9 Ornithine: At the Crossroads of Multiple Paths to Amino Acids and Polyamines

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9.1 Abstract

After the 20 amino acids that make up the proteins in all living organisms, ornithine perhaps occupies the most critical position among the non-protein amino acids. It sits at the crossroads of interconversions of glutamate and arginine on the one hand and the production of proline, polyamines and several alkaloids on the other: all products of tremendous importance to living cells. While ornithine is typically not an abundant amino acid, the metabolic flux of nitrogen through this amino acid is presumably quite rapid because of the cellular contents of the products for which it serves as the substrate. Our current knowledge of the regulation of ornithine biosynthesis is rather limited and mostly dependent upon the research targeted at understanding arginine and proline metabolism, and to some extent polyamine and alkaloid biosynthesis. Whereas most of the ornithine biosynthesis in animals occurs on the way to the production of glutamate, proline and putrescine from dietary arginine, in plants the pathway works in the reverse order, i.e. glutamate, the first product of nitrogen assimilation is the primary source of arginine, proline and putrescine. An understanding of the regulation of ornithine metabolism could help us in the genetic manipulation of plants for stress tolerance (via manipulation of proline and γ-aminobutyric acid, and the polyamine pathway), as well as in nutritional improvement (the cellular contents of important amino acids – arginine and citrulline among others). Based upon the recent observations of a strong connection between polyamine biosynthesis, ornithine metabolism and glutamic acid metabolism, it can be proposed that ornithine may act as a controlling metabolite to enhance nitrogen assimilation and, consequently, increased carbon assimilation.

9.2 Introduction

In addition to the 20 amino acids employed by nature for protein synthesis, numerous other amino acids are produced in plants, either as intermediates of metabolic pathways or for specific physiological roles in the life of a plant (Wagner and Musso, 1983; Bell, 2003). Ornithine (Orn) is such an amino acid that is not involved in protein synthesis, is often present in relatively small quantities in plants and animals compared with its related amino acids (e.g. glutamate, Glu; arginine, Arg; and proline, Pro), but plays a critical role in overall amino acid metabolism. While its importance as a key intermediate in the biosynthesis of Arg, Pro, polyamines (PAs), Glu (particularly in animals) and several alkaloids is well established in plants, no direct physiological

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functions have been assigned to Orn (Bell, 2003; Slocum, 2005). In animals, Orn is synthesized predominantly from Arg obtained through dietary sources (Morris, 2006, 2007), whereas in plants it is mainly synthesized from Glu, the entry point of N assimilation, via a series of intermediates (Fig. 9.1). Although the enzymology of Orn biosynthesis from Glu (in plants) and from Arg (in animals) has been well characterized, the regulation of its biosynthesis and its steady-state

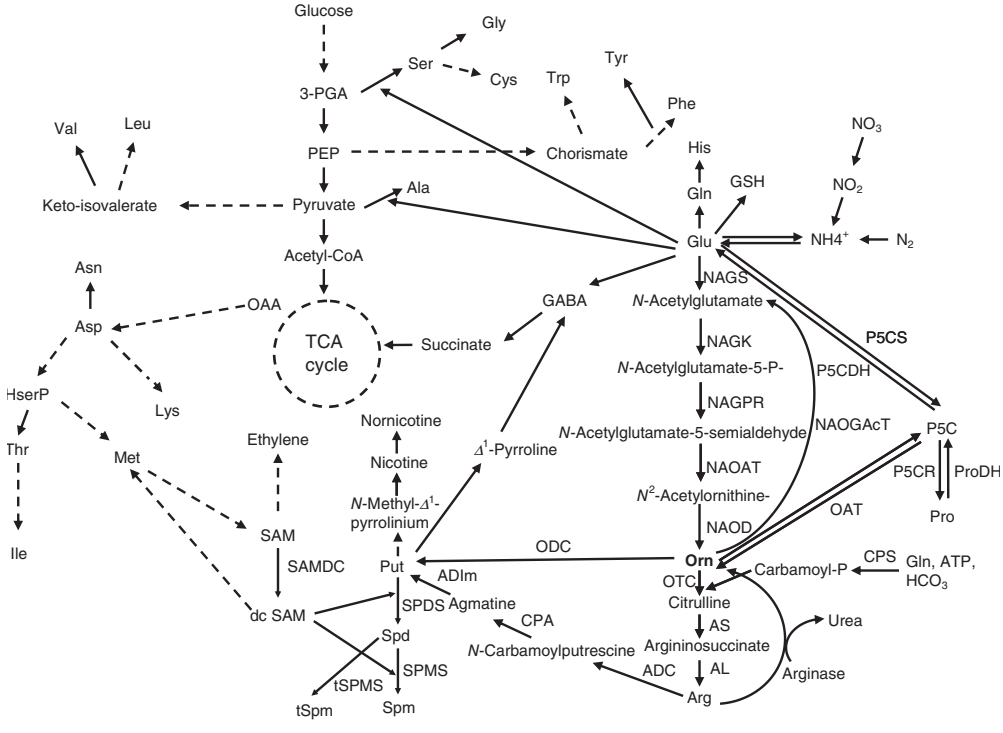

Fig. 9.1. Abbreviated pathway for the biosynthesis and metabolism of ornithine (Orn) in plants in connection to polyamines, amino acids, tricraboxylic acid (TCA) cycle metabolites and alkaloids. Abbreviations of enzymes, with EC numbers: ADC, arginine decarboxylase (EC 4.1.1.19); ADIm, agmatine deiminase (EC 3.5.3.12); AL, argininosuccinate lyase (EC 4.3.2.1); arginase (EC 3.5.3.1); AS, argininosuccinate synthase (EC 6.3.4.5); CPA, *N*-carbamoylputrescine amidohydrolase (EC 3.5.1.53); CPS, carbamoyl phosphate synthetase (EC 6.3.5.5); NAGK, *N*-acetylglutamate kinase (EC 2.7.2.8); NAGPR, *N*-acetylglutamate-5-phosphate reductase (EC 1.2.1.38); NAGS, *N*-acetylglutamate synthase (EC 2.3.1.1); NAOAT, N^2-acetylornithine aminotransferase (EC 2.6.1.11); NAOD, N^2-acetylornithine deacetylase (EC 3.5.1.16); NAOGAcT, acetyl-Orn-Glu acetyltransferase (EC 2.3.1.35); OAT, ornithine δ-aminotransferase (EC 2.6.1.13); ODC, ornithine decarboxylase (EC 4.1.1.17); OTC, ornithine transcarbamoylase (EC 2.1.3.3); P5CDH, Δ^1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12); P5CR, Δ^1-pyrroline-5-carboxylate reductase (EC 1.5.1.2); P5CS, Δ^1-pyrroline-5-carboxylate synthetase (EC 2.7.2.11/1.2.1.41); ProDH, proline dehydrogenase (EC 1.5.99.8); SAMDC, *S*-adenosylmethionine decarboxylase (EC 4.1.1.50); SPDS, spermidine synthase (EC 2.5.1.16); SPMS, spermine synthase (EC 2.5.1.22); tSPMS, thermospermine synthase (EC 2.5.1.79).
Other abbreviations: Ala, alanine; Asn, asparagine; Asp. Aspartate; Cys, cysteine; dcSAM, decarboxylated *S*-adenosylmethionine; GABA, γ-aminobutryric acid; Gln, glutamine; Glu, glutamate; Gly, glycine; GSH, glutathione; His, histidine; HserP. homoserine phosphate; Ile, isoleucine; Leu. Leucine; Lys, lysine; Met, methionine; OAA, oxaloacetic acid; P5C, Δ^1-pyrroline-5-carboxylate; PEP, phosphoenolpyruvate; 3-PGA, 3-phosphoglyceric acid; Phe, phenylalanine; Pro, proline; Put, putrescine; SAM, *S*-adenosylmethionine; Ser, serine; Spd, spermidine; Spm, spermine; The, threonine; Trp, Tryptophan; tSPM, thermospermine; Tyr, tyrosine; Val, valine. (Modified from Mohapatra *et al*., 2010b.)

cellular content have not been well understood. For animals, several reviews have indicated that cellular Orn levels respond to various physiological and clinical conditions of the organism; however, in plants such studies have been rather limited. Several recent studies (Roosens *et al*., 2002; Mohapatra *et al*., 2010b; DeBoer *et al*., 2011, 2013; Majumdar *et al*., 2013; Gholami *et al*., 2014) have implicated Orn as a regulatory molecule in plants for the biosynthesis of several amino acids and PAs, including its own biosynthesis and accumulation. Even though under normal conditions Orn concentrations in plants are often quite low, it can be argued that cells are capable of synthesizing it in large quantities in order to meet the demand for the biosynthesis of its products (i.e. Glu, Arg, Pro, PAs and alkaloids), which frequently are present in several-fold higher concentrations than Orn itself. This chapter summarizes different aspects of Orn metabolism in plants, including its biosynthesis and utilization, cellular contents, genetic manipulation of its biosynthesis through mutants and genetic engineering, and some of its regulatory roles. Limited comparisons of Orn metabolism in animal or microbial systems are made where appropriate. Finally, a model is proposed for the regulation of the metabolism of Orn and the associated flux through the Glu-Orn- Arg/Pro/PA pathways.

