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Review article

Control limits for accumulation of plant metabolites: brute force is no substitute for understanding

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Summary

Which factors limit metabolite accumulation in plant cells? Are theories on flux control effective at explaining the results? Many biotechnologists cling to the idea that every pathway has a rate limiting enzyme and target such enzymes first in order to modulate fluxes. This often translates into large effects on metabolite concentration, but disappointing small increases in flux. Rate limiting enzymes do exist, but are rare and quite opposite to what predicted by biochemistry. In many cases however, flux control is shared among many enzymes. Flux control and concentration control can (and must) be distinguished and quantified for effective manipulation. Flux control for several 'building blocks' of metabolism is placed on the demand side, and therefore increasing demand can be very successful. Tampering with supply, particularly desensitizing supply enzymes, is usually not very effective, if not dangerous, because supply regulatory mechanisms function to control metabolite homeostasis. Some important, but usually unnoticed, metabolic constraints shape the responses of metabolic systems to manipulation: mass conservation, cellular resource allocation and, most prominently, energy supply, particularly in heterotrophic tissues. The theoretical basis for this view shall be explored with recent examples gathered from the manipulation of several metabolites (vitamins, carotenoids, amino acids, sugars, fatty acids, polyhydroxyalkanoates, fructans and sugar alcohols). Some guiding principles are suggested for an even more successful engineering of plant metabolism.

Rate limiting steps: do they exist? Yes, but they are not quite as expected

As most research in plant biotechnology aims at manipulating the amount of selected metabolic products, the question arises: what limits their accumulation? The dominant textbook wisdom was – and partially still is – that in every metabolic pathway there is a reaction limiting the flux (defined as the 'rate limiting step' (RLS) of the pathway), and therefore ultimately the accumulation of downstream metabolites. As a sample quote of this belief, take the following from Ishihara *et al.* (2008) 'Anthranilate synthase activity, which controls metabolic flux in the Trp pathway …'. A similar quote is found in Nieto *et al.* (2009).

Let us first clarify the relationship between flux and metabolite accumulation. If flux remains unchanged, a metabolite concentration can increase only if other metabolite(s) in the pathway decrease; a constant flux imposes a constraint on the achievable fold accumulation of any metabolite. On the contrary, when flux increases, the end product (as well as other intermediates) can accumulate several folds. It must be noted that even small changes in flux (say a few percent of usual flux) may lead to a mM increase of a metabolite if the accumulation is protracted for many days.

The idea of the RLS can be likened, in its simplest and extreme form, to a tollgate on a highway (Figure 1): the number of cars processed in unit time is independent from the number of cars waiting in the queue or of those beyond the gate. In metabolic terms, this comparison is untenable, not only because the rate of an enzymatic reaction obviously depends on the amount of substrate (the number of cars waiting at the gate), but also on the amount of product (the number of cars beyond the gate). Many biotechnologists may have in mind more sophisticated concepts, but the core is still wrong and there are theoretical reasons as well as experimental evidence to challenge the general applicability of the RLS concept. Why has such an idea gained widespread acceptance even if it was wrong? A partial answer might be that enzymes have been studied for many years as purified, isolated components and usually in the absence of significant amount of product(s) in order to avoid the reverse reaction to occur. The same was true for the reverse reaction, when studied.

If such an experimental attitude is comprehensible, because it makes life easier to the experimenter, it has probably contributed to fix the idea in people's mind that reverse reactions have negligible rates in metabolic pathways. This is far from the truth. Measurement of *in vivo* concentration for several metabolites suggests in fact that many reactions proceed in the presence of significant amount of both substrate(s) and product(s), and many of them are near equilibrium (e.g. Bennett *et al.*, 2009; Canelas *et al.*, 2011; Yuan *et al.*, 2009); therefore reverse reaction rates are all but negligible in several cases.

Coming back to the starting question: what is limiting the accumulation of a metabolite? The trivial answer is: 'it depends'

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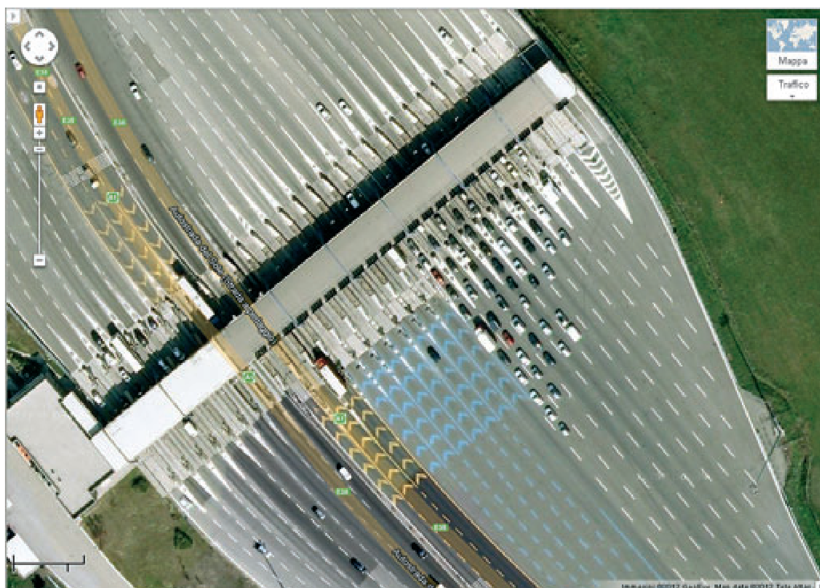

Figure 1 The ‘toll’ view of metabolic reactions. Image of the toll gate in Melegnano (Milan) with queues of cars waiting to pay the toll. Copyright 2012 by GeoEye. Map data copyright 2012 by Tele Atlas.

and namely it depends on the specific reaction, the enzyme and the metabolic context in which it is embedded. Very generally, the rates of enzyme catalyzed reactions depend on and can be limited by three terms (as described by Rohwer and Hofmeyr, 2010, with minor differences): (i) a rate capacity term proportional to the amount of enzyme (E_t) and turnover number (k_{cat}; I shall return to this later on); (ii) the degree of saturation/binding of the enzyme with substrate(s), product(s) and effector(s), which depends on the relevant metabolite concentrations and the specific kinetic constants; (iii) a term dependent on the reaction driving force (‘mass action’ term), that is the displacement from equilibrium (expressed as ρ or its logarithmic form ΔG); Limiting ourselves, for the sake of simplicity, to a uni-uni reaction, the rate can be written as follows:

$$
\begin{aligned}
v &= \left(\frac{V_f}{K_s}\right) \cdot \left[\frac{1}{1 + (S/K_s) + (P/K_p)}\right] \cdot \left\{S - \frac{P}{K_{eq}}\right\} \\
&= (v_{cap}) \cdot [\Theta] \cdot \{v_{ma}\} \qquad (1)
\end{aligned}
$$

in order to distinguish rate capacity (v_{cap}) from saturation (Θ) and mass action (v_{ma}) (Hofmeyr, 1995; Rohwer and Hofmeyr, 2010).

