Opportunities for New Methods of Genetic Improvement of Grapevines

Alan Wei, Ph.D. and James A. Stamp, Ph.D.

Dr. **James A. Stamp** is a Sebastopol, California scientist who specializes in the critical evaluation of vineyard performance issues and grapevine nursery plant material quality and propagation. He has more than 25 years of viticulture experience and established Stamp Associates after founding Novavine grapevine nursery, working in the plant biotech industry and completing a post-doctorate at UC Davis. Stamp Associates advises growers and winemakers in the U.S. and overseas in the establishment and management of high-quality, pathogen-tested vineyards. Contact Dr. Stamp at *james@jamesstamp.net.*

Dr. **Alan Wei** is the general manager of Agri-Analysis LLC, located in West Sacramento. He has more than 25 years' industry experience in developing microbial detection technologies, ranging from high sensitivity ELISA, PCR, qPCR, high throughput screening and in-field methods. He has more than 20 issued United States' Patents in this and related areas. Dr. Wei enjoys interacting and working with growers to learn from them and help identify and solve problems for them. Agri-Anlysis is a laboratory that tests for Red Blotch and other grapevine viruses to help growers protect their investments. Contact Dr. Wei at *apwei@agri-analysis.com.*

FOR THOSE TASKED WITH securing clean stock for the establishment of (healthy) virus-test negative vineyards, it is frustrating to complete a new spring planting while vines in adjacent established vineyards show fall reddening. In some cases entire neighboring vineyards may turn red, or it may be single vines located through the edge rows of a particular block. The chances are that these vines are reddening due to the presence of either Grapevine Leafroll Virus type 3 (GLRaV-3) or Grapevine Red Blotch-associated Virus (GRBaV). Other viruses, especially other leafroll types and Corky Bark virus (GVB), may be involved in the development of reddened foliage, but GLRaV-3 and GRBaV are most likely responsible because they are so rapidly and effectively transmitted by, respectively, mealybug and treehopper insect vectors. Of course, the likelihood is that white varietals are similarly infected with leafroll and Red Blotch, but their disease status is hidden because they do not possess the chemical pathways to synthesize red anthocyanin pigments.

Identification of clean "virus test negative" grapevine stock is something that can be accomplished by careful examination and testing of nursery stock mother blocks. Clean stock can be identified by examining and testing **CDFA**-certified nursery Increase Blocks (*www.cdfa.ca.gov/plant/pe/nsc/docs/regs/ccr_3024_grapevine.pdf*) in October/November of the year prior to grafting. Late fall is the time when the Increase Block (IB) scion vines are most likely to show symptoms of stress (virus, mechanical, insect, nutritional, etc.), and it is a good time to eliminate vines, rows and blocks that do not appear healthy. Appropriate testing of apparently healthy vines will permit identification of clean stock that can be used for propagation. When selecting non-certified scion materials for propagation, the chances of finding infected vines are greater. However, careful observation and rejection of questionable stock can result in the identification of clean, virus test negative material. Historically, CDFA-certified stock has proven to be contaminated with economically important viruses (Stamp, 2010; Stamp and Wei, 2013, 2014).

If clean material of a particular selection cannot be identified, submission of dormant cuttings to the virus elimination program at **Foundation Plant Services** at **UC Davis** will result in the production of virus test negative plants in approximately three years.

Grapevine species are highly heterozygous and readily cross with close and distant genetic relatives, thus resulting in the varieties that are cultivated worldwide today. The genetic variability inherent in grapevine stock is the underlying reason why grapevines are propagated vegetatively: to maintain the unique genotype of fruiting varietals.

Targets for Non-traditional Improvements in Grapevine

Foodstuffs containing the products derived from genetically modified organisms (GMO's) abound in U.S. chain supermarkets (***Time*** magazine 2015), and many fast food restaurants typically serve portions containing some GMO food derivative. So what's all the fuss? Why is there this seemingly insurmountable kickback against bringing useful and popular technology to one of the, arguably, most technologically primitive plant species cultivated? Technologically primitive? Not all grape products are equal. Considered unacceptable in wine varieties, variability and unique genetics are desired in table grapes where novelty is key and large seedless berries of distinctive flavor or off-season harvest readiness are highly sought after.

The techniques of winegrape propagation are virtually unchanged since Roman times when varieties such as Shiraz (Syrah, Hermitage) were already distinguished. The Romans and their forebears understood that maintenance of distinct varietal properties was only possible by vegetative propagation. Striking of cuttings from favored vines occurs readily—and with little improvement this is still the basis of 21st century grapevine propagation.

The technology for winegrape improvement by using non-traditional techniques has existed since the late 1980s, and it is the perceived public rejection of GMOs that has prevented the commercialization of this technology in grapevines. Desirable winegrape improvement targets are many, and the methods that would be used have been proven in a wide range of crops, including potato, soybean and papaya. Perhaps one of the simplest targets for crop improvement is the development of resistance to important viruses, and of course, this would also be one of the top targets for grapevines.