9.3 Ornithine Biosynthesis and Utilization

The biosynthesis of Arg has been well studied in bacteria and fungi (Cunin *et al*., 1986; Davis, 1986) with only a limited focus on Orn (Slocum, 2005). In plants (and bacteria), Orn is synthesized mostly from Glu (Fig. 9.1); the first enzyme believed to regulate the entire pathway is *N*-acetyl-Glu synthase (NAGS; EC 2.3.1.1). *N*-acetyl-Glu kinase (NAGK; EC 2.7.2.8) then converts acetyl-Glu into *N*-acetyl-Glu-5-phosphate which, via the intermediates *N*-acetyl-Glu-5-semialdehyde and N^2-acetyl-Orn, is converted into Orn. The enzymes involved in these steps are *N*-acetyl-Glu-5-P reductase (NAGPR; EC 1.2.1.38), N^2-acetylOrn aminotransferase (NAOAT; EC 2.6.1.11) and N^2-Acetyl-Orn deacetylase (NAOD; EC 3.5.1.16), respectively (reviewed by Slocum, 2005; Kalamaki *et al*., 2009a,b) (Fig. 9.1). Alternatively, N^2-acetyl-Orn is converted to Orn by acetyl-Orn-Glu acetyltransferase (NAOGAcT; EC 2.3.1.35), which conserves the acetyl group to generate *N*-acetyl-Glu from Glu. Under special circumstances (e.g. protein hydrolysis during seed germination), Orn can be synthesized from Arg via arginase, the by-product being urea; this pathway is analogous to the production of Orn in animals (Morris, 2006, 2007). In support of the earlier studies showing Arg production from Glu in plants, Bhatnagar *et al*. (2002) and Majumdar *et al*. (2013) have demonstrated that in cell cultures of poplar (*Populus nigra* × *maximowiczii*) and seedlings of *Arabidopsis thaliana*, respectively, Arg → Orn conversion does not occur even under condi tions of Orn depletion in the cells.

Among the different enzymatic reactions required for Orn biosynthesis from Glu, only the reaction catalysed by the enzyme NAGK was considered to be the rate-limiting and the key regulatory step for Orn production in plants (Kalamaki *et al*., 2009a,b). This enzyme was shown to be feedback inhibited by cellular Arg concentration in the context of the overall N status of cells (Burillo *et al*., 2004; Sugiyama *et al*., 2004). Presumably, this inhibition involved a group of ubiquitous proteins called P_{II} proteins (reviewed in Chellamuthu *et al*., 2013; Huergo *et al*., 2013; see also Section 9.5) through the formation of a complex with NAGK to activate it (Beez *et al*., 2009); P_{II} proteins also help to overcome the negative effect of Arg-mediated feedback inhibition of NAGK (Beez *et al*., 2009). Kalamaki *et al*. (2009a) later demonstrated that transgenic overexpression of a tomato (*Solanum lycopersicum* Mill. cv. Ailsa Craig) *SlNAGS1* gene in *A. thaliana* caused a twofold to ninefold increase in cellular Orn in the rosette leaves of pre-bolting plants. This increase in Orn was accompanied by a small increase in citrulline (Cit) and a significant decrease in Arg content in several of the transgenic lines. The transgenic plants with higher Orn content showed better germination, increased seed viability and tolerance to salt (NaCl) or drought stresses. In contrast, Brauc *et al*. (2012) have recently shown that transgenic expression of the homologous *Arginase 2* (*AtARGAH2*) gene under the control of a *Cauliflower mosaic virus* (CaMV) *35S* promoter surprisingly exhibited an approximately threefold decrease in Orn accompanied by a ~1.3-fold increase in putrescine (Put) and Pro contents in the leaves of transgenic *Arabidopsis* plants. The other amino acids that

were affected in the *ARGAH2* overexpresser lines were: alanine (Ala), tyrosine (Tyr), valine (Val), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), and lysine (Lys), which increased; and asparagine (Asn), glutamine (Gln), glycine (Gly) and histidine (His), whose concentration was lowered. The transgenic plants showed greater tolerance to the pathogen *Botrytis cinerea*. The authors suggested a possible role for urea accumulation or other stress-related metabolites in the *ARGAH2* overexpresser lines in enhancing basal resistance of the plants to disease.

Surprisingly, silencing (through T-DNA insertion) of *ARGAH2* had similar effects on PAs and amino acids as observed in the overexpresser lines, with the exception of Tyr and methionine (Met), both of which decreased in the silenced lines compared with the wild type (WT) plants. In another study, overexpression (*35S* promoter) of *AtARGAH1* and *AtARGAH2* (again in *Arabidopsis*) resulted in increased sensitivity to abiotic stresses in the transgenic plants, suggesting possible complex and differential regulation of Arg in connection with Orn responding to biotic and abiotic stresses (Shi *et al.*, 2013). As Orn is an intermediate metabolite of the PA and the amino acid biosynthetic pathways rather than a terminal product, the biosynthesis of Orn is likely to be regulated by increased demand for it for use in the production of Put, Arg or Pro, depending on environmental or intracellular conditions. Increased biosynthesis of Orn is often observed by its rapid consumption into the allied pathways rather than it being accumulated in the cells (Mohapatra *et al.*, 2010b; Majumdar *et al.*, 2013). Furthermore, as indicated by its low cellular content, it can be surmised that the Orn titre in the cells is tightly monitored as well as regulated (Mohapatra *et al.*, 2010b; Majumdar *et al.*, 2013). More details on this aspect of Orn metabolism are covered later (see Section 9.9).

Arginine is also a source of nitric oxide (NO), which plays an important role in germination, defence responses, hormonal signalling, root growth and flowering (Crawford, 2006; Grün *et al.*, 2006); its role in plants has recently been reviewed by Mur *et al.* (2013). Taken together, Arg and Orn metabolism are of central importance in plant biology, but the regulation of gene expression and the enzymes of Glu/Orn/Arg biosynthesis are poorly understood at present.

9.4 Cellular Contents

Numerous reports on the cellular contents of Orn in a variety of plant(s)/tissues have been published. The indications are that Orn contents vary widely in different species and are subject to regulation by a multiplicity of growth conditions. Only in rare instances is Orn among the abundant amino acids (Tables 9.1 and 9.2). In our studies with several species (Table 9.2), Orn contents were rarely above 1–2% of the total amino acid pool in the tissue (versus the dominant related amino acids e.g. Arg, Pro or Glu, whose contents are several fold higher than those of Orn). Cyr and Bewley (1989) (Table 9.1) reported seasonal variation of Orn content in the roots of leafy spurge (*Euphorbia esula*); during the summer, for example, Orn content was ~3 μmol g^{-1} dry weight (DW) (~8% of the soluble amino acid pool), whereas during winter a significant increase in Orn content (~18 μM g^{-1} DW) was observed, when it became the predominant amino acid (~20% of the total soluble amino acid pool). The data presented in Table 9.2 support the results of Cyr and Bewley (1989) in that significant variations in the different amino acids are common in relation to environmental conditions and/or in different tissues within the same species.