Analyzing how the different terms contribute to the actual rate is by no means intuitive because they multiply themselves; however, Rohwer and Hofmeyr (2010) proposed a logarithmic graph which makes things easier to interpret; unaccustomed readers are referred to their paper as a necessary introduction to this section.

The equation can be recast by moving K_s from the first to the second term:

$$
\begin{aligned}
v &= (V_f) \cdot \left[\frac{(1/K_s)}{1 + (S/K_s) + (P/K_p)}\right] \cdot \left\{S - \frac{P}{K_{eq}}\right\} \\
&= (V_f) \cdot [\Sigma] \cdot \{v_{ma}\} \qquad (2)
\end{aligned}
$$

where $(V_f) = k_{cat} \cdot E_t$. Note that the first two terms in Eqn 2 (V_f and Σ) correspond to those defined by Rohwer and Hofmeyr (2010) (v_{cap} and Θ), but differ by the constant K_s.

A graph in log–log scale with the representation of the rate and its three terms is presented in Figure 2. The log values are additive and it is easier to understand the different contributions.

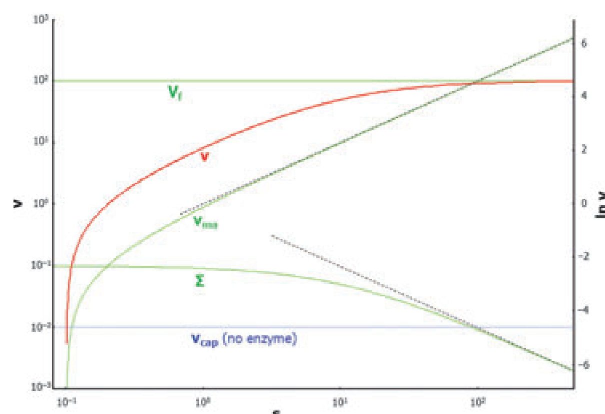

Figure 2 Rate of a reversible enzymatic reaction plotted in log-log scale, as a function of substrate concentration. The rate is equal to the product of the three terms maximal rate (V_f), enzyme saturation (Σ) and mass action (v_{ma}), when expressed as linear values (left axis), or to their sum when using the corresponding logarithmic values (right axis). The trend lines (dashed) of v_{ma} and Σ have equal slopes in absolute value. For comparison, the dotted line is the rate capacity (v_{cap}) for a chemical reaction with a kinetic constant four orders of magnitude smaller. As the distance from equilibrium increases (i.e. at increasing *s*) *v* approaches V_f. Parameters are as in Rohwer and Hofmeyr, 2010. A similar graph can be drawn where the rate is a function of product concentration.

In contrast to chemical reactions (Rohwer and Hofmeyr, 2010), enzyme-catalyzed reactions tend to saturate (the maximal rate V_f or V_r, depending on the direction of the reaction) as the distance from equilibrium increases, because the term Σ becomes smaller as the substrate increases and counteracts the corresponding increase in the mass action term (v_{ma}) (Figure 2). In simple terms it means that the net rate has an intrinsic limit at which molecules can be processed: once that the enzyme is saturated with the substrate, even when the reverse reaction is negligible for the lack of product, a maximal number of substrate molecules will be processed per enzyme in unit time; one must always remember that the maximal rate of enzyme catalyzed reactions is usually orders of magnitude larger than the rate in the absence of

enzyme. Therefore if an enzyme is catalyzing a reaction far from equilibrium ($\rho \leq 0.1$), the limiting factor will not be the distance from the equilibrium, but rather the amount (E_t), the turnover number (k_{cat}) or the saturation (Σ) of the enzyme. Such reactions are deemed kinetically controlled reactions and the control on short time scales is mediated usually by a change in the degree of activation (modulation of k_{cat}) rather than of saturation (modulation of K_m) (Lipchock and Loria, 2010). A clear example is Rubisco, which catalyzes two reactions very far from equilibrium, and as in normal, stable conditions its quantity is usually non-limiting and it can be almost halved without large effects on photosynthetic flux. This is possible because the reduction in E_t is largely compensated by the increased degree of activation (reviewed in Quick, 1994; Stitt and Schulze, 1994). Only when enzyme levels fall below 50%, the flux starts to be more severely reduced. The simple belief that a highly regulated enzyme catalyzing a reaction far from equilibrium must be a RLS is contradicted by facts. As Rubisco represents a substantial investment by plants in terms of carbon and nitrogen, how does one escape the conclusion that plants could save part of this investment by making less? If its amount was halved, plants in changing conditions (light, water, CO_2…) may fix less carbon. Having more than strictly necessary is a costly investment which however provides flexibility (adaptability).

At the other extreme one finds near equilibrium ($\rho > 0.9$) enzymatic reactions, whose rates are overwhelmingly determined by the distance from equilibrium and are thus deemed as ‘thermodynamically controlled’. Their V_{max} are often several orders of magnitude greater than the actual pathway flux and (often) lack of regulatory mechanisms acting upon them. This led to the widespread belief that such enzymes cannot limit the flux, but experimental evidence runs against this belief in some cases, e.g. aldolase, transketolase and the ADP/ATP translocator in potato plastids (this was discussed extensively in Morandini, 2009). Near-equilibrium reactions do not necessarily need control mechanism to prevent the reaction from running wild (a situation which leads to metabolite excesses). If one of these reactions would run temporarily faster than the flux, its driving force would diminish, and as we know such reactions are limited by the driving force, their rate will adjust.

Conversely, the V_{max} for reactions far from equilibrium is, obviously, still larger than the flux, but, despite being of a similar order of magnitude, it is not limiting in itself. In this case control mechanisms are necessary and sufficient to limit the reaction rate. These are in place primarily to avoid metabolite excesses, not for flux control.

Rate limiting steps are rare; flux control is usually shared among many enzymes

In order to discuss sensibly about regulation, it is necessary to specify the sort of control at stake (flux or metabolite) and to quantify it using indices called ‘control coefficients’. These describe the change in flux or metabolite concentration caused by a small change in enzyme quantity, as proposed within the framework of Metabolic Control Analysis, to which readers are referred (for a book, see Fell, 1997).

Flux control in metabolic pathways is often shared among many enzymes (Curien *et al.* (2009); Moreno-Sánchez *et al.*, 2008; Niederberger *et al.*, 1992; Schaaff *et al.*, 1989). There are very few cases of single steps that are strong flux controllers (C^J close to 1) and there are good reasons for their paucity: one reason is the control by demand, which explains control properties of many biosynthetic pathways (see below), while the other one is the avoidance of dangerous situations such as low energy charge/hypoxia (Morandini, 2009). In other cases, near equilibrium enzymes have significant C^J (0.2–0.3) because they are already at high concentration in a cell and further increases would drain cellular resources from other processes (Goelzer and Fromion, 2011) without substantial gains.

