Chapter 11

COTTON HARVEST-AIDS AND BIOTECHNOLOGY: THE POSSIBILITIES

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INTRODUCTION

The success of any crop improvement program relies on sufficient genetic variability to introduce or improve desired traits. Traditionally, when the required variability is not present, it must be induced by mutations or bred with related species for characteristics that are absent in the cultivated species (Bajaj, 1998). Such methods normally take several years to accomplish; however, genetic engineering and other forms of biotechnology can provide an approach that allows hybridization among different species in a shorter time frame, as well as transferring a greater variety of genetic information in a more precise, controlled manner. Biotechnology also may be used to

facilitate or enhance traditional breeding programs. This is adventitious for the cotton industry, as many wild species of *Gossypium* are available to provide sources of genetic variability (Prentice, 1972).

Advances in the use of biotechnology for crop improvement have led to dramatic increases in acreage of genetically enhanced cotton over the last few years (Anonymous, 2001b). U.S. cotton farmers planted genetically enhanced seed on more than 11 million acres in the 2000 growing season (Anonymous, 2001c). In that year, genetically enhanced cotton acres compromised 69 percent of total cotton acreage (Anonymous, 2001a).

Transgenic technology – The most successful approach for insect resistance in cotton (and other important agronomic crops) has been through the use of the bacterium, *Bacillus thuringiensis (Bt),* which produces proteins toxic to some of the insects most damaging to cotton. Commercially introduced by Monsanto Company in 1996, Bollgard® cotton varieties are genetically engineered to code for a delta endotoxin of *Bt.* Bollgard varieties provide effective control of tobacco budworm, pink bollworm, and salt marsh caterpillar, and suppression of several other lepidopterous pests, e.g., bollworm, loopers, and beet armyworms. However, *Bt* toxins currently available are ineffective against insects such as whitefly, thrips, boll weevil, and lygus bug; research continues for improving protection of cotton from insect attack. In addition, questions persist about the *Bt* toxin and its insect specificity and development of resistance to the toxin by target insect populations.

Another successful trait introduced into cotton is one that confers resistance to the herbicide glyphosate (Roundup®). Roundup Ready® (Monsanto Company) has demonstrated excellent tolerance to Roundup Ultra® (glyphosate) herbicide up to the four-leaf stage. Approved in 1996 and first commercially grown in 1997, Roundup Ready cotton varieties tolerate both topical and post-directed applications of Roundup herbicide. Some of these transgenic varieties also possess the Bollgard gene for insect protection. Although Roundup Ready cotton has been successful, concerns with fruit abortion and excessive cavitation on these cotton varieties have been voiced (Edmisten and York, 2000).

Bromoxynil-resistant cotton (BXN®) (Stoneville Pedigreed Seed Co.) was the first transgenic cotton variety released, but it has not met with the same initial success as have the Bollgard and Roundup Ready traits. However, as the advantages of transgenic technology become more evident, BXN cottons will gain much greater acceptance in certain production areas of the U.S. Cotton Belt.

BXN varieties contain a gene that produces an enzyme (nitralase) that gives these transgenic varieties the ability to metabolize bromoxynil, a broadleaf herbicide. This allows Buctril® (bromoxynil) herbicide (Aventis Group) to be applied post-emergence for topical control of most broadleaf weeds found in cotton fields (e.g., cocklebur, common ragweed, and all species of morningglory) (York and Culpepper, 2000). Cotton varieties with the BXN trait were introduced to farmers in 1995. In 1997, the Environmental Protection Agency announced its decision to deny the company's petition to extend the use of the herbicide Buctril on gene-altered cotton for the 1998 growing season (Kantz, 1998). The decision was based on the company's failure to meet certain risk assessment guidelines for bromoxynil, as prescribed by the Food Quality Protection Act. However, in May, 1998, registration of Buctril on BXN cotton cultivars finally was approved (Byrd, 1998).

In addition to the single-gene transgenic varieties, grower demand for multiple-gene, or "stacked," varieties is increasing. An example is Stoneville Pedigreed Seed Co.'s ST 4892BR™ variety, which stacks the protection of Bollgard and the weed control attributes of Roundup Ready.