In a detached leaf assay with rice (*Oryza sativa*), Yang *et al.* (1999) reported that Orn content was higher under dark treatment (~10 nmol g^{-1} fresh weight (FW); 0.03% of total soluble amino acid pool) compared with light treatment (3 nmol g^{-1} FW; 0.01% of total soluble amino acid pool) (see Table 9.1). In 4-year-old saplings of white spruce (*Picea glauca*), cellular contents of Orn in the root, stem and buds were about 0.4% of the total soluble amino acids (Durzan, 2010). In poplar (*P. nigra* × *maximowiczii*) cell cultures, Orn was found to be ~1–4 nmol g^{-1} FW, constituting <0.1% of the total soluble amino acids (Mohapatra *et al.*, 2010b) (see Table 9.1). In cashew (*Anacardium occidentale*) leaves, Orn was about ~2 μmol g^{-1} DW, contributing to ~3% of the total soluble amino acids (da Rocha *et al.*, 2012; see Table 9.1). Likewise, seedless grapes obtained from different rootstocks showed Orn contents of 7 to 28 μg g^{-1} DW, constituting ~2–4% of the total soluble amino acids; the contents varied with the variety and the time of analysis (Jogaiah *et al.*, 2013). Cellular concentrations of Orn also varied among different tissues/organs of *Erythroxylum coca* plants;

Table 9.1. Cellular contents of glutamine (Glu), arginine (Arg) and ornithine (Orn) in different plant species and parts under different conditions.

Plant species/ conditions	Amino acid concentration (variable units, fresh weight, FW, or dry weight, DW)			Tissue	Reference
	Glu	Arg	Orn		
Anacardium occidentale	~15 (μmol g^{-1} DW)	~2 (μmol g^{-1} DW)	~2 (μmol g^{-1} DW)	Leaves	da Rocha *et al.*, 2012
Arabidopsis thaliana	~7.5 (nmol mg^{-1} FW)	~0.5 (nmol mg^{-1} FW)	~3 (nmol mg^{-1} FW)	Rosette leaves	Adio *et al.*, 2011
Arabidopsis thaliana	3.48 (μmol g^{-1} FW)	5.25 (μmol g^{-1} FW)	0.7 (μmol g^{-1} FW)	Whole seedlings	Majumdar *et al.*, 2013
Erythroxylum coca	47.35 (nmol mg^{-1} DW)	11.53 (nmol mg^{-1} DW)	0.11 (nmol mg^{-1} DW)	Leaves	Docimo *et al*., 2012
	46.32 (nmol mg^{-1} DW)	15.3 (nmol mg^{-1} DW)	0.17 (nmol mg^{-1} DW)	Buds	
	47.65 (nmol mg^{-1} DW)	107.19 (nmol mg^{-1} DW)	0.56 (nmol mg^{-1} DW)	Stems	
	15.07 (nmol mg^{-1} DW)	9.02 (nmol mg^{-1} DW)	0.34 (nmol mg^{-1} DW)	Roots	
Euphorbia esula summer season	~6 (μmol g^{-1} FW)	~3 (μmol g^{-1} FW)	~2.5 (μmol g^{-1} FW)	Roots	Cyr and Bewley, 1989
winter season	~15 (μmol g^{-1} FW)	~5 (μmol g^{-1} FW)	~17.5 (μmol g^{-1} FW)	Roots	
Oryza sativa light	5589.9 (nmol g^{-1} FW)	191.2 (nmol g^{-1} FW)	2.9 (nmol g^{-1} FW)	Leaves	Yang *et al.*, 1999
dark	6070.3 (nmol g^{-1} FW)	717.7 (nmol g^{-1} FW)	10.3 (nmol g^{-1} FW)	Leaves	
Populus nigra × *maximowiczii*	818 (nmol g^{-1} FW)	522 (nmol g^{-1} FW)	3 (nmol g^{-1} FW)	Cells	Mohapatra *et al*., 2010b

stems had the highest levels of Orn (~0.6 μmol g^{-1} DW), followed by roots (~0.3 μmol g^{-1} DW), buds (~0.2 μmol g^{-1} DW) and leaves (~0.1 μmol g^{-1} DW) (see Table 9.1). Overall, Orn constituted about 0.1–0.3% of the total soluble amino acids among different tissues (Docimo *et al*., 2012). In 12-d-old seedlings of *Arabidopsis*, Orn content was only 0.7 μmol g^{-1} FW (≤0.5% of the total soluble amino acids (Majumdar *et al*., 2013; see Table 9.1), whereas Kalamaki *et al*. (2009a,b) had reported Orn contents to be as high as ~50 μg 100 mg^{-1} FW (about ~4 μmol g^{-1}) in *Arabidopsis* leaves transformed with tomato *Sl-NAGS* versus about 0.4 μmol g^{-1} in the WT plants.

9.5 Mutants of Ornithine Biosynthesis

Whereas mutants of several genes in the Glu-Orn-Arg/Pro pathway have been studied in several plants, the studies do not cover all of the genes involved in the pathway. This is in contrast to a significant body of knowledge about inherited mutations of this pathway in animals and humans (Section 9.7). A few of the mutants that have been reported in detail are described here briefly.

A group of highly conserved proteins called P_{II} proteins are found in all living organisms and are believed to act as signalling molecules to activate NAGK in plants and cyanobacteria by forming a P_{II}-NAGK complex and thus participating in the control of Glu → Arg pathway (reviewed by Chellamuthu *et al*., 2013; Huergo *et al*., 2013). In *Arabidopsis*, P_{II}-deficient mutants P_{II}v1 and P_{II}s2 (T-DNA insertion in the P_{II} homologue *GLB1* gene) when grown in 1 mM nitrate medium (i.e. subjected to N deficiency) followed by resupply of different forms of N, showed a significant decrease (up to 70%) in Orn, Cit and Arg

Table 9.2. Cellular contents of glutamate (Glu), arginine + serine (Arg + Thr), proline (Pro), γ-aminobutyric acid (GABA) and ornithine (Orn) in the leaves of different forest tree species at different sites.

Site/plant type and species (n)[a]	Mean ± SE amino Acids (nmol g^{-1} fresh weight)[b]					Reference
	Glu	Arg + Thr	Pro	GABA	Orn	
HBEF (Hubbard Brook Experimental Forest), New Hampshire, USA						
RS-CY (20)	230 ± 19	132 ± 30	31 ± 4	352 ± 65	11 ± 4	Minocha *et al.*, unpublished
RS-PY (20)	214 ± 16	81 ± 18	37 ± 8	237 ± 36	11 ± 3	Minocha *et al.*, unpublished
SM (30)	277 ± 25	292 ± 21	68 ± 5	283 ± 22	5 ± 1	Minocha *et al.*, 2010
YB (30)	797 ± 72	536 ± 111	28 ± 3	298 ± 21	22 ± 4	Minocha *et al.*, 2010
AB (30)	243 ± 47	196 ± 46	34 ± 3	1039 ± 80	10 ± 4	Minocha *et al.*, 2010
Teakettle, California, USA						
JP (30)	768 ± 54	1095 ± 461	138 ± 13	287 ± 31	15.78 ± 5.62	Minocha *et al.*, 2013
SP (30)	771 ± 42	680 ± 246	156 ± 13	163 ± 13	9.09 ± 1.96	
WF (30)	153 ± 11	172 ± 39	24.4 ± 2.2	504 ± 26	5.25 ± 1.04	
Harvard Forest						
RP-control (3)	174 ± 11	NA	81 ± 6	109 ± 6	1 ± 1	Bauer *et al.*, 2004
RP-chronic-N (6)	773 ± 60	4072 ± 1233	174 ± 48	753 ± 95	55 ± 14	
Monagahela National Forest, West Virginia, USA						
QR (10)	718 ± 84	249 ± 44	270 ± 54	830 ± 95	NA	Minocha, R. *et al.*, unpublished
BC (10)	3883 ± 357	1014 ± 149	1323 ± 227	703 ± 85	NA	
TP (10)	2006 ± 173	304 ± 81	77 ± 7	1098 ± 152	1 ± 0	
SM (10)	1613 ± 94	469 ± 65	299 ± 74	1014 ± 100	3 ± 1	
Czech Republic						
NS-CY (15)	176 ± 13	166 ± 16	22 ± 1	284 ± 20	NA	Minocha, R. *et al.*, unpublished
NS-PY (15)	206 ± 13	32 ± 32	30 ± 3	315 ± 21	NA	
Juneau, Alaska, USA						
SS-CY (4)	85 ± 16	7 ± 4	52 ± 6	59 ± 11	7 ± 1	Minocha, R. *et al.*, unpublished
WH-CY (6)	117 ± 4	0 ± 0	10 ± 2	103 ± 11	1 ± 1	

Continued

Table 9.2. Continued.

