

# The Next Generation of Biotechnology for Apple Improvement and Beyond, the Tale of TALENs and TALEs

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Apple production in New York State and elsewhere requires several time consuming and labor intensive operations such as pruning, spraying and harvesting. The rising

**“TALENs (transcription activator-like effector nucleases) are a genetic tool that allows precise insertion of specific genes, for modification or replacement of genes in apple at their native genomic location without involving any other sources of DNA beyond *Malus* (apple). They are very versatile tools for improving disease, insect and stress tolerance, crop quality and yield. Looking forward, apple growers (and growers of many other crops) can expect improved varieties developed using TALEN technology, which will be equivalent to those developed by conventional breeding.”**

labor and chemical costs not only squeeze the industry's profit margin, but also threaten the industry's sustainability.

Why pruning? Most apple trees tend to overgrow their allotted space and the canopy gets too dense. Pruning helps maintain good light distribution within the canopy and maintains the trees in their allotted space for

high production of quality fruits. Properly pruned trees produce fruit earlier and live longer.

Why thinning? Like many other plants, apple produces an excessively large number of flowers, fruits and seeds for survival and spreading. Spraying chemicals such as naphthaleneacetic acid (NAA), carbaryl, and/or 6-benzyladenine (BA) can remove excessive fruitlets (up to 90%) to optimize fruit load. However, fruit thinning remains one of the most unpredictable operations in apple production (Robinson, 2012).

Why spraying pesticides? There are many damaging diseases (e.g. apple scab, fire blight) and insects (e.g. apple maggot, codling moth) in apple orchards. Chemical control has been an important means to keeping these pests at bay.

Why harvesting by hand? Apples are easily damaged when mechanically harvested using tree or limb shakers used in other fruit crops. In addition, fruits are set at varying positions within the canopy and machines smart enough to pick apple fruits individually have long been sought, but remain unavailable.

All of these labor intensive and costly operations are driven by the genetic characteristics of apples and the orchard condi-

tions. In other words, the way apples in general and each variety grows, develops, and responds and adapts to the orchard environment dictates when, what and/or how the orchard is pruned, thinned, sprayed and harvested. Apple varieties differ naturally in growth, development and resistance. For example, 'Wijcik McIntosh' grows slower and branches less than its mother variety 'McIntosh', requiring much less pruning. 'Gala' and its sports are more difficult to thin than 'Golden Delicious', and more sensitive to apple scab than 'Liberty'. To thin 'Gala', thinner chemicals must be delivered with a precisely measured amount and strict timing. To control apple scab on 'Gala', frequent sprays of fungicides are needed.

Each of these characteristics, such as less branching, hard to thin, and resistance or susceptibility to diseases, is called a trait in genetics and breeding. The varietal variations in a trait or traits are caused by the variation in genes that control the trait.

Are there ways to bring a set of desirable genes into one variety that would require minimal pruning, that would shed the unwanted fruitlets so that no chemical thinning is needed, and that would repel damaging insects and be immune or resistant to major diseases so that pesticides are no longer necessary? In theory, this is possible through both conventional breeding and genetic engineering, but the technical challenges make the task nearly impossible with conventional breeding. In contrast, there are fewer technical difficulties to take on the task using genetic engineering, and it has been proven doable in apple (Aldwinckle and Malnoy 2009). However, some of the undesirable points associated with existing biotechnology have resulted in public concern and opposition by several groups. The controversial points are the random insertion of a transgene in the apple genome along with a selectable marker gene and other DNA fragments.

A new emerging plant biotechnology, called transcription activator-like effector (TALE) nucleases (TALENs), is perceived to have great potential for genetic improvement of plants without negative aspects of existing biotechnology. Below is a brief introduction to this emerging biotechnology, including what it is, how it is developed, what we have been doing with this new biotechnology and what the limitations are in apple improvement.

TALENs are a protein with two components: The first component is a designed transcription activator-like (TAL) effector (TALE) that mimics the phenomenal natural TAL effector proteins secreted by the plant bacterial pathogen *Xanthomonas*. The second is a popular nuclease that can cut DNA in cells. TALENs can be used as a tool to site specifically "edit" the genome of many species. The applications of TALENs include gene mutation/

disruption, gene therapy/replacement, and gene addition. So far, several successful cases using TALENs as a clean “cut and paste” tool to site-specifically “edit” the genome have been reported in cells of various organisms, including yeast, *C. elegans* (a worm), zebra fish, mammals and plants. TALENs hold the promise of revolutionizing the fields of biotechnology and genomics studies. It won't be too long until TALENs affect our daily lives. Because of the wide applicability of TALENs, the highly accredited journal Nature Methods named the TALENs as its 2011 “Method of the Year” (Nature Methods Editorial 2012).

