https://doi.org/10.17221/46/2017-CJGPB

The Progress of Genetic Improvement in Alfalfa (*Medicago sativa* L.)

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Abstract

Kumar T., Bao A.-K., Bao Z., Wang F., Gao L., Wang S.-M. (2018): The progress of genetic improvement in alfalfa (*Medicago sativa* L.). Czech J. Genet. Plant Breed., 54: 41–51.

Alfalfa (*Medicago sativa* L.) is a perennial and outcrossing species, widely grown as a forage legume for hay, pasture and silage. The genetic engineering approaches involve the transfer of useful or novel gene(s) into alfalfa to improve desired traits. The recent development of genetic engineering is extensively applied to basic and applied research for alfalfa improvement, including improvement of herbicide resistance, reinforcement of the resistance to biotic and abiotic stresses, production of novel compounds, improved yield for industrial and/or pharmaceutical proteins and renewable energy sources. On the other hand, alfalfa forage needs to possess additional fermentable carbohydrates, proteins with a balanced amino acid profile that are gradually degraded in the rumen of domestic livestock, and zero anti-nutritional factors, which are the major concerns of recent interest in alfalfa. However, an advance of transgenic approach is contentious, requiring vigilant experimental methods and design to contest bio-safety challenges. More importantly, the technology of clustered regularly interspaced short palindromic repeats (CRISPR) is rapidly growing and might be a game player or changer in alfalfa. The present review can enable us to identify the proper direction, get familiar with new research methods and success of genetic engineering technology in alfalfa, to produce maximally improved cultivars.

Keywords: CRISPR/Cas9 technology; forage yield and quality; genetic engineering; stresses tolerance, transgenic alfalfa

Alfalfa (*Medicago sativa* L.) is a perennial legume species of the family Fabaceae and has been extensively used for forage crop since the beginning of known history. Alfalfa originated from the vicinity of Iran and was introduced into North America by the European colonists during the 17th century. It is also formally called "Queen of the grasses" and is cultivated worldwide as forage crop on over 32 million hectares (Tesfaye *et al.* 2006; Kumar 2011). Being a high-yielding biomass species, it is used for green chop, grazing, hay, and silage production over a wide range of soil and climate conditions. In addition, alfalfa possesses a deep rooting system and also establishes a symbiotic relationship with the soil bacterium *Sinorhizobium meliloti*, which fixes nitrogen (N) from the air and thus provides N for the plant and increases soil nitrogen fertility for subsequent crop rotations. One of the most typical characteristics of alfalfa is its high nutritional value as animal feed with 15–22% of crude proteins (CP) as well as ten different kinds of vitamins and abundant minerals (McCoy & Walker 1984; Soto-Zarazua *et al.* 2016).

Moreover, alfalfa contains low fibre, relatively higher proteins in comparison with other forage crops, which helps in superior intake of the forage. In addition to the traditional uses of alfalfa as an animal and livestock feed, it is also used as a biofuel, bioremediation, soil conservation and natural biofactory, for the production of novel pharmaceutical compounds, industrial enzymes and important proteins such as lignin peroxidase, cellulase, phytase

https://doi.org/10.17221/46/2017-CJGPB

and alpha-amylase and so on (Tesfaye *et al.* 2005; Kineman *et al.* 2010; Li & Yuan 2013).

The current problems manifested in cultivation and utilization of alfalfa forage are: biotic and abiotic stresses, seed yield depression, anti-quality traits, poor digestibility of hay and inefficient nutrient utilization. Fortunately, there have been lots of works on genetic improvement of the above traits in alfalfa.

Differing from conventional breeding methods transgenic technology permits the correct and precise transfer of one or a few desirable genes. Genetic engineering has emerged as the most powerful tool to increase yield productivity without losses caused by weeds, pests and pathogens. The first genetic transformation of alfalfa was reported in the mid-1980s (Shahin *et al.* 1986). Since then, transformation of alfalfa using *Agrobacterium* mediated transformation and other methods has become a routine exercise in many research laboratories. Here we summarized these advances.