Site/plant type and species (*n*)[a]	Mean ± SE amino Acids (nmol g^{-1} fresh weight)[b]					Reference
	Glu	Arg + Thr	Pro	GABA	Orn	
Invasive plants						
MV (10)	1001 ± 43	237 ± 22	55 ± 5	729 ± 24	11 ± 2	Minocha, R. *et al.*, unpublished
AA (10)	2242 ± 83	1566 ± 139	339 ± 25	910 ± 38	3 ± 0	
GM (10)	1732 ± 36	483 ± 34	2474 ± 233	300 ± 17	1 ± 0	
Maine, USA						
BF-CY (15)	148 ± 16	104 ± 9	17 ± 1	495 ± 41	NA	Minocha, R. *et al.*, unpublished

[a]Abbreviations used by site/plant type and in order of occurrence: *n* = number of replicates.
HEBF: RS-CY = red spruce (*Picea rubens*) – current year foliage; RS-PY = red spruce (*Picea rubens*) – 1-year-old year foliage; SM = sugar maple (*Acer saccharum*); YB = yellow birch (*Betula alleghaniensis*); AB = American beech (*Fagus grandifolia*).
Teakettle: JP = Jeffrey pine (*Pinus jeffreyi*); SP = sugar pine (*Pinus lambertiana*); WF = white fir (*Abies concolor*).
Harvard Forest: RP = red pine (*Pinus resinosa*).
Monagahela National Forest: QR = red oak (*Quercus rubra*): BC = black cherry (*Prunus serotina*); TP = tulip poplar (*Liriodendron tulipifera*); SM = sugar maple (*Acer saccharum*).
Czech Republic: NS-CY = Norway spruce (*Picea abies*), current year foliage; NS-PY = Norway spruce (*Picea abies*), 1-year-old foliage.
Juneau, Alaska: SS-CY = Sitka spruce (*Picea sitchensis*), current year foliage; WH-CY = western hemlock (*Tsuga heterophylla*), current year foliage.
Invasive plants: MV = Japanese stiltgrass (*Microstegium vimineum*); AA = tree of heaven (*Ailanthus altissima*); GM = garlic mustard (*Alliaria petiolata*).
Maine: BF-CY= balsam fir (*Abies balsamea*), current year foliage.
[b]NA, not available.

compared with the WT plants. Other major amino acids, such as Gln, Glu, aspartate (Asp) and Asn were also reduced, albeit to a lesser extent (15–50%) in response to ammonium resupply (Ferrario-Méry *et al.*, 2005, 2006). Microarray analysis of Arg metabolism genes, in contrast, did not show any differential expression in the P_{II} mutants compared to the WT under the same conditions. These results together suggest that Orn/Arg biosynthesis is significantly and specifically affected under excess N conditions, and is perhaps not subject to transcriptional regulation of the pathway genes. These results are consistent with our studies on poplar cells that showed a several-fold increase in Glu → Orn flux in response to increased demand for Orn production, but no change in the expression (analysed by microarrays and quantitative PCR) of most genes in the Glu-Orn pathway was seen (Page *et al.*, 2007, 2012).

In *Arabidopsis*, mutants of the *TUMOR PRONE5* (*TUP5*) gene (*tup5-1*) encoding the enzyme NAOAT had significantly lower (31–85%) Arg content in 11-d-old seedlings when grown under long-day (LD) conditions but not in the dark; the WT plants did not show this response (Frémont *et al.*, 2013). Lower cellular Arg in the mutant was accompanied by a significant increase (as % of WT) in several amino acids including, Gly (by ~800%), Gln (by ~450%); Asn, Val, tryptophan (Trp) and Phe by ~300%; Asp, threonine (Thr), Ala, Tyr and Ile, by ~200%; and Met and Lys by ~150%. Moreover, this overall increase of amino acids in the mutant varied with developmental stage in that only Gln and Asn increased in the 17-d-old seedlings. The authors suggested that the observed increase in Gln and Asn could be a compensation for cellular Arg (as an N sink) as these two amino acids are important in N storage and transport in plants. It was further proposed that the overall increase in amino acids in the mutant was perhaps a consequence of reduced protein synthesis, again due to Arg becoming a rate-limiting factor. Besides, the *tup5-1* mutant plants showed a blue light-dependent short-root phenotype (that could be restored by exogenous supply of Arg or its precursors) resulting from arrested cell division at the meristem region, which appeared to lack an organized structure and had no starch granules in the columella cells. The null mutants showed a decrease in gamete viability, accompanied by embryo lethality. Whereas overexpression of the *TUP5* gene in the *tup5-1* background was able to restore Arg content to the levels in the WT plants, no significant increase in Arg content was observed in the transgenic plants overexpressing this gene in the WT background. It can thus be argued that this step might not be rate limiting for Orn/Arg biosynthesis in *Arabidopsis*. The cellular Orn content in neither the mutant nor the overexpresser plants was reported.

A T-DNA insertion mutant of the N^{δ}-acetylOrn transferase gene (*nata1-1*) in *Arabidopsis* showed the absence of N^{δ}-acetylOrn in all organs but with no visual phenotype and no significant effect on PAs or amino acids in the mutant plants (versus the WT) under normal conditions of growth (Adio *et al.*, 2011). Methyl jasmonate (MeJa), a phytohormone that has been shown to induce the expression of N^{δ}-acetylOrn transferase in plants (Zimmermann *et al.*, 2004; Yan *et al.*, 2007), significantly increased N^{δ}-acetylOrn and caused a decrease in Put and spermidine (Spd) contents in the WT plants. There was also a concomitant increase in Put and agmatine (Agm) and a decrease in Spd contents with no N^{δ}-acetylOrn detected in the *nata1-1* mutant compared with its corresponding control. The amino acids that increased significantly upon MeJa treatment in the *nata1-1* mutant plants were Asp, Glu, Gly, His, Ala, N^{δ}-acetylOrn, Pro, Ile and Leu. In contrast, constitutive (*35S* promoter) expression of *NATA1* in the *nata1-1* background significantly decreased Pro content (by fivefold). There was a concomitant decrease in Orn and a significant increase in Thr, Val, Ile and Phe in the overexpressers, but without a significant change in total amino acid contents. Different biotic stresses, e.g. feeding on plants by *Myzus persicae* (the green peach aphid) or infection with *Pseudomonas syringae* increased the expression of *NATA1* accompanied by accumulation of N^{δ}-acetylOrn in the infected plants. Transient expression of *NATA1* or incorporation N^{δ}-acetylOrn in the aphid diet had a negative impact on insect reproduction, suggesting a role for N^{δ}-acetylOrn in tolerance to biotic stress.

The conversion of L-Arg to L-Orn is catalysed by the enzyme arginase (L-Arg amidinohydrolase; EC 3.5.3.1), which produces urea as a by-product; the urea is then rapidly recycled by urease to produce ammonia (Polacco and Holland, 1993).

During seed germination, Arg (a storage form of N) is catabolized by arginase to provide nourishment to the developing embryo. In *Arabidopsis*, mutations of either of the two arginase genes (*argah1-1* and *argah2-1*) show an increase in the formation of lateral and adventitious roots in the seedlings, accompanied by increased accumulation of NO (Flores *et al*., 2008). The mutants exhibit enhanced accumulation of NO upon exposure to the synthetic auxin naphthaleneacetic acid (NAA) as well. Also, increased activity of β-glucuronidase (GUS) was observed in the root tips, lateral roots and hypocotyl under the control of an auxin-responsive promoter *DR5* when expressed in the *argah* background. NO in plants has been shown to be positively correlated with root formation (Hu *et al*., 2005; Lombardo *et al*., 2006) and is inducible by auxin. These observations, together with Arg-induced NO accumulation and increase in adventitious root formation in the mutants (versus WT plants), suggest that Arg is a potential source of NO regulating root development in concert with auxin (Flores *et al*., 2008).

