

Polyamine- and Amino Acid-Related Metabolism: The Roles of Arginine and Ornithine are Associated with the Embryogenic Potential

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The mechanisms that control polyamine (PA) metabolism in plant cell lines with different embryogenic potential are not well understood. This study involved the use of two *Araucaria angustifolia* cell lines, one of which was defined as being blocked, in that the cells were incapable of developing somatic embryos, and the other as being responsive, as the cells could generate somatic embryos. Cellular PA metabolism was modulated by using 5 mM arginine (Arg) or ornithine (Orn) at two time points during cell growth. Two days after subculturing with Arg, an increase in citrulline (Cit) content was observed, followed by a higher expression of genes related to PA catabolism in the responsive cell line; whereas, in the blocked cell line, we only observed an accumulation of PAs. After 14 d, metabolism was directed towards putrescine accumulation in both cell lines. Exogenous Arg and Orn not only caused a change in cellular contents of PAs, but also altered the abundance of a broader spectrum of amino acids. Specifically, Cit was the predominant amino acid. We also noted changes in the expression of genes related to PA biosynthesis and catabolism. These results indicate that Arg and Orn act as regulators of both biosynthetic and catabolic PA metabolites; however, we suggest that they have distinct roles associated with embryogenic potential of the cells.

Keywords: Amino acid biosynthesis • *Araucaria angustifolia* • Conifer embryogenesis • Embryogenic potential • Polyamine gene expression • Polyamine metabolism.

Abbreviations: ADC, arginine decarboxylase; ALDH, aldehyde dehydrogenase; Arg, arginine; Cit, citrulline; CPM, counts per minute; CuAO, copper-containing amine oxidase; GABA, γ -aminobutyric acid; NO, nitric oxide; ODC, ornithine decarboxylase; Orn, ornithine; OTC, ornithine carbamoyltransferase; PA, polyamine; PAO, polyamine oxidase; PCA, principal component analysis; Put, putrescine; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; SAM, S-adenosylmethionine; SE, somatic embryogenesis; Spd, spermidine; SPDS, spermidine synthase; Spm, spermine; SPMS, spermine synthase; TLC, thin-layer chromatography.

Introduction

Polyamines (PAs) are small aliphatic amines, which have multiple functions and are considered essential for cell survival; their presence is universal in living organisms (Minguet et al. 2008, Silveira et al. 2013, Minocha et al. 2014, Masson et al. 2017). Their positively charged structures at cellular pH allow an electrostatic interaction with various macromolecules, such as DNA, RNA, phospholipids, hormones and proteins; therefore, they are able to influence and regulate various developmental processes (Baron and Stasolla 2008, Minocha et al. 2014).

Three common PAs in plants are putrescine (Put), spermidine (Spd) and spermine (Spm). The diamine Put is synthesized directly from ornithine (Orn) by ornithine decarboxylase (ODC; EC 4.1.1.17) or from arginine (Arg) by arginine decarboxylase (ADC; EC 4.1.1.19) via two additional steps (Bais and Ravinshankar 2002). The co-existence of ADC and ODC in some plant species may be related to their different contributions to stress, development and tissue-specific processes (Vuosku et al. 2006). The triamine Spd and tetra-amine Spm are synthesized by the sequential addition of aminopropyl groups to Put, using S-adenosylmethionine (SAM), the product of SAM decarboxylase (SAMDC; EC 4.1.1.50), and the enzymes spermidine synthase (SPDS; EC 2.5.1.16) and spermine synthases (SPMS; EC 2.5.1.22), respectively (Tiburcio et al. 1997, Vuosku et al. 2012). PA catabolism is mediated by PA oxidases (PAOs; EC 1.5.3.3), copper-containing amine oxidase (CuAO; EC 1.4.3.6) and aldehyde dehydrogenase (ALDH; EC 1.2.1.3) (Cheng et al. 2015).

PA metabolism is part of a network of highly interdependent pathways that are central to nitrogen metabolism (Page et al. 2016, Wuddineh et al. 2018) and are interconnected with other pathways, such as those related to the biosynthesis of amino acids (Majumdar et al. 2016), ethylene (Lasanajak et al. 2014) and nitric oxide (NO) (Tun et al. 2006). The accumulation of PAs may play a role in the protection of cells against damage from reactive oxygen species (ROS) (Salo et al. 2016); a major product of their catabolism, i.e. hydrogen peroxide, could also

be the source of ROS damage to cells. Increased metabolic conversion of Arg or Orn into Put may considerably affect the pool of other amino acids and metabolites in the cell (Majumdar et al. 2016), along with changes in the expression of a broad spectrum of genes (Page et al. 2016). The diverse functions of PAs are thought to require their homeostasis through regulation of their biosynthesis, catabolism and transport (Kusano et al. 2008), processes that are under complex mechanisms of control, including post-translational regulation (Fortes et al. 2011, Majumdar et al. 2016). Although significant progress has been made in understanding the regulation of PA biosynthesis and signal transduction, little is known about the molecular processes associated with the multiple modes of action of PAs (Anwar et al. 2015, Majumdar et al. 2016, Page et al. 2016).

In plants, PAs are known to play roles in cell division, flowering and fructification, programmed cell death, senescence, rooting, response to biotic and abiotic stress, and embryogenesis (Bais and Ravinshankar 2002, Kuehn and Phillips 2005, Floh et al. 2007, Kuznetsov and Shevyakova 2007, Gemperlová et al. 2009, de Oliveira et al. 2017). PA metabolism has been associated with both zygotic embryogenesis and somatic embryogenesis (SE) in many plant species (Bastola and Minocha 1995, Minocha et al. 1999, Astarita et al. 2003c, Minocha et al. 2004, Silveira et al. 2004, Vuosku et al. 2006, Steiner et al. 2008, Gemperlová et al. 2009, Vuosku et al. 2012, Jo et al. 2014, Salo et al. 2016, de Oliveira et al. 2017). Small changes in PA levels and amino acid content related to PA biosynthesis have been observed at different developmental stages in SE, from the induction of embryogenic cultures to embryo germination (Andersen et al. 1998, Minocha et al. 1999, Astarita et al. 2003a, Astarita et al. 2003b, Silveira et al. 2004, Pieruzzi et al. 2011). In this context, due to the similarity to zygotic embryogenesis, SE represents an effective model to study factors that affect embryo development (von Arnold et al. 2002, Jo et al. 2014, Elbl et al. 2015, dos Santos et al. 2016, Salo et al. 2016, Navarro et al. 2017).

While protocols for SE have been described for many conifer species (Klimaszewska et al. 2016), they have not been as well established for *Araucaria angustifolia* (Brazilian pine), an endangered conifer species that grows in the southern part of Brazil. A lack of knowledge of the underlying genetic programs and biochemical pathways that regulate embryogenesis in this species has limited in vitro development to only a few mature somatic embryos (Jo et al. 2014, Elbl et al. 2015, dos Santos et al. 2016, Navarro et al. 2017). However, studies of molecular processes and biochemical activities using comparative transcriptomics (Elbl et al. 2015), proteomics (dos Santos et al. 2016), and metabolism of PAs (Jo et al. 2014, de Oliveira et al. 2015) and carbohydrates (Navarro et al. 2017) in different embryogenic cell lines have been reported. Additional studies have involved analyses of transcripts (Elbl et al. 2015) and protein profiles (Silveira et al. 2008, Balbuena et al. 2011), and the content of ABA (Silveira et al. 2008), IAA (Astarita et al. 2003a), amino acids (Astarita et al. 2003b, de Oliveira et al. 2017) and PAs (Astarita et al. 2003c, de Oliveira et al. 2017), all during zygotic embryogenesis of this species.

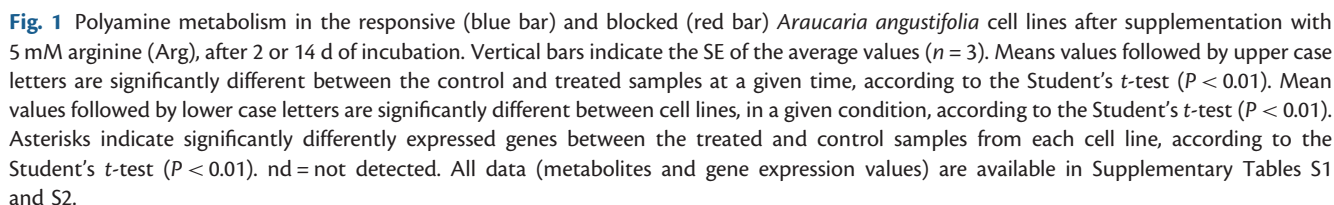
The mechanisms that control PA metabolism in cell lines with different embryogenic potentials are not yet clearly understood. In *Pinus nigra* (Noceda et al. 2009) and *P. sylvestris* (Salo et al. 2016), a high Put concentration was found to be associated with inability to induce somatic embryo production, and higher levels of Spd were observed during cell proliferation and maturation in *Picea abies* (Minocha et al. 2004, Malá et al. 2009). Thus, a better understanding of the mechanisms that regulate PA metabolism in embryogenic cell lines with differing embryogenic capacities would be of considerable value for improving the experimental and growth conditions used for SE.

