Co-ordination of leaf minor amino acid contents in crop species: significance and interpretation

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Abstract

The question of whether general control of amino acid synthesis exists in plants remains to be resolved. It is not known whether there is overall co-ordination of the biosynthesis of amino acids that are formed through distinct pathways. In this work, amino acid contents were measured in a large number of samples taken from wheat, potato and barley leaves under different photosynthetic conditions. The variability in total soluble amino acid contents between samples was approximately 6-fold in wheat and potato. Subtracting the major amino acids from the total soluble amino acids showed that the variability in summed minor amino acid contents was approximately 20-fold. This variability was not correlated with short-term changes in primary carbon and nitrogen metabolism, and only poorly correlated with total leaf amino acids. By contrast, striking linear relationships between the contents of most minor amino acids were observed, demonstrating that the contents of many minor amino acids vary in concert. These observations show that amino acid contents are co-ordinated across biosynthetic families. While these data might be interpreted as an indication of cross-pathway regulation of the expression of key biosynthetic enzymes, the impact of factors such as protein degradation and storage cannot be ignored.

Key words: Amino acid synthesis, barley, co-ordination of biosynthesis, leaves, minor amino acids, potato, wheat.

Introduction

Plants synthesize amino acids from inorganic compounds and use them to generate a wide variety of products, including proteins, pigments, nucleotides, enzyme co-factors, hormones, structural components, and defensive agents. In most non-leguminous crop plants, the dominant organ in amino acid synthesis and distribution is the leaf, which uses the energy and carbon skeletons produced by photosynthesis to assimilate nitrogen into primary amino acid products (Gln and Glu). The amino groups of these products are then allocated through transamination reactions to form the host of amino acids necessary for protein synthesis and other purposes. Because of their centrality to cellular metabolism and their involvement in multiple metabolic pathways, amino acids are a key focus of the developing techniques of metabolite profiling (Maimann et al., 2000; Roessner et al., 2000, 2001).

The leaf contents of minor amino acids vary in a co-ordinated fashion

The soluble amino acids in plants can be divided into two groups. The major amino acids are normally present in high concentrations, and usually include those most closely linked to primary carbon metabolism and nitrogen assimilation, while the minor amino acids are generally less abundant. Defined by these criteria, the minor amino acids include most if not all of the nine that are essential in the human diet. There is, therefore, considerable interest in plant contents and the factors that influence these contents. In this work the extent of variation in soluble amino acids in leaves from nitrogen-sufficient wheat and potato under defined conditions has been explored. Attached leaves were introduced into infrared gas analysis chambers and photosynthesis monitored at a given irradiance, CO2 concentration, or O2 concentration. On attainment of the steady-state rate of photosynthesis (25–35 min illumination), metabolism in the monitored...
section of the leaf was rapidly quenched by the technique of freeze-clamping with aluminium tongs pre-cooled in liquid N$_2$. Analysis of amino acid contents in the samples showed that eight amino acids (Glu, Gln, Gly, Ser, Asp, Asn, Ala, Thr) contributed 85–97% and 61–90% of the total amino acids detected in wheat and potato, respectively. These experiments were designed to test the relationships between the major amino acids and photosynthesis, and the contents of several of these amino acids were found to be related to the rate of photosynthetic processes (data not shown). The analyses, however, measured all the common protein amino acids except Cys and Pro. Figure 1 shows that total soluble minor amino acids in wheat and potato varied 20-fold, while the variation in total amino acids was approximately 6-fold. In both plants, total minor amino acid contents were independent of photosynthetic rates (Fig. 1). In wheat, summed minor amino acids correlated poorly with total amino acid contents, though a stronger correlation was observed in potato (Fig. 1). Plotting each individual minor amino acid against total amino acids likewise gave poor or no correlations (data not shown). If, however, the eight major amino acids were subtracted, and individual minor amino acids were plotted against the summed minor amino acids, clear correlations were obtained in both wheat and potato, with most of the points for many of the minor amino acids falling close to a straight line passing close to the origin (Fig. 2). Thus, most minor amino acids varied in proportion to each other, but correlated relatively poorly with total amino acids. The correlation between minor amino acids was particularly evident for branched chain and aromatic amino acids (left-hand panels for both plants), though clear correlations were also observed for Trp, Lys, Arg (in wheat), and His (in potato). Met was the only amino acid for which no correlation was observed in either plant.