So it seems that the amount of enzymes is tuned to minimize, as far as compatible with other constraints, the C^J values in order to avoid the presence of RLSs. For instance, plants adjust Rubisco levels when shifted to new environmental conditions in order to reduce its flux control coefficient (Quick, 1994). Every time an enzyme becomes more limiting its C^J rises, flux is reduced and the driving force of the reaction increases, which means that the metabolites around the enzyme will tend to vary, with substrate (s) increasing and product(s) decreasing (Morandini *et al.*, 2005). This translates in metabolite imbalances, which are not desirable for several reasons (see end of section ‘Flux control and concentration control are different concepts’).

Parallel activation of several steps is not only the best option to modulate fluxes but is also highly desirable for metabolite homeostasis (Fell and Thomas, 1995; Naqvi *et al.*, 2010). *In vivo*, the activation on short time scales is achieved using mechanisms modulating k_{cat} or K_m rather than the amount of enzyme (E_t), and this requires post-translational modifications (through thioredoxins, kinases…etc.) or changes in pH, ion concentration or allosteric effectors. When the change in flux required is much larger than the flux bearing capacity in the fully active state (V_{max}), then there is no way to augment flux other than increasing the total enzyme concentration by protein synthesis. Many biotechnologists still try to achieve flux changes by expressing single, supposedly limiting, enzymes. These manipulations are, of course, easy to perform, but tend to disrupt metabolite homeostasis, particularly when the introduced enzymes are highly overexpressed or modified in order not to respond any longer to feedback inhibitors. This is particularly troubling because feedback inhibition functions to maintain metabolite homeostasis (Hofmeyr and Rohwer, 2011).

Flux control and concentration control are different concepts

Increasing *in vivo* enzyme quantity/activity causes both flux changes, whose extent is described by the flux control coefficient (C^J) of the enzyme, and metabolite concentration changes, especially proximal ones; also in this case, the extent of the change in concentration is described by the concentration control coefficient (C^S), one for each metabolite. C^Js have received much more attention than C^S, for the understandable reason that fluxes are primary targets of manipulation to increase end product accumulation.

Most C^Js are expected to be small (≈ 0.1) and are in fact small, as there is ample evidence that single enzyme manipulations, even of purported RLS, usually entail small increases in flux, despite large increases in expression level and/or enzymatic activity. In this section, I shall concentrate on recent, paradigmatic evidence, as older literature was reviewed previously (Morandini, 2009; Morandini and Salamini, 2003; Morandini *et al.*, 2005) and in particular on the manipulation of the following biosynthetic pathways: vitamin C (vitC), vitE, vitB9 and carotenoids. The most paradigmatic examples of each pathway are listed in Table 1.

Table 1 Single enzyme manipulations affecting metabolite homeostasis

Metabolic pathway	Examples	Metabolite homeostasis disturbances	Details	More examples reported by/discussed in
VitC	Cronje *et al.* (2012)	++	Up to 10-fold drop in myo-inositol 10- to 20-fold increase in L-Gulonate.	Bulley *et al.* (2012) (table S1)
	Bulley *et al.* (2012)	NR	Enzyme activity 10 000-fold for tomato	Imai *et al.* (2012)
VitE	Karunanandaa *et al.* (2005)	+	Several fold changes in tocochromanols forms	Valentin and Qi (2005) (table 2)
	Zhang *et al.*, 2012;	+		
Folate	Hossain *et al.* (2004)	+++	Up to 1250-fold increase in pterins	Hanson and Gregory (2011) (table 1)
	Storozhenko *et al.* (2007)	++	Up to 89-fold increase in pABA	
Carotenoid	Rosati *et al.* (2000)	+	β-carotene content increased 7-fold, overall 1.7-fold increase	Fraser *et al.* (2009) (table 1) Bai *et al.* (2011) (table 1)
Lipid	Voelker *et al.* (1996)	+*	Incorporation stimulated by increased	Weselake *et al.* (2009) (table 1)
	Moire *et al.* (2004)	+*	demand	Lu *et al.* (2011); Ruiz-López *et al.* (2012b)
AA	Zhu and Galili (2004)	+[†]	Strong accumulation of Lys needs block	Azevedo and Arruda (2010) (table 1)
	Wakasa *et al.* (2006)	NR	of degradation. Effects on yield/viability	Kirma *et al.* (2012)
	Dubouzet *et al.* (2007)	+	in all cases	

The examples refer to pathways discussed in the text where the effects on flux are much smaller than the effects on gene expression or enzyme activity. Usually, large effects are seen on metabolite homeostasis, when measured, next to the engineered step.
NR, not reported.
*Unincorporated fatty acids degraded.
[†]Free aa degraded by catabolic pathway.

Vitamin C

The separate overexpression of six genes in the vitC *de novo* biosynthetic pathway (Zhou *et al.*, 2012) led to the modest increases in vitamin, despite some genes being highly overexpressed (up to 42-fold). When transgenes were combined in couples, increases were greater even with a substantial reduction in the overexpression factor. An easy prediction is that combination of more genes will cause an even greater accumulation, even at reduced overexpression levels. Also Bulley *et al.* (2012) reported significant vitC increases by expressing GDP-L-galactose phosphorylase: 2- to 3-fold in potato tubers, up to sixfold in fruits of tomato and twofold in strawberry. The gene expression/enzyme activity levels were however increased up to 30-fold/up to 10 000-fold for tomato (and the highest overexpressors had no seeds) and in the 100-fold/10-fold range for strawberry. The observed small changes in flux compared to the large changes in activity mean that the C^Js of those enzymes are low. Similar results were obtained for several genes in other species: tomato (Cronje *et al.*, 2012; Zhang *et al.*, 2011), tobacco (Badejo *et al.*, 2009; Imai *et al.*, 2012), rice (Liu *et al.*, 2011), potato (Hemavathi *et al.*, 2010; Qin *et al.*, 2011; Upadhyaya *et al.*, 2011) and Arabidopsis (Wang *et al.*, 2012b); see also Table 1 and recent reviews (Fitzpatrick *et al.*, 2012; Hancock, 2009; Smirnoff, 2011; Zhang *et al.*, 2007). As examples of the large effects on metabolite concentration, see for instance the large drop in myo-inositol (up to 10-fold) and the large increase in gulonate in transformants expressing myo-inositol oxygenase, despite the modest changes in vitC content (Cronje *et al.*, 2012).

Vitamin E

As far as vitE (tocochromanols) biosynthesis is concerned, again, single gene manipulations provide evidence for small C^Js of individual enzymes, especially in seed metabolism, because increases in flux are small. The following quote by Farré *et al.* (2012b) reveals the betrayed expectations: 'Because HPPD catalyzes the first step in the pathway, we expected to see an overall increase in tocochromanol synthesis, which was not the case'. Similar results were obtained by overexpression of several other enzymes in different species (Collakova and DellaPenna, 2003; Kanwischer *et al.*, 2005; Rippert *et al.*, 2004; see Table 1). Only the simultaneous expression of more than one biosynthetic gene (van Eenennaam *et al.*, 2003; Karunanandaa *et al.*, 2005; Naqvi *et al.*, 2009, 2011a; Raclaru *et al.*, 2006) was able to achieve flux increases more comparable to activity increases. More papers are cited in recent reviews (DellaPenna, 2005; DellaPenna and Pogson, 2006; Mène-Saffrané and DellaPenna, 2010; Valentin and Qi, 2005, 2010).

