, patterns of expression, particularly in endocarp tissue, were similar to that seen in *Arabidopsis* (Dardick et al., 2010). These genes were analyzed in a plum series of tissues from initial floral bud set

Fig. 4. Various sources of plums without stones: (A) fruit from 'Sans Noyau'; (B) fruits from 'Stoneless' from the USDA National Clonal Germplasm Repository (NCGR), Davis, CA. (C–E) 'Stoneless' from ARS in Parlier, CA, grown in Kearneysville, WV. Each picture represents a different year, demonstrating the variation in the amount of stone obtained depending on the environmental conditions around pollination.

Fig. 5. Fruit derived from F₁ seedlings of 'Stoneless' grown in Kearneysville, WV. The fruit in the upper left corner has a complete stone formed (Normal), whereas the remaining five fruit have partial or almost no stone at all (Abnormal) demonstrating the dominance and variation of the stoneless trait.

Fig. 6. Fruit cross-sections from a normal stone cultivar, *Cacanska lepotica* (A) and from 'Stoneless' (B) stained with 0.025% Toluidine blue O. The blue is the result of the interaction with lignin, which is beginning to be formed in endocarp cells surrounding the seed cavity. The number of cell layers forming the endocarp in 'Stoneless' is only a fraction of that seen in the normal stone cultivar *Cacanska lepotica*.

in early summer to May samples taken the next year, when the stone began to harden at ≈ 252 d after the first collection in July. The accumulation patterns of the RNAs again showed that plum fruit development was similar to that of *Arabidopsis* with flower and carpel transcription factors being expressed before and up to pollination and the endocarp forming transcription

factors being expressed around the time of pollination to shortly afterward (Fig. 7).

From expression profiles of these genes it appears that the time of endocarp formation is within 10 d after pollination. This is similar to the time when endocarp formation in 'Stoneless' is affected by temperature, resulting in more or less stone being formed. RNAs were

sampled in the flanking time, from 10 d before pollination to 8 d past pollination for whole carpels and fruitlets as well as ≈ 25 d past pollination for endocarp tissue for both 'Stoneless' and two normal stone cultivars. This comparison of RNA expression has found over 2000 genes being expressed significantly different between 'Stoneless' and normal stone cultivars (data not shown). These differences are either at one time in development or in the overall expression of the RNA higher or lower in the 'Stoneless' fruit. It is not clear yet which of these differences are important to stone development. These data will be further analyzed and compared first to understand the gene activity necessary for making endocarp and second, to identify specific genes or pathways associated with the differences in 'Stoneless'.

To genetically engineer a stoneless plum, we could prevent those endocarp cells from forming the hardened lignin. Modifications of lignin in this manner have been used to modify wood formation with goals for making low pulp wood or easier to extract biofuel (Boerjan et al., 2003). This could then be used to manipulate only those processes in an otherwise high-quality fruit cultivar to obtain a soft and potentially edible stone. Alternatively, we could change the formation of the endocarp cells that eventually lignify either by eliminating them like in the stoneless mutant of Mr. Burbank or changing them into mesocarp cells. This also has precedence in the literature because there is a natural mutation in oil palms that has no endocarp layer of cells as a result of the absence of a functional transcription factor *SEEDSTICK* (Singh et al., 2013). When one copy of the gene is present, a thin endocarp is formed and when two copies are present, a normal hardened endocarp is present. There are also mutants in the dehiscent fruit of *Arabidopsis* that eliminate or convert the endocarp to mesocarp tissue (Ferrandiz, 2002). These examples can also be considered as a proof of concept for the molecular aspect of making a stoneless plum through genetic engineering.

ACCELERATING TIME TO FRUITING

The approaches to perfecting Mr. Burbank's stoneless tree all require time to evaluate because of the necessary wait for the transition of non-fruiting juvenile trees to mature trees that will fruit. Our first F₁ population has not completely fruited even after 7 years. One approach that Mr. Burbank used was to continually graft seedlings onto mature fruiting trees, reducing the time to flowering to only a few years or even in the next season. Not only did he speed up the process, but he was able to minimize the space needed by grafting many seedlings on a single tree (Fig. 8A). We have taken a different tack by using genetic engineering to create a germplasm source that flowers and fruits continuously in the greenhouse (Srinivasan et al., 2012). The gene *FLOWERING LOCUS T* from poplar was introduced into plum, which caused the transformed plums to flower and fruit within