What is the source of the variation in minor amino acids?

The synthesis of many of the minor amino acids has been shown to be subject to feedback end-product control (Azevedo et al., 1997; Morot-Gaudry et al., 2001). One view of the significance of such control is that it allows synthetic rates to be matched to requirements, so that concentrations should be maintained within a relatively narrow range, at least within those compartments where synthesis takes place. The extent of the variation observed may, therefore, be considered surprising. Control experiments showed that, at most, 10% of this variation could be explained by systematic errors of the method.

Assuming a total soluble protein concentration of 20 mg mg$^{-1}$ chlorophyll and a mean amino acid formula weight of 120 Da, the total amino acid content of soluble

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**Fig. 1.** Relationship between summed minor amino acids (His, Arg, Tyr, Trp, Met, Val, Phe, Ile, Leu, Lys) and (A) net photosynthesis and (B) total protein amino acids (minor amino acids + Glu, Gln, Asp, Asn, Gly, Ser, Ala, Thr) in leaves of wheat (*Triticum aestivum* var. Cannon) and potato (*Solanum tuberosum* var. Désirée). Plants were grown in soil containing slow-release complete fertilizer under supplemented natural light (wheat) or artificial light (potato) in controlled temperature rooms, and watered daily. Material was sampled in five experiments with each plant, all carried out on different days. The graphs show values for 99 (wheat) and 77 (potato) independent extracts, each from a different leaf. For wheat, samples were taken from the middle sections of the 3rd and 4th leaves of 6- to 8-week-old plants. For potato, samples consisted of half of the leaf distal from the petiole, taken from comparable leaves on plants 8 weeks after tuber planting. In all cases, samples were obtained by freeze-clamping of leaves during steady-state photosynthesis at defined irradiance, CO$_2$ and O$_2$ concentrations. Samples were stored at $-70$ °C until extraction of amino acids. Leaf samples were pulverized in a mortar to a fine powder in liquid N$_2$. The powder was then ground in 1 ml absolute ethanol to the mortar to extract residual material. The combined extract was immediately vortexed and 1 ml added to 1 ml water containing $a$-aminobutyrate as an internal standard (this amino acid was not detectable in leaf extracts of any of the plants). Extracts were vigorously and rapidly mixed then incubated 6 min at 70 °C followed by 60 min at 4 °C. Extracts were clarified by centrifugation and aliquots of the supernatant were dried under vacuum, redissolved in HPLC-grade water, filtered, and amino acids were quantified on a Waters Alliance® HPLC system by fluorimetric detection of o-phthalaldehyde derivatives, using a method adapted from that described previously (Noctor and Foyer, 1998).
leaf protein would equal 167 μmol mg⁻¹ chlorophyll, which is about 10-fold higher than the highest value for total soluble amino acids observed in wheat (Fig. 1). Because the pools of soluble minor amino acids are relatively small, leaf contents of these amino acids might be expected to be disproportionately affected by protein degradation. Experiments were therefore carried out to examine the possibility that variable rates of proteolysis during the extraction procedure could have contributed to the correlations observed. Four sets of four potato samples were extracted with or without a heating step, in the presence or absence of bovine serum albumin (BSA) added to 20-fold excess over endogenous leaf protein. No significant differences in any of the minor amino acid contents were observed between these treatments. Summed minor amino acid contents (μmol mg⁻¹ chlorophyll, n = 4) were 1.07 ± 0.09 (no BSA, no heating step), 1.13 ± 0.14 (+ BSA, no heating step), 1.26 ± 0.10 (no BSA, + heating step), and 1.15 ± 0.13 (+ BSA, + heating step). These results suggest that proteolytic activity during the extraction did not make an appreciable contribution to the measured contents of minor amino acids.