In the present study, we used two distinct *A. angustifolia* embryogenic cell lines to investigate several key topics of fundamental importance for understanding the roles of Arg and Orn in Arg|Orn|PA metabolism and somatic embryogenesis. We measured cellular PA and amino acid contents, the incorporation of labeled precursors, along with a quantitative real-time PCR (qRT-PCR) analysis of key genes involved in the Arg|Orn|PA pathway. We investigated whether Arg or Orn levels changed not only in association with PA and amino acid profiles, but also with the expression patterns of the related genes. This allowed us to address whether the participation of these precursors in this pathway is correlated with embryogenic capacity. The results suggest that Arg and Orn could play distinct roles in the Arg|Orn|PA pathway, associated with the cell growth phase and embryogenic potential of the cultures. This information should help with optimization of SE conditions, by mimicking the biochemical and molecular changes that occur during zygotic embryogenesis.

Results

Supplementation with Arg or Orn changes PA levels, independent of the embryogenic potential of cell lines

Suspension cell cultures of embryogenic cell lines with different embryogenic potentials but similar growth curves after subculture to fresh medium (see Supplementary Fig. S1) were established in order to evaluate their metabolic response to supplementation with 5 mM Arg or Orn in terms of free PAs and amino acids. The two cell lines selected are identified as 'blocked' (cultures incapable of developing somatic embryos) and 'responsive' (cultures capable of forming cotyledonary embryos) in the same medium, and grown under the same conditions (see the Materials and Methods for details). Samples were collected after 2 and 14 d of cell proliferation, representing the lag phase and the exponential growth phase, respectively. In addition to differences in embryogenic potential, the two cell lines used in this study have different PA profiles, especially with regard to Put abundance (Fig. 1; Supplementary Table S1), which was the dominant PA in the responsive cell line vs. the blocked cell line, at both time points. In the responsive cell line, Put content was followed by Spd and Spm at both times of analysis. In blocked cell line at 2 d, Spd was the main PA, followed by Put and Spm, while at 14 d, Put was the most abundant.



Orn were distinct from those of the respective controls (liquid medium without Arg or Orn supplementation) (PC1 explained 62–70% of the total variance) (Supplementary Fig. S2). After 2 weeks, while the Orn samples were distinct from the control in the responsive cell line, the Arg samples were not (Supplementary Fig. S2A). In the blocked cell line, the Arg- and Orn-treated samples were distinct from the control (Supplementary Fig. S2B).

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both cell lines after 2 d as well as after 14 d of incubation with 5 mM Arg. However, this increase was more pronounced in the blocked (2-fold) than in the responsive cell line (Fig. 1). The supplementation with 5 mM Arg resulted in differences in the levels of Spd in the responsive cell line, but not in the blocked line, when compared with the control (Fig. 1). A significant difference in Spm content by addition of Arg was observed only after 14 d of incubation (Fig. 1). Also, Spm content was lower at 14 d compared with that observed at 2 d, for both control and Arg supplementation.

With respect to 5 mM Orn treatment, after 2 d of supplementation with Orn, the Put content was higher (vs. the control) in the blocked cell line (almost 4-fold) and lower in the responsive line (Fig. 2). After 14 d, Put content was lower than at 2 d in both cell lines, and it was higher in the blocked line (>20-fold vs. control) than in the responsive line (2-fold increase vs. control). Supplementation with 5 mM Orn resulted in increased Spd and Spm levels in the blocked cell line at both time points; however, in the responsive cell line, they were lower after 2 d, and higher at 14 d, compared with the control.

In summary, the Arg and Orn treatments resulted in small but significant changes in the Spd and Spm contents, and greater changes in Put contents. In general, supplementation of Arg or Orn promoted a similar effect in the PA contents in both cell lines, when comparing the treatments (Supplementary Fig. S3).

The Arg|Orn|Cit pathway is affected by supplementation with Arg or Orn

The two cell lines also differed in their amino acid profiles as revealed by PCA; the two lines had opposite metabolic profiles at the 2 and 14 d time points (PC1 explained 99.1% and 80.2%, respectively) (Supplementary Fig. S4A, B). This variation was specific for each time point for each cell line. At the 2 d time point, alanine, γ -aminobutyric acid (GABA), glutamine and glutamate were the main amino acids detected in both cell lines, and they were significantly higher in the blocked than in the responsive cell line (see Supplementary Fig. S4C). However, in contrast to 2 d, at 14 d, the cellular content of amino acids was quite different, with alanine, asparagine, glutamine, glycine, lysine, Orn, phenylalanine, serine and valine (Supplementary Fig. S4D). At this point, most amino acids were significantly higher in the responsive than in the blocked cell line. We observed that the two precursors for Put biosynthesis (Arg and Orn) were present at significantly higher levels in the blocked cell line than in the responsive cell line at 2 d, whereas at 14 d their levels were similar between them.

Since the two cell lines differ in amino acid contents, and Arg and Orn are substrates for Put as well as several other amino acids, we hypothesized that exogenous Arg and Orn should directly affect the levels of other amino acids associated with the PA biosynthetic pathway (Figs. 1, 2; Supplementary Fig. S3; Supplementary Table S1). In the control medium, Orn content was higher than the Arg content in both cell lines, the former representing approximately 5% of the total pool of amino acids, while Arg was present at <1%. In the blocked cell line, Orn varied

from 1% to 20% (at 2 and 14 d, respectively) and Arg <2% of the total amino acid pool (Supplementary Table S1). Overall, the contents of Arg and Orn in the control medium were higher in the blocked than in the responsive cell line at 2 d, but were similar at 14 d (Figs. 1, 2).

In addition to the increasing cellular Put content together with the accumulation of Arg and Orn absorbed from the medium, the higher levels of amino acids were observed at 2 d of culture in both cell lines (Figs. 1, 2). Addition of exogenous Arg did not affect the levels of endogenous Orn, while citrulline (Cit) levels were significantly higher at both time points (12- to 166-fold, respectively), indicating a lower conversion of Arg into Orn through arginase action, and probably a higher conversion into Cit, either via NO synthesis or via the Orn pathway (Fig. 1). The exogenous Arg also resulted in changes in the levels of other amino acids that participate as substrates in Arg and Orn biosynthesis, notably glutamate, glutamine and aspartate (Fig. 1). After 2 d of incubation, the contents of these three amino acids increased (3- to 4-fold) in the responsive cell line, while they decreased (6- to 7-fold) in the blocked cell line. However, at 14 d, their levels were lower in both cell lines compared with the 2 d time point. GABA is a catabolic product of Put, and it represented 3.5–4.5% of the amino acid pool in the cell lines used here. Following supplementation with 5 mM Arg, GABA levels increased 4.3-fold in the responsive cell line after 2 d (Fig. 1), indicating either increased Put catabolism or its biosynthesis from glutamate, via glutamate decarboxylase, whereas in the blocked cell line, GABA abundance decreased 6.3-fold (Fig. 1). After 14 d, GABA levels were generally lower than those observed at 2 d, although only a slight decrease (1.3-fold) was observed in the responsive cell line and an increase (4.7-fold) was detected in the blocked cell line after Arg addition (Fig. 1).

In contrast to Arg treatment, supplementation with 5 mM Orn resulted in an increase in endogenous Arg content (Fig. 2) after 2 d in the responsive (87-fold) and blocked cell lines (4-fold), followed by an increase in Cit levels (56-fold in the responsive and 5-fold in the blocked cell line). As observed in treatment with Arg, the supplementation of Orn increased the levels of aspartate, glutamate and glutamine in the responsive cell line, while it decreased them in the blocked cell line. After 14 d of supplementation with 5 mM Orn, profiles of other amino acids were similar to that observed in 5 mM Arg treatment in both cell lines, except for Cit, whose content was higher in the responsive cell line, similar to that observed at 2 d (Fig. 2).

Overall, the exogenous Arg treatment resulted in greater changes in the endogenous Arg and Cit contents; on the other hand, exogenous Orn promoted a significant increase in Orn, aspartate, glutamine, glutamate and GABA (Supplementary Fig. S3).