In plants, the TALENs based biotechnology provides several major improvements over the existing biotechnology used in many genetically modified organisms (GMOs), which include:

1. Gene modification/replacement that is site specific and precise, and can be done without introducing any foreign DNA.
2. No presence of a selection marker, such as the Kanamycin resistance gene.
3. If a foreign gene must be introduced, it is inserted to a predefined genomic location.

For growers, the TALENs based biotechnology means that better varieties of field crops, vegetables and fruits are coming. Although TAL effectors were discovered in *Xanthomonas*, TALENs can be used to improve resistance to most other diseases in plants. In fact, TALENs are a tool that is very versatile, and using it for improving crop quality, yield and stress tolerance is very feasible. Looking forward, growers of apples, berries, corn, grapes, soybeans, tomatoes, wheat and many others crops will spray much less for disease control while harvesting more high quality fruits, vegetables and grains.

### The Tale of the TALEs

The development of the TALENs based biotechnology demonstrates another great story of discovering the secrets of nature. In this case, we learned it through studying the molecular structure of TAL effector proteins secreted by the bacterium *Xanthomonas*, a plant pathogen that causes diseases in nearly 400 plant species, including bean, grape, pepper, rice, tomato, and stone fruits. TALE proteins function to wreak havoc in the host plant, leading to an environment where the bacterium can readily grow.

Earlier plant pathogenicity studies show that bacterium *Xanthomonas* has a needle like structure that can penetrate the plant cell wall to deliver TALE proteins into plant cells (Fig. 1). The delivery process is similar to a vaccination, but it is meant to harm the plant.

Inside the plant cells, the TALEs from the bacterium work very precisely. It appears that this bacterium evolved a method of controlling the basic mechanism of gene expression and regulation. The expression of any particular gene is normally controlled by the DNA sequence immediately upstream of the gene, called promoter (Fig. 2). The promoter sequence functions like a switch for turning on or off the gene downstream. When the promoter is bound with a transcription activator from the plant itself, the gene is turned on and a specific protein is made benefiting the plant. However when the TALE of the *Xanthomonas* bacterium is inserted into the plant to disrupt the plant defense, the TALEs from the bacterium only bind to the promoter sequence of the susceptibility genes that are master regulators of many other decrease responsive genes (Fig. 2). Thus the *Xanthomonas* bacteria

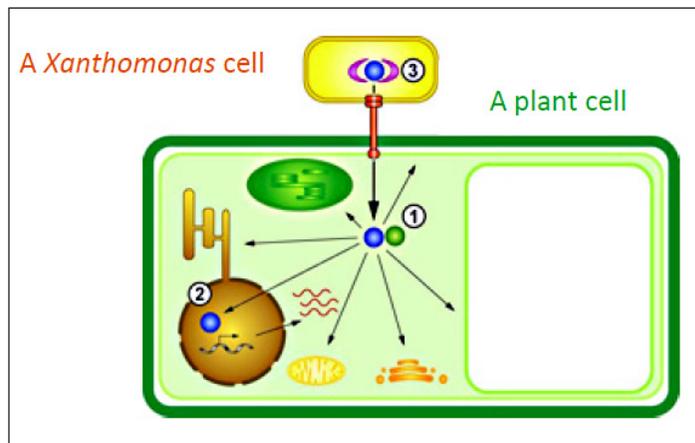


Figure 1. A diagram of *Xanthomonas* injecting TAL effector proteins (in blue) into a plant cell (Adapted from [http://www.biologie.uni-halle.de/im/1291887664\\_936\\_0.jpg](http://www.biologie.uni-halle.de/im/1291887664_936_0.jpg))

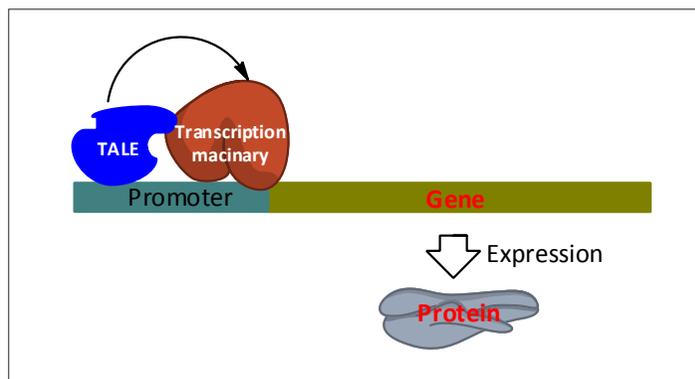


Figure 2. Gene and gene expression control. Effector proteins, such as TAL effectors (TALE) from *Xanthomonas*, bind to the promoter of a target gene to control gene expression.