Also in this case larger effects on metabolites concentration are detected next to the manipulated enzyme (reviewed in Della Penna, 2005). Some of the single site manipulations are still a success, because there is a sharp increase in α-forms at the expense of γ-forms, a shift that improves overall vitE activity (e.g. Zhang *et al.*, 2012).

Folate

Similar examples can be drawn from work on the vitB9 (folate). Extensive compilations of older literature and recent attempts on folate biofortification are available (Bekaert *et al.*, 2008; Hanson and Gregory, 2011; see Table 1). The overexpression of a bacterial GTP cyclohydrolase-1 in *Arabidopsis* raised the levels of neopterin (metabolic intermediates close to the engineered enzyme) in transgenic plants 'up to 1100-fold higher than the corresponding levels in nontransgenic plants' Hossain *et al.* (2004). Expressing a mouse GTP cyclohydrolase in tomato caused again an upsurge in pterins and little flux increase (Díaz de la Garza *et al.*, 2004), while the expression of a chicken enzyme in lettuce allowed a significant accumulation of folate (Nunes *et al.*, 2009) but intermediates were not measured. Expression of two enzymes from the plastidial and the cytosolic branches did

ameliorate the situation (Díaz de la Garza *et al.*, 2007). Also Storozhenko *et al.* (2007) overexpressed the same enzymatic activities (from *Arabidopsis*) in rice, achieving a remarkable increase in folate, ranging from 15 to 100 times higher than in control plants. Single transformants showed larger metabolite excesses than double transformants. Interestingly, Waller *et al.* (2010) reported that the expression 'of three downstream pathway genes […] increased by up to 7.8-, 2.8-, and 1.7-fold, apparently in response to the build-up of specific folate pathway metabolites'. Thus the substantial accumulation of folate reported in some publications might be due to a partial parallel activation stimulated by feedforward mechanisms rather than high flux control coefficients of the enzymes targeted for manipulation.

Carotenoids

Carotenoid engineering in plants for biofortification and feed supplements production has been extensively reviewed (Bai *et al.*, 2011; Beyer, 2010; Farré *et al.*, 2011; Fraser *et al.*, 2009; Misawa, 2009; Wurtzel *et al.*, 2012). This metabolism is more complex because several enzymes can perform more than one reaction in the pathway (several perform two, one bacterial enzyme even 6, see figure 2 of Bai *et al.*, 2011 for details) implying that apparent single enzyme manipulations are, in fact, multiple ones. Also the induction of endogenous enzyme (e.g. lycopene β-cyclase, Aluru *et al.*, 2008) presumably by feedforward mechanisms could play an additional role.

Many groups reported very significant increases, even many thousands fold compared to control tissues, in maize, potato, wheat, carrot, cassava, canola, tomato, sweet potato, kumquat and banana (Aluru *et al.*, 2008; D'Ambrosio *et al.*, 2004; Diretto *et al.*, 2007; Failla *et al.*, 2012; Fujisawa *et al.*, 2009; Kim *et al.*, 2012; Lu *et al.*, 2006; Maass *et al.*, 2009; Naqvi *et al.*, 2009; Sayre *et al.*, 2011; Zhu *et al.*, 2008; for more references: Bai *et al.*, 2011).

Most of these works were indeed transformation with several genes, often including a carotenoid desaturase performing 6 reactions by itself. In one case, a combinatorial approach was used (Farré *et al.*, 2012a; Zhu *et al.*, 2008) allowing the creation of many different phenotypes. The same group achieved also the accumulation of three different vitamins in corn by using the same combinatorial approach (Naqvi *et al.*, 2009). See also Hasunuma *et al.* (2008) and Misawa (2009) for the engineering of astaxanthin production. Another remarkable achievement is the expression of the cytosolic mevalonate pathway (six genes) in tobacco plastids (Kumar *et al.*, 2012) which increases carotenoid content simply by providing more precursors.

The fact that many of the examples mentioned above are multisite modulations explains the substantial flux increases achieved. Several examples of single enzyme manipulation in tomato are referred to in table 1 of Fraser *et al.* (2009), with the relative increase in total and specific carotenoids. Among single enzyme manipulations, for instance, Rosati *et al.* (2000) transformed tomato with a licopene β-cyclase and improved β-carotene content sevenfold, but with a limited flux increase (overall carotenoid content increased only 1.7-fold); also Maass *et al.* (2009) reported a 10-fold increase in carotenoid content of *Arabidopsis* calli, but 'a change of the pattern of accumulated carotenoids, as xanthophylls decreased relative to β-carotene and carotene intermediates accumulated'. These examples are quite reminiscent of those obtained for manipulation of the tocopherol pathway mentioned above.

Even though this paper does not cover conventional breeding or the use of molecular markers to facilitate crop development, it is clear that the combination of metabolic engineering with natural allelic variation allows a wider manipulation of carotenoids content (Naqvi *et al.*, 2011b). The contribution of classical breeding could be very significant, provided that natural variation for metabolite composition exists in a crop (e.g. Harjes *et al.*, 2008; Yan *et al.*, 2010). It is clear that classical breeding can easily cause multisite modulations.

The selected examples are sufficient to suggest a wider acceptance of multisite manipulation of metabolism (Fell and Thomas, 1995), except for those cases where flux control rests mainly on the demand. There are indeed good reasons to maintain metabolite homeostasis, because its breakdown has consequences which are listed below. First, increasing the concentration of certain intermediates automatically depletes the concentration of other ones – this concept is usually referred to as 'mass conservation'; for instance accumulating a lot of sugar phosphates (or phospho-acids) reduces the free phosphate pool (discussed in Morandini, 2009). Similar arguments can be brought concerning other potentially limiting intracellular metabolites (e.g. S or N containing ones). Second, some intermediate may be (come) toxic and/or induce the formation of toxic compounds by spontaneous reactions whose rates are directly proportional to the concentration, for instance methylglyoxal (and aldehydes in general) and free glucose, see Rabbani and Thornalley, 2012; Thornalley, 2003; Yadav *et al.*, 2008; for a mevalonate pathway toxic intermediate see Kizer *et al.*, 2008. Third, maintaining the metabolites relatively constant also avoids alteration in fluxes departing from the manipulated pathway, because a drop (or a rise) of a metabolite concentration decreases (or stimulates) the rate of the reactions. Other metabolic constraints that shape the responses to manipulation (e.g. oxygen-dependent energy supply and cellular resource allocation, discussed in Morandini, 2009 and below) are often overlooked; this is a pity as many responses are mounted to counteract the negative effect of metabolite concentration extremes, both high and low ones. These negative consequences appear faster and are much more relevant than osmotic buildup.

In short: flux control and concentration control are different concepts and must be addressed in different ways; manipulations aiming at flux usually end up disturbing metabolite homeostasis, with negative consequences.