Expression of Arg|Orn|PA metabolism-related genes is affected by Arg and Orn supplementation

Since the two cell lines used in this study showed different PA profiles, we compared the expression of genes involved in Arg|Orn|PA biosynthesis and catabolism in them. Specifically,

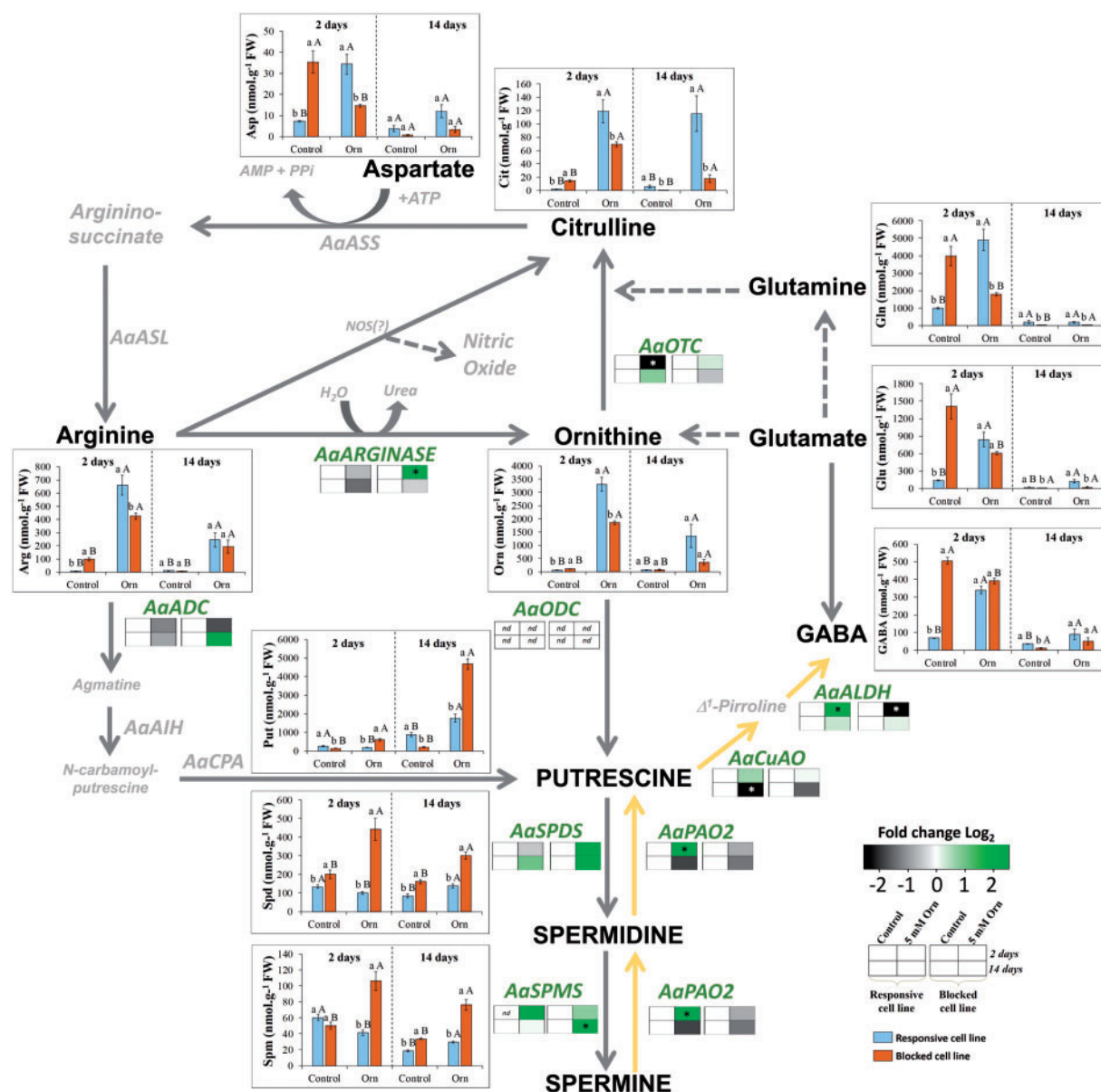


Fig. 2 Polyamine metabolism in the responsive (blue bar) and blocked (red bar) *Araucaria angustifolia* cell lines after supplementation with 5 mM ornithine (Orn), for 2 and 14 d. Vertical bars indicate the standard error of the average values ($n = 3$). Mean values followed by upper case letters are significantly different between control and treated samples at a given time, according to the Student's *t*-test ($P < 0.01$). Mean values followed by lower case letters are significantly different between cell lines, in a given condition, according to the Student's *t*-test ($P < 0.01$). Asterisks indicate significantly differently expressed genes between the treated and control samples from each cell line, according to the Student's *t*-test ($P < 0.01$). nd = not detected. All data (metabolites and gene expression values) are available in Supplementary Tables S1 and S2.

we examined the expression of *AaADC*, *AaODC*, *AaSPDS*, *AaSPMS*, *AaARGINASE*, *AaOTC*, *AaPAO1*, *AaPAO2*, *AaCuAO* and *AaALDH*. These genes, which we detected as participating in Arg|Orn|PA metabolism, have been previously identified and characterized in zygotic embryos and megagametophytes of *A. angustifolia* (see de Oliveira *et al.* 2017), and their expression changed during zygotic embryogenesis. Even though their transcripts have been detected, based on their presence in the *A. angustifolia* transcriptome database (Elbl *et al.* 2015), the mRNA levels of the *AaODC* and *AaPAO1* genes were very low and, in a

quantitative PCR analysis, were only detected after 50 cycles under any condition tested, which, for the purposes of this study, we considered to be below the cut-off threshold of detection.

First, we investigated the gene expression profiles under control conditions (Fig. 3). At 2 d, two genes involved in PA catabolism (*AaCuAO* and *AaALDH*) and one gene involved in Spd biosynthesis (*AaSPDS*) were expressed at significantly higher levels in the blocked than in the responsive cell line (Fig. 3A). After 14 d, the relative expression of most of the

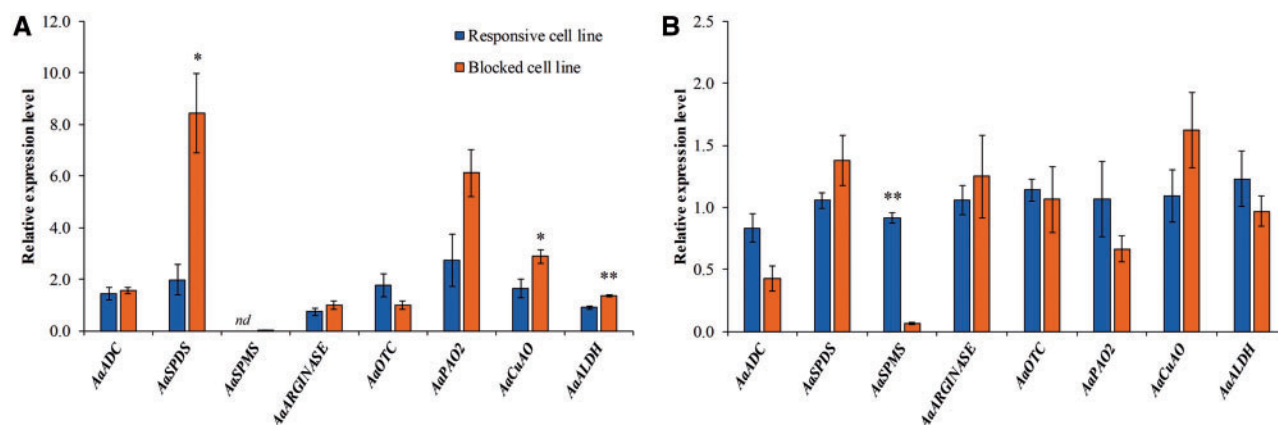


Fig. 3 Relative expression of genes related to the Arg|Orn|PA metabolic pathway, in responsive and blocked *Araucaria angustifolia* cell lines incubated for 2 d (A) or 14 d (B). Vertical bars indicate the standard error of the average values ($n = 3$). Statistically significant differences between the blocked and the responsive cell line are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$) at a given time, according to the Student's t -test.

genes tested was similar between the two cell lines, with the exception of AaSPMS, which was expressed at significantly lower levels ($P < 0.01$) in the blocked cell line than in the responsive cell line (Fig. 3B).

To elucidate further the effect of Arg or Orn on *A. angustifolia* PA metabolism, we analyzed the expression of the genes described above after supplementation with 5 mM Arg or Orn. The expression level of each gene was calculated relative to its expression in the control cultures. We noted that AaSPMS expression was not detected in the responsive cell line at 2 d under control conditions by qRT-PCR after 50 cycles. However, after supplementation with Arg or Orn, expression was detected; therefore, the equivalent data points are shown as absolute values in the heatmap.