Gene Research – New developments in gene identification and transformation technologies will assist in the development of more transgenic applications, such as cotton plants possessing novel genes involved with fiber modifications, parental gene expression, and key physiological pathways. For example, the National Science Foundation awarded a federal grant for a three-year cotton gene research project, focusing on the triggering mechanism of fiber development, to be headed by the University of California-Davis (Geissinger, 1999). The National Science Foundation also is funding a unique study on the expression of parental genes in plant polyploids (where more than one parental genome is present). A research team has been assembled under a grant to study what, if any, impact parental gene expression contributes to the success of important polyploid crops such as canola, cotton, corn, potatoes, and wheat (Fannin, 2000).

Research focusing on plant genomes also is in progress. Independently investigating drought- and freezing-tolerance mechanisms, another University of California-Davis research team is working on manipulating complex pathways through key regulatory genes, as opposed to the typical genetic engineering of single genes or a small number of genes to synthesize a particular compound (Amber, 2000).

Monsanto Company has conducted research on development of a "Technology Protection System" (TPS) or "terminator" gene. Transgenic varieties incorporate the TPS gene, in which – when the plant nearly is mature – the introduced plant gene becomes active, stopping the seed from making the protein required to produce new plants (Nixon, 1999). However, the company has altered the project's goal. Monsanto Company now is working on other "gene-protection technology," which would discourage farmers from planting seeds from a previous crop by inactivating only the specific gene responsible for the value-added biotech trait (Pro Farmer Editors, 1999).

Genetic engineering to confer useful agronomic traits to cotton is likely to lower the cost of production, improve yield and quality, and promote environmentally friendly farm practices (Bajaj, 1998). To date, biotechnology has not been commercially applied to the area of cotton harvest aids. However, this may change as stricter regulations are established regarding chemical use and as costs increase. The potential exists to manipulate physiological processes to enhance harvest-aid efficiency. This chapter explores these possibilities as well as briefly describing some of the technology that could be used to achieve physiological goals.

PHYSIOLOGICAL ASPECTS SUITABLE FOR GENETIC MANIPULATION

Specific combinations of hormones and their relative concentrations are important regulators of plant growth and development. In early studies, genes from *Agrobacterium tumefaciens* were shown to alter the levels of cytokinin and auxin in plants, demonstrating that the ratio of these hormones can control root and shoot production (Klee *et al.*, 1987; Medford *et al.*, 1989). Many physiological processes directly affected by hormonal signals are triggered by environmental circumstances. In these cases, production of the hormone does not involve changes in gene expression. Therefore, genetic manipulation at the level of hormone production is very complex and, in fact, may not be entirely useful. However, development of the receptor (protein) that the hormones bind to usually is genetically regulated and is active only in certain tissue at certain times. Therefore, enhancing the cotton harvesting process by genetically manipulating hormonally regulated physiological aspects of the plant may be a key area for future research.

REGULATION OF ABSCISSION/SENESCENCE BY ETHYLENE AND AUXIN

Regulation of abscission within cotton plants will greatly enhance harvest efficiency and fruit retention. As with many other plant processes, the process of abscission is not simple. Although auxin and ethylene play the major roles in abscission, gibberellin, abscisic acid, and cytokinins also have important effects. The promotion of abscission by gibberellin, abscisic acid, and cytokinin results from stimulating ethylene production, while auxin appears to be mediated, at least in part, by phytochrome. The phytochrome molecule senses changes in day length and produces a signal transduction cascade that causes the plant to start the process of senescence and abscission (leaf drop). The start of the abscission process usually is noted by a marked decrease in auxin levels within the leaf.

In general, ethylene enhances abscission by promoting the formation of an abscission zone. Abscission occurs in specific cells at the base of some petioles, leaves, floral organs, and fruit; however, not all plant parts have abscission layers or exhibit ethylene-enhanced abscission. Such is the case with cotton plants in which abscission zones form in the leaves, but not in mature cotton fruit. This allows ethylene-releasing compounds to be used on mature cotton plants, defoliating them without causing fruit drop.

The abscission zone that forms at the base of fruit, flowers, and leaves consists of one or more layers of thin-walled cells. Just before abscission occurs, certain cells within the abscission layer (the cells farthest from the stem) are digested by cellulases and pectinases. In addition to increases in cell wall-degrading enzymes, there is an unequal pattern of growth within the abscission zone, resulting in leaf, fruit, or flower drop. This process can be delayed by high levels of auxin.