Flux control for several 'building blocks' of metabolism is placed on the demand side; increasing demand can be very successful

Why are some single enzyme manipulations more effective than others at increasing flux? Clearly, some enzymes are more 'limiting' (better: have higher C^Js) but why is it so? In this section I try to explain this behaviour at the light of supply-demand analysis. This is a theoretical framework (Hofmeyr and Cornish-Bowden, 2000; Hofmeyr and Rohwer, 2011; Rohwer and Hofmeyr, 2008) to describe how the processes providing a metabolite (the 'supply') integrate with those consuming it (the 'demand').

Many metabolic pathways supply cells with 'building blocks' [aminoacids (aa), nucleotides, sugars and activated sugars, fatty acids, glycerol and several carbon skeletons] and cofactors (mainly ATP and NADPH) to synthesize macromolecules and namely: proteins, nucleic acids, starch and other carbohydrates, lipids and

other cell constituents (e.g. lignin). It is sensible to assume that when different building blocks are not being used, their synthesis slows down to avoid build up, an event detrimental in several ways. Therefore, mechanisms must exist which harmoniously link synthesis and consumption (supply and demand). The harmonious link is achieved when the demand for a metabolite is nearly saturated by the metabolite itself, (using a more formal wording: when the demand elasticity is low compared to supply elasticity, see Hofmeyr and Rohwer, 2011); see Curien *et al.* (2009) for an example describing the molecular mechanisms allowing control by demand. In this way, the demand holds the majority of flux control, and the supply rate drops when consumption is reduced, but simultaneously the demand process looses most of the control over the concentration of the linking metabolite. In the words of Hofmeyr and Rohwer (2011), 'the functions of flux and concentration control are mutually exclusive in the sense that if one block [totally] controls the flux, it loses any influence over the magnitude of variation in the concentration of the linking intermediate'.

A prime example of flux control by demand is ATP production (glycolysis and/or respiration), whose flux is controlled by ATP demand in *Escherichia coli* and probably in many cells under normal conditions (Ebert *et al.*, 2011; Koebmann *et al.*, 2002a,b). An even more convincing example has been just published (Celton *et al.*, 2012a,b) where the authors achieved a remarkable 8- to 22-fold increase in NADPH consumption over anabolic demand using a engineered NADPH-dependent butanediol dehydrogenase coupled with increased concentrations of its substrate acetoin (0, 100, 200 and 300 mM) in the growth medium. Only at the highest acetoin concentration, the growth rate was reduced by 60%, while at the lowest concentration (100 mM) there was an increase in NADPH consumption of eight times, but no changes in expression of the genes involved in the main NADPH-producing pathways (the pentose phosphate and acetate pathways). Larger increases in NADPH demand resulted in the induction of several pentose phosphate pathway genes. Thus, yeast cells are able to cope with an increase in NADPH demand equivalent to eight times the anabolic demand by increasing the flux through these two pathways, and this was essentially mediated by metabolic control. This means that the cofactor flux control resides in the demand block, not only in wild type cells, but also in cells at 100 mM acetoin, despite a huge increase in demand. As a consequence, the control of NADPH concentration must reside in the supply block and I predict that the NADPH concentration is minimally affected in cells exposed to 100 mM acetoin, becoming substantially smaller only at higher demand levels.

It is reasonable to expect that the fluxes producing other building blocks of metabolism will be controlled by demand, and I try next to substantiate this belief using the recent literature on the metabolic engineering of four different types of building blocks (aa, lipids, Acetyl-CoA and sugars) for the synthesis of more complex molecules. As expected, in most cases, supply enzymes are not limiting in unmodified living systems.

Aminoacids

One of the areas where the supply-demand concept allows a better understanding of the results is the improvement in the aa composition of several crops, most notably maize, which is particularly poor in Lys and Trp. The starting point was the discovery of the maize mutant *opaque-2* (*o2*) which shows a significant increase in Lys (+70%) and Trp content (Mertz *et al.*, 1964). The mutation mainly affects the synthesis of the 22-kDa α-zein (Jones *et al.*, 1977). This simple observation should have prompted the recognition that the mutation simply increased the demand, because the reduction in the 22-kDa α-zein, devoid of Lys and poor in Trp was compensated by an increase in albumins and globulins, other storage proteins, leaving the supply unchanged; hence supply was not limiting. Essentially the same result, a large increase in Lys, was obtained by transgenesis through selective inhibition of zein isoform synthesis (Huang *et al.*, 2004, 2005, 2006). Another piece of evidence was that Lys translocated to developing seeds was in excess and a substantial fraction underwent degradation in the endosperm (Silva and Arruda, 1979). On the contrary, many tried to develop and express isoenzymes less sensitive to feedback inhibition by Lys (so called 'desensitized' enzymes) at various point of the pathway in the hope to divert extra carbon. Limited success was obtained (several reviews are available: Azevedo and Arruda, 2010; Kirma *et al.*, 2012; Ufaz and Galili, 2008) at increasing free Lys several fold in leaves, much less in seeds. As the great share of aa is incorporated into proteins, the overall increase was usually negligible. When free Lys concentration rises in seeds, catabolism kicks in and degradation occurs. The reduction in the level of a bifunctional Lys-degrading enzyme, lysine-ketoglutarate reductase/saccharophine dehydrogenase allowed a large rise in free Lys. Combining the block in degradation with the expression of the desensitized biosynthetic enzyme CordapA, the desired large rise in free Lys (40-fold) in maize seeds was achieved (Frizzi *et al.*, 2008). Similar results were obtained in *Arabidopsis* (Zhu and Galili, 2003, 2004).

A recent review however recognized that 'significant enhancement of lysine levels specifically in developing seeds by genetic engineering also causes major problems associated with inefficient seed germination and plant growth.' (Kirma *et al.*, 2012).

Swapping to Trp, overexpression in rice calli and plants of a feedback-insensitive form of anthranilate synthase led to a large increase in free Trp (up to 300-fold) as well as total Trp (up to 14-fold). Trp accumulation led however to poor germination and weak seedling growth (Dubouzet *et al.*, 2007), reduced spikelet fertility, germination and seed yield (Wakasa *et al.*, 2006) and possibly also reduced seed weight in the highest Trp-accumulating line (see table 1 of Wakasa *et al.*, 2006).

The incapacity to distinguish between the different types of control (concentration control and flux control) and to recognize that control mechanism such as feedback inhibition are designed to maintain metabolite homeostasis is a recipe for failure because people keep targeting one control for the other. The available data do not highlight large accumulation of intermediates in aa pathways, but it is unclear how many of these are effectively measured in metabolomic experiments and how much undergoes degradation. For instance, a novel indole compound, possibly derived from indole glycerol-3-phosphate, an intermediate of the Trp biosynthetic pathway, was detected in rice calli (Morino *et al.*, 2005), while Ishihara *et al.* (2007) mention that anthranilate in transgenic rice expressing a feedback-insensitive anthranilate synthase was 'only several times greater than that in the wild type'.

