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Different Contributions of the Various Isoenzymes to the Flux in the Aspartate-derived Amino Acid Pathway in *Arabidopsis thaliana*

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Abstract

Since isoenzymes were first discovered, their physiological role has generated interest and discussion, and study of the flux distribution between isoenzymes in a real pathway, studied with real parameters, should shed light on this role. The aspartate-derived amino acid pathway from plants constitutes an excellent system for understanding the role of isoenzymes, as well as the effects of regulatory mechanisms such as feedback inhibition and allosteric interactions, because there are several branch-points, numerous isoenzymes, and different allosteric control mechanisms (inhibition, activation, antagonism and synergism). It is responsible for the distribution of the carbon flux from aspartate into the branches for synthesis of lysine, threonine, methionine and isoleucine. A mathematical model of the core of the pathway in the chloroplasts of *Arabidopsis thaliana* was constructed, and as kinetic data from the literature are often inadequate for kinetic modelling, we combined kinetic measurements obtained *in vitro* with purified enzymes, in near-physiological conditions, with *in vitro* reconstitution

and numerical simulation. The model accurately predicts the experimentally observed behaviour, and shows that the isoenzymes contribute unequally to the flux and its regulation. The effects of some isoenzymes knockouts are also studied.

Introduction

The recognition by Markert and Møller half a century ago of multiple forms of enzymes catalysing the same reaction in the same cell or organism, i.e. isoenzymes, led to hopes of a better understanding of cellular metabolism through this phenomenon [1]. The discovery of the first isoenzymes thus raised the question of what physiological advantage derives from having more than one type of protein able to catalyse the same reaction. Different protein species can be generated by many different mechanisms, including different gene splicing. The term ''isoenzyme'' applies normally to proteins that are coded by different genetic loci, the evolutionary results of gene duplication or of gene duplication and fusion [2].

Isoenzymes tend to perform their functions in distinct ways, and they may differ in affinity and specificity for substrates or cofactors, in their response to allosteric effectors, subcellular localization, susceptibility to dietary and hormonal treatment, or time of appearance during differentiation [2]. The demonstration of one or several different properties can be used to explain the physiological advantages of a particular set of isoenzymes. For example, the different half-saturation values of the vertebrate hexokinases for glucose (and other kinetic parameters) may represent a regulatory device to handle overloads of dietary glucose [3, 4]. Especially intriguing are the differences in isoenzyme expression between related species. For example, although there are four hexokinase isoenzymes in rat liver (A, B, C, D), this is not a universal characteristic of mammalian liver or even of the livers of other rodents or of pig, which, like rat, is omnivorous. A system of three hexokinases is more common (ACB, ACD, ABD) and systems of two are also found (AB, AC) [5 – 7].

Studies on the metabolism of microorganisms revealed the existence of isoenzymes in the allosteric regulation of branched pathways. Their role in the synthesis of amino acids derived from aspartate was especially well investigated in *Escherichia coli*, particularly from the point of view of their allosteric properties [8]. At that time the global regulation of entire metabolic networks had not been clarified in a quantitative sense. It was assumed that there was a logical reason behind the existence in *E. coli* of three aspartokinases, each of them inhibited by a different amino acid product of the pathway. In some cases the same amino acid also repressed the corresponding gene expression.

One might be tempted by the idea that isoenzymes, especially if they have different properties, will be found at branch points of metabolic sequences. However, studies over many years show that isoenzymes are not restricted to highly regulated steps or to branch points in

metabolism [9]. Reactions that are not at crossroads, and ones without obvious regulatory significance, are also catalysed by isoenzymes, and in several cases there are no differences in properties [2].

Recognition of kinetic differences within a group of isoenzymes should not be considered sufficient support on its own for speculations about the involvement of the isoenzymic system in regulation, even in the presence of a plausible correlation. However, the possibility of simulating a metabolic pathway offers a powerful tool for analysing the role of isoenzymes.

Aspartate Metabolism in Chloroplasts of *Arabidopsis thaliana*

The aspartate pathway of thale cress, *Arabidopsis thaliana*, provides an excellent opportunity for testing and developing ideas about metabolic regulation, the role of isoenzymes and the organization of metabolic pathways. In particular, it allows a test of one of the classical ideas of metabolic regulation, commonplace in textbooks, that the most highly regulated step is the one that controls the flux. There have been many theoretical discussions of such ideas [10 – 13] but almost none of them have been based on studies of real non-linear pathways, with quantitative experimental data obtained in physiologically relevant conditions.