Chlorophyll was used as a basis for expression of leaf contents because the rapid-quench method used to stop metabolism precluded accurate weighing. The variation in chlorophyll content per unit fresh weight was at most 2-fold in the leaves used. Although a small part of the variation might be explained by differences in chlorophyll contents between leaves, the poor correlation between minor amino acids and total amino acids discounts such an effect as a predominant influence on the correlations shown in Fig. 2. Furthermore, leaf fresh weight can vary considerably, particularly throughout the day–night cycle and as a function of water availability to the plant. The use of chlorophyll as a basis for expression avoids detection of changes in leaf amino acid contents that could be ascribed principally to altered leaf water status.

There was no clear effect of the different short-term experimental conditions (high versus low light, CO₂ or O₂) on leaf contents of the minor amino acids. It was also possible to discount significant concentration gradients within leaves; for potato, the sampled material was always taken from the same part of the leaf (see legend to Fig. 1). For wheat, samples were

![Fig. 2. Correlation between contents of minor amino acids in wheat and potato leaves. Data are from the same experiments as Fig. 1. Each individual minor amino acid is plotted against the sum of minor amino acids. Peaks were quantified by reference to a quadratic standard curve generated using mixed standards containing amino acids in proportions as close as possible to those in leaf extracts. The amounts of amino acids in the leaf extracts were, in all cases, within the range of the standards, which varied 120-fold in concentration. Variation due to the method (including that due to chlorophyll estimation) was estimated by repeated independent extraction, drying, derivatization, and injection of homogenous pulverized leaf powder. This procedure gave a coefficient of variance (relative standard deviation, n = 4) for each amino acid of 3–28% (wheat) and 13–31% (potato). Control experiments, in which ethanolic extracts of leaf material were diluted with water containing known amounts of amino acids, gave recovery quotients of between 90% and 105% for all amino acids except His (72%) and Met (73%).](https://academic.oup.com/jxb/article-abstract/53/370/939/537246)
taken from the middle part of the leaf, and the intra-leaf concentration gradients of amino acids in the wheat leaves analysed were insignificant (data not shown). Some of the variation can probably be attributed to diurnal effects. Each of the ten experiments in which the data were generated was carried out within a window of 4–6 h in the middle of the 14 h photoperiod, and other experiments showed that the contents of some minor amino acids could vary several-fold over this time (Foyer and Noctor, 2002). However, leaf-to-leaf variation in extracts from leaves sampled at the same time of day was often as great or greater. It should be noted that the relative standard deviation in the mean contents of each set of 4–6 extractions was similar to those reported for leaf minor amino acids analysed using other methods (Maimann et al., 2000). It is concluded that the observed variation reflects co-ordinated variability in planta, chiefly as a result of leaf-to-leaf variation.

What causes the co-ordination of leaf contents?

Regardless of the source of the variation in amino acid contents, the remarkable feature of the data is that the contents of most amino acids vary in concert. Investigations of amino acid metabolism have generally concentrated on factors responsible for regulating the synthesis of amino acids within specific biosynthetic groups (Shaual and Galili, 1993; Singh and Shaner, 1995). Metabolite profiling has recently allowed the report of correlations between the contents of such amino acids (Ile and Leu, Lys and Met) in potato tubers (Roessner et al., 2001). As far as is known, the present data are the first to show a more or less general correlation across biosynthetic families although, interestingly, no correlation between Met and Lys contents was observed.

The absence of a correlation with photosynthesis (Fig. 1) clearly suggests that the variation in minor amino acids could not be traced to short-term changes in primary carbon and nitrogen assimilation. This is also borne out by the maintenance of the correlation in a barley mutant with a severe lesion in nitrogen recycling (Fig. 3). In these experiments the mutant, which has very low glycine decarboxylase activity (Blackwell et al., 1990), showed an accumulation of Gly up to 75% of total amino acids within 30 min illumination, accompanied by severe depletion of Glu, Gln, and Ser, as well as inhibition of photosynthesis (data not shown). Despite this, the correlation between the minor amino acids was similar to the wild-type (Fig. 3) and, in both cases, independent of changes in photosynthesis or contents of the major amino acids (data not shown).