Besides *ARGAH*, dysfunction of an *OTC* (*Orn transcarbamoylase*) gene in *Arabidopsis* (a T-DNA insertion mutation) showed increased sensitivity (reflected in reduced germination and FW) of the plants to high Orn concentration in the medium, in contrast to Arg or Cit (Quesada *et al*., 1999); once again, no information was available on the cellular concentration of Orn or other related compounds in this mutant line. Two venosa (*Ven*) mutants, *ven3* and *ven6*, that carry mutations in the large and small subunits of the enzyme carbamoyl phosphate synthetase (CPS; EC6.3.5.5; involved in Cit biosynthesis), respectively, show leaf reticulation and a decrease in mesophyll cell numbers in the leaves (Mollá-Morales *et al*., 2011). The metabolites that were significantly affected in the mutant plants were increase in *N*-acetylOrn (35-fold), L-Orn (elevenfold), and decrease (<50% of WT) in Cit/Arg contents; this was accompanied by increased sensitivity to exogenous Orn compared with the WT plants. Both the defects in the mesophyll cells and the reticulated phenotype were rescued by exogenous application of Cit, yet this amino acid was unable to restore smaller leaf size in the *ven3*/*ven3 ven6*/*ven6* double mutant plants. These results together support a regulatory role for Cit in determining leaf morphology in plants. Changes in other metabolites observed in the *ven* mutants were associated with amino acid biosynthesis and the TCA (tricarboxylic acid) cycle (Mollá-Morales *et al*., 2011).

9.6 Genetic Manipulation of Ornithine Metabolism and its Impact on Amino Acids and Other Related Compounds

The steady state cellular content of a metabolite is the sum total of its biosynthesis and utilization in different pathways. Of course, its transport out of the cells as well as its conjugation with or incorporation into macromolecules (e.g. for the 20 amino acids involved in protein biosynthesis) would also significantly affect its cellular content at any time. Orn is neither significantly transported from one organ to the other nor is it incorporated into proteins or any other macromolecule. For Orn, while its biosynthesis in plants occurs largely from Glu, its utilization involves multiple pathways, the three major ones being the biosynthesis of Arg (for use in protein synthesis, with some for PA biosynthesis via arginine decarboxylase, ADC), Pro (also for protein biosynthesis and for storage as an osmolyte) and the PAs via Orn decarboxylase (ODC) for their numerous physiological functions. Under conditions when Orn is produced from Arg instead of Glu, the biosynthesis of Glu and Pro are important consumers of Orn. Thus, the cellular content of Orn is subject to manipulation both through its increased biosynthesis (upregulation) and/or its increased utilization (depletion). As described above, upregulation of Orn biosynthesis via overexpression of *NAGS* gene has apparently been attempted with some success (Kalamaki *et al*., 2009a,b); however, other genes of the pathway have not been successful in upregulating Orn biosynthesis. Enhancement of Orn biosynthesis via increased utilization into the PA or the Arg biosynthetic pathways have been quite effective in modulating its rate of biosynthesis, but without increased accumulation. Several laboratories, including ours, have used this approach, with the primary target of modulation of PAs in plants via the overexpression of both the *ADC* and *ODC* genes.

The alteration of Orn metabolism through the genetic manipulation of Orn aminotransferase

(OAT; EC 2.6.1.13) has been reported in plants as Orn serves as a major precursor for Pro biosynthesis as well (see Verslues and Sharma, 2010, and references therein). Overexpression of an *Arabidopsis* OAT in *Nicotiana plumbaginifolia* increased Pro content by threefold in the transgenic plants (Roosens *et al.*, 2002). The transgenic plants showed overall increased tolerance to osmotic and NaCl stresses with higher seed germination and increased biomass production under the influence of these stressors compared with the WT plants. The authors did not report the overall effect of OAT manipulation on the contents of Orn, PAs or other amino acids in the transgenic plants. It should be pointed out though that among the different fates of Orn into Put, Arg and Pro, the reaction carried out by ODC to make Put from Orn is the only known rate-limiting step in which the substrate Orn becomes limiting and not the enzyme ODC (Pegg and McCann, 1982; Hickok *et al.*, 1986; Majumdar *et al.*, 2013).

In another study, overexpression of an *O. sativa* OAT cDNA (*OsOAT*) under a constitutive maize ubiquitin promoter in Japonica rice showed significant increase in Pro content in both normal and drought conditions (You *et al.*, 2012). The other amino acids that increased significantly in the transgenic plants were Glu, serine (Ser), Lys and Met, concurrently with a significant decrease in Arg content. Transgenic plants with higher Pro and glutathione (GSH) contents showed significant tolerance to drought, osmotic, and oxidative stresses, mainly through enhanced reactive oxygen scavenging capacity.

Transgenic manipulation of Orn metabolism through overexpression of a mouse *ODC* (m*ODC*) in poplar (*P. nigra* × *maximowiczii*) cell cultures under a constitutive (CaMV 2x*35S*) promoter resulted in the alteration of a majority of the proteinaceous amino acids in the transgenic cells, especially those that are synthesized from Glu (Mohapatra *et al.*, 2010b). The transgenic cells showed threefold to sevenfold increase in Put content, with only small increases in Spd and spermine (Spm). Increased biosynthesis of Put was accompanied by a parallel increase in its catabolism, thus leading to increased accumulation of γ-aminobutyric acid (GABA; see Chapter 7); this must have created a significant increase in the metabolic flux of Orn consumption as well as its production. While the Orn content of cells was quite low to begin with (less than 0.1% of the total soluble amino acid pool), and it became even lower in the transgenic cells, its increased utilization as a substrate by the transgenic mODC had little effect on its utilization for the biosynthesis and accumulation of Arg. The increased flux of Orn into Put via ODC, while still not meeting the demand of transgenic mODC, resulted in elevated cellular Arg (albeit small). The observed change in cellular Arg accumulation was apparently the direct result of its increased biosynthesis from Orn, and not due to its reduced utilization in either protein biosynthesis or Put production via the ADC pathway. If anything, both these activities were higher in the transgenic cells, where a deficit of Orn was created by its overconsumption by mODC. Besides, there was no back conversion of Arg into Orn, which is biochemically possible (see above). Significant changes in the contents of Pro (also a part of the Glu-Orn-Arg-Pro-Put pathways) were also observed concurrently. Accompanying this increased flux of Orn consumption (and production), there was a significant increase in total soluble protein, as well as in total cellular N and C contents, in the transgenic cells overproducing Put via the ODC pathway (Bhatnagar *et al.*, 2001, 2002; Mohapatra *et al.*, 2010a). The increase in GABA in this case could be attributed to its overproduction via enhanced Put catabolism (Bhatnagar *et al.*, 2002; Shelp *et al.*, 2012; Shao *et al.*, 2014).

The above observations in cell cultures of poplar have been replicated in whole plants of *A. thaliana* using both a constitutive and an inducible (oestradiol mediated) system to regulate m*ODC* expression. The results again showed significant changes in the major amino acids of the Glu-Arg-Pro pathway in the transgenic plants under both situations (Fig. 9.2), indicating a rapid metabolic flux adjustment in Orn production in response to its rapid utilization (Majumdar 2011; Majumdar *et al.*, 2013). Here, it should be pointed out that *Arabidopsis* is an exception in the plant kingdom in that it does not have a native *ODC* gene and Put is exclusively produced via ADC from Arg (Hanfrey *et al.*, 2001), which itself is entirely produced from Orn (Slocum, 2005). Once again, neither the production of Put from Arg nor the protein content in young seedlings was affected in spite of a 20- to 40-fold increase in Orn biosynthesis for increased Put production via the transgenic mODC step.