The aspartate pathway is a branched pathway producing four amino acids (lysine, methionine, threonine and isoleucine) and in the reported study [14] we worked with a core of 13 enzymes. *Arabidopsis* has isoenzymes at several points, which respond unequally to effectors (Fig. 1). It has bifunctional enzymes and many different regulatory interactions, such as inhibition, activation, synergism and antagonism. Under physiological conditions the flow goes from aspartate to the other amino acids, but some reactions have equilibrium constants that favour the opposite direction. This is especially true for the reaction catalysed by aspartokinase (AK), where the equilibrium constant strongly favours the reverse reaction [15].

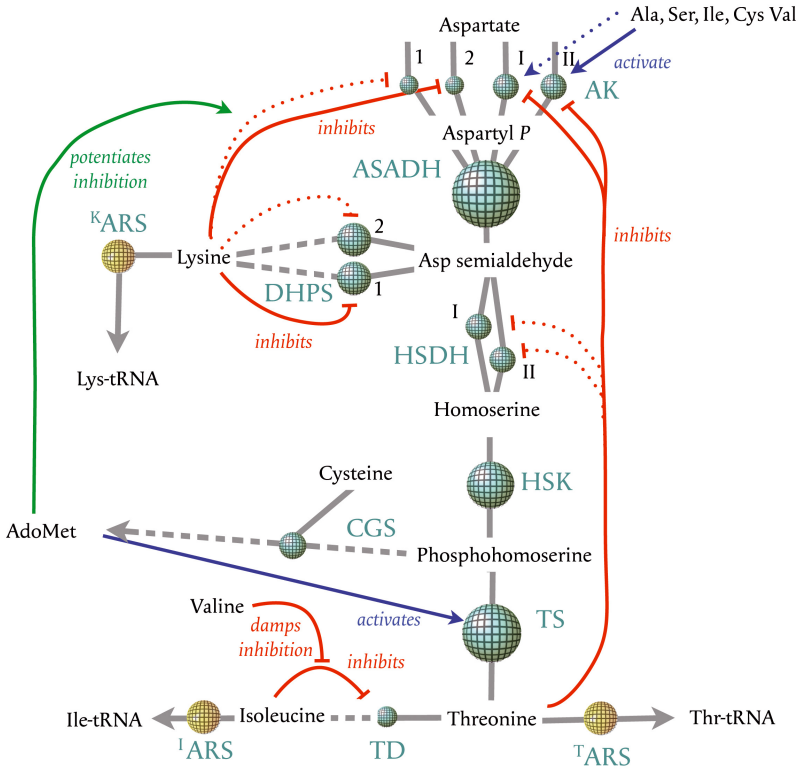

Figure 1. Model of aspartate metabolism in *Arabidopsis thaliana.* The pathway was modelled as a system of 13 enzymes (not including the three aminoacyl tRNA synthetases), as shown. The activation of AKI and AKII by five amino acids was not considered. Each turquoise sphere represents the concentration of the enzyme in question and should be considered three-dimensional, i. e. concentrations are proportional to the implicit volumes, not to the diameters, so ASADH, for example, has 46 times the activity of AK1. The activities of the three aminoacyl tRNA synthetases (yellow spheres) were treated as adjustable parameters.

Presence of isoenzymes

In the chloroplast there are four isoenzymes of aspartate kinase, which are designated in a most unfortunate way, as AK1, AK2, AKI and AKII. A fifth isoenzyme, AK3, exists but was not taken into account in the model because it is restricted to vascular tissues [16]. There are two isoenzymes of homoserine dehydrogenase, HSDH I and HSDH II; two of threonine synthase, TS 1 and TS 2, of which only TS 1 (referred to in the rest of the paper just as TS) exists in the chloroplast; and two of DHDPS, DHDPS 1 and DHDPS 2. As discussed below, all of these isoenzymes differ in substrate and effector affinity and the aspartate kinases also differ in effector specificity. A striking feature of this pathway is the

presence of bifunctional enzymes: AKI and HSDH I are activities of the same protein, and the same applies to AKII and HSDH II. This bifunctionality has been conserved since the divergence of plants and bacteria, the two structural domains being quite independent [17].