The supplementation with 5 mM Arg or Orn revealed contrasting patterns of gene expression (Figs. 1, 2; Supplementary Fig. S5). After 2 d of growth in the presence of exogenous Arg, an increase in the expression of all the tested genes related to PA metabolism in the responsive cell line was observed (0.35- to 2.54-fold), while in the blocked cell line most showed decreased expression (up to -1.19 -fold) (Fig. 1; Supplementary Fig. S5A, C). Interestingly, expression of genes involved in PA catabolism (AaPAO2, AaCuAO and AaALDH) increased at this time point after supplementation with Arg, while in the blocked cell line it decreased.

After 14 d, an increase in the expression of the biosynthetic genes AaADC, AaSPMS and AaSPDS was observed in the responsive cell line supplemented with Arg, while the expression of genes involved in PA catabolism decreased: AaPAO2 (-1.03 -fold), AaCuAO (-0.27 -fold) and AaALDH (0.12 -fold) (Fig. 1; Supplementary Fig. S5B). Expression of genes involved in Arg|Orn biosynthesis and degradation changed by approximately 0.5 for both AaOTC and AaARGINASE (Fig. 1; Supplementary Fig. S5B). Compared with the 2 d time point, these changes were smaller. In contrast, the blocked cells showed higher expression of genes involved in PA biosynthesis (AaADC, AaSPDS and AaSPMS), albeit only 0.87- to 2.16-fold; however, the expression of genes related to PA catabolism decreased (AaCuAO) or did not change (AaPAO2 and

AaALDH) (Fig. 1; Supplementary Fig. S5D), which correlated with the high accumulation of Put.

We also observed changes in gene expression in cells treated with 5 mM Orn at 2 d. In the responsive cell line, there was a decrease in the expression of genes involved in PA biosynthesis (AaADC and AaSPDS) and amino acid biosynthesis (AaARGINASE and AaOTC), while genes involved in PA catabolism were expressed at higher levels than in the control treatment (Fig. 2; Supplementary Fig. S5A). In the blocked cell line, expression of genes involved in PA catabolism was lower (AaPAO2 and AaALDH) or similar (AaCuAO) in parallel with a slightly lower GABA content, while the expression of PA biosynthetic and Arg|Orn degradation genes was higher (AaSPDS, AaSPMS, AaARGINASE and AaOTC) together with higher PA levels (Fig. 2; Supplementary Fig. S5C).

After 14 d of growth in 5 mM Orn, the PA catabolism genes (AaPAO2 and AaCuAO) showed lower expression in both cell lines, as did genes involved in Arg degradation (AaARGINASE) (Fig. 2; Supplementary Fig. S5B, D). In contrast, AaADC showed opposite profiles in the two cell lines, with lower expression in the responsive cell line and higher expression in the blocked cell line.

Comparing only the effect between Arg and Orn treatments, in general Orn resulted in a decrease of gene expression in the responsive cell line, while an increase was observed in the blocked cell line (Supplementary Fig. S6). The greatest changes in gene expression were found at 2 d after Arg or Orn treatments.

Effects of exogenous Arg or Orn on ADC and ODC enzymatic activities

To investigate whether the activities ADC or ODC, enzymes involved in Put biosynthesis, were affected by adding 5 mM Arg or Orn to the growth media, we measured the rate of decarboxylation of L-[U- 14 C]Arg (ADC activity) and L-[1- 14 C]Orn (ODC activity) in extracts from responsive and blocked cells, following 2 and 14 d of incubation, with or without amino acid supplementation. The supplementation with Arg or Orn had no significant effect on ADC activity in either cell line (Fig. 4A, C); the ADC activity in the blocked cell line

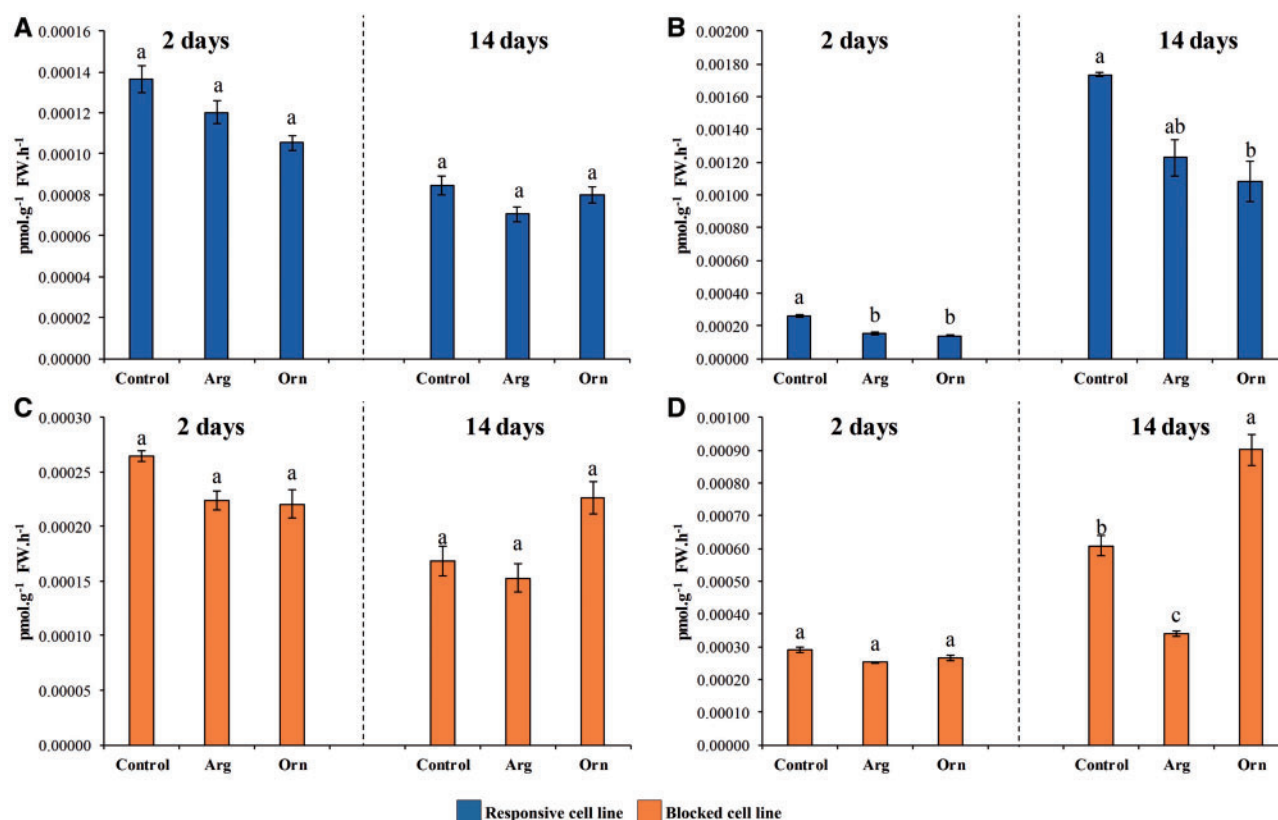


Fig. 4 Enzymatic activity assays of arginine decarboxylase (ADC) (A, C) and ornithine decarboxylase (ODC) (B, D) in responsive and blocked *Araucaria angustifolia* cell lines treated, or not, with 5 mM Arg or Orn, for 2 or 14 d. The activities were expressed as $\text{pmol g}^{-1} \text{FW h}^{-1}$ of CO_2 released. Vertical bars indicate the standard error of the average values ($n = 3$). Statistically significant differences ($P < 0.01$) among treatments at a given time are indicated by different letters, according to the Student's *t*-test.

was almost twice as much as in the responsive cell line. On the other hand, ODC activity in the responsive cell line supplemented with both Arg and Orn was lower than in the control (Fig. 4B) at both time points. However, in the blocked cell line, the ODC activity was lower at 14 d of incubation with Arg, but increased with supplemental Orn (Fig. 4D).