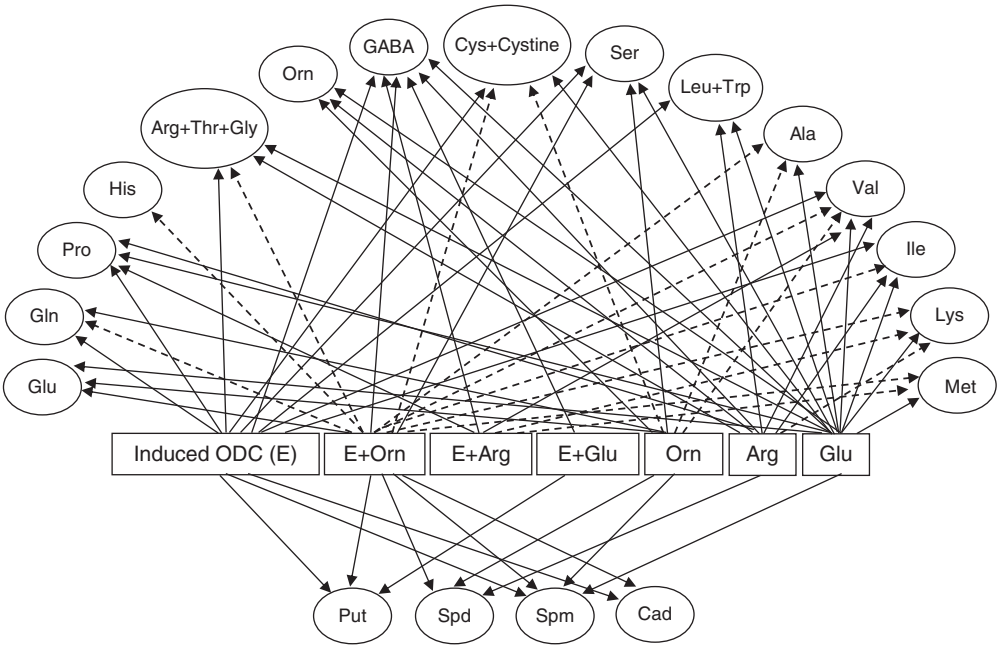

Fig. 9.2. Effects of exogenous Orn, Arg and Glu in uninduced and induced mouse ornithine decarboxylase (m*ODC*) transgenic *Arabidopsis thaliana* seedlings on amino acids and polyamines. Solid lines indicate increase and dotted lines indicate decrease (data are based on nmol g^{-1} FW basis; $P \leq 0.05$). Key: Ala, alanine; Arg, arginine; Cad, cadaverine; Cys, cysteine; E, induced ODC; GABA, γ-aminobutryric acid; Gln, glutamine; Glu, glutamate; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Pro, proline; Put, putrescine; Ser, serine; Spd, spermidine; Spm, spermine; The, threonine; Trp, Tryptophan; Val. Valine. (Modified from Majumdar *et al*., 2013.)

As with poplar cells, total N and C in the transgenic plants were also higher concomitant with the production of high Put.

The experiments with inducible mODC expression revealed how rapidly the metabolic flux from Glu to Orn (and then Put) could be induced. On short-term (24 h) induction of m*ODC* in 12-d-old *Arabidopsis* seedlings, significant increases in Gln, Pro, Arg, GABA, cysteine (Cys), Ser, Leu + Trp and Val were observed, whereas constitutive ODC activity in the same aged seedlings resulted in increase of GABA, Met, Val and Thr (Majumdar *et al*., 2013). These differences in the response of inducible versus constitutive expression of the transgene are perhaps due to the long-term homeostatic adjustment of cellular metabolism in the constitutive transgenics. In both instances (i.e. under inducible or constitutive expression of m*ODC*), the transgenic seedlings showed up to a 40-fold increase in Put with only small changes in Spd and Spm contents; these results reinforce the argument that there is a tighter homeostatic regulation of higher PAs (i.e. Spd and Spm) than Put in plants. Increased Put production (inducible or constitutive) in the seedlings also showed increased catabolism of Put (Shao *et al*., 2014), a result similar to that seen in poplar cells (Bhatnagar *et al*., 2001, 2002). In mature plants, the amino acids whose contents increased in 2x*35S* versus the WT plants in different organs included Glu, Gln, Leu, Val and His in the buds, and Pro and Ile in the rosette leaves. Conversely, Glu and Thr in the leaves, Ala in the buds, Orn in the flowers, and Arg, Orn, and His in the siliques were somewhat higher in the WT than in the transgenic plants. Notably, Glu content was significantly higher in the buds of the transgenic plants versus the buds of WT plants, but it was significantly lower (than WT) in rosette leaves and siliques (fruits) of the transgenic plants. Also, Orn was almost undetectable in many organs except the siliques and flowers. In general,

total soluble amino acids were higher in the reproductive organs, among which flowers recorded the highest amount, compared with the rosette leaves in both the transgenic and the WT plants. All mature organs that were tested contained significant amounts of cadaverine (Cad, derived from Lys by the activity of mODC), which was actually even higher than Put in many organs. Supplementation of the growth medium with exogenous Orn further enhanced Put accumulation, indicating that in spite of its increased production, the demand for mODC was still not fully met (Majumdar, 2011; Majumdar *et al.*, 2013).

Our studies have led us to conclude that most of the changes in amino acids in the *ODC* overexpressing cells/plants are perhaps a compensation for the rapid consumption of Orn by the ODC enzyme, thereby creating a positive metabolic pull (a vacuum) to convert more Glu into PAs; this, we suggested, causes more N assimilation into Glu (Mohapatra *et al.*, 2010b; Majumdar 2011; Majumdar *et al.*, 2013). We further believe that this increase in N assimilation leads to increased C assimilation, thus providing an effective approach to increasing plant biomass through manipulation of the PA pathway.

In addition to overexpression, the inhibition of Put production via the mutation of *ADC* and other PA biosynthetic genes has been reported in plants, but no true mutants of the *ODC* gene are available (Watson *et al.*, 1998; Hanzawa *et al.*, 2000; Imai *et al.*, 2004a,b; Urano *et al.*, 2004, 2005; Kakehi *et al.*, 2008). Other approaches to reducing PA production in plants have included RNAi technology and chemical inhibitors. As Orn is both a direct and an indirect (via Arg) precursor of PA biosynthesis, alteration of PA biosynthesis would be expected to directly affect Orn metabolism. In tobacco (*N. tabacum*), inhibition of ODC activity via the RNAi approach showed downregulation of both *ODC* transcripts (by fourfold) and enzyme activity (~threefold) in transgenic hairy root cultures (DeBoer *et al.*, 2011). A slight increase of *ADC* transcripts was observed with a concomitant increase in ADC activity in response to ODC downregulation. Decreased activity of ODC altered alkaloid composition in the transgenic plants, in which the major alkaloid, nicotine, was decreased, but the second major alkaloid, anatabine, was increased, both in intact plants and in hairy root cultures. MeJa, which typically stimulates nicotine biosynthesis in tobacco, did not have any effect in the *ODC* RNAi plants; rather, the anatabine content was increased upon treatment with MeJa. Another study conducted by the same group (DeBoer *et al.*, 2013) showed that downregulation of *ODC* expression, again through constitutive RNAi, prevented the increase in anabasine in *N. glauca* hairy root cultures in response to wounding (another effect of MeJa) when compared with the vector-only controls, suggesting a possible role of ODC in elevating anabasine content in response to wound stress in this species. Besides affecting amino acid biosynthesis, the silencing of ODC was shown to affect several TCA cycle metabolites that are directly or indirectly derived from the diamine Put (DeBoer *et al.*, 2011, 2013). Arguably, Orn flux in these plants must have been altered because it is the primary precursor for the biosynthesis of both alkaloids (both via the ADC or ODC pathways) and several amino acids; however, no direct measurements of this or other amino acids were reported in these studies.

In plants, the conversion of Orn to Pro occurs via two steps, i.e. Orn $\rightarrow \Delta^1$-pyrroline-5-carboxylate (P5C) $\rightarrow$ Pro, while in some bacteria (e.g. *Agrobacterium* spp.) Orn can be directly converted to Pro by Orn cyclodeaminase (OCD; Trovato *et al.*, 2001). The expression of *AtOCD* was enhanced by Pro rather than its substrate Orn, and induced expression of *AtOCD* was especially observed under low water potential (Sharma *et al.*, 2013). In *Arabidopsis*, a T-DNA insertion mutant (*Atocd*) of the *AtOCD*-like gene, as well as *AtOCD*-RNAi transgenic plants, were shown to have increased (by ~15%) Pro accumulation, without any change in the expression of Pro catabolic genes, under low water potential conditions. Both the mutant and the overexpresser lines of *AtOCD* (*35S* promoter) showed a significant increase in Orn and a significant decrease in cellular oxalate contents only under low water potential compared with the WT plants, suggesting an indirect effect of *AtOCD* expression on redox status in plants under water stress conditions (Sharma *et al.*, 2013).