Enzyme concentrations

The enzyme concentrations are not all the same: AKI and AKII are more abundant than AK1 and AK2, and there is 46 times as much ASADH as AK1, and about ten times as much as the four AK isoenzymes together.

Complexity of regulatory interactions

There are many and diverse regulatory interactions (Fig. 1). Lysine inhibits AK2 and DHDPS 1 strongly, and AK1 and DHDPS 2 weakly; however the weak inhibition of AK1 is highly potentiated by *S*-adenosylmethionine, the universal methyl donor, methionine as such exerting no regulatory interaction [18]. *S*-adenosylmethionine does not exert a direct feedback inhibition of its branch derived from phosphohomoserine, but it activates threonine synthase 1. The synergistic amplification by *S*-adenosylmethionine of the weak inhibition of AK1 by lysine allows demand for *S*-adenosylmethionine to regulate its production.

Isoleucine inhibits threonine deaminase, but valine damps this inhibition. Threonine in turn inhibits the two bifunctional aspartate kinases strongly, and the two homoserine dehydrogenases weakly; however HSDH I is more strongly inhibited than HSDH II, which is not at all inhibited by threonine in physiological conditions [17] The inhibition by threonine of AKI and AKII is counteracted by alanine, cysteine, isoleucine, serine and valine, which activate AKII strongly and AKI weakly [17]. Several of these effectors bind at ACT domains [19, 20]. Of all these effectors, alanine and threonine are much more abundant than the others in *Arabidopsis* leaf chloroplasts [17].

The complexity of this metabolism and of the regulatory interactions prevents an easy understanding of the function played by any given isoenzyme relative to another, and of the importance of the dual controls by lysine, threonine and *S*-adenosylmethionine.

There are several questions that can be asked. Which enzymes control the flux? Are these in the supply block (most of the enzymes) or the demand block (the aminoacyl tRNA synthetases)? How is the flux partitioned among the isoenzymes? Does every isoenzyme carry some flux? How well does the system regulate the flux in response to varying demand? How is the total flux distributed between the different branches? How independent are these branches? For the bifunctional enzymes, does AKI carry the same flux as HSDH I, and likewise for AKII and HSDH II? To answer these and other questions it is necessary to simulate the network as a whole.

Modelling

For doing a suitable modelling a substantial amount of experimental data is required. Literature data are usually inadequate for kinetic modelling, because the kinetic parameters have not been obtained in the appropriate physiological conditions (pH, presence of products and effectors, etc.) [21], and often the precise conditions of measurement are not specified. Important parameters are often missing, especially those for the reverse reaction. If the equilibrium constant is very high and greatly favours the reaction in the physiological direction, the reverse reaction can be neglected, but inhibition by products must still be taken into account [22].

Fortunately, for the aspartate pathway in chloroplasts of *Arabidopsis* a considerable amount of information has accumulated over the years on the kinetic parameters of the different enzymes and on the physiological concentrations of enzymes and metabolites [17, 23 – 28]. This information allowed the construction of a detailed kinetic model for simulating and predicting the dynamic response of the pathway in conditions relevant to those *in vivo* [14].

To model a system such as the one represented in Figure 1, a system of equations is needed, together with a simulation program and, in particular, a great number of experimental measurements in conditions relevant to physiological conditions. As a core of 13 enzymes are considered, 13 catalytic constants, 13 enzyme concentrations, 63 other kinetic parameters, and 5 fixed concentrations (of ''external'' metabolites) are required, which means a total of 94 numerical values to be measured. This calculation suggests that it may be almost impossible to have a realistic simulation, but that is too pessimistic, as this type of modelling is typically very robust, in the sense that two-fold or even larger errors in the values of the parameters typically have little quantitative effect on the predictions, and no qualitative effect.

The existence of two types of aspartate kinases, two isoenzymes specifically evolved for synthesis of lysine and maybe methionine (AK1, AK2) and two (AKI, AKII) for the synthesis of threonine and isoleucine may suggest the possibility of channelled routes to these amino acids. However, there is no evidence of channelling, and all indications are that there is a single pool of aspartyl-phosphate derived from the action of the four aspartate kinase isoenzymes, and this is what was assumed in the modelling.

