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Valorizing faba bean for animal feed supplements via biotechnological approach: Opinion

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ABSTRACT

Despite the high protein content, faba bean valorisation, as a protein supplement for the livestock, is hampered by the presence of anti-nutritional compounds in the seeds, such as vicine and convicine (v-c). Conventional methods had limited success in improving the faba beans nutritional quality, and hence, transcriptomic approaches, such as the identification of the genes related to the v-c biosynthesis/transport can be looked upon. These genes can be knocked out in a seed-specific manner using the RNA interference (RNAi) technology, to create faba bean varieties which synthesize alkaloids but hinder their accumulation within the seeds. Recently, a single marker conferred low v-c phenotype in faba bean, however, such reports warrant further investigations. Successful accomplishments would facilitate the faba bean valorisation for the food and feed, and therefore, negating the European dependency on the soya meal import. Moreover, it will be quite significant for legume supported cropping system in Europe.

1. Introduction

The European dependency on the import of protein rich food and feed materials led to an European Union parliament motion for putting more effort in the research and development of legumes (Visser et al., 2014). The meals based on protein sources, such as soybean, are a great source of essential amino acid, for e.g., lysine. Lysine is the first limiting amino acid among pigs and second among poultry birds. European self-sufficiency is just 3% in terms of soymeal, whereas, it is used for the 64% of the protein-rich feed material (Bues et al., 2013). Therefore, alternative for soybeans, such as faba beans, should be looked to overcome such dependency on soymeal. The breeding for animal nutrition is being focused in Europe, where subsidies aim to increase the faba bean production, and decrease the imports of vegetable protein, particularly soybean.

Faba bean (*Vicia faba*) is one of the oldest crop grown by humans, and fourth important legume used as a source of protein in the human diet. Moreover, it is quite important as a fodder and forage crop for animals, and for available nitrogen (A.K. Singh et al., 2013). Faba bean is essential for the legume supported cropping system in Europe, as it has the highest positive N balance (19.8 kt) (legume future report 1.5/2014, www.legumefutures.de). In spite of its potential, the total area of faba bean cultivation has steadily decreased in many countries over the years (A.K. Singh et al., 2013), primarily as these protein-rich cotyledons also contain anti-nutritional compounds (ANCs), such as vicine and convicine (v-c). These ANCs cause hemolytic anemia and favism in humans whose erythrocytes are deficient in glucose-6-phosphate dehydrogenase (G6PD). Besides they lower growth rate and feed efficiency, and cause the enlargement of the liver and the pancreas in the chickens. They also reduce fertility among birds and pigs (Rizzello et al., 2016).

The removal of v-c is one of the most important breeding priorities as they decrease digestibility and biological value of the protein in animal feeding (Gnanasambandam et al., 2012). Such an approach in faba bean would make it suitable to replace soybean as the protein source for the food and feed. This, in turn, can promote self-sufficiency of the European Union in the import of the plant protein rich products (Köpke and Nemecek, 2010). Reduced v-c accumulation in the faba bean have been reported, but complete removal of these compounds have not been achieved yet (Crépon et al., 2010; Pulkkinen et al., 2015). This is the major limiting factor for the utilisation of this crop.

Although several attempts are being made to eliminate these ANCs by the conventional breeding (Coda et al., 2015), and the marker-assisted selection (Khazaei et al., 2015a), the results are not satisfactory.

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A single quantitative trait loci (QTL) on the faba bean chromosome 1 has been reported for v-c concentration (Webb et al., 2016), but involvement was not ascertained for a low v-c content on different germplasm (Khazaei et al., 2015a, 2015b). Recently, there is a report of a high-throughput low-cost KASP (Kompetitive Allele Specific PCR) marker for the low v-c concentration in the faba bean. This might be very useful in distinguishing the low v-c phenotypes, and can be used for further breeding programs towards crop improvement (Khazaei et al., 2017).

