

New Challenges and Opportunities of Protein Engineering in Plants

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Abstract

The present increase of the human population provokes higher demands for food, combustible and raw materials that may cause short-medium term famine and scarcity. The use of enzyme engineering techniques in plants could contribute to palliate these global problems. Enzyme engineering is changing the chemical and pharmaceutical industries and opens new possibilities, although until now the use of these techniques in plants is very limited. The new techniques employed in Plant Sciences, such as transformation of herbaceous crops and trees, the increasing number of full sequenced plant genomes, and the chemical diversity of plants can support a rapid development of enzyme engineering in plants. In the future, the application of enzyme engineering techniques could increase the crop yield or facilitate the biofuel production.

Enzyme engineering brings our society with an excellent opportunity to advance. The use of these techniques in chemical and pharmaceutical industries has changed our world, and opens new possibilities [1]. Enzyme engineering techniques have been widely used in microorganisms, improving chemical processes due to their diversity in species, lifestyles and metabolic reactions, which make possible the discovery of new enzymes having new interesting characteristics (e.g. an enzyme with a known function more thermostable), or catalyzing new reactions [2]. Moreover, the expression of recombinant proteins in bacteria is routinely done.

But in plants, the use of protein engineering techniques is very limited at this moment. Maybe the main reason of this has been the difficulty to make transgenic crop plants, and the limited number of full sequenced genomes compared to microorganisms until now. Although at present, it is possible to transform almost all crop plants [3], including some trees like poplar [4] and pine [5,6], and the number of full sequenced genomes of plant species is highly increased in the last years; thanks to next generation sequencing techniques [7]. In the present moment, we have enough technology to attend the protein engineering in plants.

The plant domestication constituted the major revolution of all in the human history [8]. Now, the plants are essential for the humanity in different ways. They support the human nutrition, but also are the source of raw materials (wood, pulp, etc), chemicals (biofuels, etc) and pharmaceuticals (acetylsalicylic acid, etc). Nowadays, the human population is increasing, so the demands for food, combustible and raw materials are also increasing, which can cause short-medium term famine and scarcity [9]. The use of enzyme engineering techniques in plants could contribute to palliate these global problems by improving crop yield and qualities.

The Genome Era: Identification of New Genes/Enzymes

The genome era makes possible to identify new genes and protein functions, in a way never thought, until this moment. In plants, it is estimated that the number of protein of unknown function is higher than in the rest of organism, constituting over 50% of the total [10]. A great portion could encode for enzymes and transporters. Many of these proteins of unknown function are part of conserved families shared between bacteria, plants, and other eukaryotes [11]. On this way, the comparative genomics becomes an excellent tool to predict functions and guide experimental validation [10,12].

In plants, this amount of proteins of unknown function can constitute a near unlimited source of new enzymes with new catalytic

capacities, which can be used for enzyme and metabolic engineering. It is known that plants have an enormous chemical diversity, which confers a great potential to work with enzymes with new functions [13]. Obviously, the diversity of secondary metabolites in plants must be correlated with an exceptional number of enzymes catalyzing the synthesis and degradation of these metabolites. The modification of these new enzymes involved in the secondary metabolism, and the combination with metabolic engineering techniques could lead to obtaining plants, which can be better exploited by the industry.

Translation from Microorganism Platforms to Plants

Because they are manageable and have a great biomass production capacity (by their capacity to assimilate CO₂), the plants are good candidates to be transformed with modified enzymes from bacteria. So far this possibility has not been much followed, although it is proposed to be used together with metabolic engineering in the production of plants for the use in bioremediation [14]. This methodology has an enormous potential to improve plants, or for the modification and generation of metabolic pathways of interest. These approaches allow the introduction of new routes in plants or the optimization of existing routes, for example, increasing the thermostability or the catalytic capacity of the enzymes. There are some examples of plant transformation with non-modified genes from microorganisms that may confer new or different capabilities. The endogenous tobacco RubisCO in the chloroplasts was replaced by the RubisCO from the α -proteobacterium, *Rhodospirillum rubrum* [15]. The transgenic tobacco plants were fully autotrophic and reproductive, although under CO₂ supplementation. In other case, the *Lactuca sativa* plants have been transformed with gene coding for the asparagine synthetase A (EC 6.3.1.1) from *Escherichia coli* (AS-A) [16]. This enzyme mainly uses ammonium to produce asparagines, a key amino acid in the nitrogen and carbon metabolisms of plants. On the contrary, the

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Received November 15, 2012; Accepted November 17, 2012; Published November 24, 2012

Citation: Cañas RA (2012) New Challenges and Opportunities of Protein Engineering in Plants. Enz Eng 1:e106. doi:10.4172/eeg.1000e106

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asparagine synthetase of plants mainly uses glutamine as N donor to form asparagine. The transgenic plants with AS-A had new traits, such as a higher leaf number, wider leaf surface and seed germination, formation and development of leaves, bolting and flowering which occurred earlier than the wild-type [16]. The metabolic profile of the transgenic plants also changed accumulating inulins, a polysaccharide beneficial for human health with industrial and medical uses [17,18].

Only with the use of metabolic engineering, the transformation of plants with non-modified enzymes from microorganisms is enough to obtain interesting results. The use of these techniques, together with the enzyme engineering, could revolutionize the agriculture and the chemical and pharmaceutical industries. An example is the modification of bacterial glyphosate N-acetyltransferases, improving its efficiency four orders and conferring an increased tolerance of *Arabidopsis*, tobacco and maize to the herbicide glyphosate, when the engineered enzymes were expressed in the plants [19].