In nature, excess N absorbed from atmospheric acidic deposition, soil fertilization or constitutive overexpression of a gene in the N pathway can cause accumulation of N in the form of free amino acids, including Orn and PAs, thus causing a shift in the overall C and N metabolite ratios. Nutrient imbalances (Ca:Al and Ca:Mn ratios)

and leaching of Ca and Mg from the soil is often observed under these conditions as a result of lowering of soil pH. If the conditions persist, a decline in growth is observed (Minocha *et al*., 2000; Bauer *et al*., 2004; Magill *et al*., 2004). These conditions may be reversed by the addition of Ca or by removal of the source of excess N (Wargo *et al*., 2002; Minocha *et al*., 2010). Hence, the accumulation of certain amino acids (including Orn) is indicative of their role in ammonia (excess N) detoxification. It was suggested by Kalamaki *et al*. (2009a,b) that engineering plants to constitutively accumulate a non-toxic metabolite such as Orn, that can readily be converted to osmo-protective molecules (e.g. Pro or PAs) upon stress induction, might present an alternative way of increasing abiotic stress tolerance in plants. These authors directly demonstrated improvement in stress tolerance by the accumulation of Orn via overexpression of the tomato *SlNAGS1* gene in *Arabidopsis*. They also proposed an interesting indirect role of Orn accumulation before the exposure of the plants to abiotic stress, during which Pro could be quickly produced on demand, thereby endowing the plant with future protection from abiotic stress. Relatively high levels of Pro in flowers and other reproductive structures (~60-fold higher than those in other organs) in several plants has been suggested to play a role in the protection of these parts from potential osmotic stress (Mattioli *et al*., 2009). It was further suggested that such high levels of Pro were synthesized from Orn by Δ^1-pyrroline-5-carboxylate synthetase (P5CS; EC 2.7.2.11/1.2.1.41) with the help of Pro transporter; under such conditions, a rapid flux of Orn → P5C pathway (see Fig. 9.1) will be essential.

9.7 Ornithine Biosynthesis and Functions in Animals

Like plants, in animals also, the cellular concentration of Orn is much lower than that of Arg or Glu. However, in contrast to plants, Orn biosynthesis in animals occurs solely from Arg by cleavage carried out by the enzyme arginase (Morris, 2006, 2007). Further metabolism of Orn is carried out by two enzymes, namely OAT to make Cit and Orn transcarbamoylase (OTC; EC 2.1.3.3) to make Pro. High activity of ODC and reduced activities of OAT and OTC have been correlated with rapid growth of hepatomas and associated tumorigenesis (Weber *et al*., 1972; Tomino *et al*., 1974). A similar effect of increased ODC activity on OAT was observed under short-term induction of ODC in rat colon, which was accompanied by an increase in cellular Orn and Arg besides increased Put. Reduced OAT activity has been shown to result in higher accumulation of Orn in both OAT-deficient humans and adult mice with inhibited OAT activity. However, increased arginase activity in skin tumour cells showed an elevated Orn level as well as ODC activity, thus increasing Put production. Han *et al*. (2001) suggested that such changes in Orn or Arg concentrations in the cells trigger the conversion of a normal cell to a tumour phenotype. While the accumulation of Orn will allow more of it to be available for PA biosynthesis, the depletion of Orn might create metabolic flows in pathways, especially the PA and the amino acid biosynthetic pathways that are connected through Orn.

Demand of Arg, a semi-essential amino acid for mammals, can be met either through endogenous biosynthesis or dietary intake. Besides being a major substrate for PA biosynthesis (via Orn), Arg plays diverse roles in other metabolic pathways, e.g. urea, creatine and NO biosynthesis. Male Swiss albino mice when fed with Arg-rich dietary supplements for short periods did not show dramatic changes in the overall free PA pool (Teixeira *et al*., 2002). In long-term studies, the major changes observed were in Put content at 15 d and Spd at 30 d; the effects differed among different organs. It was concluded that endogenous Arg biosynthesis was the major contributing factor in maintaining PA pools in the tissues. In another study to elucidate the role of exogenous Orn on Arg metabolism, piglets fed with ^{14}C-Orn showed no significant changes in the plasma Arg concentration or the flux of Arg or Arg synthesis from Orn; Orn had a greater effect on Pro biosynthesis (Urschel *et al*., 2006). The addition of Orn to an Arg-deficient diet had a significant effect on Orn and Arg metabolism, more so than supplementation with dietary α-ketoglutarate. Analyses of Arg in various organs in mice revealed that decreased Arg level in plasma resulted in a reduced Gln level in the muscle, accompanied by increased renal Arg and gut Cit (Vissers *et al*., 2007a,b). Reduced plasma Arg concentration was also observed in

tumour-bearing mice and human cancer patients (Vissers *et al.*, 2005). The feeding of Orn to young rats through the diet caused a significant increase in tissue protein synthesis by positively affecting growth hormone levels (Tujioka *et al.*, 2012).

In animals, Orn biosynthesis is also critical for the disposal of excess ammonia in the form of urea, thereby detoxifying the cells of excess N. The N in Orn, however, remains intact, as for urea production, the N comes from ammonia and Asp (Krebs, 1973). So endogenous levels of Orn apparently limit the production of urea in animals, and the exogenous supply of Orn is the most effective way to promote urea formation (Sikorska *et al.*, 2010).

Ornithine is a commonly used nutraceutical supplement to increase muscle strength and cardiovascular activity (http://www.tysonnutraceuticals.com/L-Ornithine.htm). In order to investigate the role of L-Orn in alleviating fatigue, a study was conducted with healthy individuals in which Orn was administered orally before exercise (Sugino *et al.*, 2008). Individuals with L-Orn supplements were able to overcome fatigue by increasing their efficiency of energy consumption with efficient excretion of cellular ammonia. Other positive effects of Orn are related to its ability to produce NO. In an earlier study by Alhonen *et al.* (2002), the increased production of PAs from dietary Orn was shown to stimulate hepatic regeneration in rats. Dietary supplementation with Orn also led to enhanced wound breaking strength and collagen deposition (Sikorska *et al.*, 2010).

There are numerous genetic disorders known in humans that are related to the Arg-Orn-Glu-or the urea cycle pathway (Leonard and Morris, 2002; Ficicioglu and Yudkoff, 2013) and many of them are manageable by dietary supplementation of amino acids. In disorders such as citrullinaemia, argininosuccinic aciduria and argininaemia, Orn insufficiency and an excess of ammonia also occur (Zieve, 1986). It is recognized that the presence of insufficient Orn for normal urea cycle responsiveness leads to vulnerability to ammonia toxicity under conditions of protein deficiency and malnutrition (Zieve, 1986). A recent review (Bai *et al.*, 2013) has revealed that supplementation with L-Orn-L-Asp (LOLA, a stable salt of two nonessential L-amino acids) could help hepatic encephalopathy patients in the improvement of disease by reducing the serum ammonia concentration compared with the placebo/no-intervention controls. Ammonia excess also occurs in the normal liver when amino acid mixtures lacking Orn, Arg or Cit are introduced/infused or when the gastrointestinal tract contains an excess of protein, urea or NH_4^+; e.g. after a gastrointestinal haemorrhage. It may also occur with the injection of specific amino acids such as Gly, or ammonium salts, urea and urease (Zieve, 1986).

9.8 Exogenous Supply of D- and L-Ornithine

Ornithine is available in two racemic forms – D-Orn and L-Orn, which are interconvertible by the enzyme Orn racemase (EC 5.1.1.12), and are found in several organisms, including humans (Chen *et al.*, 2000). Alternatively, D-Orn can be converted into D-Arg and then into L-Arg by Arg racemase (EC 5.1.1.9; see http://www.genome.jp/kegg-bin/show_pathway?mlo00472) for normal metabolism. Some microbes directly metabolize D-Orn into (2*R*,4*S*)-2,4-diaminopentanoate (by D-Orn 4,5-aminomutase; EC 5.4.3.5), a pathway that allows them to utilize D-Orn as a source of N (Yonaha *et al.*, 1975). Therefore, D-Orn is sold on the market as a nutraceutical for the same usage as L-Orn or Arg; however, in some studies, differences in the physiological effects of the two enantiomers have been reported.

Besides rapid utilization of intracellular Orn and its impact on PAs, amino acids, the TCA cycle metabolites etc., the exogenous supply of Orn (L/D forms) also affects related metabolites in living cells. In intact leaves or leaf discs of cashew plants, exogenous application of L-Orn (10 mM) showed a significant increase in the cellular content of Pro (daRocha *et al.*, 2012). In 12-d-old *Arabidopsis* seedlings, short-term treatment (24 h) with exogenous L-Orn (0.1 mM) resulted in significant increases in Glu, Gln, Orn and Ser (Majumdar *et al.*, 2013). Another study on the effect of D- and L-Orn on cell viability and other associated metabolites in tobacco (*N. tabacum* L. cv. Burley 21) cell suspension cultures showed differential effects of the two forms of Orn on amino acid biosynthesis and the TCA cycle (Gholami *et al.*, 2014). Cell viability decreased significantly with increasing concentration of L-Orn

(beyond 1 mM), whereas D-Orn did not have any such effects. The amino acids that showed a linear increase with increase in L-Orn concentrations (1, 5, 10 mM) were L-Orn, Cit, Glu, Gly, Pro, Leu, Lys, Tyr, Phe, Val, Ile and Trp, with the highest increase observed in L-Orn (40-fold) and Cit (tenfold) with 10 mM L-Orn treatment; there was no change in the same amino acids when treated with the corresponding concentrations of D-Orn. The TCA cycle metabolites that were affected (positively) most by L-Orn were galactonate and malate, in addition to the alkaloid nicotine. In contrast, D-Orn caused a significant increase in Arg content (80-fold higher than the control) and also in urea, lactic acid and glucose.