The use of planting materials from virus-tested certified nursery stock is currently the primary virus control option. These certified stocks are derived from materials originated from foundation vineyards maintained by Foundation Plant Services at UC Davis. In vineyards where infected vines are present, management strategies rely on the elimination or rogueing of virus-infected vines and the reduction of insect vector populations (mealybugs and tree hoppers) through the application of systemic insecticides. The level of mealybug control required to limit virus spread is not known although encouraging results were recently reported (Wallingford et al, 2015). Management of leafroll viruses and their mealybug vectors remains challenging due to a lack of recognized host resistance (Oliver and Fuchs, 2011).

Very recently and disturbingly, leafroll virus 3 and Red Blotch have been detected in CDFA Protocol 2010-certified Foundation vines at FPS's Foundation Block west of UC Davis and in Protocol 2010 rootstock Increase Block vines under cultivation at a California nursery.

New Plant Breeding Technologies

Although "traditional" genetic engineering techniques developed in the 1980s were revolutionary at the time and resulted in products such as "Roundup-ready" crops, the public backlash against these approaches has soured the opportunities for a new generation of techniques—collectively referred to as New Plant Breeding Technologies, or "green biotechnology" (Costa et al, 2017). These technologies do not rely on the introduction of foreign genes into the target organism but rather the clever control of expression of native genes that theoretically could have been introduced through traditional breeding. These methods, called cisgenesis or gene editing, are ideal for improvement of highly heterozygous crops with long juvenile phases, such as grapevines, and result in the targeted expression of native genes far more rapidly than achievable through standard breeding.

Pierce's Disease Resistance

Pierce's disease (PD), caused by the bacterium *Xylella fastidiosa* (Xf), is an important disease of grapevines in California and many winegrowing regions. In the vineyard, Xf is spread by sharpshooters: insect vectors that feed on infected vegetation and then inject the bacterium into the sap of healthy grapevines. The disease is also graft transmissible if the scion and/or rootstock materials are infected with the bacteria. The bacterium multiplies in the xylem, eventually reducing the movement of water throughout the vine, resulting in disease and/or death.

Conventional breeding methods can be accelerated by modern molecular tools such as PCR-based marker-assisted selection (MAS), for the rapid identification and selection of new varieties. Using this technique, Dr. **Andy Walker**'s group at UC Davis has developed several new Pierce's Disease (PD) resistant varietals (Riaz et al, 2009). By scouring North America for wild and weedy *Vitis* species Walker identified candidate grapevine germplasm possessing natural resistance genes against Xf. The Mexican grape species *Vitis arizonica* was found to be the most promising candidate, possessing a single dominant PD-resistant gene. By using the molecular techniques of MAS, Walker was able to backcross wild species of grapevine with popular varieties, such as Cabernet Sauvignon, Chardonnay and Pinot Noir. By repeated backcrossing and by testing and selection of seedlings for PD-resistant traits, Walker was able to create PD-resistant grapevine plants that were up to 97 percent genetically identical to the backcrossed varietal parent. These plants are now undergoing trials, and wines derived from them have been favorably reviewed. Because PD is such a serious problem in many premium wine regions of California there is strong demand for these resistant plants, especially for replanting diseased vineyard rows adjacent to riparian habitat. The hope is to establish PD-resistant border rows to prevent the movement of the disease from these rows inward to non-resistant traditional varieties

Biocontrol of Pierce Disease

Bacteriophage, also known informally as phage, is a virus that infects and replicates within a bacterium to eventually kill the bacteria. Phage therapy has been used to treat or prevent pathogenic bacterial infections in human medicine, food and agricultural applications. For example, the **USDA** and **FDA** have approved the use of several lytic phages specifically for bacterial pathogens, such as *Escherichia coli* O157:H7, *Salmonella spp.* and *Listeria monocytogenes* in foods, produce and on food processing surfaces (Sharma, 2013). It is quite likely that the fresh salads at the local market have been treated by a bacteriophage product. Since *Xylella fastidiosa*, the casual agent of Pierce's Disease is a bacterium, why not treat PD with bacteriophages?

Researchers at Texas A&M University evaluated the therapeutic and prophylactic efficacy of a phage cocktail composed of four virulent (lytic) phages for control of PD. Xf levels in grapevines were significantly reduced in therapeutically or prophylactically treated grapevines. PD symptoms ceased to progress one-week post-therapeutic treatment and symptoms were not observed in prophylactically treated grapevines. Cocktail phage levels increased in grapevines in the presence of the host. No phage-resistant Xf isolates were obtained, and Xf mutants selected for phage resistance in vitro did not cause PD symptoms. These results show that phages have great potential for biocontrol of PD in grapevine and other economically important diseases caused by *Xylella fastidiosa* (Das et al 2015).