Labeled Arg and Orn associated with PA metabolism

To better understand the PA metabolic pathways in each cell line, we measured the incorporation of radioactivity from ^{14}C -labeled precursors (i.e. $\text{L-[U-}^{14}\text{C]Arg}$ for PAs and amino acids or $\text{L-[1-}^{14}\text{C]Orn}$ for amino acids only) along with 5 mM cold Arg or Orn, at two time points (2 and 14 d after incubation). Dansyl-PAs and amino acids were separated by thin-layer chromatography (TLC), and the radioactivity associated with chromatographed spots corresponding to the three PAs (Put, Spd and Spm) and four amino acids (Arg, Orn, Cit and GABA) was measured to establish whether the ^{14}C -labeled precursors were incorporated into PAs and/or other amino acids that are products of the pathway (Supplementary Table S3). Incorporation through $\text{L-[1-}^{14}\text{C]Orn}$ was analyzed in the case of Arg and Cit, since the label from the precursor $1\text{-}^{14}\text{C}$ in Orn is lost by the action of decarboxylase enzymes. Therefore, unlike with $\text{L-[U-}^{14}\text{C]Arg}$, PA catabolism could not be studied with $\text{L-[1-}^{14}\text{C]Orn}$.

The metabolic and incorporation rate data were used to generate a schematic overview of the Arg|Orn|PA metabolic pathway for each cell line, highlighting the regulation through Arg and Orn. This analysis allowed the identification of Arg distribution (Fig. 5) and their participation in the changes observed in PA and amino acids contents, either for biosynthesis, catabolism or accumulation, and, in the case of Orn (Fig. 6), in Arg or Cit.

The two supplemented amino acids had different effects on the Arg|Orn|PA metabolic pathway in the two cell lines and at the two time points. In the responsive cell line at the 2 d time point, the distribution of ^{14}C through $\text{L-[U-}^{14}\text{C]Arg}$ among Orn, Cit and GABA [based on counts per minute (CPM) $\text{g}^{-1} \text{FW}$] was similar (Fig. 5A). $\text{L-[U-}^{14}\text{C]Arg}$ was directed towards the biosynthesis of Cit by the NO biosynthesis pathway, or via Orn, by degradation of Arg, as well as to GABA, of which an increase was also observed at this time (Figs. 1, 5A). Among the PAs, the incorporation of $\text{L-[U-}^{14}\text{C]Arg}$ was higher in Spd, followed by Put and Spm (Fig. 5A; Supplementary Table S3). In contrast, in the blocked cell line, $^{14}\text{C} \text{Arg}$ was directed towards Cit and Put (Fig. 5C). $^{14}\text{C} \text{Cit}$ was higher in the presence of Arg; however, our data suggest that conversion of Arg into Cit can occur via Orn. To support this hypothesis, we compared the labeled precursor incorporation rate with the gene expression and biochemical data (Figs. 1, 5C; Supplementary Table S3). Although *AaOTC*

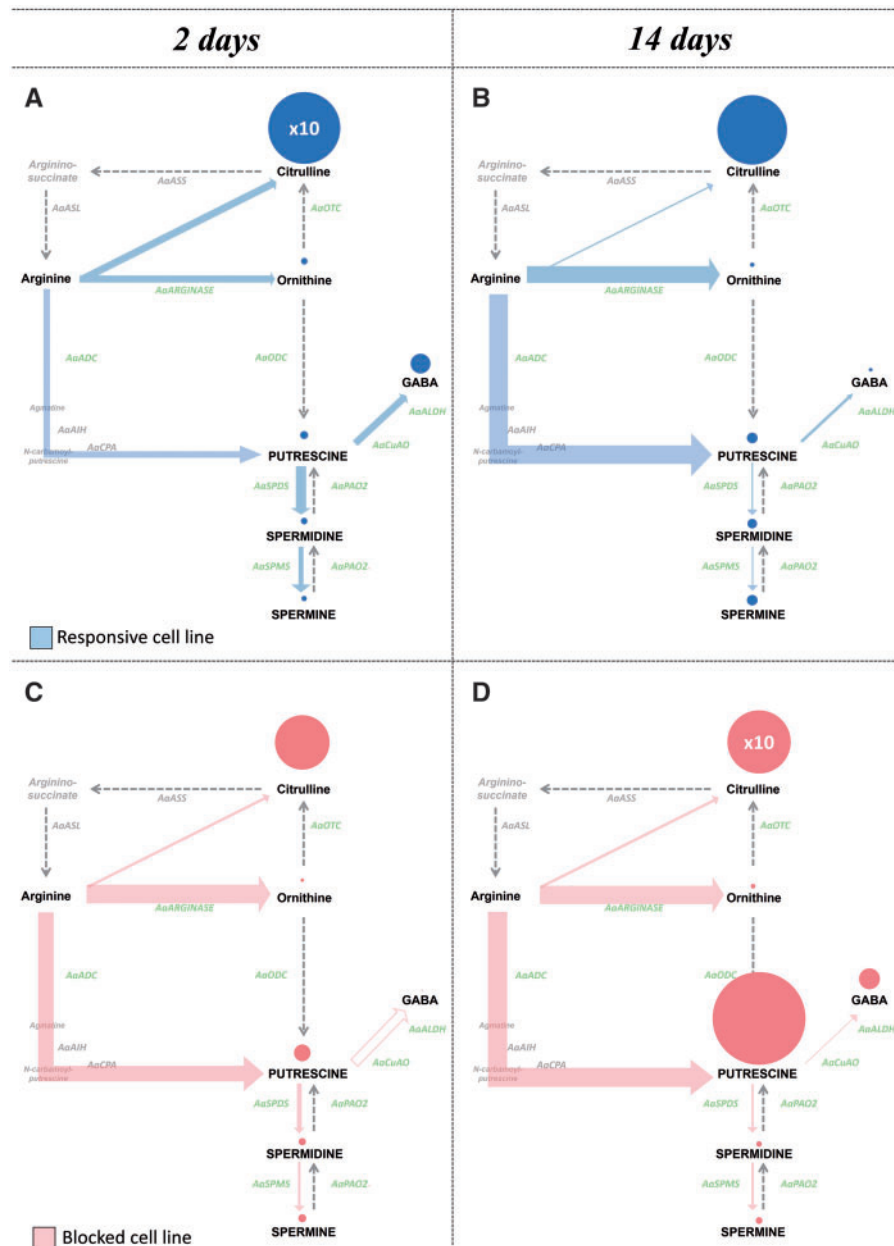


Fig. 5 Schematic overview of the changes in polyamine (PA) biosynthesis pathways after supplementation with 5 mM arginine (Arg) in the responsive (A, B) and blocked (C, D) *Araucaria angustifolia* cell lines after 2 or 14 d of incubation. The endogenous contents of amino acids and free PAs are depicted by the diameter of the circle, whereas the ¹⁴C incorporation rate through L-[U-¹⁴C]Arg is depicted by the thickness of the corresponding arrows in the pathway. The contents of amino acids and PAs are depicted proportionally to the control, as a percentage. The incorporation rate is represented by the percentage distribution of labeled precursor into PAs [counts per minute (CPM) values of putrescine + spermidine + spermine = 100%] or amino acids [CPM values of citrulline + ornithine + γ-aminobutyric acid (GABA) = 100%]. CPM values and the incorporation rates are available in Supplementary Table S3. Due to space limitations, the diameter of citrulline is shown 10-fold higher, as indicated in the figure.

expression was decreased by Arg supplementation at the 2 d time point, higher L-[U-¹⁴C]Arg incorporation was detected in Orn than in Cit, even though Orn content showed less of a change. No radioactive signal was detected in GABA from L-[U-¹⁴C]Arg in the blocked cell line (Supplementary Table S3).

After 14 d, the supplemented [¹⁴C]Arg was directed to Cit (via Orn) and Put biosynthesis in both cell lines (Fig. 5A, D). At this time, we detected greater ¹⁴C incorporation into GABA in the blocked cell line than in the responsive cell line (Fig. 5B, D).

Most of the L-[1-¹⁴C]Orn was found to be converted into Arg (Fig. 6A, C). The supplementation of Orn after 2 d increased the Arg and Cit levels, but the presence of ¹⁴C in Cit was only detected in the responsive cell line. Similar to what was observed following Arg supplementation, exogenous Orn promoted an increase in GABA levels in the responsive cell line, and in PAs in the blocked cell line. After 14 d, the two cell lines showed a similar Arg|Cit incorporation profile (Fig. 6B, D).