Concentrations of external metabolites

The values of [aspartate] = 1.5 mM, [cysteine] = 15 µM, [*s*-adenosylmethionine] = 20 µM and [valine] = 100 µM are those measured experimentally and are treated as constant during the time where the model is valid (2 hours). The same applies to the concentrations of ATP, ADP, NADPH, NADP, inorganic phosphate and pyruvate, which are considered fixed concentrations because their levels are assumed to be regulated elsewhere in the metabolism.

$$v_{\mathrm{AK1}} = [\mathrm{AK1}] \cdot \frac{5.65 - 1.6[\mathrm{AspP}]}{1 + \left[[\mathrm{Lys}] / \left(\frac{550}{1 + [\mathrm{AdoMet}]/3.5}\right)\right]^2}$$

$$v_{\mathrm{HSK}} = [\mathrm{HSK}] \cdot \frac{\left(\frac{2.85[\mathrm{ATP}]}{54 + [\mathrm{ATP}]}\right) \cdot [\mathrm{Hser}]}{12 + \frac{40}{1 + [\mathrm{ATP}]/80} + [\mathrm{Hser}]}$$

$$v_{\mathrm{TS1}} = [\mathrm{TS1}] \cdot \frac{\left(\frac{0.42 + 3.5[\mathrm{AdoMet}]^2/73}{1 + [\mathrm{AdoMet}]^2/73}\right)[\mathrm{PHser}]}{\left[\frac{250\left(\frac{1 + [\mathrm{AdoMet}]/0.5}{1 + [\mathrm{AdoMet}/1.1}\right)}{1 + \frac{[\mathrm{AdoMet}]^2}{140}}\right]\left(1 + \frac{[\mathrm{P_i}]}{1000}\right) + [\mathrm{PHser}]}$$

Figure 2. Illustration of the rate equations required for the modelling. The rate expressions for AK1, HSK and TS 1 are shown. Corresponding expressions were also required for the other steps in the system.

Kinetic equations

The three equations shown in Figure 2, which correspond to AKI, HSK and TS 1, illustrate the sort of equations required for modelling the system. They are not mechanistic equations, but empirical ones derived from experimental data, and validated in reconstituted experiments *in vitro*. Each of the first two equations requires 5 measured parameters (including the Hill coefficient for AK1, $h = 2$,) but TS 1 requires no fewer than 12 parameters.

Internal metabolites

The concentrations of metabolites aspartyl-*p*, aspartate semialdehyde, homoserine, lysine, phosphohomoserine, threonine and isoleucine were calculated on the basis of the kinetic equations of the enzymes by using the simulation program COPASI [28]. All the fluxes were calculated at the same time as the internal concentrations. The calculated internal metabolite concentrations are, within experimental error, the same as those measured in chloroplast stroma in light conditions, for those concentrations that were high enough to allow detection. The low calculated concentration of aspartyl-*p* agrees with the fact that this concentration is below the level of experimental detection.

Flux Distribution in the Reference State

The model allowed us to calculate the flux through the different branches of the network and to establish how much flux is carried by each isoenzyme. The analysis refers to a state that we define as the *reference state*, which corresponds to the metabolite and enzyme concentrations measured in chloroplast stroma in light conditions (pH 8.0). It showed a different flux distribution between the different isoenzymes and in branch points. The flux tends to be unequally distributed between the different isoenzymes and in various respects is quite different from what one might expect: For this sort of system intuition is not sufficient and can be misleading. At the step of aspartate phosphorylation the lysine-sensitive AK1 and AK2 account for most of the flux (88%), while the threonine-sensitive AKI and AKII account for very little, AK1 being the one that carries most of the flux (73%). There is also an important difference between the dihydrodipicolinate synthase isoenzymes: DHDPS 2 accounts for 28% of the total flux whereas DHDPS 1, which is strongly inhibited by lysine, carries only 3%.

Contrary to what intuition would suggest, the two activities of each of the bifunctional enzymes AKI-HSDH I and AKII-HSDH II, carry very different amounts of flux: 32% for HSDH I but only 3% for AKI, 37% for HSDH II but only 9% for AKII. Notice that there is no major difference between the fluxes carried by the two homoserine dehydrogenase isoenzymes.

Another striking feature is the fact that at the bifurcation point at phosphohomoserine the flux to *S*-adenosylmethionine, 0.058 μM/s (5.7% of the total flux) is much smaller than the flux to threonine (63%). The activation of TS by *S*-adenosylmethionine probably avoids a "branch-point effect" [29] whereby small changes in the synthesis of threonine would perturb the regulation of *S*-adenosylmethionine production, but the simulations themselves did not deal with that because *S*-adenosylmethionine was defined as an external metabolite at fixed concentration. The model explains why despite the activation of TS by *S*-adenosylmethionine an inverse relationship between its concentration and that of threonine was observed experimentally.