A **gene** is a DNA molecule made up of four nucleotides or bases: adenine (A), cytosine (C), guanine (G) and thymine (T) that encode genetic information.

A **genetic code** comprises three DNA bases. For example, the genetic code ATG encodes for methionine, an essential amino acid we cannot make and must obtain from our diet.

**Gene expression** is referred to the process in which a gene is transcribed and then translated into a protein (consisting of amino acids).

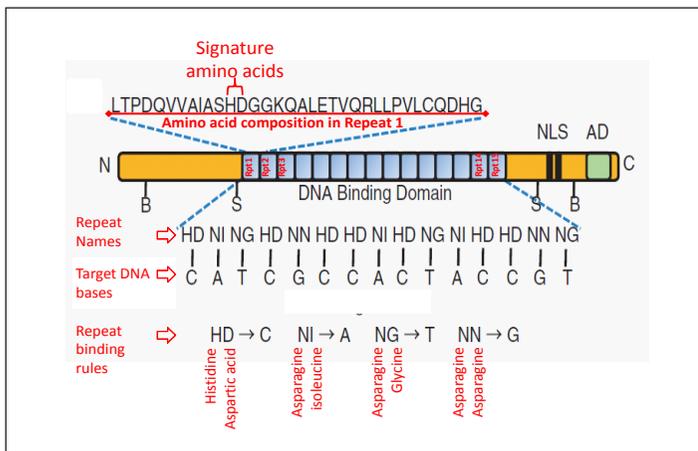
A **promoter** is a DNA sequence immediately upstream of a gene that controls the downstream gene expression.

**Transcription machinery** is referred to a complex of enzymes that synthesizes mRNA (messenger RNA) from a gene before a protein is made.

exploit the plant own gene expression/regulation mechanism for its own benefit.

There are tens of thousands of genes in plant genome. Therefore, what guides the TALEs from *Xanthomonas* to bind to the promoter sequences of the master susceptibility genes without missing the targets? To resolve this mystery, scientists have recently sequenced the genes encoding TALEs and uncovered the molecular structure of TALEs (Fig. 3). This work shows that the TALE proteins contain a nuclear localization signal (NLS), a transcriptional activation domain (AD) and a DNA binding domain (Fig. 3). The NLS allows TALEs to enter the nucleus inside a plant cell (Fig. 1) while the AD domain stimulates the transcriptional machinery for transcription or gene expression (Fig. 2).

Since the TALE functions to bind to DNA, the DNA binding domain is the most logical place concealing the mystery of guiding TALEs to their target, the master susceptibility genes (Fig. 3). This



**Figure 3.** A typical structure of TALEs containing a nuclear localization signal (NLS), a transcriptional activation domain (AD) and a DNA binding domain. The DNA binding domain comprises multiple repeats that are identical except for their two signature amino acids near the middle. To name the repeats, the two signature amino acids are conveniently used. For example, the first repeat is named as HD (histidine, aspartic acid). For each repeat, the two signature amino acids specify which DNA base (A, T, G or C) to bind. The rule governing the binding is simple, i.e. repeat HD binds to base C, repeat NG (asparagine, glycine) to T, repeat NN (two asparagines) to G and repeat NI (asparagine, isoleucine) to A (adapted from Cermak et al. 2011).

turns out to indeed be the case. There are many repeat units that vary slightly in the DNA binding domain, and each repeat unit follows a simple rule to specifically bind to one of four DNA bases A, C, G and T (Fig. 3) (Boch et al. 2009; Moscou and Bogdanov 2009). This is similar to an array of keys assigned with a specific letter on a keyboard for typing. Therefore, the content, number and order of the repeats in the DNA binding domain determine the specific binding site of TALEs.

The greatest lesson that nature has taught us here is the simple rule of “one repeat unit binds to one DNA base”. It is this simple rule that allows us to mimic the *Xanthomonas* TALE’s and design and engineer our own TALEs for binding to a site we want in the genome. It is also this simple rule that allows us to combine the TALEs we design with a nuclease that cuts DNA to create TALE nucleases (TALENs), thereby the TALENs based biotechnology as briefly described above. The TALENs based biotechnology is turning out to be a versatile tool for genome editing, suitable for a wide range of applications in humans, animals and plants.