Different physiological responses of tobacco cells treated with different Orn enantiomers were seen under salt stress (250 mM NaCl). Growth of these cells was adversely affected by L-Orn under normal conditions, with further deterioration under salt stress, whereas the same concentration (1 mM) of D-Orn improved cell growth and ameliorated salt stress. This was apparently achieved through the activation of antioxidant enzymes (namely superoxide dismutase, catalase and peroxidase), increase in Pro content and a lowering of H_2O_2 production in the cells. Interestingly, the same concentration (1 mM) of D-Orn had a positive effect on Put content in these cells when grown under normal conditions. These results together suggest a potential role via unknown mechanisms of D-Orn in plant cells activating the antioxidant system under stress situations (Ghahremani *et al*., 2013).

9.9 Modelling of Ornithine Metabolism and Associated Flux: Ornithine as a Regulatory Molecule

There are two key steps involved in the regulation of steady state metabolic flux in a cell: a sensory mechanism to regulate flux rate; and the actual control of flux rate. The sensory aspects often require molecular mechanisms that can assess the need to respond to the changing environment and then send messages to the regulatory mechanisms. For decades following the early models of transcriptional control, it was believed that most metabolic pathways are regulated through changes in the concentration of the enzyme(s) that are involved in the specific metabolic step(s), which produce a certain metabolite. Kochanowski *et al*. (2013) have recently summarized the various scenarios under which metabolic flux regulation could occur in microbes at the level of transcription, translation, post-translational protein modifications and metabolites (substrates and products). The discovery of feedback regulation of enzyme activities, the complexities of certain reactions involving multiple substrates, cofactors and coenzymes, which were either limiting or non-limiting, and the stimulation of catabolism by certain metabolites themselves acting as the substrate, made the study of flux rates rather complex.

There are three things that become apparent from the published literature on the regulation of Orn metabolism in plants: (i) rate of its biosynthesis is dependent upon the activity of NAGS, the first enzyme in the pathway; (ii) this enzyme must somehow respond to the need for Orn utilization in multiple pathways (Pro, Arg and PA biosynthesis) because Orn is not the end product of the pathway; and (iii) the other enzymes of the so-called Orn pathway (i.e. NAGK, NAGPR, NAOGAcT, NAOAT and NAOD) are often constitutively available. If one assumes that the biosynthesis of Orn is not regulated by the need for its accumulation nor by a feedback regulation by its products (Arg, Pro and PAs), one can then argue that Orn biosynthesis is largely regulated by the rate of its utilization. The experimental evidence for this line of reasoning comes from the studies involving: (i) genetic manipulations (both upregulation and downregulation via transgene expression, the RNAi approach and mutations) of the cellular contents of the three major products of Orn; and (ii) considerable (many fold) variations in the cellular contents of the three products under conditions of abiotic stress without significant changes in cellular Orn content.

The two main driving forces to increase or decrease metabolic flux through a pathway are: (i) an upsurge in demand for the product(s); and (ii) a need to ameliorate the accumulation of an intermediate. In multi-nodal pathways, such as those involving Orn utilization into the production of Arg, Pro, PAs and alkaloids, at a given time the increase in demand may come from any one or more products. Based upon (i) the results of our own studies with transgenic manipulation

of the PA pathway, where the Orn → Put step was targeted, and (ii) several other studies where increased PA biosynthesis in response to abiotic stress is often accompanied by an increase in Pro and Arg as well, it appears that increases in Arg, Pro and PAs often occur concurrently under several conditions. There is also a concomitant increase in GABA accumulation under the same conditions, which can be produced either directly from Glu by Glu decarboxylase (GAD; EC 4.1.1.15) or through the catabolism of Put by diamine oxidase (DAO; EC 1.4.3.6) (Shelp *et al.*, 2012). That makes Glu the primary substrate being used in all of these reactions, so that its demand must be under strict coordination, and its steady state production/availability must be ensured. One obvious common metabolite for all but the GAD node of this pathway is the intermediate Orn. Thus, it can be hypothesized that as long as Glu is not limiting, the Pro, Arg, PA and GABA (and perhaps alkaloid production, where it occurs) pathways can be upregulated concurrently if Orn is presumed to play a regulatory role; this argument has recently been made by Majumdar *et al.* (2013). It is further argued that upregulation of any of the terminal products of these nodal pathways would increase the demand for Orn (by acting as a sink to draw Orn consumption), which would force the flux of Glu to Orn to increase. As Glu is critical for a multitude of metabolic pathways, this situation could also induce mechanisms to increase the production of Glu in plants, perhaps directly from N assimilation. Under normal growth conditions in plants, the C needed for increased Glu production will of course come from the products of photosynthesis.

Therefore, a question that can be raised is: 'Could Orn act as a controlling metabolite to enhance total N uptake through which total C sequestration could also be positively affected'? This would enhance the total biomass of the vegetative organs in the plant, providing a potential way to increase bioenergy production. Increased Glu → Orn flux would also enhance the production of the critical amino acids involved in protein biosynthesis, in plant stress response and, most importantly, in affecting the oxidative state of the cells, suggesting pleotropic effects of such single-step manipulation on overall cellular metabolism. While increased biomass production (i.e. faster growing plants) through genetic alteration of the major nitrogenous compounds (Pro, Arg, Glu and PAs) could be play a positive role in benefiting from the genetic manipulation of plants for the production of renewable energy, increased use in phytoremediation, reduction in atmospheric CO_2 and perhaps also in N pollution; the cautionary adverse aspect of this approach could be the potential changes in the balance of N-rich nutrients for food and feed crops. Our recent analyses of metabolomic changes in WT and the high Put poplar cells have indicated that the genetic manipulation of a single step (i.e. Orn consumption via overexpression of ODC) causes major changes not only in the nitrogenous metabolites but also in numerous other metabolites, including those associated with the TCA cycle (Page *et al.*, unpublished). This is presumably due to the metabolic connection of Put to the various C metabolites via the succinate-GABA shunt (reviewed by Shelp *et al.*, 2012). In the next generation of genetically engineered plants aimed at modifying their nutrient values, this would require a careful analysis of the entire metabolome of the final product, i.e. the transgenic plant. The key questions as to how Orn would control its own biosynthesis without accumulation (flux rate), and what mechanism is involved in Orn sensing and the signal transduction pathway still pose a future challenge for the scientific community.

9.10 Conclusions

Despite the fact that Orn is not involved in protein synthesis, is often present in relatively small quantities and has no direct and specific role in any known physiological function in plants, it plays a critical role in overall N metabolism that directly affects several critical functions in plant growth, development and stress responses. Due to its presence at the crossroads of three important metabolic branches, namely the biosynthesis of Arg, Pro and the PAS (and also alkaloids in some plants), the flux of Orn biosynthesis (from Glu) must be stringently regulated to meet its demands without it accumulating in large quantities. The published literature so far indicates that under the conditions of changing demands for Orn utilization into the various metabolic sub-pathways, most of this regulation occurs via post-translational events for regulation of enzyme activity rather than the level of transcription of the

various genes that code for these enzymes. Furthermore, increased demand of Orn that leads to increased utilization of Glu appears to result in replenishing Glu availability to the other competing pathways via its increased biosynthesis. As increased Glu biosynthesis involves additional N as well as C, one can postulate that genetic manipulation of an increased flux of Glu into Pro, Arg and/or PAs could increase the assimilation of both N and C from the external environment. Overall, the increased biosynthesis of Pro, Arg, and PAs (and perhaps of GABA, which is also produced directly from Glu via GAD and from the catabolism of Put as well) would not only increase the stress tolerance of plants (which is a well-known phenotype associated with high Pro, Arg, PAs and GABA) but also enhance the assimilation of N and C in the plants. Unfortunately, the mechanism by which the flux of Glu into Orn is regulated is not currently understood.

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Amino Acids in Higher Plants

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