Fig. 7. Relative amounts of mRNA for transcription factors associated with floral bud formation, carpel formation, and endocarp formation during the time before (floral buds) and at pollination (carpels) as well as 20 d later in the endocarp tissue alone. There are three periods of peak expression: pre-pollination, pollination, and post-pollination. Ap1 = *APETELA1*; lfy = *LEAFY*; rpl1 = *REPLUMLESS*; FUL = *FRUITFUL*; ALC = *ALCATRAZ*; IND = *INDEHISCENT*; AG1 = *AGAMOUS*; shp = *SHATTERPROOF*; stk = *SEEDSTICK*; pin3 = *Pin formed 3*; NST = *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1*. Results were obtained using quantitative polymerase chain reaction with triplicate samples and quantification by the $\Delta\Delta$ Ct method using 26S RNA as the standard gene.

the first year. There is no juvenility period, no vernalization required, and no long-day, short-day effect resulting in the plums flowering all year-round in the greenhouse. We can then pollinate the early-flowering plums with 'Stoneless' pollen to move the system into a yearly breeding cycle. The early-flowering trait segregates as a single locus so half of the seedlings will flower early, and half (according to our genetics) will have the stoneless trait. The fourth of the seedlings that contain both can then be crossed with high-quality fruit cultivars to recombine in the fruit quality traits necessary. These crosses can be repeated as many times as necessary to obtain the necessary quality traits while still keeping only the stoneless and early-flowering seedlings. Then only those that are not early flowering (half stoneless and half normal) will be planted out and evaluated for combination of stoneless and high fruit quality.

SEEDLESSNESS

In terms of convenience food, once the stone is gone, the seed itself presents a problem because it can be bitter as a result of the presence of varying amounts of amygdalin, which can be converted to cyanide on digestion (Bolarinwa et al., 2014). Future plans are to incorporate seedlessness to have a pitless fruit. Mr. Burbank mentioned that he was able to obtain pitless fruit, although not with the fruit qualities he desired (Burbank, 1914a) suggesting that it is possible to produce fruit that contain neither stone nor seed. The generation of seedlessness becomes another project with similar types of approaches as stonelessness, although in fact there are consumers who quite like the taste of the seed as is the case for this ice cream recipe (New York Times, 2000).

CONCLUSION

Through standard hybridizations, Luther Burbank created a nearly stoneless plum with high fruit quality from a bush-like plum bearing fruit containing a partial stone with bad flavors and of very small size. This was an amazing feat, especially for the time because there was little understanding of the potential complexities of genetics (Stansfield, 2006). Mr. Burbank was able to coax a nearly complete stoneless phenotype from this partial stone and obtain good fruit size and flavors, traits that usually revert to the more wild-type phenotype of small and bad flavors. We have obtained what are most likely remnants of this breeding feat and have begun to implement both a traditional and molecular breeding program to ultimately obtain Mr. Burbank's goal of a completely stoneless plum with the high fruit quality traits. Having seen many seasons of fruit on the parent 'Stoneless' and now fruit on the first F_1 generation, this has become an even more incredulous accomplishment. The stoneless trait appears to be dominant, which certainly makes it easier to follow, but the degree of stoneless varies in the

Fig. 8. Both pictures represent techniques to accelerate the juvenility period of plums to allow fruiting sooner. (A) Picture of Burbank's "Many Plums on One Tree.Several hundred cultivars may be found growing from a single trunk" (Burbank, 1914a). (B) An early-flowering plum from the FasTrack system that is 1 year old and is fruiting and flowering in the greenhouse.

F₁ population making it difficult to score and varies by year. It is not clear if this is an effect of different backgrounds or modifiers of the stoneless trait. We also found out that the trait appears to be environmentally affected in its expression such that some years, it is not stoneless at all. For Mr. Burbank to follow that would have been extremely difficult. It also may be part of the reason his releases were not popular in that they may not have been stoneless every year depending on the climate.

Mr. Burbank had been known to have a keen eye in the selection of parents and progeny such as the breeding of the blue poppy from red poppies (Burbank, 1914d). We do not have that same eye; hence, we are incorporating a 21st century technique of molecular markers to identify traits invisible in the seedlings. To this end, the genome of 'Stoneless' has been sequenced to identify polymorphisms to use for those markers.

To speed up the breeding cycle, Mr. Burbank grafted seedlings on mature trees to get them to fruit faster. We have used a technology that uses a genetically engineered early and continually flowering plum. This will allow us to advance the number of breeding cycles for leaving behind possible negative traits, which Mr. Burbank said was harder to do than to obtain completely stoneless fruit. In our last approach to obtaining Mr. Burbank's goal, we are going totally 21st century in identifying targets at the molecular level to manipulate. Although we have not advanced the generation of

Burbank's stoneless plum, we have now developed tools with which to attempt to improve on his amazing feat of a stoneless plum.

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