The enhancement of herbicide resistance

Weeds reduce the quality of alfalfa hay, lower the potential selling price and may be a threat to the animal health. Dodder that belongs to the *Cuscuta* species is an annual parasitic weed infesting alfalfa, which may cause up to 20% losses in forage production. Among the poisonous weeds that have been found in alfalfa hay is oleander belonging to the *Nerium oleander* species, a nitrate accumulator, foxtail (*Hordeum murinum* L.), pyrrolizidine alkaloid producing plants and bristly oxtongue (*Picris echioides* L.). Therefore it is better to choose herbicide resistant crops. Although alfalfa is susceptible at the establishment stage, it competes with weeds at other stages well, and so it is challenging for alfalfa producers to grow weed-free alfalfa. To meet the high quality of forages as demanded in the dairy industry, perfect weed control is compulsory for field growers. To cope with this requirement glyphosate is the broad-range and most commonly utilized herbicide, able to control different weeds including dodder, nutsedge, and quackgrass. It was discovered by Monsanto in 1970. Monsanto brought it under the trademark Roundup™, Roundup Ready Alfalfa (RR® Alfalfa), a genetically modified variety of alfalfa developed in 2005 by Forage Genetics International (FGI) using a gene construct owned by Monsanto, has a single bacterial gene inserted into the alfalfa DNA that codes for the C4-5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme instead of EPSPS enzyme, the C4-EPSPS is similar to the naturally occurring enzyme in both structure and function, except that it is unaffected by glyphosate. In plants with the C4-EPSPS enzyme, the shikimate pathway can produce the aromatic amino acids necessary for plant growth and survival even in the presence of glyphosate, because mammals do not synthesize their own aromatic amino acids either via the shikimate pathway or via EPSPS enzyme metabolic pathway systems (Cole 1985). Glyphosate-resistant (GR) alfalfa became available in 2005, and was planted in 2006. Production was allowed for the period of 6 years, depending upon location and production of seed, or due to the perennial nature. Furthermore, the source of the transgene was present in the landscape until 2011, whereas GR alfalfa was deregulated for the second time (ISAAA 2016). Furthermore, the characterization and expression of a plant-optimized variant of *glycine oxidase* (*GO*) one with (*GOTP*+) and one without (*GO TP*–) the pea rbcS plastid transit peptide were used from *Bacillus subtilis,* which efficiently degrade glyphosate in transgenic alfalfa tested for both *in vitro* and *in vivo* scenario (Nicolia *et al.* 2014). More recent studies on glyphosate tolerant alfalfa have been available for sale in the US for years the HarvXtra™ reduced lignin, downregulation of the monolignol biosynthetic enzyme hydroxycinnamoyl coenzyme A: *shikimate hydroxycinnamoyl transferase* (*HCT*), results in higher digestibility and offers 15 to 20% increase in the yield of alfalfa which is currently on the market (Gallego-Giraldo *et al.* 2011, 2014) and hence is likely to be accepted by farmers.

Interestingly, recent co-transformation of an oxidative responsive gene, *CsALDH12A1*, and *CsLEA* from a desert grass *Cleistogenes songorica* for drought and salt stress associated with the *bar* gene for herbicide resistance get more attention in transgenic alfalfa (Duan *et al.* 2015; Zhang *et al.* 2016b).

The reinforcement of insect, pest and disease resistance

Insects and pests cause a significant reduction in the yield and quality of forage. Certain insects such as alfalfa weevil (*Hypera postica* G.), spotted alfalfa aphid (*Therioaphis maculate* B.), potato leaf hopper (*Empoasca fabae* H.), pea aphid (*Acrythosiphon pisum* H.) and blue alfalfa aphid (*Acyrthosiphon kondoi* S.), are considered to maximize forage yield and quality (Liu *et al.* 2008). Severe infestation affects