The demand for amino acids (in the form of the activities of the three AA tRNA synthetases) was adjusted to obtain concentrations of intermediate metabolites close to those measured *in vivo* (Reference state). Metabolic control analysis [10, 30, 31] was used to identify the most sensitive steps and metabolites of the network. Most of the control of the common flux is shared between the 3 AA-tRNA synthetases, which represent the cellular demand for the amino acids, and not at the highly regulated AK step, confirming the hypothesis of Kacser and Burns [10], later developed in detail [11], that the effect of feedback inhibition is to *withdraw* the control from the first step and to transfer it to the demand. This is important as an illustration that controls and regulation are not the same thing. However, in the reference

state there is still some control associated with AK1, which is at the ''supply'' end of the pathway, with the consequence that AK1 contributes significantly to the maintenance of the steady state of threonine. This is highlighted in the knockout simulations analysed below.

How Well is the System Regulated in Practice?

If lysine is consumed in substantial amounts by competing pathways does that make it less available for making protein? The modelling showed that the flux of threonine to protein is not affected at all in this condition and the flux from lysine to protein is affected only slightly. There is an equivalent result if threonine is consumed in substantial amounts by competing pathways: the flux of lysine to protein is not affected at all and the flux of threonine to protein is affected only slightly. We can ask the same question in relation to methionine by modulating the concentration of *S*-adenosylmethionine, with a similar answer. The system thus appears to be very robust in relation to *increases* in demand. However, if the demand for the three end-products is too low the system is not able to cope and there is no steady state. This result is interesting in the light of the results obtained with knockouts of the three AA-tRNA synthetases, shown below. Although amino acid catabolism was not explicitly included in the model the simulations of high demand for threonine and lysine suggest that the results would not probably be affected qualitatively if it had been.

It follows that the different branches of the network respond independently to fluctuations in the demand for their end-products, even though they share common steps. The kinetic independence of the two domains of the bifunctional AK-HSDHs, and the different response of isoenzymes to effectors contributes to this independence. However, as discussed below, knockouts in one branch can affect enormously the metabolite concentrations of others.

Knockouts in silico

With the idea of shedding some light in relation to the physiological role of isoenzymes, in particular of those that carry almost no flux, some knockout experiments were done *in silico*, as illustrated in Table 1. It is striking that a knockout of AK1 still allows 80% of the total flux, even though this isoenzyme carries 73% of the flux in the reference state. In this condition, therefore, the other three isoenzymes can compensate for the knockout. Even if both lysine-sensitive aspartate kinases are eliminated, leaving only AKI and AKII active, two isoenzymes that account only 12% of the flux in the wild type, the resulting total flux is still 74% of that in the wild type: The two threonine-sensitive isoenzymes can compensate for loss of the other two.

Table 1. Effects of knock-outs on fluxes and intermediate concentrations. The top line (“wild-type”) shows the common flux (through aspartate semialdehyde dehydrogenase) and the concentrations of threonine, lysine and isoleucine in the reference state, and the one rows show the same variables when the activities of the enzyme or enzymes in the left-hand column are set to zero

Step	*JAK = JASADH*	[Thr]	[Lys]	[He]
Wild type	1.016	296	69.2	58.8
AK1	0.812	92.7	54.6	32.9
AK2	0.974	223	65.7	52.3
AK1 and 2	0.749	70.3	50.6	26.4
AK2, I and II	0.869	122	58.2	39.2
AKI	1.010	285	68.8	58.0
AKII	0.978	228	66.0	52.9
AK I and II	0.869	206	64.8	50.6
DHDPS 1	1.025	330	66.8	61.3
DHDPS 2	1.141	7760	37.9	179.2
DHSPS 1 and 2	- no steady state -			
Lys^{tRNA}	0.543	104	5×10^{8}	35.5
Thr^{tRNA}	0.757	11400	81.9	203
Ile^{tRNA}	0.789	6600	79.0	2×10^{13}

It is important to notice that although the flux through aspartic semialdehyde dehydrogenase is not very much affected if AK1 is knocked out, the threonine concentration is greatly affected, as it falls to only 31% of the wild-type value with the AK1 knockout, and to 24% with the double knockout: this illustrates the general property that metabolite concentrations are more susceptible to perturbations than fluxes [22, 32]. This decrease in the threonine concentration relieves the inhibition of AKI and AKII, allowing them to replace AK1 and AK2.