The enzymes responsible for abscission are genetically regulated (Salisbury and Ross, 1992). For example, levels of mRNA molecules coding for cellulase have been found to increase following increases in ethylene production (Ruperti *et al.*, 1998). Ethylene has been shown to increase the steady state level of endopolygalacturonase mRNA in the abscission zone of peach trees (Bonghi *et al.*, 1992) and increases a protein kinase in the abscission zone of some plant species (Sessa *et al.*, 1996).

Abscission-specific genes have been identified in cotton that may be modified through genetic manipulation (Peterson *et al.*, 1996). A study by Del Campillo and Bennett (1996) suggests that abscission in tomatoes is a multistep process

involving both activated and repressed cellulase genes, and that the relative importance of each cellulase in the process of abscission depends on the physiological conditions under which abscission takes place. Bean-leaf abscission has been correlated with the *de novo* accumulation of a cellulase and mRNA accumulation (Koehler *et al.*, 1996). In this study by Koehler *et al.*, genes encoding the bean leaf abscission cellulase were isolated and partially sequenced. One study actually has identified three separate polygalacturonases that are expressed in tomato leaf abscission and flower expression, each with a different temporal expression (Kalaitzis *et al.*, 1997). Several other studies have identified genes that are involved in the process of abscission, some of which are promoted by ethylene (Taylor *et al.*, 1991; Tucker *et al.*, 1991; Coupe *et al.*, 1995; Gonzalez-Bosch *et al.*, 1997).

Although the majority of research has been conducted on other crop species, this information may be used by molecular biologists interested in cotton leaf abscission. Knowing that abscission results from many genetically regulated events and that specific genes have been identified, it may be possible to use biotechnology to regulate these events, thus regulating abscission and improving harvest efficiency. Some points of regulation may be genes involved in the production of cellulase, and ethylene and auxin activity. It also is conceivable that a plant could be genetically regulated to prevent formation of an abscission zone in young squares, flowers, or bolls, thereby preventing premature abscission and potential losses in yield. Another possibility is to modify the cotton plant in such a way that zone forms at maturity or from a day-length signal, so natural defoliation could occur without the application of harvest-aid chemicals.

BOLL DEVELOPMENT

Uniform boll development is desirable for proper cotton harvest; however, the indeterminate nature of the cotton plant results in unequal maturation of cotton bolls. At harvest, chemicals can be applied to the plant to cause as many bolls to open as possible.

The process of boll opening is similar to the formation of an abscission zone during the defoliation process. The harvest-aid chemical, ethephon, increases the natural ethylene level in mature closed bolls, causing them to open. Premature use of ethephon may cause the opening of immature bolls containing fiber inferior to that of bolls that were set earlier (Kerby and Ruppenicker, 1989).

Crop uniformity is a management objective influenced by every aspect of production. Weather and insect pests cause the greatest variations in crop maturity, from delayed plantings with poor stands to irregular fruit set during the season. Management options help reduce the impact of these natural factors. A more uniform boll set accomplishes two important management goals. First, it provides more open bolls for a timely once-over harvest. Second, the fiber within the bolls will be of uniform quality (Hake *et al.*, 1996).

Several studies with plant species other than cotton have identified genes related to fruit ripening that may aid in improving uniformity in boll development. In a study by Hadfield *et al.* (2000) on melon fruit, cDNAs corresponding to mRNAs – whose abundance is ripening-regulated and fruit-specific– were identified. One of these mRNAs encodes for a protein corresponding to 1-amino-cyclopropane-1-carboxylic acid (ACC) oxidase, an important enzyme in the ethylene biosynthesis pathway. The other identified mRNAs encode for proteins involved in amino acid biosynthesis and seed storage. Several other studies have identified additional ripening-related genes (Rebers *et al.*, 1999; Sato-Nara *et al.*, 1999; Zegzouti *et al.*, 1999).

REGROWTH

Cotton is a perennial plant grown as an annual. If the cotton plant is exposed to available soil moisture and warm temperatures following defoliation, it will resume growth by sprouting new vegetation. Regrowth vegetation is difficult to defoliate, because the juvenile tissue does not form abscission zones.