Lipids

Another area where supply-demand analysis would have helped is lipid/triacylglycerol (TAG) accumulation (reviewed in Barthole *et al.*, 2012; Chapman and Ohlrogge, 2012; Lu *et al.*, 2011; Rogalski and Carrer, 2011; Weselake *et al.*, 2009). The RLS idea strikes again, as exemplified by the following quote: 'As in

bacteria, fungi and animals, both *in vitro* and *in vivo* evidence indicates that acetyl-CoA carboxylase (ACCase) is a key rate-determining step that controls FA biosynthesis. ACCase activity is under complex regulation …' (Chapman and Ohlrogge, 2012). Yet, overexpression of ACCase yielded a disappointing small, if at all true, flux increase (Roesler *et al.*, 1997). Examples of similar failures due to overexpression of supply enzymes can be Madoka *et al.* (2002) and Tomlinson *et al.* (2004), but for a *bona fide* example of the contrary see Vigeolas *et al.* (2007).

Several examples of successful increase in lipid content have been reported by increasing demand (Bouvier-Navé *et al.*, 2000; Maisonneuve *et al.*, 2010; Oakes *et al.*, 2011; Petrie *et al.*, 2012; Taylor *et al.*, 2001, 2009b; Zheng *et al.*, 2008) especially mediated by augmenting the diacylglycerol transacylase (DGAT) activity, the major TAG biosynthetic enzyme (for more examples, see Table 1).

When a change in composition without substantial increase in overall content is the desired target, this is achieved by expressing the required biosynthetic enzymes (be they thioesterases, desaturases, elongases or hydroxylases) as well as the acyltransferases or other activities that are able to incorporate the specific fatty acids into lipids/TAG (Burgal *et al.*, 2008; Hoffmann *et al.*, 2008; Li *et al.*, 2010; Mietkiewska *et al.*, 2004; Nguyen *et al.*, 2010; Ruiz-López *et al.*, 2012a; Sayanova *et al.*, 2012; Taylor *et al.*, 2009a; Truksa *et al.*, 2006; Wilkes, 2008; Wu *et al.*, 2005; additional examples in Ruiz-López *et al.*, 2012b). Very interestingly, the increased preference for hydroxyacids by a DGAT or a phospholipid:diacylglycerol acyltransferase from *Ricinus* stimulated the incorporation of ricinoleic acid into TAG from 17% to respectively 30% and 25% of total seed lipid (Burgal *et al.*, 2008; Kim *et al.*, 2011), and similar situations were reported for lauric (Knutzon *et al.*, 1999) and vernolic acid (Li *et al.*, 2010). When the supply of fatty acids is not matched by an increase in demand, a futile cycle of fatty acid degradation via β-oxidation and sucrose re-synthesis is triggered (Moire *et al.*, 2004; Poirier *et al.*, 1999; Voelker *et al.*, 1996).

Also in the case of lipids, the parallel activation of many genes, especially using transcriptional regulators coordinating genes involved in TAG synthesis, achieved remarkable flux increases, even in vegetative tissues (Andrianov *et al.*, 2010; Gao *et al.*, 2009; Naqvi *et al.*, 2010; Pouvreau *et al.*, 2011; Sanjaya *et al.*, 2011; Shen *et al.*, 2010; Slocombe *et al.*, 2009; reviewed in Baud and Lepiniec, 2010; Lu *et al.*, 2011; Weselake *et al.*, 2009). Both positive and negative regulators of oil content have been identified (*LEC1* and *LEC2*, *WRI*, *PKL* and *ASIL1*) and exploited to this purpose.

An often overlooked factor limiting lipid accumulation in *Arabidopsis* seeds is oxygen, because hemoglobin overexpression boosts lipid accumulation by 40% in absolute values per seed and as percentage of seed dry weight (Vigeolas *et al.*, 2011). The haemoglobin maintained a higher ATP/ADP ratio even under low (4%) external oxygen. However strange it may appear, seeds of various species experience an internal O_2 concentration in the 2–4% range (v/v) (see references in Vigeolas *et al.*, 2011). Given the importance of the energy charge to sustain the metabolism at large, supplying more oxygen to heterotrophic tissues may improve the accumulation of several compounds (see also below).

Polyhydroxybutyrate

A third area where to apply the concepts developed in the supply-demand analysis is the production of polyhydroxybutyrate (PHB) and related polymers, reviewed in Mooney (2009); Poirier and Brumbley (2010); Suriyamongkol *et al.* (2007) and van Beilen and Poirier (2008, 2012). Being a compound not produced by plants and having a very short biosynthetic pathway composed of only three reactions, it was relatively easy to establish the pathway in plants. After the first report of expression in the cytosol with poor yield, the genes were expressed in plastids, boosting the accumulation of PHB up to 14% of the dry weight (10 mg/g fwt) (Nawrath *et al.*, 1994), a value 100 times larger. Interestingly, based on the consideration that the flux of acetyl-CoA to PHB was comparable to the flux for lipid biosynthesis, the author suggested that 'a mechanism exists to enhance synthesis of acetyl-CoA in response to demand'. In a plant cell there are five different pools of acetyl-CoA (plastidial, peroxisomal, mitochondrial, cytosolic and nuclear) produced by the degradation of different sources: carbohydrates, lipids and amino acids. Acetyl-CoA in plastids (Oliver *et al.*, 2009) derives from sugar catabolism and, to a lesser extent, from acetate; it feeds mainly lipid (both membrane and extracellular lipids such as cuticle and suberin) and aa biosynthesis, and it is therefore sensible to assume regulation by demand. An improvement in PHB yield was obtained by Bohmert *et al.* (2000) who achieved a remarkable value of 42 mg of PHB/gFW (i.e. ca. 40% of dry weight) in *Arabidopsis*, with the best lines. Not surprisingly, there was "a strong positive correlation between the accumulation of PHB and the expression of the three *phb* genes in each line", but also a negative correlation with plant growth. The highest accumulators grew very poorly, were chlorotic and sterile. This result in terms of yield was achieved by using a single plasmid bearing three constructs instead of crossing transformants expressing single genes; variable copy number, segregation and cosuppression are presumably responsible for the difference in expression and yield. The demand for acetyl-CoA is so high in these new transformants that supply cannot cope any longer, causing the extreme phenotype observed at the highest PHB yield. In this case, it is advisable to increase acetyl-CoA supply through new enzymatic activities. My suggestion is to overexpress pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase in the plastid to bypass the pyruvate dehydrogenase step. In this way, plastidial pyruvate would be oxidized to acetaldehyde and then to acetate and finally converted into acetyl-CoA; such a pathway seems to be working in pollen and roots (Mellema *et al.*, 2002; see Oliver *et al.*, 2009 for further references).

A very recent paper reported the production of tobacco plants accumulating up to 17.3% dry weight PHB in leaf and 8.8% of the total biomass (Bohmert-Tatarev *et al.*, 2011), a very promising result achieved by optimization of transgene expression cassettes and integration in the plastidial genome. Transgenic plants showed slower growth, reduced biomass, delayed flowering and some chlorosis, but were fertile and the construct was stably inherited. Also in this case augmenting the acetyl-CoA supply could be beneficial to alleviate the phenotype.