For the dihydrodipicolinate synthase isoenzymes, the result obtained with the knockout of DHDPS2, the isoenzyme weakly inhibited by lysine, is striking: there is a slight increase in the total flux (to 112%), which may be explained by the decrease in the concentration of lysine, which is now 55% of the reference value. Conversely, the isoleucine and threonine concentrations are dramatically increased, isoleucine to 305% and threonine to 2600%, in agreement with the observation that the concentration of threonine is very unstable [14]. So although the amount of lysine would probably still be sufficient to satisfy the plant needs, the huge increase in threonine concentration would be toxic. This result illustrates the point that one needs to be cautious in relation to the interpretation of knockout effects, as pointed out by Cornish-Bowden and Cárdenas [33]: In this case the damaging effect is not so much the decrease in lysine but the huge increase in threonine. As could be expected, knockout of both isoenzymes of dihydrodipicolinate synthase prevents a steady state from being attained. However, as note below, the model does not include catabolism, and in the living plant this loss of steady state might not occur.

Conclusions and Perspectives

There have been some excellent simulations of metabolic systems, such as erythrocyte metabolism [34], or glycolysis in *Trypanosoma brucei* [35], and sucrose metabolism in sugarcane [36] but these have not addressed the role of feedback inhibition in branched pathways. Studies of feedback inhibition have, on the other hand, referred to models of invented pathways with hypothetical data [11, 12].

For the first time, therefore, we can describe metabolic regulation in a detailed model of a real system with measured kinetic parameters. Most of the control of the network is on the demand steps (AA-tRNA synthetases), and not at the severely regulated AK, confirming that the effect of feedback inhibition is to transfer the control from the first step of a pathway to the demand steps [10,11], and showing the difference between control and regulation.

The presence of isoenzymes makes the system very robust and contributes to the constancy of the flux; enzymes that carry almost no flux in the reference state can take care of it if another isoenzyme is missing. Although the branches appear independent in relation to variations in demand for the different amino acids, because the fluxes are not disturbed, the steady state concentrations of certain metabolites, such as threonine, could be highly disturbed in the absence of certain isoenzymes. In addition, the existence of isoenzymes could be seen as an evolutionary strategy for being able to respond to a wide range of effectors, which would be more difficult with just one enzyme, in which case a partial effect of effectors is required (cumulative inhibition or activation). The alternative for allowing the action of several different effectors being regulation by covalent modification (metabolic cascades), which in addition allows a higher sensitive response.

The system appears to have evolved to cope with increases in demand, as the demand for lysine or threonine can increase considerably without major perturbation [14]. However, decreases in demand below a certain threshold considerably altered metabolite concentrations, as illustrated in Table 1 with the knockouts at the demand step, and could even prevent a steady state from being attained. Notice, however, that the simulated knockout studies do not consider amino acid catabolism, and in the living plant it is possible that catabolic reactions would prevent the huge changes of concentrations (especially of threonine) that we see in the simulations. It will be interesting to incorporate this in future studies, as well as the interactions that may exist with other amino acid pathways, such as amide amino acid metabolism and pyruvate metabolism. The latter is important, because part of the isoleucine molecule derives from pyruvate, and as this is also the case for valine it may be related to the regulatory effect of valine on the inhibition of threonine deaminase by isoleucine, which appears puzzling if considered in isolation.

As pointed out by Mazat and Nazaret [37] this modelling of the global network of the branched pathway derived from aspartate [14] should not be seen as an end. On the contrary, it should provide a basis for the interpretation of changes that can be produced in the plant. Furthermore it will provide a framework to extend similar simulations to other organisms; it would be interesting if the study done in *E. coli* in relation to the control of threonine synthesis [38] could be extended.

Abbreviations

AK, aspartate kinase;
HSDH, homoserine dehydrogenase;
DHDPS, dihydrodipicolinate synthase;
ASADH, aspartic semialdehyde dehydrogenase;
TD, threonine deaminase;
TS, threonine synthase;
AA-tRNA synthetase, aminoacyl-tRNA synthetase

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