Inter-pathway co-ordination of biosynthetic rates?

Leaf amino acid contents are likely to be determined by the interplay of several processes (Fig. 4). Most attention has focused on rates of amino acid synthesis. General control of minor amino acid synthesis in plants is controversial, and relatively few studies support cross-talk between the different pathways. Specific inhibition of isopropylmalate dehydrogenase, involved in Leu synthesis, was reported to cause accumulation not only of the other branched-chain amino acids but also of Tyr and Phe (Wittenbach et al., 1994). Indeed, the closest inter-family correlation observed is between the shikimate pathway amino acids and the branched-chains (Fig. 2). Inhibition of His synthesis was shown to elicit enhanced transcripts for enzymes involved in the formation of amino acids synthesized via other pathways (Guyer et al., 1995). On the other hand, it has been argued that general control would be physiologically inappropriate as many of the protein amino acids are required for purposes other than protein synthesis (Zhao et al., 1998). This point might be especially relevant in the case of the amino acids synthesized through the shikimate pathway, particularly in tissues laying down cell wall and strengthening tissue or where defensive components are required.
co-ordinated synthesis of amino acids may be effected through factors such as the [Gln]/[2-oxoglutarate] ratio, as in bacteria and fungi (Ferrario-Méry et al., 2000). Therefore, although metabolic control may act effectively to insulate amino acid synthesis from the major processes of primary metabolism in the short-term, longer-term changes in the status of photosynthetic products could be transmitted far and wide through changes in gene expression.

The potential influence of different rates of protein degradation

As noted above, a second process that could affect amino acid contents is protein degradation. To obtain the data presented here, all material was taken from photosynthetically active source leaves. Nevertheless, accumulation of ornithine and to a lesser extent, γ-aminobutyrate, was observed in one barley and one wheat extract with relatively high minor amino acid contents. Although these amino acids were negligible in all other extracts, including those which had equally high contents of minor amino acids, it remains possible that differences in rates of remobilization of protein may contribute to the co-ordination in minor amino acid contents. In maize roots, senescence induced by carbon starvation involves degradation of protein, accompanied by a marked accumulation of remobilized nitrogen in the form of free Asn (Brouquisse et al., 1992). In the present study, Asn contents were at least as variable as any of the minor amino acids, but no correlation was observed between them (data not shown).

Compartmentalization and transport of amino acids

If only part of the co-ordinated variation can be attributed to differences in protein remobilization, and feedback inhibition dictates that synthetic rates should not vary hugely in the compartment where synthesis occurs, then it must be concluded (1) that amino acids can accumulate significantly in compartments other than their site of synthesis and (2) that leaf contents may be a poor indicator of biosynthetic capacity. Comparatively little is known about the phloem content of minor amino acids, though the concentration of Val in the phloem sap was shown to be higher than the estimated overall leaf cytosolic concentration (Riens et al., 1991). Inter-leaf differences in vascular tissue contents could contribute to the co-ordinated variation observed here.

Many of the minor amino acids are synthesized principally or exclusively in the chloroplast, and their transport to and accumulation in the cytosol could allow synthesis to continue unabated. Another possibility is that a considerable portion of free amino acids are sequestered in the vacuole, and stored remote from their site of formation and utilization. Heldt and co-workers

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**Fig. 4.** Scheme showing factors affecting leaf amino acid contents.

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potato leaves, however, these amino acids showed a strong correlation with overall minor amino acid contents (Fig. 2).

Met plays a key role in C₁ metabolism (Azevedo et al., 1997), which could explain why the contents of this amino acid varied independently of other minor amino acids (Fig. 2). Since this method did not measure Cys contents, the possibility cannot be excluded that the lack of co-ordination of Met contents could also reflect distinct controls over the sulphur-containing amino acids. There is, however, evidence for co-ordination of the pathways of nitrogen and sulphur assimilation (Smith, 1980; Koprivova et al., 2000).