In what appears to be a promising research track, Dr. **Don Hopkins**' research group at the **University of Florida** is studying the protection or "inoculation" of uninfected grapevines from PD by injecting them with a benign strain of *Xylella fastidiosa* (Hopkins, 2005). Field trials are currently underway in California and in southwestern USA.

Powdery Mildew Resistance

Grapevine powdery mildew (PM) caused by the fungal pathogen *Erysiphe necator*, is a major fungal disease in California and all grape-growing regions of the world. At present, fungicide applications are the main control method for powdery mildew but are costly, labor intensive and environmentally unsustainable. **Agri-Analysis, Inc.** has frequently found fungicide-resistant strains of *E. necator* in client samples: resistant to demethylation inhibitors (DMI) and quinone outside inhibitors (QOI). Dr. **Walt Mahaffe**'s group at **USDA Agriculture Research Service** surveyed Napa Vineyards for QOI-resistant PM and found over 85 percent of samples contain QOI resistant *E. necator* and more than 62 percent were also resistant to at least some DMIs (e.g. mycobutinol and tebuconezol). Needless to say *E. necator* is a rapidly evolving fungal pathogen with significant economic impact in the wine industry. Dr. Andy Walker's group at UC Davis has investigated powdery mildew resistance in multiple accessions of the Chinese grapevine species *Vitis piasezkii*. Chinese *Vitis* species have attracted attention from grape breeders because of their strong resistance to powdery mildew and their lack of negative fruit quality attributes that are often present in resistant North American species. Walker discovered two distinct powdery mildew R loci designated Ren6 and Ren7 in multiple accessions of this Chinese grape species. Their location on different chromosomes to previously reported powdery mildew resistance R loci offers the potential for grape breeders to combine these R genes with existing powdery mildew R loci to produce grape germplasm with more durable resistance against powdery mildew (Pap et al, 2016). However, there may not be a single magic bullet against PM.

"Even with plant resistance you will still need to use fungicides to protect the investment in the resistant varieties. *E. necator* is constantly evolving and has circumvented everything we have thrown at it to date. I doubt this will change in the future. Look at any other crop that deploys plant resistance traits—it is an arms race. The pathogen gets around the trait, and breeders develop another. The difference is we can't afford to replant very often," said Dr. Walt Mahaffee.

Innovative "green" biotechnologies to breed virus-resistant new crops can complement traditional breeding and genetic engineering methods and address their inherent limitations. Resistance can be achieved by a number of methods, including interference (RNAi), CRISPR and anti-viral antibodies. Some examples of the most recent developments in plant improvement are described below. These examples are not exhaustive, and interested readers should read more comprehensive reviews by Costa et al, (2017).

Fanleaf Virus Resistance

The human immune system consists of two fundamental pillars—the innate, general defense and the acquired, specialized defense. Both systems work closely together and take on different tasks to defend against foreign substances, such as viruses and toxins. Antibodies are a vital component of both pillars, especially in acquired defense. Plants, on the other hand, do not possess antibodies to eliminate viral infection although they have an innate defense system. Advances in recombinant antibodies have made it possible to introduce crop resistance by the expression of pathogen-specific antibodies that can bind and neutralize the virus and therefore hinder its ability to propagate inside plant cells.

Although traditional antibody approaches have been attempted for plant protection with limited success, the research community has been reinvigorated by a new class of antibodies called nanobodies derived from the camelid (Llama) family. These are single-domain antigen-binding fragments of heavy-chain only antibodies. Since their discovery in 1994, there has been intense interest in their use as therapeutic drugs for various human diseases, including cancers and infectious diseases. Recently, French researchers identified a set of nanobodies specific to grapevine fanleaf virus (GFLV) that confer strong resistance to GFLV upon stable expression in the model plant *Nicotiana benthamiana* and also in grapevine rootstock 41B. They showed that resistance was effective against a broad range of GFLV isolates but not against any isolates of its close relative *Arabis* mosaic virus. They also demonstrated that virus neutralization occurs at an early step in the virus life cycle, prior to cell-to-cell movement (Hemmer C. 2018).

"Nanobodies are exciting in that they are one-tenth of the size of a traditional antibody. This means they can be more readily expressed in plants. Despite their small molecular size, they are resistant to organic solvent and heat. Some nanobodies have been shown to maintain activity even after exposure to 100° C heat treatment. These properties make nanobody-mediated resistance to plant viruses an attractive anti-viral strategy," said Distinguished Professor **Bruce D. Hammock** who runs a nanobody research program aimed to develop next generation diagnostic and therapeutic tools in the Department of Entomology at UC Davis. Funded by a USDA SBIR grant, Agri-Analysis has developed a set of nanobodies for the viral coat protein of GLRaV-3. Work is in progress to use these nanobodies for the possible protection of grapevines from the GLRaV-3 virus.