Crop Yield Increase: Photosynthesis Engineering (RubisCO), NUE

One of the main challenges in biology is to cover the nutritional needs of a human population in constant growth. The enzyme engineering can play an important role as a tool to achieve the crop yield increases needed to meet the created and future demands. In this sense, the modification and improvement of Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO; EC 4.1.1.39) is a main objective. RubisCO is the key enzyme of the Calvin-Benson-Bassham pathway, the enzyme responsible for the assimilation of atmospheric CO₂ into organic molecules [20]. The carboxylase activity of RubisCO produces two molecules of 3-phosphoglycerate from ribulose 1,5-bisphosphate (RuBP), CO₂ and H₂O. However, the enzyme also catalyzes a competing oxygenase reaction, converting O₂ and RuBP to one molecule of 3-phosphoglycerate and 2-phosphoglycolate. The oxygenase activity decreases the overall performance in CO₂ fixation [21]. Some plants minimize the RubisCO oxygenase activity with systems, to increase the concentration of CO₂ in the chloroplasts of the cells where it acts on RubisCO in C4 plants (e.g. maize and sugarcane) [20]. Although most of the crops are C3 plants with high rates of photorespiration; they expend large resources in photorespiration that slows down their growth and yield. Therefore, the RubisCO engineering, through the increase in its affinity for CO₂, may be crucial for the improvement of the yield of crops with C3 metabolism.

In microorganisms, there have been numerous attempts to improve the RubisCO [21,22]. In plants, the number of works is limited until now. The RubisCO plant transformation is not trivial, since it has two subunits. The large subunit (L) is coded by a gene present in the genome of the chloroplast. The small subunit (S) is coded by several genes present in the nuclear genome [23]. Noteworthy is the work of Whitney et al. [24] who have achieved great results, both in the transformation of plant chloroplasts with genes coding the RubisCO L subunit, and in modifying the properties of RubisCO by directed mutagenesis. They have also linked RubisCO subunits, obtaining an active recombinant RubisCO that could facilitate RubisCO transformation works in plants [23,25]. They also have found that the plastomic replacement of the tobacco RubisCO L subunit gene with one from sunflower produced transgenic tobacco plants with an active hybrid RubisCO, able to support the plant growth [26]. Finally, they have established that the activity of a RubisCO from a very different phylogeny, bacteria, can be integrated into chloroplast photosynthetic metabolism, without prohibitive problems [15].

But, it is not only possible to improve photosynthesis through modification of RubisCO. It is known that plant photosynthesis declines, when the temperature exceeds its optimum range. This reduction in photosynthesis is due to the RubisCO deactivation by the inhibition of RubisCO Activase (RCA), under moderately elevated temperatures. *Arabidopsis* plants transformed with genes coding a modified thermostable RCA had improved photosynthesis and growth rates, under heat stress that could be transferred to crop plant [27].

The improvement of the Nitrogen Use Efficiency (NUE) is also very important for increasing crop yield. The N is a limiting factor for growth and yield, so in the last century, the use of nitrogen fertilizers has been largely increased [28]. As a consequence, a great increase in the production costs and serious environmental problems such as eutrophication have occurred [28]. Therefore, improving NUE has become a fundamental subject of study in Plant Sciences. However, there have been no enzyme engineering approaches for improving NUE, where it could have a very significant role. The change in the characteristics of key enzymes of nitrogen metabolism of plants, such as glutamine or asparagine synthetases, could improve NUE. In this regard, although protein engineering techniques were not carried out, it has been shown that *Lactuca sativa* plants transformed with the gene coding for the asparagine synthetase A from *Escherichia coli* (AS-A), which has different characteristics to the asparagine synthetase of plants, which exhibit an improved NUE [16].

Secondary Metabolism: Lignin

Due to the current energy and global environmental crisis, the discovery of new renewable energy sources and raw materials are essential for today's society. In this context, the production of plant biomass for obtaining biofuels, biopolymers and chemicals is an alternative, which is extensively studied in recent years [9]. The modification in the activity or functions of the enzymes involved in the lignin synthesis could be of great importance to develop the biofuel industry. One of the problems to the efficient use of cellulosic fibers in agricultural and industrial applications is the lignin, particularly in the conversion of cellulosic biomass to liquid biofuels [29]. The use of protein engineering to reduce or modify the content, and/or quality of lignin in the crops used in the biofuel industry, is a major challenge today. At this moment, there is no much work about it except for the article by Zhang et al. [30]. They described catalytic improvement of an artificial monolignol 4-O-methyltransferase, created by iterative saturation mutagenesis. This new enzyme is expressed into *Arabidopsis*, reducing the lignin levels of transgenic plants, which are able of producing higher saccharification yields [30]. This is very interesting because it uses enzyme engineering techniques over the plant, not in a chemical process, which is very promising for the industry.

In this line, the modification of plant secondary metabolic enzymes is not too much explored, although there is enormous chemical diversity of plants. The most successful example is the engineering of the sesquiterpene synthases. In a first work, the plasticity residues were probed in the active site of a promiscuous sesquiterpene synthase, and finally seven specific and active synthases were obtained, producing specific and very different products [31]. In a later work, all the possible substitution combinations of nine amino acid residues in the active site of a sesquiterpene synthase were explored, resulting in the correlation between the substitutions and the sesquiterpene product spectra [32]. Unfortunately, these results were not applied over plants for diversifying plant metabolism.

Conclusion

The enzyme engineering opens new possibilities to solve classic and present problems in Plant Sciences. The new techniques to generate transgenic plants in herbaceous crops and even in trees, the increasing number of full sequenced plant genomes, and the chemical diversity of plants constitute a great source for enzyme engineering works. In the future, the application of enzyme engineering techniques could increase yield of crops (RubisCO activity or NUE increases), or facilitate biofuel production (lignin amount modifications).

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