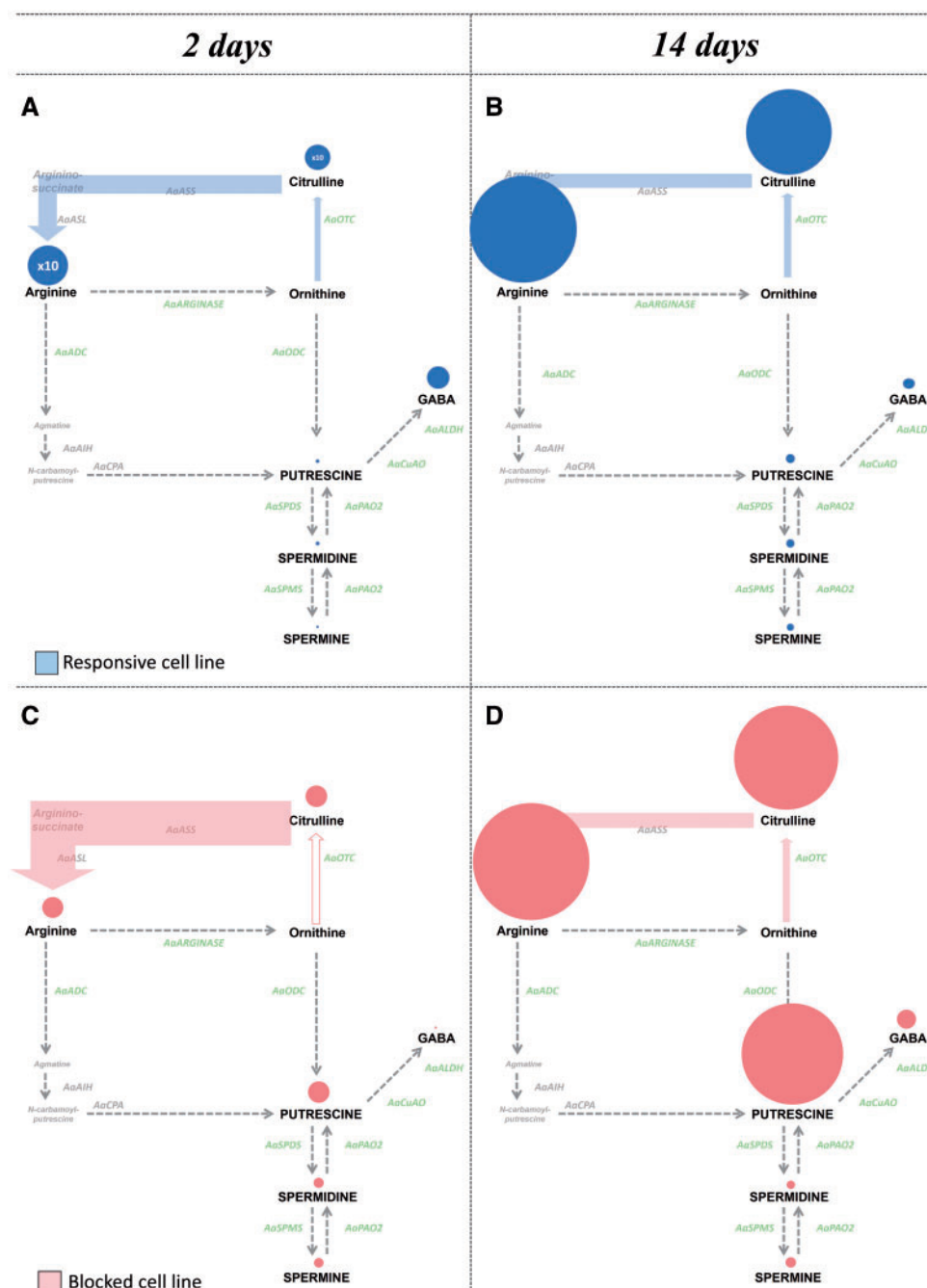


Fig. 6 Schematic overview of the changes in the polyamine (PA) biosynthesis pathways after supplementation with 5 mM ornithine (Orn) in the responsive (A, B) and blocked (C, D) *Araucaria angustifolia* cell lines, after 2 or 14 d of incubation. The endogenous amino acid and free PA contents are depicted by the diameter of the circle, whereas the ^{14}C incorporation rate through L-[1- ^{14}C]Orn (into Cit or Arg) is depicted by the thickness of the corresponding arrows in the pathway. The amino acid and PA contents are depicted proportionally to the control, as a percentage. The incorporation rate is represented by the percentage distribution of labeled precursor into amino acids [counts per minute (CPM) values of arginine + citrulline = 100%]. CPM values and the incorporation rates are available in Supplementary Table S3. Due to space limitations, the diameters of Cit and Arg are shown 10-fold higher, as indicated in the figure.

Discussion

Elucidation of the regulation of PA and amino acid metabolism in plants is of major interest due to the fundamental role they play in responses to biotic and abiotic stress, interaction with other macromolecules and pathways, and development,

including SE (Vuosku *et al.* 2012, Minocha *et al.* 2014, Muilu-Mäkela *et al.* 2015, Salo *et al.* 2016). Treatments that modify cellular PA levels, such as genetic manipulation and exogenous application of PAs or amino acids, or inhibitors of ADC and ODC activities, can help reveal the regulation of the interactive Arg|Orn|PA metabolic pathways, and offer the possibility of

studying stress response in plants and the generation of somatic embryos (Minocha et al. 1999, Minocha et al. 2004, Majumdar et al. 2016). In the present study, *A. angustifolia* cell lines with different embryogenic potential were utilized to analyze this pathway. The results showed distinct PA and amino acid profiles, and differences in the expression of genes related to the associated metabolic pathways.

The importance of Arg and Orn as precursors for Put has been well established in a variety of plant species (Bhatnagar et al. 2001, Bais and Ravinshankar 2002, Bhatnagar et al. 2002); however, relatively little is known about these pathways in non-model species, such as *A. angustifolia*. In our system, the response to supplementation with these amino acids depended on the cell growth phase. Lower Put content and Put/(Spd + Spm) ratios were observed after 2 d of culture in the medium supplemented with Arg or Orn as compared with longer term treatment for 14 d. The Put/(Spd + Spm) ratio has been correlated with embryogenic development in this species, and is considered to be a biochemical marker of the developmental stage that changes with cell division and elongation (Minocha et al. 1999, Minocha et al. 2004, Silveira et al. 2004). The maximum difference in Put content was observed after 14 d of culture, which is the period of the exponential growth phase of these cell lines (Silveira et al. 2006). Several studies have demonstrated a relationship between Put levels and a high cell division rate, consistent with a role for Put in the cell proliferation phase, while Spd and Spm have been more associated with cell differentiation (Minocha et al. 1999, Niemi et al. 2002, Silveira et al. 2006, Carone et al. 2010, Vuosku et al. 2012). In *A. angustifolia* cell lines, the increase in Put content was higher in the blocked cell line (that has no embryogenic potential) than in the responsive cell line (high embryogenic potential). It has previously been shown that distinct cell lines can show different PA profiles, which can also be associated with embryogenic potential (Jo et al. 2014).

In embryogenic cultures of *A. angustifolia*, Put is reported to be the predominant PA, followed by Spd and Spm (Silveira et al. 2006, Jo et al. 2014). In the present study, the conversion of Put into Spd or Spm constituted only a small fraction of the total Put content in the cells. Some of this increase in Put may have come from reverse conversion of Spm to Spd to Put, since an increase in AaPAO2 expression in the responsive cell line was observed after 2 d with Arg supplementation. This condition was not observed in the blocked cell line at this time point, which differentiates the two cell lines.

There is no significant increase of ADC or ODC activities by the addition of amino acids, except by the supplementation of Orn in the blocked cell line, which increased the ODC activity after 14 d. These results suggest that: (i) an inhibition of the enzymes by the increased Put levels, via feedback inhibition of the enzyme product; or (ii) the increase in Put occurred as a result of Spd catabolism, reflected in an increase in AaPAO2 expression. It is known that ADC is the prime regulatory enzyme of Put biosynthesis in zygotic embryogenesis and/or SE in *P. sylvestris* (Minocha et al. 2004, Vuosku et al. 2006, Gemperlová et al. 2009, Vuosku et al. 2012). In *A. angustifolia*, both ADC activity and AaADC expression are important for Put

biosynthesis during zygotic embryo development (de Oliveira et al. 2017). In the present study, using direct measurement of enzyme activity, we observed that ODC was the main pathway for Put biosynthesis during *A. angustifolia* cell proliferation. However, exogenous addition of Arg and Orn to proliferating cell lines promoted differential expression of AaADC, while AaODC transcripts were below the cut-off detection threshold, as was also seen earlier for zygotic embryos of *A. angustifolia* (de Oliveira et al. 2017). The lack of correlation between PA contents, enzymatic activity and transcript levels may be a consequence of complex post-transcriptional and metabolic regulation of this pathway (Carbonell and Blázquez 2009, Page et al. 2012, Majumdar et al. 2016, Wuddineh et al. 2018).