Regrowth of foliage after defoliation of a cotton plant is not desirable because of its potential to interfere with harvest and to stain the cotton fiber (Hake *et al.*, 1996). Excessive regrowth vegetation must be desiccated before harvest, requiring additional harvest-aid chemicals. Additional chemical treatments often are insufficient to prevent staining during harvest and storage.

Off-color or stained cotton is marketed at a discounted value. Additional cleaning to remove stained fibers is not practical, because of the reduced quality of the cotton and increased processing costs. Newly formed leaves also will add to the trash content. Excess trash requires that the cotton be passed through multiple gin cleaners, reducing the amount of fiber (i.e., some fiber is lost during each cleaning). Bringing clean cotton to the gin benefits the producer by reducing lint losses and preserving fiber quality.

In the specific case of regrowth in cotton after defoliation, the hormones of most concern are those involved in shoot formation (auxin, gibberellic acid, and ethylene). For example, high levels of auxin present in late-season regrowth will make new foliage less likely to defoliate when subsequent defoliation compounds are applied, because of the lack of abscission-layer formation. One method of controlling regrowth in cotton plants after defoliation may occur at the level of receptor formation. Hormone receptors are proteins that the hormone binds to in order to cause a plant response (e.g., regrowth). Regulation at the level of hormone production would not be practical, as many environmental circumstances also can cause hormone production without gene involvement. Regulation of receptor formation would prevent a particular hormone from causing a response, regardless of hormone concentration.

ENHANCED ABSORPTION OF HARVEST AIDS ON LEAF SURFACES

The cuticle of the leaf protects it from excessive water loss and also serves as a deterrent to chemical entry. Environmental conditions affect the thickness of the cuticle as well as its composition. For example, research has demonstrated that, under hot, dry conditions, cotton leaf cuticle thickness increased by 33 percent, and uptake of defoliant was reduced by 34 percent (Oosterhuis *et al.*, 1991). The general practice of adding surfactants or spreaders to the spray solution can increase the contact of the defoliant with the leaf surface, while, under conditions that favor a thick waxy layer, the addition of crop oils to the spray solution increases chemical entry and improves defoliation.

Although a relatively thick cuticle is desirable throughout most of the life of the plant to reduce water loss, a thinner cuticle at the time of defoliant application would be beneficial. If a cotton plant could be developed that reduces its waxy layer as it reaches full maturity, chemical defoliants could enter the leaf more easily. This would result in the use of smaller quantities of defoliants, surfactants, and oils. Some studies related to pathogen attack on leaf surfaces have identified genes that code for proteins (enzymes) that aid in the degradation of pectic polymers. These enzymes include several pectinolitic enzymes and pectin methylesterase (Gaffe, 1997; Shevchik, 1999). It may be possible to identify these genes in cotton or to introduce them into cotton plants to induce a change in leaf wax composition and thickness as the plant gets closer to the defoliation period.

INCREASED RETENTION OF SQUARES, FLOWERS, AND BOLLS

A greater number of retained squares produces more flowers, which results in more harvestable bolls. Squares, flowers, and young bolls (<10 days post anthesis) will abscise because of many factors. Some of these factors include insect attack, water stress, nutrient stress, and poor weather conditions (Kerby and Hake, 1996). A possible point of regulation for increasing retention is to develop plants that do not form abscission zones in the flowers, squares, and young bolls, or that form them at a slower pace.

Important points of regulation would be to control or to stop the presence of cellulase activity in young flowers, squares, and bolls. Localized regulation in these areas is desired, as foliage still would require abscission zones and cellulase activity for defoliation to occur. The most likely successful point of regulation is in the site-specific control of cellulase production and other enzymes involved in the formation, degradation, and separation of the abscission zone.

The physiological processes mentioned here generally are thought to be closely linked to cotton defoliation practices. However, modifications in water-stress tolerance, insect and herbicide resistance, growth characteristics, and fiber quality are areas that may assist the harvesting process by providing a healthy plant that produces a high-quality cotton crop. The following section discusses possible techniques that may help in improving the physiological processes that have been noted.

USE OF BIOTECHNOLOGY TO ACHIEVE PHYSIOLOGICAL GOALS

Significant progress has been made in biotechnology in general, accompanied by an increase in its uses for the improvement of cotton. "Biotechnology" has been defined as "the collection of industrial processes that involve the use of biological systems" (King and Stansfield, 1990). Some of the most dynamic techniques relating to agriculture are the sequencing of plant genomes, comparative mapping across species with genetic markers, and objective-assisted breeding after the identification of candidate genes or chromosome regions for further manipulations (Ortiz, 1998).