### Application of the TALENs Based Biotechnology in Apple

There is no doubt that the TALENs based biotechnology is readily applicable to apple. My group has begun to target two genes that we identified recently: one is the *Ma* gene controlling fruit acidity levels (Xu et al. 2011), the other is the *Co* gene controlling columnar growth habit (Bai et al. 2012). We will soon include the *Vf* gene for apple scab resistance (Malnoy et al. 2008) in our work. Our initial objective was to test how well the TALENs we designed would target the selected genes in apple cells. But the long-term goal is to use a series of custom TALENs to modify or replace a set of genes in popular apple varieties so that desirable traits, such as minimal pruning, self thinning, resistance to major insects and diseases, high keeping quality and others,

will be expressed. Optimistically, this goal can be accomplished in the next 5 to 10 years if the key apple genes controlling these desirable traits are identified. So far, such characterized key apple genes are limited to a handful. Therefore, uncovering genes that have significant effects on apple growth, development, resistance to diseases and insects, and fruit quality is currently the major limit to using the TALENs based biotechnology for apple improvement.

Recently there has been encouraging progress towards identifying key genes of great impact on traits of horticultural or economic importance. This has been stimulated by the publication of the first draft of the apple genome sequence in August 2010 (Velasco et al. 2010). For example, my program has identified two such key genes *Ma* and *Co* (Bai et al. 2012; Xu et al. 2011). However, more genomic studies are needed to provide desirable genes that can be utilized with TALENs technology. In the case of apple scab resistance, as many as 19 genes have been discovered (Wang et al. 2012), and two (*Vf* and *Vr2*) have been isolated thus far (Galli et al. 2010; Malnoy et al. 2008). These latest development in the field of genomics should now allow us to discover other apple genes controlling important function, such as the remaining apple scab resistance genes, faster and more cost-effectively. When more apple scab resistance genes are isolated, site-specific modification or replacement of these genes in susceptible varieties with TALENs will provide multiple gene protection against apple scab.

### Conclusions

The advances in TALEN technology and faster gene discovery now make grower investments in biotechnology much more feasible. The sentiment or notion that gene discovery belongs to basic research and is not affordable to the industry ought to be left behind in this era where the apple genome has been sequenced. Investment in more apple genomic studies will eventually reap returns to the industry with a surprisingly high rate.

It should be emphasized that TALENs technology will allow inserting specific genes, for modification or replacement of genes in apple will occur at their native genomic location without involving any other sources of DNA beyond the *Malus* (apple) species. As a result, the improved apple varieties using TALENs will be absolutely equivalent to those developed by conventional breeding. Current concerns about existing GMOs varieties will not apply to the upcoming TALENs improved varieties.

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## New York Vegetable Expo Gets New Name and Look!



It began simply as The Vegetable Conference.

Today, the NYS Vegetable Growers Association (NYSVGA) Board of Directors is proud to unveil a new name and logo for what has become the largest gathering of fruit, flower, and vegetable growers in New York-- The Empire State Producers Expo.

"I can remember years ago attending the conference with my father up in Niagara Falls, New York," recalls NYSVGA President Mark Henry, of Eden Valley Growers in Eden, New York. "Jean Warholc organized a fine show. But I doubt she ever imagined how big the Expo would become."

"For the last several years we've added a new contributing organization every year, and the name of the Expo was growing accordingly. We were working with The Empire State Fruit and Vegetable Expo and Direct Marketing Conference and Becker Forum. Try putting that on a logo!" laughs Expo Director Jeanette Marvin. "We decided to create one name that would say it all-- The Empire State Producers Expo."

The Empire State Producers Expo runs January 21 - 24, 2013 at the On Center in Syracuse, New York. The Expo includes the one-day Becker Issues Forum, the three-day Trade Show and three days of concurrent educational sessions. Contributing organizations include: the NYSVGA, Cornell Cooperative Extension, Empire State Potato Growers Inc., NYS Berry Growers Association, NYS Flower Industries, Inc., NYS Horticultural Society, Farmers' Direct Marketing Committee, and Cornell University. The Expo offers both DEC and Certified Crop Adviser credits.

The Expo Trade Show includes over 164 companies representing a variety of products and services from equipment and inputs to software, packaging, bakery supplies and much, much more.

**Watch for more Expo updates, registration details and the full schedule on**  
**[www.nysvga.org](http://www.nysvga.org)**