It has been reported that the co-existence of ADC and ODC in the Put biosynthetic pathway may relate to their differential contribution to stress responses, development processes and tissue specificity (Tiburcio et al. 1997, Vuosku et al. 2006, de Oliveira et al. 2017); however, a specific role for either of the two enzymes in embryogenesis has yet to be established. It has been implied that ODC is particularly active in cell proliferation, whereas ADC is involved in embryo and organ differentiation and stress response (Kevers et al. 2000, Vuosku et al. 2006). Our data suggest that the *A. angustifolia* cell lines preferably use the ODC pathway for Put biosynthesis during embryogenic cell growth (de Oliveira et al. 2015).

The changes in the expression profiles of PA catabolism genes in response to supplementation with Arg/Orn, which were more active in the responsive cell line than in the blocked line, indicate that these responses may be associated with their embryogenic potential in *A. angustifolia*. This is an important point to consider for future studies, since PA oxidation by CuAOs and PAOs contributes to the regulation of PA homeostasis, thereby generating catabolic products, which have been linked to several other biological functions of PAs (Cona et al. 2006, Angelini et al. 2010, Moschou et al. 2012). For example, hydrogen peroxide (H₂O₂), a product of PA catabolism (Moschou et al. 2012), is an important signaling molecule during oxidative metabolism, and associated with the responsive cell line in *A. angustifolia* (Jo et al. 2014). Whether these responses can actually regulate the embryogenic potential of these two cell lines would need to be tested in future studies.

Another Put product, i.e. GABA, is generated by the actions of CuAO and ALDH (Majumdar et al. 2016, Page et al. 2016). An alternative pathway for GABA biosynthesis, that has been well characterized by Shelp's group, is via direct decarboxylation of glutamate by glutamate decarboxylase. Its metabolism in plants is complex, since various associated enzymes are spatially compartmentalized in the cell (Shelp et al. 2012). Moreover, it is not known whether GABA biosynthesis and catabolism are regulated at the transcriptional level or post-transcriptionally (Majumdar et al. 2016). While the importance of GABA during embryo development has been suggested earlier (Aragão et al. 2015, de Oliveira et al. 2017), the relative contribution of its metabolism in maintaining PA homeostasis in plants is not known (Majumdar et al. 2016).

Our results using radiolabeled Arg reveal that Put catabolism leading to GABA formation is different in the two *A. angustifolia*

cell lines when grown in the presence of exogenous Arg or Orn. In the responsive cell line after 2 d of supplementation, a portion of the pool of Arg was directed to GABA biosynthesis, whose content also increased, followed by an increase in *AaCuAO* and *AaALDH* expression. In contrast, in the blocked cell line, Arg supplementation resulted in a decrease both in cellular GABA contents and in *AaCuAO* and *AaALDH* expression, indicating that PA catabolism was not activated by this precursor. At 14 d of growth, *AaCuAO* and *AaALDH* expression and the GABA content were lower in both cell lines, compared with 2 d treatment. These data suggest that a possible signaling as a result of PA catabolism occurs mostly after 2 d of Arg supplementation.

In addition to being direct precursors for PAs in *A. angustifolia*, Arg and Orn also interact with other amino acid pathways. In most land plants, Arg can be converted into Orn, by arginase activity, and then utilized by ODC in Put biosynthesis (Bais and Ravinshankar 2002). In the present study, Arg conversion to Orn was observed by calculating the incorporation of [^{14}C]Arg into Orn; however, minimal changes were detected in [^{14}C]Orn levels in both cell lines, which is consistent with studies showing that Orn can act as a regulatory molecule, and that its levels tend to remain stable (Majumdar *et al.* 2013).

The cell lines used in this work had higher Orn than Arg levels, a similar profile to that observed in *A. angustifolia* zygotic embryos (de Oliveira *et al.* 2017). Addition of Orn to the culture medium resulted in its conversion to Arg, via Cit and argininosuccinate, and an increase in Arg levels. Biochemical and labeled precursor incorporation data indicated that: (i) this conversion is higher in the responsive than in the blocked cell line after 2 d of supplementation with exogenous Orn; (ii) after 14 d, both cell lines showed similar profiles in this pathway, with a higher accumulation of Arg and Cit. Interestingly, *AaARGINASE* did not differ significantly between the two cell lines when grown in the control medium but was affected differently in response to Arg or Orn supplementation. The presence of additional Orn may have caused an increase in *AaARGINASE* expression in the blocked cell line after 14 d because of additional Arg being formed from Orn (also supported by the incorporation of [^{14}C]Orn into Arg). A similar response (i.e. higher *AaARGINASE* expression) in the responsive cell line after 2 d in the presence of Arg may be due to an increase in its uptake.

Cit is an intermediate product in NO biosynthesis, but it can also be synthesized from Orn, through the action of OTC (Page *et al.* 2012, Majumdar *et al.* 2016). It has been suggested that Cit can act as a hydroxyl radical scavenger and a strong antioxidant as well as a source of nitrogen; its levels are associated with drought tolerance (Akashi *et al.* 2001, Slocum 2005, Kusvuran *et al.* 2013). The direct increase in Cit content as a result of Arg supplementation is particularly interesting, since the oxidation of Arg also produces NO (Crawford 2006, Flores *et al.* 2008). The importance of NO production for embryo development in association with the maintenance of polarity (embryonic-suspensor cells) in pro-embryogenic masses in *A. angustifolia* has been described earlier (Silveira *et al.* 2006). Furthermore, NO is biochemically related to PA metabolism through Arg, a common precursor in this biosynthetic route. Thus, alteration in NO

homeostasis may affect PA bioavailability and vice versa, through an as yet uncharacterized mechanism (Silveira *et al.* 2006, Tun *et al.* 2006, Filippou *et al.* 2013, Tanou *et al.* 2014). The overlapping roles of PAs and NO raise the question of their mechanisms of interaction during plant development (Silveira *et al.* 2006, Tun *et al.* 2006). Based on our findings, it would be interesting to study this interaction in species showing poor SE responses, by regulating NO biosynthesis and Cit levels using Arg, with the goal of optimizing in vitro somatic embryo development. Importantly, the increased Cit content observed after 2 d of Arg supplementation were in the range of those reported in *A. angustifolia* zygotic embryos (de Oliveira *et al.* 2017), and so probably represent physiologically relevant conditions.

It has been proposed that Orn may not only be a key regulator of PA biosynthesis, but may also regulate the inter-related pathways involving glutamate conversion to Arg and proline (Page *et al.* 2007, Page *et al.* 2012, Majumdar *et al.* 2013, Majumdar *et al.* 2016, Wuddineh *et al.* 2018). However, Arg is also known to be an essential metabolite involved in nitrogen distribution (Silveira *et al.* 2006, Tun *et al.* 2006, Flores *et al.* 2008, Brauc *et al.* 2012, Shi *et al.* 2013, Winter *et al.* 2015). The results presented here on Orn supplementation are consistent with the suggested regulatory roles of Orn on PA accumulation and the Arg|Orn|Cit pathway. However, Arg supplementation had different effects on the two cell lines: the activation of PA catabolism in the responsive cell line leading to an increase in GABA content and the expression of related genes, and promotion of the accumulation of PAs in the blocked cell line. The effects of Arg supplementation were mainly seen after 2 d of culture. Thus, it can be proposed further that both Orn and Arg are important regulators of the Arg|Orn|Cit|PA biosynthetic pathway, perhaps depending upon their embryogenic capacity.

Conclusions

Our study provides new insight into the Arg|Orn|PA metabolic pathway in two cell lines with contrasting embryogenic potential. The present study demonstrates a potential regulation of this pathway through supplementation of Arg and Orn in the medium, providing an opportunity for unraveling their complexity as well as laying the foundation for further dissection of the cross-talk patterns between the PA pathway and the embryogenic capacity in conifers. The supplementation with Arg or Orn revealed changes in both biosynthesis and catabolism of PAs, by changing the contents of PA and amino acids and gene expression profiles. While Arg promoted PA catabolism and an increase in GABA as well as Cit content, Orn, on the other hand, had more effect in PA biosynthesis. Our study also revealed that the two distinct cell lines are different in relation to PA biosynthesis and catabolism: a high activity in PA catabolism was detected in the responsive cell line, whereas in the blocked cell line we observed an accumulation of PAs. These conclusions together may lead to the design of growth conditions for cell lines to enhance their somatic embryo development potential.