https://doi.org/10.17221/46/2017-CJGPB

the forage quality and re-growth strength of multicut varieties. Larvae of the weevil are considered to be most damaging which cause skeletonization of leaves and stunted growth, consequently reduced forage yield and quality as well. Some of the annual species are regarded to show disease or pest resistance and environmental stress tolerance. For example, *M. scutellata* and *M. rugosa* show resistance to alfalfa weevils and aphids (Chandra 2009), while sexual incompatibility to *M. sativa*. It is hard to transfer these traits by conventional breeding (Mizukami *et al.* 2006). Modern biotechnological approaches such as embryo rescue, somatic hybridization and in-planta gene transfer method can be employed for interspecific hybrids and shifting of the traits. Somatic hybrids of *M. sativa* and *M. falcata* were produced by the polyethylene glycol (PEG) mediated protoplast fusion method (Mendis *et al*. 1991; Weeks & Rommens 2008). Alternatively, genetic transformation of alfalfa with *Bt* (*Bacillus thuringiensis*) gene has been proven to be an effective strategy (McCaslin *et al.* 2002). Some studies indicated that *Bt* crystalline proteins have been safer to humans, animals and non-target pests (Kumar *et al.* 2008; Rule *et al.* 2014). Alfalfa showed high resistance against weevil and beet armyworm when expressing *Cry1C* encoding a *Bacillus thuringiensis* δ-endotoxin as compared to the wild type (Strizhov *et al.* 1996). Another study revealed that alfalfa can be made resistant against weevil by expressing the synthetic *Cry3a* gene into alfalfa genome (Tohidfar *et al.* 2013). Other *Bt* crystal proteins and pyramiding of genes (*Cry1F*, *Cry1A.105*, and *Cry2Ab2*) expressed in SmartStax Corn Hybrids against *Lepidopterans* were found to be an effective strategy (Rule *et al.* 2014). Alfalfa production has been drastically impacted by pathogens. About 20% of the losses of US alfalfa hay crop are owing to different diseases, which may account for up to $1 billion (Nutter *et al.* 2002). Anthracnose, caused by the fungus *Colletotrichum trifolii*, is one of the destructive diseases of alfalfa. Thus, integrated pest management (IPM) is an optional and crucial step toward production of high quality forages, mainly for market export purposes. Equivalent defence responses were observed against *C. trifolii* in *M. truncatula* and other annual *Medicago* species (Torregrosa *et al.* 2004). Yang *et al*. (2008) reported that map-based cloning of the *RCT1* (resistance to *C. trifolii* race *1*) gene for R protein encoding broad-spectrum anthracnose was transferred to the alfalfa and the results indicated that the host resistance has potential against the fungal *Colletotrichum*, and also helped to understand translational research progress from *M. truncatula* into alfalfa plant. Whilst transgenic alfalfa was tested for fungal chitinase gene resistance to antifungal activity, the endochitinase gene (*ECH42*) yielded encouraging results in vegetative organs and root exudates, the chitinase activity of root exudates in transgenic plants was profoundly 7.5–25.7 times higher than in the control counterpart (Tesfaye *et al.* 2005). Significant and consistent efforts are being urgently needed to take advantage of *M. truncatula* as a model plant to characterize legume-pathogen interactions (Tivoli *et al.* 2006). Since most of the alfalfa pathogens are the same as pathogens of *M. truncatula*, it is expected that *M. truncatula* may serve as a tool for searching resistance genes for many common diseases of alfalfa, and that the functional disease resistance will be maintained when genes are moved across the species by transgenic technology (Yang *et al.* 2008; Wu *et al*. 2016).

The enhancement of tolerance to abiotic stress

Drought and salinity stress are the major environmental limiting factors for plant growth, development as well as survival, and lead to enormous yield losses each year. On the other side, higher plants have evolved intricate mechanisms to rapidly adapt to harsh environment. In the last few years, stress physiology in crops has become one of the central issues of plant biologists and more attention has been paid to mechanisms of plant stress tolerance, including biochemical metabolisms, morphological variations, and gene expression. These investigations further provide new methods to improve plant stress tolerance and prevent crop yield losses (Castroluna *et al.* 2014).

The plant abiotic stress is a quantitative character regulated by polygenes. Stress resistance is an important indicator for evaluation of plants. Extensive studies including: functional protein, osmotic, signal transduction, and transcription factor related genes have been conducted. More specifically, researchers have examined target genes from various glycophytes, halophytes and xerophytes that can increase salt and drought tolerance and also enhance water use efficiency (McKersie *et al*. 1996; Winicov 2000; Bao *et al.* 2009, 2016; Jiang *et al.* 2009; Kumar 2011; Kumar *et al.* 2014). Some of the useful genes for abiotic stress tolerance have been well expressed in

https://doi.org/10.17221/46/2017-CJGPB

alfalfa (Suárez *et al.* 2009; Jin *et al.* 2010; Zhang & Wang 2015). Recently the more realistic efforts were achieved by co-expressing *ZxNHX* and *ZxVP1-1* encoding tonoplast Na+/H+antiporter and H+-PPase from the xerophyte *Zygophyllum xanthoxylum*. Transgenic alfalfa showed improved growth and enhanced tolerance to drought, salt and phosphate deficiency, increased productivity, and forage quality in T_1 generation compared with wild type plants, both in a greenhouse and in field conditions (Bao *et al.* 2016; Kang *et al.* 2016).