Resources – This section briefly describes some of these techniques and tools that could be applied toward achieving the physiological goals

previously discussed. A number of excellent resources are available (see the Literature Cited section at the end of this chapter), if more information is desired. Examples of such resources include:

Bajaj, Y.P.S. 1998. *Biotechnology in Agriculture and Forestry, 42: Cotton.* Springer Verlag, New York.

Bains W. 1998. *Biotechnology from A to Z.* Oxford University Press, New York.

Maniatis T., J. Sambrook, and E.F. Fritsch. 1989. *Molecular Cloning : A Laboratory Manual* (Three-Volume Set). Cold Spring Harbor Laboratory Press, New York.

Mather J.P., and P.E. Roberts. 1998. *Introduction to Cell and Tissue Culture: Theory and Technique (Introductory Cell and Molecular Biology Techniques).* Plenum Publishing Corp., New York.

Paterson, A.H. 1997. *Molecular Dissection of Complex Traits.* CRC Press LLC, Boca Raton, FL.

PLANT GENOMICS/MOLECULAR MARKERS

Plant genomics, the science that seeks to understand how genes enable a plant to carry out its functions as a living organism, is a newly emerging field based on the developing technology of gene sequencing. The information derived from studies of plant genomics will enable scientists to investigate how the diversity of functions in all plants is related to simple changes in individual genomes (Delaney *et al.*, 1998). The field effectively began in 1989 with the initiation of the Multinational *Arabidopsis* Genome Research Project (Clutter, 1999). Ultimately, plant genomics may be applied to modifying plants for optimal performance. For example, more information may be available on why plant-resistant genes are clustered together and how they may be manipulated (Paterson, 1997). Commercial crops from this new research area even may be available within the next few years (Gwynne, 1999).

Linkage is a familiar concept in genetics that dates back to the early studies on *Drosophila* (fruit fly), when it was shown that combinations of genes tended to be inherited as groups, linked together because of proximity to one another on the same chromosome (Watson *et al.*, 1992). As linkage relationships are identified as a result of the increasing number of known genetic markers for plant chromosomes, chromosome maps can be constructed. Markers found to be

linked to important agronomic characteristics also can be used to select for those characteristics in breeding programs. Some of the techniques used to manipulate and analyze genomes already are well established, while a great deal of ingenuity and energy is being expended in devising new methods to overcome the technical difficulties inherent in tackling entire genomes (Watson *et al.*, 1992).

GENETIC TRANSFORMATION

Some of the major limitations of genetically transforming agronomically important crops are the extreme difficulty of isolating and maintaining viable protoplasts, the inefficiency of current transformation methods, and, in particular, the inability to regenerate complete fertile plants from transformed cells (Smith, 2000). *Agrobacterium*-mediated transformation and particle bombardment of target tissue, followed by regeneration through somatic embryogenesis, are two techniques commonly used to transform cotton (Peeters and Swennen, 1998). *Agrobacterium*-mediated transformation is useful for introducing single genes, such as those responsible for many insect or herbicide resistances (Umbeck *et al.*, 1987), while particle bombardment allows for the introduction of multiple genes. A third technique involves the direct DNA uptake into protoplast, analogous to plasmid transformation of bacterial cells.

***Agrobacterium*-mediated transformation** is the method most commonly used to genetically alter cells of dicotyledonous plants. *Agrobacterium tumefaciens* is a naturally occurring pathogenic bacteria in the soil that has the ability to transmit a tumor-inducing plasmid into an adjacent living plant cell. Strains of *A. tumefaciens* carrying the plasmid may be genetically engineered artificially (without causing tumor induction) to introduce foreign genes of choice into plant cells (King and Stansfield, 1990). The process of gene transfer from *A. tumefaciens* to plant cells is quite complex and involves a number of procedures, including bacterial colonization, induction of the bacterial virulence system, and T-DNA transfer and integration into the plant genome (de la Riva *et al.*, 1998).

