

Abstract

Lysine is one of the most important essential amino acids but its content is limited in most food and feed crops. In higher plants, lysine is synthesized through the aspartate pathway and two enzymes, aspartate kinase (AK, EC 2.7.2.4) and dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52), are known as the key enzymes of the feedback inhibition mechanism involved in the regulation of lysine biosynthesis. According to all studies up to now, plant DHDPS enzymes are strongly inhibited by lysine and in nearly all plants lysine-sensitive isoforms of AK are predominant compared to threonine-sensitive isoforms. However, not all plants have low lysine content. The grain legumes are known as a source of lysine supplement to balance food and feed due to their relatively higher content of lysine. This suggests important differences in lysine metabolism between legumes and other plant species.

Our results with the model legume *Medicago truncatula* demonstrate that regulation of lysine biosynthesis in leguminous plants indeed has unique properties. DHDPS activity extracted from *M. truncatula* as well as several grain legumes is less sensitive to feedback inhibition by lysine compared to non-legume plants. The sensitivity of *M. truncatula* DHDPS to feedback inhibition is temporally and developmentally regulated. Accordingly, we cloned two differentially expressed *dhdps* cDNAs, one encoding a lysine sensitive and one encoding a lysine insensitive DHDPS. This demonstrates for the first time the existence of a naturally feedback insensitive form of this enzyme in plants. AK activity extracted from *M. truncatula* is also less sensitive to feedback inhibition by lysine compared to non-legume plants.

To define the carbon flux in leguminous plants, we have transferred the bacterial feedback insensitive *ak* gene (mutated *LysC* gene from *E. coli*) into *M. truncatula*. Overaccumulation of lysine rather than threonine in transgenic *M. truncatula* indicated that in leguminous plants the metabolite flux is directed in favour of lysine biosynthesis.

Traditionally, grain legumes are added to cereals in food and feed for balancing the diet, but the increasing use of crystalline lysine as a feed additive indicates that natural lysine content in grain legumes is unable to cover the nutritional needs. Thus, it is obvious that crops with enhanced lysine content have an important economic value in the market.

With the aim of enhancing the lysine content in the seeds of the grain legume pigeonpea [*Cajanus cajan* (L.) Millsp.] through genetic engineering, a highly efficient *in vitro* regeneration procedure was established using seedlings grown on B5 medium supplemented with 10 mg l⁻¹ 6-benzylaminopurine, which induced differentiating callus formation in the cotyledonary node region. The calli were transferred on B5 medium with 0.2 mg l⁻¹ 6-benzylaminopurine to obtain shoot induction. Elongated shoots were then further cultured on a B5 hormone-free medium for rooting. Using this regeneration system, transgenic pigeonpea plants harbouring the *dhdps-r1* gene from a mutant *Nicotiana sylvestris*, which encodes a DHDPS enzyme insensitive to feedback inhibition, were obtained both by particle bombardment and *Agrobacterium tumefaciens*-mediated gene transfer. The *dhdps-r1* gene was driven by a phaseolin or an Arabidopsis 2S2 promoter. The presence of the transgenes in the pigeonpea genome was confirmed by PCR and Southern hybridisation. The transgenic rooted plants were successfully transferred to soil in the greenhouse. PCR assays of T1 progenies confirmed that the transgenes were stably transmitted to the next generation.

In 11 transgenic pigeonpea lines analyzed, a 2- to 6-fold enhanced DHDPS activity in immature seeds at late stage of maturation was found in comparison to wild type. The overexpression of *dhdps-r1* led to enhance the content of free lysine in the seeds of pigeonpea from 1.6 to 8.5 times compared with wild type. However, this was not reflected in an increase in total seed lysine content. This might be explained by a temporal discrepancy between maximal expression of *dhdps-r1* and the rate of amino acid incorporation into storage proteins. Assays of the lysine degradative enzyme lysine-ketoglutarate reductase in these seeds showed no co-ordinated regulation of lysine biosynthesis and catabolism during seed maturation. All transgenic plants were fertile and produced morphologically normal seeds.