Other attempts in sugarcane, poplar, soybean and switchgrass have been published (Anderson *et al.*, 2011; Dalton *et al.*, 2011; Matsumoto *et al.*, 2011; Petrasovits *et al.*, 2012; Schnell *et al.*, 2012; Somleva *et al.*, 2008; Tilbrook *et al.*, 2011), several of which achieved reasonable polymer content, but again, with some impairment in phenotype as content increases. The development of an inducible system for gene expression (Kourtz *et al.*, 2007) seems an effective way to alleviate part of the negative burden, probably because it avoids the interference with early phases of growth, where acetyl-CoA is crucial for lipid biosynthesis and membrane formation. Indeed, only leaves developing post-induction were chlorotic and stunted. The

system has already been applied to poplar with very interesting results (Dalton *et al.*, 2011). It is important here to remind that the overall PHB yield, not tissue content, is a relevant economical parameter (Tyo *et al.*, 2010), and that a sick plant with a high PHB content may show a lower overall productivity per hectare. On the contrary to bacteria, thus, the limitation in the best transgenic plants may be more on the supply side, whose capacity must then be increased.

A tale of sinks and sources

Photosynthesis in leaves is regulated by photosynthate demand, that is, photosynthetic rates decrease when sugar production exceeds intracellular utilization (or export to the phloem and ultimately to the sinks (Paul and Foyer, 2001; Stitt, 1991)). This was shown in several cases when the source-sink status of the plant was manipulated for instance by removing fruits or leaves (Azcon-Bieto, 1983; Blechschmidt-Schneider *et al.*, 1989; Ceppi *et al.*, 1987; Legros *et al.*, 2009; van Oosten *et al.*, 1997; Paul *et al.*, 1991; Plaut *et al.*, 1987). The regulation is mediated by mechanisms sensing the rise in intracellular sugars (reviewed in Rolland *et al.*, 2006) linked to the reduced export (Ayre, 2011). The decrease in photosynthetic rates is a safety measure to avoid the building up of metabolites (remember free reducing sugars are quite reactive molecules and their concentration should stay low) and the regulation is functional at maintaining metabolite homeostasis. If one desires to increase flux, it seems more sensible to try to increase sink strength rather than tampering with signals or signal transduction elements. Whether increasing flux by increasing sink strength is always a feasible strategy, it remains to be extensively tested, but there are indications that this is, at least in certain crops and conditions, possible. As tissues of choice I suggest aboveground, photosynthetic organs (leaves, culms), because heterotrophic tissues like potato tubers and sugar beet taproots, which depend on respiration for fuelling the biosynthetic machinery, may be already limited by oxygen supply (discussed in Morandini, 2009 and below). Very interesting in this respect are the findings about the plastid-localized NTRC protein which is involved in long-distance redox signalling, linking redox regulation, carbohydrate flux and starch synthesis in both source and sink tissues (Michalska *et al.*, 2009), and that sugar levels modulate the activity of the SUT1 transporter of potato via redox regulation (Krügel *et al.*, 2008; both are reviewed in Slewinski and Braun, 2010).

Sugars and fructans

A remarkable achievement in the context of demand was the doubling of total sugar content in sugarcane (Wu and Birch, 2007) by targeting a bacterial sucrose isomerase (Goulter *et al.*, 2012) that drives the conversion of sucrose into isomaltulose through a thermodynamically favoured reaction. What was once thought as an upper limit for sugar accumulation that resisted around 40 years of breeders' efforts (Jackson, 2005) was easily overcome by a single bacterial gene expressed at low levels. Unfortunately, the trait seems to be rather unstable because the same transformants, as well as new ones, did not reach comparable levels in subsequent years, either in greenhouse or open field trials (Basnayake *et al.*, 2012). The most reasonable explanations for this are transgene silencing (Mudge *et al.*, 2009) and protein instability (Wu and Birch, 2007), but only further analysis will explain it in detail. Despite any claim to the contrary about what is limiting photosynthesis, it is clear that, at least in sugarcane, photosynthetic flux can increase and energy conversion rates can improve if a new carbon sink is created. A very similar strategy was also recently published by the same group (Hamerli and Birch, 2011).

Other interesting attempts at increasing flux are the engineering of enzymes for fructans, fructose polymers derived from sucrose (reviewed in Banguela and Hernández, 2006; Cairns, 2003; Ritsema and Smeekens, 2003; van Arkel *et al.*, 2012). The polymers accumulated at high levels in some cases, but only when the enzyme was targeted to the vacuole and sucrose concentrations in this compartment were high enough (Stoop *et al.*, 2007; Sévenier *et al.*, 1998; see table 1 of van Arkel *et al.*, 2012 for a summary). The engineering of a fructan synthetic pathway in a high-sucrose maize background boosted inulin accumulation from 3.2 to 41 mg/g kernel (Stoop *et al.*, 2007). This was interpreted as the result of substrate competition between starch and inulin production (see Ritsema and Smeekens, 2003), but it could be also interpreted as the requirement for a sufficiently high concentration to attain a significant enzyme rate. The starting enzyme 1-sucrose:sucrose 1-fructosyltransferase has, in fact, a high K_m for sucrose (50–600 mM, discussed in Ritsema and Smeekens, 2003) which may prevent significant fructan accumulation in crops with low vacuolar sucrose. Moreover, fructans can be degraded by the same biosynthetic enzyme as well as by vacuolar invertases. Several plants showed good accumulation of fructan polymers, and in one case they represented 40% of the root dry weight, but this happened without substantial flux increases in carbon assimilation or even with a decrease in other polymers (starch in potato). In other words, what was achieved was a substantial conversion of sucrose into fructans (Gerrits *et al.*, 2001; Hellwege *et al.*, 1997, 2000; van der Meer *et al.*, 1994; Sévenier *et al.*, 1998; Weyens *et al.*, 2004).

One of the most probable factors limiting accumulation in underground organs is energy consumption, due to the chemistry and compartmentalization of the pathway. The fructan synthesizing enzymes use sucrose as an activated sugar to promote polymerization and release glucose in the vacuole. This means that one molecule of sucrose is hydrolyzed for each fructose monomer added to the polymer and therefore glucose, continuously produced, must be exported back to the cytosol, retransformed into sucrose and pumped back to the vacuole to sustain the synthesis (see the rise in glucose, for instance, reported in: table 1 of Weyens *et al.*, 2004; or by Hellwege *et al.*, 2000; tables 2 and 3; Gerrits *et al.*, 2001; Sévenier *et al.*, 1998; table 1). This entails a larger energy requirement per polymerized hexose unit and could well explain the decrease in carbon yield. The decrease in starch yield in potato caused by futile cycles which were triggered by altered metabolite homeostasis was discussed at length previously (Morandini, 2009). If increasing oxygen availability can boost lipid content in *Arabidopsis* seeds (Vigeolas *et al.*, 2011), which are exposed to air and surrounded by photosynthesizing, oxygen-producing tissues during the day, much more so is expected for heterotrophic tissues in underground organs. Therefore I repeat again the plea (Morandini, 2009) for engineering haemoglobin overexpression to engineer an oxygen reservoir for improving energy production capacity.