Besides drought and salt tolerance, winter hardiness is another important and economic agronomic trait for the improvement of alfalfa productivity. A negative influence on winter hardiness and biomass yield obscures alfalfa crop improvement (Brummer 2004). McKersie *et al.* (2000) improved winter hardiness using the superoxide dismutase (*SOD*) gene and found increased forage yield with significantly improved winter survival in transgenic alfalfa. Similarly, increased tolerance to acid and aluminium soil was described by Tesfaye *et al.* (2001) expressing a malate dehydrogenase gene in alfalfa. Calderini *et al.* (2007) introduced the stay-green phenotype into alfalfa by senescence-specific cytokinin production. Wang *et al.* (2014) produced transgenic alfalfa plants expressing *AtNDPK2* with enhanced tolerance to high temperature, drought and salt stresses and transgenic plant grows better partially through increased expression of auxin-related genes for indole acetic acid (IAA) under normal growth conditions compared to wild type plants. *GsZFP1* encodes a Cys_2/His_2-type zinc-finger protein, obtained from the wild legume *Glycine soja*, while overexpression of *GsZFP1* in transgenic line induced higher expression of stress-responsive marker genes, *MtCOR47*, *MtRAB18*, *MtP5CS*, and *MtRD2*, in transgenic alfalfa it results in superior drought tolerance (Tang *et al.* 2013).

Production of novel compounds

In comparison with the animal cells, plant cells can also provide a low-cost and more human-friendly system for the production of commercially new and useful recombinant proteins. Recently, advancement in the field of bio-pharming has been tremendously employed to produce a large variety of important pharmaceutical products like monoclonal antibodies and blood substitutes (Vlahova *et al.* 2005). Transgenic alfalfa can be used to produce industrial enzymes in the bioreactor, an easy and significantly cheaper way in contrast to the expenses for constructing new fermentation amenities (D'Aoust *et al.* 2004). Austin-Philips and Ziegelhoffer (2001) produced the feed enzyme phytase in transgenic alfalfa. Glycosylation studies have revealed that alfalfa has potential for producing recombinant glycoproteins with homogeneous glycosylation patterns. Vlahova *et al.* (2005) employed transgenic alfalfa as a new expression model to produce the human recombinant protein lactoferrin, an iron-binding glycoprotein with antiviral activity with broad-spectrum capability to arrest the replication of a wide range of human and animal RNA and DNA viruses. Thus alfalfa may become a model system for molecular bio-farming.

Quality traits improvement

In the US, dairy operators' demand has been increasing for higher quality alfalfa, even at more cost on yield. Recent studies suggested that partially replacing conventional soybean meal (SBM) as a protein source with low-fibre alfalfa meal in the laying-hen diet can positively influence yolk quality without adversely affecting productive traits (Laudadio *et al.* 2014).

More importantly, forage digestibility has been a vital goal of forage breeders to improve the effective quantity and quality of presently available feeds to animals. The cell wall of plants has been distressed to be a limiting factor for forage ingested by ruminants. The lignin is a polymer compound comprising hydroxylated and methoxylated phenylpropane units, and it consists of two main components of monolignols, monomethoxylated guaiacyl (G) and dimethoxylated syringyl (S), polymerized into five dissimilar linkages. Any significant change or reduction in the concentration of cell wall components may enhance both intake and energy density in forage crops. Likewise, increasing digestibility of the cell wall would improve more energy. Lignin biosynthesis pathway genes for most of the enzymes have been identified and many of their functions have been downregulated in transgenic plants (Baucher *et al.* 1999; Shadle *et al.* 2007). As the matter of concern, alfalfa contains a higher fibre proportion of lignin than other grasses, which results in lower digestibility (40 to 50%) compared to 60 to 70% fibre digestibility (Buxton & Redfearn 1997). The first lignin gene for cinnamyl alcohol dehydrogenase (CAD) has been downregulated in alfalfa by Baucher *et al.* (1999) and Nair *et al.* (2004). However, these studies did