Particle bombardment (or biolistics) is the technique whereby microscopic particles of tungsten or gold, coated with genetically engineered DNA, are explosively accelerated into cells (Forbes *et al.*, 1999). Transformation efficiencies are affected by the attributes of the particles used, surface properties of the bombarded tissue, and turgor pressure of the cell. A variety of particles and acceleration systems are available to introduce genetic material into cells.

CLONAL PROPAGATION

The cotton plant is propagated by seed and is cultivated as an annual crop. Deterioration of varieties occurs because of natural crossing and mechanical mixtures during the ginning process. Clonal techniques could be helpful in maintaining varietal purity. In addition, transgenic plant production, regardless of method, requires the ability to regenerate plants from single (or a small number of), isolated transfected cells (Old and Primrose, 1989). Clonal propagation, a tissue culture technique, allows plant cells and tissue to be regenerated into mature, fertile plants. Two such clonal propagation methods are somatic embryogenesis and protoplast cultures.

Somatic embryogenesis is a complex process of making artificial (cloned) seeds using an asexual means of reproduction. The process has been a significant achievement in plant tissue culture as a target for genetic engineering and for the production of synthetic seeds. This method also has greater potential for inexpensive, large-scale propagation than current methods (e.g., seeds, macropropagation, and micropropagation) (Thompson, 1998). The phenomenon of somatic embryogenesis has been reported in about 300 species of plants (Bajaj, 1998). However, regeneration through somatic embryogenesis is genotype-dependent (Trolinder and Chen, 1989). Somatic embryogenesis in cotton first was observed in suspension cultures of the wild species, *G. klotzschianum* (Price and Smith, 1979), with considerable progress being made since this first observation (Gawel and Robacker, 1995).

Protoplast cultures. Protoplasts – cells whose walls have been removed – have proved suitable for gene transfer in a number of agricultural crops (Bajaj, 1994). With the right combination of the plant hormones, auxins and cytokinins, transformed protoplasts can be induced to regenerate cell wall and callus, as well as whole plants (Smith, 2000).

Applications of protoplast technology are limited, as many species of economic importance fail to regenerate with this method (de Marco and Roubelakis-Angelakis, 1996). In cotton, although protoplasts have been isolated by a number of researchers (Firoozabady and DeBoer, 1986; Chen *et al.*, 1989; Peeters *et al.*, 1994), the regeneration of complete plants is a comparatively recent development (Bajaj, 1998).

SUMMARY

The genetic engineering of plants has facilitated the production of agronomically desirable crops that exhibit increased resistance to pests, herbicides, pathogens, and environmental stress, and enhancement of qualitative and quantitative crop traits (Gasser and Fraley, 1992). Commercially available transgenic cotton varieties include Bollgard, Roundup Ready, and BXN traits. New developments in gene identification and transformation technologies will assist in increasing the number and type of transgenics on the commercial market.

A number of cotton research projects, not yet at the commercial development stage, are investigating novel avenues of genetic engineering. Examples discussed in this chapter include gene research projects focused on improving cotton fiber quality (Geissinger, 1999), the impact of parental gene expression (Fannin, 2000), manipulating complex pathways (Amber, 2000), and a "gene protection" technology (Pro Farmer Editors, 1999).

To date, biotechnology has not been commercially applied to the area of cotton harvest aids. The future may be different, as stricter safety regulations and policies are established, and as costs of chemicals and their application increases. Fortunately, the potential exists to manipulate many physiological processes, resulting in enhanced harvest-aid efficiency. Some of these physiological processes include abscission/senescence, boll development, regrowth of foliage, absorption quality of the leaf surface, and retention properties of squares, flowers, and bolls.

Genetic engineering to confer useful agronomic traits to cotton is likely to lower the cost of production, improve yield and quality, and promote environmentally friendly farm practices (Bajaj, 1998). Along with these many benefits, though, comes the potential for adverse ecological effects, because of the often-sustained expression of the engineered traits in the genetically engineered (transgenic) plant and the persistence of the transgenic plant or plant residue in the environment (Donegan and Seidler, 1998). Other concerns include reduction of genetic diversity, new pest emergence, changes in ecosystem dynamics, chemical contamination, and genetic pollution (Charest and Duchesne, 1995). However, with careful monitoring and responsible handling of the advancements possible from genetic engineering, benefits to society may be achieved with minimal environmental risk.

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