Faba bean is partially allogamous, and its progenitor species is not known (Gnanasambandam et al., 2012). Furthermore, its large genome (~13 Gb, diploid) poses substantial challenges for the effective development and implementation of the genetic markers (Kaur et al., 2014). Within the fifth framework of EU FABA project, marker linked to the growth and the nutritional value of the faba bean have been identified, which lead to a 10–20 fold reduction in the v-c contents (Gutierrez et al., 2006). However, the plant, as a whole, lacked the ability to synthesize the alkaloids, which act as protective agents, rendering them more susceptible to the insects and some pathogens. Hence, using modern agricultural biotechnology techniques becomes indispensable in producing faba bean plants which can biosynthesize alkaloids, and at the same time, refrain their accumulation within the seeds. The molecular mechanism of the genes involved in the synthesis, transportation and accumulation of v-c has not been yet fully understood (Ray et al., 2015). We suggest biotechnology approaches for the identification of the key genes involved in v-c biosynthesis, and their transport into the faba bean seeds by a contemporary transcriptomic approach. An RNAi mediated silencing of these genes is also suggested in order to eliminate alkaloids in the seeds using seed-specific promoters. Hence, this approach has the potential to meet the following objectives.

- Identification of v-c biosynthesis genes in faba beans.
- Cloning of the genes important for v-c biosynthesis and their transport into the seeds, and their subsequent transformation in a model plant for expression studies.
- Functional characterization of v-c biosynthetic and regulatory gene (s), which could be knocked out by RNAi to prevent v-c accumulation in seeds.
- Confirmation of v-c free faba bean transgenic plants by molecular biology and biochemical methods, in order to obtain homozygous and stable transgenic lines.
- Nutritional quality and toxicology evaluation, followed by field trials in a containment facility and valorisation of v-c free faba bean for animal feed.

2. Methods

2.1. Identification of genes that regulate v-c biosynthesis in faba bean

A strong correlation between the induction of the alkaloid content of the plumules in the faba beans, by coupling ascorbic acid to the salt treated seeds, has been demonstrated (Awatif et al., 2014). The combination of the ascorbic acid with the salt treatment result as a trigger to induce the alkaloid biosynthesis pathway. This idea suggests that genes involved in the alkaloid biosynthesis pathway of the faba bean can be encountered by a differentially expressed gene cloning approach.

Recent large-scale transcriptomic approach was unable to encounter the genes for v-c biosynthesis (Ray et al., 2015). The suppression subtractive hybridization (SSH) method seems a better choice to identify the candidate gene (R.K. Singh et al., 2013, 2015). SSH may be performed using the mRNA of a control faba bean as the "driver population", and the mRNA from an elicited plant (with ascorbic acid and salt treatment) as the "tester population". These two populations of mRNA should be hybridized two times in the presence of excessive "driver". The driver represents the common transcripts in both the population. It facilitates the identification of the uncommon transcripts from tester population which are supposedly specific for the alkaloid biosynthesis. This subtracted library information would serve as a preliminary resource to elucidate the genes involved in alkaloid biosynthesis in the faba beans in the subsequent tasks.

Identified gene sequences may be analysed using bioinformatics tools from the National Centre for Biotechnology Information (NCBI) and the European Molecular Biology Laboratory (EMBL) databases. To assign putative functions to the non-redundant cDNAs, the sequences may be compared with other hits in the Gene Bank databases, using BLASTX, and expressed sequence tags database (dbEST), via BLASTN tools. The maximum probability threshold of 10^{-5} can be set for a sequence match, and the cDNA sequences with the best BLASTX matches to the genes with known functions, may be annotated. Appropriate functions can be assigned to the non-redundant genes based on the annotation generated for other plant species and organisms in the genome database.

2.2. Cloning of full-length genes of the alkaloid biosynthesis pathway

In order to obtain the full-length sequence of the desirable genes a rapid amplification of cDNA ends (RACE) method may be used. For each hypothetical gene, a subsequent amplification may be carried out both from the 5' and 3' directions with gene-specific primers. This can be followed by sequencing and assembling. The assembled cDNA sequences and the predicted gene structure for each gene may be aligned with the corresponding genomic sequences using DDS/GAP2 program.