https://doi.org/10.17221/46/2017-CJGPB

not provide any definitive information on the factors associated with the digestibility of forage. Reddy *et al*. (2005) developed transgenic alfalfa lines encoding cytochrome P450 enzymes, namely cinnamate 4-hydroxylase (C4H), coumaroyl shikimate 3-hydroxylase (CSH) and coniferaldehyde 5-hydroxylase (C5H), with altered lignin content and composition which catalyze reactions in the formation of monolignol, G-unit, and S-unit components, respectively. Transgenic alfalfa lines with downregulated caffeic acid 3-O-methyltransferase (COMT) and caffeoyl CoA 3-O-methyltransferase (CCoAOMT) genes show a strong negative relationship between lignin content and rumen digestibility, while there is no significant relationship between lignin composition and digestibility traits (Guo *et al.* 2001; Li *et al.* 2008).

Fibre intake and digestibility affect the forage quality. The rate of digestion of alfalfa fibre is threefold higher than that of grasses. Alfalfa has the lack of fermentable carbohydrates as compared to the corn silage crop (Britto & Broderick 2006). In order to further improve digestibility of alfalfa, the alternative strategy is to selectively increase the rapidly fermentable carbohydrate component such as pectin in the cell wall. Noticeably the rapid starch fermentation is necessary to increase the pectin percentage in alfalfa. Any addition of cellulose content without increment of lignin may also result in a cell wall matrix with a higher degree of ruminal degradation. More recently an experiment on fibrolytic enzyme application with ferulic acid esterase (FAE) producing bacterial inoculants at the baling shows a potential method for enhancing the performance of lambs fed baled alfalfa hay. Replacing or substitution of at least one part of the cell wall fraction with another fast digestible polysaccharide may have the superior influence on fibre digestibility and quality (Aboagye *et al.* 2015).

Alfalfa is a rich source of protein not only of crude protein (CP) but also most of the CP fraction content in which sulphur-containing amino acids become a more demanding factor especially for animals kept for wool production (Pickering & Reis 1993). The maize endosperm storage protein δ zein is a hydrophobic protein containing 23% of methionine and 4% of cysteine (Kim & Krishnan 2003). Such a kind of proteins enriched with sulphur amino acids has been targeted for expression in many leguminous crops such as alfalfa, lupine and soybean. Bagga *et al*. (2004) introduced the gene conferring zein from maize into alfalfa, which resulted in stable accumulation of δ-zein in endoplasmic reticulum-derived protein bodies formed by β-zein. The transgenic alfalfa expressing the Arabidopsis cystathionine γ-synthase (*AtCGS*) gene showed up to be 32-fold, 19-fold, and 2.2-fold higher in total methionine, S-methylmethionine (SMM) and water-soluble proteins than the wild type (Avraham *et al.* 2004). Thus, transgenic alfalfa expressing higher levels of the amino acids methionine and cysteine offers a promising way to improve protein quality.

A larger amount of the consumed alfalfa proteins is degraded rapidly in the rumen, with less efficient utilization of the proteins and nitrogen losses and risks of bloat as the protein degradation is very fast, ruminal microbes are unable to utilize all of the amino acids sufficiently, causing an extra amount of ammonia. Such a loss of proteins in the form of ammonia from the rumen is termed as "ammonium overflow". A higher concentration of tannins reduces the voluntary feed uptake and nutrient digestibility, but a low or moderate concentration of tannins might improve the digestibility of protein. Some reports are available on the ability of tannins to reduce the protein degradation risk (McMahon *et al.* 2000). These paybacks have led to the success of intensive research efforts to define the chemical composition of condensed tannin (CT). Although research progress has been unhurried, the main objective has been to develop alfalfa that expresses foliar CT for better protein utilization in the rumen. Scientists are exploring possibilities for the production of hydrolysable tannins in alfalfa, having positive attributes similar to condensed tannins. A wide variety of compounds is present in forages that can reduce animal growth and performance, or may cause sickness or even loss of the animal life. These compounds are saponins, alkaloids, nitrates, oestrogens, cyanoglycosides and mycotoxins. Forage of superior quality does not contain any harmful or any anti-quality factors. The saponins are considered to be the main anti-nutritional issues in alfalfa that significantly inhibit nutrient utilization and feed conversion performance (Cheeke 1996); they also diminish microbial fermentation, imbalance nutrient digestion and adversely disturb protein biosynthesis in ruminants (Sen *et al.* 1998). They are also implicated in ruminant bloat and also cause different susceptibility in different animals (Mathison *et al.* 1999). In the future, besides addressing the existing problems of developing transgenic alfalfa, emphasis should be laid on the biosynthetic pathway and molecular mechanism to develop low-saponin cultivars of alfalfa, and it is also necessary to improve feeding

https://doi.org/10.17221/46/2017-CJGPB

formula and feeding scale by mixing with other grasses to overcome this problem. Hence, proper interventions are urgently needed to decrease the level of saponins and improve the forage quality in alfalfa.