Classical strategies to identify co-ordinating cross-talk between synthetic pathways in plants have generally employed the tactic of starving plant tissues of one amino acid, or of specifically inhibiting one biosynthetic sequence. It is not clear whether these approaches are necessarily the most appropriate to uncover cross-regulation of different biosynthetic pathways. Treatment of *Lemna minor* with an amino transferase inhibitor was shown to lead to striking accumulation of several amino acids, including Leu, Val, Ile, Lys, Tyr, and Phe (Brunk and Rhodes, 1988). The accumulation was insensitive to chlorsulfuron, an inhibitor of the synthesis of branched-chain amino acids: this observation and other data provided convincing evidence that the observed accumulation of many amino acids was due primarily to enhanced protein degradation (Brunk and Rhodes, 1988). An alternative approach, in which tobacco leaves were fed sucrose, was also shown to cause the accumulation of several minor amino acids, an effect attributed to up-regulated biosynthesis (Morcuende et al., 1998). At least one gene involved in the synthesis of minor amino acids has been shown to respond to sugar as a ‘feast’ gene (Jackson et al., 1993). In transformed poplars with high rates of glutathione synthesis in the chloroplast, the major intracellular site of leaf amino acid synthesis, increases in the leaf contents of several of the minor amino acids were observed (Noctor et al., 1998). It has been proposed that
have examined the subcellular distribution of the most abundant amino acids in several species. Riens et al. reported that about 20% of these amino acids was found in the spinach leaf vacuole with almost 50% located in the chloroplast (Riens et al., 1991). In tobacco, only 9% of major amino acids was found to be localized in the chloroplast with 38% in the vacuole and the major part in the cytosol (Heineke et al., 1994). From the study of Leidreiter et al. in potato leaves, an approximate percentage distribution can be calculated of 50% (chloroplast), 30% (cytosol), 20% (vacuole) (Leidreiter et al., 1995). It is therefore clear (1) that the vacuole contains significant amounts of amino acids and (2) that the vacuolar fraction of major amino acids can vary. Little or nothing is known about the relative subcellular concentrations of minor amino acids, but it could be that the vacuole acts as a storage buffer for all amino acids. Storage of amino acids within the leaf could be influenced by source-sink regulation so that when sink demand is lower, amino acids are preferentially retained within source leaves. Although no clear relationship was observed between minor amino acid contents and those of the major transported amino acids (Riens et al., 1991), most amino acids are probably exported from source leaves to some extent. When invertase was overexpressed the resulting accumulation of leaf carbohydrate was accompanied by marked build-up of leaf amino acids (Büssis et al., 1997), suggesting that amino acid export is closely coupled to sucrose translocation.

Conclusion

The contents of many leaf minor amino acids are co-ordinated in wheat and potato leaves. Several processes may contribute to this phenomenon, including co-ordinated rates of synthesis between different metabolic pathways. While synthetic rates can be restrained by feedback inhibition, elasticity can probably be introduced by changes in enzyme amounts as a result of modified gene expression triggered by key metabolites or the interplay between them. Whether there are transcription factors that produce co-ordinated synthetic rates of the different amino acids remains to be seen. It is becoming clear that, like metabolic pathways themselves, cellular integration networks are likely to be composed of a complex web of factors that introduce checks and balances through shared intersection components, such as that recently shown to be common to sugar-signalling and responses to abscisic acid (Huijser et al., 2000). A recent study reported that transcript abundance varied in a circadian fashion for several genes involved in nitrogen metabolism (Harmer et al., 2000). Differences between leaves in the rates of protein turnover and capacity for amino acid storage could also be key to the co-ordinated variation observed. If the regulation of synthesis, remobilization, and storage is sufficiently elastic that each process has the capacity to vary independently 3-fold, then a large enough sample size should detect a 27-fold variation in leaf contents, which would be greater than that observed here. Co-ordination will nevertheless require that each amino acid is synthesized, metabolized and stored at similar rates. If a significant part of the variation observed is due to differences in processes such as vacuolar storage of amino acids, then this would complicate analysis of synthetic pathways through metabolite profiling. While the potential power of metabolite profiling is due to the unbiased analysis of many compounds, the present data emphasize the need for large sample sizes if significant differences between genotypes are to be identified.

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References