Very recently, Banguela *et al.* (2011, 2012) expressed in tobacco a bacterial levansucrase (*LsdA*), an enzyme that catalyzes the synthesis of levan and fructo-oligosaccharides from sucrose. The transformants accumulated levan with the degree of polymerization higher than 100 fructose residues in mature tobacco

leaves, where the polymer represented between 10% and 70% (w/w) of total dry weight. The transgenic plants looked normal during growth, but older leaves bleached prematurely and became rigid due to the high turgor. The transformant at the highest concentration was not able to set seed and it was therefore lost, but the other clones showed stable expression and levan accumulation in T2 generation. It is not clear from the paper whether the levan accumulated beyond the usual dry matter, but it seems plausible that also in this case the new sink increased demand and subsequently also carbon assimilation.

Sugar alcohols

Similar attempts have been made to engineer sugar alcohols in sugarcane (Chong *et al.*, 2007, 2010) and tobacco (Sheveleva *et al.*, 1998, 2000). In sugarcane, sorbitol accumulated up to 120 mg/g dry weight in leaves, corresponding to more than half of the soluble sugars, and up to 10 mg/g dry weight in stalks without affecting sucrose accumulation in the culm. In both cases, however, there were negative effects (reduced biomass, necrosis and early senescence proportional to sorbitol levels). Less drastic phenotypes were associated with the joint accumulation of mannitol and D-ononitol (Sheveleva *et al.*, 2000) or lower levels of sorbitol (Deguchi *et al.*, 2004, 2006). As before, these new sinks were active in the cytosol of source leaves and obviously conflicted with several other metabolic pathways. Another new carbon sink deriving from an aa was described in sugarcane (McQualter *et al.*, 2005) with higher accumulation of p-hydroxybenzoic acid in leaves than in stems.

From the overall literature, it is clear that the stimulation of demand by engineering new polymers/compounds must be tuned both spatially and temporally to avoid conflict with the endogenous processes and particularly with the energy status, the more so if this happens in heterotrophic tissues and when large increases in flux are required. To this end it is important that: (i) the additional sink should preferably be located in a photosynthetic tissue and in a compartment (such as the vacuole) that minimizes conflict with basal metabolism; (ii) demand must be turned on after the completion of development and after a substantial source function has been accomplished, that is, when photosynthetic capacity is available but mostly unexploited; (iii) the K_m of the new enzyme should preferably be higher (or the V_{max} smaller) than the corresponding value of essential enzymes acting on the same substrate, so that the new demand sets in only when the intracellular substrate concentration is rising, after the endogenous fluxes (export to roots and young leaves) are decreasing. (iv) Last but not least, it may be also important to avoid or minimize degradation of the new compounds (e.g. Wu and Birch, 2011) not only to increase the levels, but also to avoid triggering futile cycles of synthesis and hydrolysis which waste resources and render the process inefficient (e.g. Sévenier *et al.*, 1998; van der Meer *et al.*, 1994).

To achieve these goals it is therefore important to choose promoters that are activated shortly before senescence or in any case after the completion of organ development. Also the choice/combination of enzymes is important. It is easy to envisage that sugarcane is the crop of choice for many of these manipulations.

Even if this may disappoint readers, manipulations of the flux to starch will not be discussed in this paper, because the subject was already partially covered in a previous review (Morandini, 2009), because of space limitations and also because there seems to be a flood of papers claiming flux increases which are difficult to reconcile among themselves and with previous literature (Debast *et al.*, 2011; Fu and Xue, 2011; Hädrich *et al.*, 2011; Kunz *et al.*, 2010; Li *et al.*, 2012; McKibbin *et al.*, 2006; Oliver *et al.*, 2008; Uematsu *et al.*, 2012; Wang *et al.*, 2012a; Weise *et al.*, 2012; Zhang *et al.*, 2008; Zuther *et al.*, 2011; see Geigenberger, 2011 for additional references). Time will clarify the issue.

To summarize, most of this section can be best paraphrased using a quote from Niebuhr (1943) 'Nothing is so incredible as an answer to an unasked question.'

Ways for increasing demand/accumulation

How is it then possible to increase demand? As mentioned, accumulation of free metabolites could be dangerous and/or ineffective. More successful means are all those in which the metabolite is effectively removed from equilibrium. One mean is to polymerize it, as it is carried out with aa and acetyl-CoA during the formation of proteins and PHB, respectively. Another is crystal formation, as it happens in the extracellular medium for some bacterial strain producing aa (for an example of crystal formation in *Arabidopsis*, see Maass *et al.*, 2009). A third one, which holds quite some promise and which has been so far unexploited, is to stimulate export to some other compartment (Morandini and Salamini, 2003).

A way to perform multisite modulations, sometimes stimulating both supply and demand, is through transcription factors. Nice, recent examples are the overexpression of *WRI*1, LEC1 and LEC2 (reviewed in Lu *et al.*, 2011; see above), master regulators of genes involved in the conversion of sucrose to fatty acids, or the development of custom transcription factors (van Eenennaam *et al.*, 2004). Another example is the OR protein (Lu *et al.*, 2006), a regulator of chromoplast differentiation whose mutation causes carotenoid accumulation in cauliflower and potato.

The DOs and DON'Ts of metabolic engineers

If the desired outcome is the modulation of concentration (e.g. quantitative conversion to α-tocopherol), then it is clear that the overexpression of single enzymes, especially desensitized ones can be pursued, but one must be careful to side effects and metabolic constraints. If however, as it is more common, the modulation of flux is the target, then I dare suggesting a few guidelines:

- Try to understand/guess if flux to product is controlled by demand. In this case, increase demand by one or more approaches.
- If, on the contrary, flux is controlled by supply, try parallel activation of supply enzymes, possibly using one construct or transcription factors or regulators.
- In this second case, avoid single enzyme manipulation and, even more, shun the temptation of using desensitized enzymes.
- In all cases be aware that one often overlooked limitation is the energy supply, especially in non-photosynthetic tissues and even more in underground organs. Avoid triggering futile cycles and try to increase energy supply.

Beyond science: regulation, the mother of all constraint

The broader application of genetic engineering to plants has allowed the generation of popular commercial traits (herbicide tolerance and insect resistance). Metabolic engineering of plants

has achieved remarkable goals in the past 10 years, being able to modulate the content of several endogenous and useful compounds, especially vitamins, aa and lipids, but little of this achieved commercialization. Many other benefits are within reach, for both developed and developing countries, among which the reduction of toxins and allergens in several crops (Gallo and Sayre, 2009; Morandini, 2010). However brighter the future might look, a substantial rethinking of current regulatory framework is required if the promised benefits are to be fulfilled (Giddings *et al.*, 2012; Gómez-Galera *et al.*, 2012; Miller, 2010; Potrykus, 2010). The overwhelming consensus is that transgenesis does not imply *per se* new or higher risks compared to conventional breeding (Arber, 2010; Parrott, 2010) and the approval cost are unjustified and determine a *de facto* block at the research stage. Renouncing to most of the potential benefits for futile reasons is a shame to our generation.

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Conflict of interest

The author declares no conflict of interest.

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