Concerns of genetically modified (GM) alfalfa

Since its inception the debate over genetically modified organisms (GMOs) has continued, to release herbicide-resistant alfalfa and to assess its effect on the forage export market, which is a big question of concern. Many growers and exporters have agreed that herbicide-resistant alfalfa would be helpful to ease the production of weed-free hay; conversely, they express their fear that associated concerns of Roundup ready (RR) alfalfa may influence the export market. Transgenic plants have been in use for animal feeding and human benefit for more than a decade. According to the 'substantial equivalence' investigations about the bio-safety of transgenic crops, the US Department of Agriculture (USDA) suggested that RR alfalfa can be safely employed in the US feed market for animals (USDA 2005).

In addition, the transgene contamination with traditional/wild type alfalfa has been a major big dilemma. Pollen grains carried out by bees allow the spreading of alien genes. Another big source of contamination is unwanted volunteer seedlings, large-scale cultivation of RR alfalfa, uncultivated and weed alfalfa may become cross contaminated by means of cross-pollination. In the light of the fact that glyphosate is a broad-spectrum herbicide, the RR alfalfa will have a superior advantage over non-GM alfalfa plants and may become a super weed. There are many regulatory authorities in the countries to keep eyes on research and development of GMOs and ensure that the introduction of GMO would not pose any threat to human health or the environment. However, after deregulation, there would be no regulatory boundaries for planting and use of transgenic varieties. Hence, stewardship programs should be developed to defend the varieties and surrounding crops. Implementation of the present strategies would not only be the means for the successful re-introduction of RR alfalfa, but also it needs to set a standard that will enable to introduce new transgenic traits into alfalfa crops.

Alfalfa as a renewable energy source

Alfalfa has been considered as a potential and sustainable cellulosic feedstock for ethanol production and yield of other industrial raw materials (Samac *et al.* 2006). Although considerable efforts of research have been taken to improve the forage value, only limited research was undertaken to improve biofuel production. Chen *et al.* (2006) investigated biomass digestibility relationships between six different transgenic alfalfa lines [gene downregulated for trans-cinnamate 4-hydroxylase (C4H), hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), p-coumaroyl shikimate 3'-hydroxylase (C3H), caffeoyl CoA 3-O-methyltransferase (CCoAOMT), ferulate 5-hydroxylase (F5H) and caffeic acid O-methyltransferase (COMT)] and found differences in the cellulase/cellobiose saccharification efficiencies of acid-pretreated cell walls of these lines. Some of the other successful studies suggested that genetic modification of lignin biosynthesis can make smooth progress of the processing of lignocellulosic materials for biofuel production (Chen & Dixon 2007; Li *et al.* 2008; Gallego-Giraldo *et al.* 2011, 2014). Another possibility will be upregulating lignin biosynthesis to increase biomass energy density rather than downregulating, in view of the fact that the lignin polymer is comparatively more reduced than polysaccharides, biomass and higher lignin content would be a better raw material or choice for gasification and the process used for biofuel production (Agrawal *et al.* 2007). Although in the process of biofuel production, the current potential problems are that the pathogens and insects must be addressed before large-scale application and production of low-lignin plants (Li *et al.* 2008). Therefore, combined and strict efforts are required to identify a cogent approach to engineer alfalfa as a bio-energy crop, and thereby to ensure the sustainability of this new agricultural paradigm.