2.3. Elucidation of the functional relevance of identified genes

Identified genes which are related to the v-c biosynthesis and their transport into the seeds may be subjected to a functional characterization in a model plant species, such as *Tobacco* and *Arabidopsis*. This would provide information on the relevance of the identified genes. Briefly, identified genes must be cloned in a plant expression vector under the control of seed-specific promoters such as legumin B4 promoter (LeB4), phaseolin promoter, or a usp-promoter (napin); or in a constitutive promoter, such as the Cauliflower mosaic virus promoter (CaMV35S) and a suitable terminator. These plasmid constructs may be then introduced into different *Agrobacterium tumefaciens* strain, for e.g., EHA105, LBA4404, and GV3101 etc. *Tobacco* and *Arabidopsis* plants should be infected with either of these bacteria, and the transformed plants may be obtained using the standard procedures. After molecular characterization, seeds and other tissues of the transgenic plants expressing these genes may be subjected to high performance liquid chromatography (HPLC) analysis to quantify the v-c (Purves et al., 2018).

2.4. Production of alkaloid-containing faba bean varieties that produce v-c free beans

The RNAi technique has rapidly gained favour as a "reverse genetics" tool to knock down the expression of targeted genes in plant species (Zhu et al., 2013). This genetic engineering approach of RNA mediated gene silencing may be employed for eliminating or reducing alkaloids in the seeds. Plant expression vectors containing RNAi cassettes of the alkaloid biosynthesis and transporter genes can be constructed under the control of seed-specific promoters mentioned above. Such an approach can be used to transform faba bean.

The lack of an efficient transformation protocol for the faba bean could be the main obstacle in this direction. It should be noted that legumes generally remain recalcitrant to *A. tumefaciens* mediated transformation. Although a few reports on *A. tumefaciens* mediated genetic transformation of the faba bean have been published, the transformation efficiency is very low (Hanafy et al., 2005; Rabha et al., 2014). Hence, a biolistic-mediated transformation could be a good choice as an alternative method for the transformation, although it has limitations. As the presence of antibiotic marker gene in transgenic plants is an environmental and ethical concern, co-transformation strategies may be incorporated to eliminate such markers from the transgenic plant.

Efficient recovery of transformed plants strictly depends on the totipotency of transformed cells. In vitro regeneration of faba bean has been extensive and challenging (Gnanasambandam et al., 2012). Hence, to overcome this challenge, cotyledonary nodes or other meristematic tissues should be used as explants.

After a successful production of the transgenic plants, transgenic lines may be analysed by gene-specific primers, followed by the southern blotting, until the establishment of stable lines is achieved. These lines should be proceeded to obtain the subsequent generation, to confirm the stability of the target genes. The stable transgenic lines should be maintained in a containment facility. Thereafter, v-c shall be quantified by a HPLC analysis to compare with the control plants (Purves et al., 2018).

2.5. *Valorisation of v-c free Faba bean for animal feed*

The faba meal produced from the new biotech varieties may also be evaluated for its usage in the animal feed industry. For this purpose, an extensive characterization of its nutritive value using chemical analyses and in vitro digestibility trials may be done to select different residues. The effects of the incorporation of v-c free faba meal would be compared with a commercial diet on (a) growth performances, i.e., feed intake, growth, feed conversion, morbidity and mortality, (b) digestibility of several diet fractions, i.e., dry matter, fibre fraction, protein, fat and starch, (c) faecal fermentation, i.e., volatile fatty acids, pH and ammonia production, (d) intestinal characterization, i.e., digestive tract morphology, histopathology and enzymatic profile, and (e) gut microbiology, i.e., bacteria count, should be evaluated by trials on rabbits or chickens, etc. These trials will allow defining the quality and the efficiency of the faba meal incorporation in commercial diets, ultimately enabling its utilisation as an ingredient in the feed industry.

3. Conclusions

The developed modified variety will improve the quality and efficiency of faba meal incorporation in commercial diets, enabling its utilisation as feed ingredient in the feed industry. It will also contribute to the sustainable legume-based cropping system, and thus, the subsequent expansion of the faba bean cultivation. This would eventually boost the European self-sufficiency in terms of a protein supplement for the livestock.

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