Future scientific challenges and perspectives

Generally, alfalfa cultivars are synthetic populations that originated by a heterogeneous combination of heterozygous genotypes, which complicates the application of genomic solutions in the breeding process (Brummer 2004). The application of genomic techniques for genetically improved alfalfa may be promising and challenging, but many of the problems can be solved by concerted and continuous efforts. Although plant genetic engineering meets such opportunities for alfalfa improvement to a certain extent, there are still many challenges at both the technical and commercial level. Gene transformation technol-

https://doi.org/10.17221/46/2017-CJGPB

ogy, appropriate use of promoters, transit peptides, choice of selectable or reporter markers, etc. are all protected by laws of international patents. There are limited and fully characterized promoters that provide high gene expression in alfalfa. Therefore, a set of constitutive, tissue or temporal-specific promoters effective in alfalfa is obligatory for the optimized expression of transgenic research. Due to the autotetraploid character, the transgene integration and gene stacking approach developed for diploid crop species like corn, soybeans and cotton etc. might be less suitable for alfalfa. New emerging breeding techniques would be needed to adopt and address these inimitable challenges in alfalfa. Developing an ideal alfalfa plant is a vital scientific future challenge, even then, public acceptance for GM crop issues may co-exist that might hinder the commercial potential of the technology in GM responsive markets.

Ideal alfalfa forage should have the better balance of proteins and rapidly fermentable carbohydrates and better use of their content. The balanced content of essential amino acids with delayed timing of protein degradation in rumen would be the desirable traits of an improved alfalfa crop (Barry & McNabb 1999). Enhancing the extent of fibre digestion by altering lignin content or composition change would be demanding too (Reddy *et al.* 2005). Ideal alfalfa must have increased cellulose quantity and decreased lignin in the cell walls of the stem. Winter hardiness traits, better water use efficiency (WUE), salt tolerance and pest resistance are also desirable characteristics to be inserted while considering ideal forage. Minimizing bloat-causing properties by engineering alfalfa with improved nutritional qualities, pest resistance, fewer cuttings characteristics, increased yield and better WUE would lead us to achieve an ideal alfalfa plant for animal feeding. However, an ideal alfalfa plant for biofuel production is supposed to have enhanced cellulase/cellobiose saccharification (Chen & Dixon 2007; Li *et al.* 2008). Above all, the ideal alfalfa should have an enhanced potential for biomass production. Furthermore, it should have quick regrowth after harvest and straight tall growth to make ease in the mechanical harvesting period (Robins *et al.* 2007). Although all these traits would be desirable and required in an ideal alfalfa, their genetic characterization and the genetic relationships between them are not yet well understood. Many of these traits are quantitative traits controlled by numerous genes; quantitative trait locus (QTL) mapping and marker-assisted selection (MAS) would be required for pyramiding of the compatible traits.

The advent of an easier way based on the bacterial type II CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) immune system has recently emerged as a wonderful technology for genome editing (Belhaj *et al.* 2013; Teotia *et al.* 2016). The CRISPR/Cas9 system can be delivered into plant cells for genome editing by a number of different means including biolistic/gene gun, virus-based guide RNA (gRNA) and *Agrobacterium*-mediated delivery. Recent advances in the CRISPR system associated genome editing in plants by focusing on application of this technology in model plants, crops, fruits, woody plants and grasses have been well reported (Li *et al.* 2014; Schuster *et al.* 2015; Basak & Nithin 2015; Michno *et al.* 2015; Ricroch & Henard-Damave 2015; Schaart *et al.* 2015; Zhang *et al.* 2016a; Meng *et al.* 2017), which can help in genome editing associated with the CRISPR system to get insights into genome modifications and functional genomics in forage crops. A web-based server has already been developed for the CRISPR/Cas9 system based on alfalfa and *M. truncatula* to identify specific sequences of promoters and terminators for optimal expression, promoters for expression of the CRISPR gRNA, and potential CRISPR/Cas9 target sites, including restriction enzyme sites that can facilitate the detection of new mutations (Michno *et al.* 2015). Michno *et al.* (2015) designed codon-optimized CRISPR/Cas9 platform to direct double-stranded breaks to the targeted loci in hairy root cells; the modified Cas9 enzyme successfully mutated target genes in somatic cells of *M. truncatula*, indicating that these new optimized tools may help to facilitate targeted mutagenesis in legumes and other plant species in future.

Acknowledgements. The authors acknowledge the financial support from the National Basic Research Program of China (2014CB138701), National Natural Science Foundation of China (31670405;31372360), and Chinese Government Scholarship Council for the Doctoral Program.

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Received for publication March 29, 2017
Accepted after corrections October 25, 2017
Published online January 12, 2018