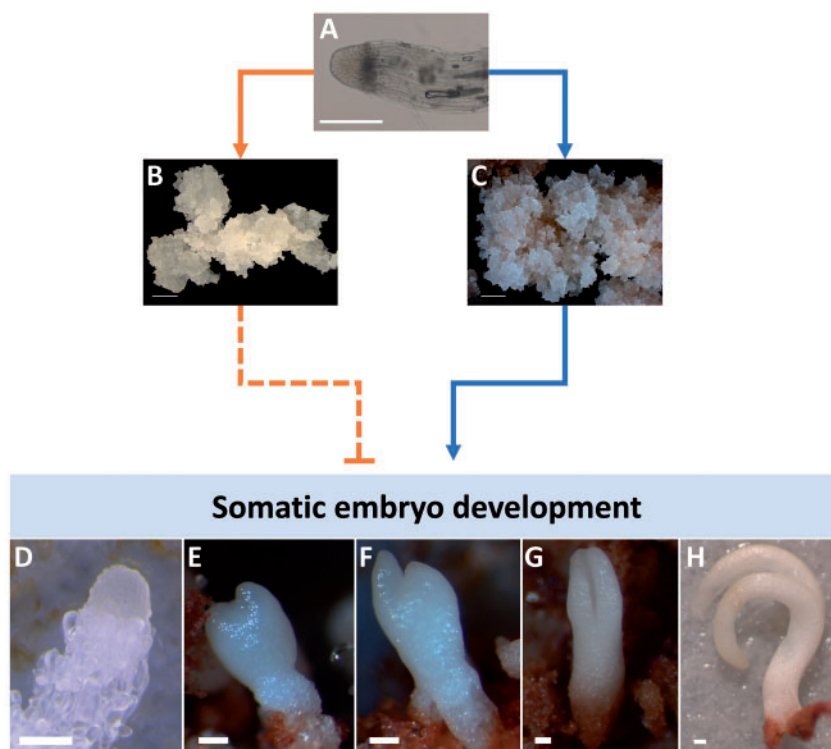


Fig. 7 Somatic embryogenesis (SE) of *Araucaria angustifolia*. (A) Immature zygotic embryo used as explant; (B) blocked cell line; (C) responsive cell line; (D) globular somatic embryo; (E–H) development of cotyledonary somatic embryo. Scale bar: (A), (D–H) = 200 μm , (B, C) = 2,000 μm .

Materials and Methods

Plant material and experimental conditions

Two *A. angustifolia* embryogenic cell lines induced (dos Santos et al. 2008) from zygotic embryos (Fig. 7A) were used in this study. Cell lines were selected as described by Jo et al. (2014), based on their different responses under maturation conditions [MSG medium (Becwar et al. 1989) supplemented with 6% (w/v) sucrose, 1.46 g l⁻¹ L-glutamine, 0.15% (w/v) activated charcoal, 1% (w/v) Gelrite and 240 μM ABA]. The selection resulted in lines that were: (i) blocked, i.e. cells were incapable of developing somatic embryos in the maturation medium (Fig. 7B); or (ii) responsive, i.e. cells were capable of producing cotyledonary embryos in the maturation medium (Fig. 7C–H). Although the two cell lines have different embryogenic potential, they have similar growth parameters such as fresh weight and dry weight. In addition, both cell lines are similar in reaching the lag, exponential, linear and stationary phases at the same time after transfer to fresh medium (Supplementary Fig. S1).

Two-week-old cultures growing on a semi-solid MSG medium, pH 5.8 containing 1.46 g l⁻¹ L-glutamine, 3% (w/v) sucrose were used for experimentation. Approximately 100 mg (FW) of each cell line were dissected into small pieces and transferred to six-well plates (Techno Plastic Products), containing 5 ml of liquid MSG medium per well (as described above, but without Gelrite®), with or without 5 mM Arg or Orn (Sigma-Aldrich). The experiment was carried out during the proliferation phase of the embryogenic cultures.

For incorporation of labeled precursors, 0.25 μCi of either L-[U-¹⁴C]Arg (specific activity 274.0 mCi mmol⁻¹, PerkinElmer) or L-[1-¹⁴C]Orn (specific activity 57.1 mCi mmol⁻¹, PerkinElmer), along with 5 mM (final concentration) of cold Arg or Orn, were added to each well.

The suspension cultures were grown in the dark, at 25 $\pm$ 1 °C, on a gyratory shaker at 110 r.p.m. They were collected into 15 ml conical tubes, after 2 and 14 d, representing the lag and exponential phase, respectively, for both cell lines. The cells were pelleted by centrifugation (11,000 $\times$ g) for 5 min at room temperature. The supernatant was discarded, and the pellets were washed three times with 2 mM cold Arg or Orn, followed by three washes with distilled water, with additional centrifugation after each wash. The pellets were weighed, frozen

in liquid nitrogen and stored at -80 °C for biochemical analysis, as described below.

Determination of free amino acids

The amino acid content was determined according to the protocol described by Santa-Catarina et al. (2006). A 100 mg (FW) aliquot of cells was homogenized in an ice-cold mortar with liquid nitrogen, mixed in 3 ml of 80% (v/v) ethanol and concentrated in a Speed-Vac. The samples were re-suspended in 1 ml of Milli-Q water, and centrifuged at 11,000 $\times$ g for 10 min. The supernatant was filtered through a 20 μm membrane (Sartorius Stedim Biotech). Amino acids were derivatized with o-phthalaldehyde and separated by HPLC (Shimadzu) on a C₁₈ reverse-phase column (5 μm $\times$ 4.6 mm $\times$ 250 mm, Supelcosil LC-18, Sigma-Aldrich). The gradient was developed by mixing proportions of 65% methanol with a buffer solution (50 mM sodium acetate, 50 mM sodium phosphate, 20 ml l⁻¹ methanol, 20 ml l⁻¹ tetrahydrofuran and adjusted to pH 8.1 with acetic acid). The 65% methanol gradient was set to 20% during the first 32 min, from 20% to 100% between 32 and 71 min, and 100% between 71 and 80 min, with a flow rate of 1 ml min⁻¹ at 40 °C. Detection and quantification were performed using a fluorescence detector (RF-20 A, Shimadzu), set at 250 nm excitation and 480 nm emission wavelengths.

Analysis of free PAs

Extraction of free PAs was performed according to Bhatnagar et al. (2001). Samples were mixed with cold 5% (v/v) perchloric acid at a ratio of 1:4 (w/v; 100 mg FW of tissue in 400 μl of perchloric acid) and stored at -20 °C until PA analysis. Prior to derivatization, the samples were subjected to three cycles of freezing (-20 °C) and thawing (at room temperature), prior to centrifugation at 11,000 $\times$ g for 10 min, and supernatant collection.

Derivatization of free PAs was performed according to Silveira et al. (2004). A 40 μl aliquot of plant extract was added to 100 μl of dansylchloride (5 mg ml⁻¹ in acetone), 20 μl of 0.05 mM diaminoheptane (internal standard) and 50 μl of saturated sodium carbonate. After 50 min incubation in the dark at 70 °C, the excess dansylchloride was converted to dansylalanine by adding 25 μl of alanine (100 mg ml⁻¹). After 30 min incubation (room temperature),

dansylated PAs were extracted with 200 μ l of toluene. The toluene phase was collected and dried in a Speed-Vac at 45°C. Dansylated PAs were dissolved in 200 μ l of acetonitrile.

PAs were separated by HPLC using a C₁₈ reversed-phase column (as described above). The gradient was developed by mixing increasing proportions of absolute acetonitrile with 10% acetonitrile in water (pH 3.5). The gradient of absolute acetonitrile was set to 0–65% for the first 10 min, 65–100% from 10 to 13 min, and at 100% from 13 min to the final 21 min, at a flow rate of 1 ml min^{−1} at 40°C. PAs were detected at 340 nm (excitation) and 510 nm (emission) wavelengths with an RF-20 A fluorescence detector (Shimadzu).

Analysis of labeled precursor incorporation

Dansylated PAs (10 μ l in acetonitrile) from L-[U-¹⁴C]Arg-treated samples (samples treated with L-[1-¹⁴C]Orn were not analyzed because L-¹⁴C from Orn is released as ¹⁴CO₂, leaving no radioactive PA) were spotted onto 20×20 cm TLC plates (silica gel 60, Merck KGaA). Plate development was performed in a solvent mix of chloroform:triethylamine [3:1 (v/v)] in a glass chromatograph chamber (Bhatnagar *et al.* 2001). When the solvent front had shifted 15 cm from the origin, the plates were air-dried and the respective PA bands were marked under UV light and collected for quantification of radioactivity.

L-[U-¹⁴C]Arg and L-[1-¹⁴C]Orn incorporation into other amino acids related to the PA biosynthetic pathway was assayed by applying 20 μ l of amino acid extract to TLC plates, and resolution in a solvent mix of *n*-butanol:acetic acid:water (4:1:1 by vol.). When the solvent front had shifted 15 cm from the origin, the plates were air-dried and the spots corresponding to Orn, Arg, Cit and GABA (from L-[U-¹⁴C]Arg) and to Arg and Cit (from L-[1-¹⁴C]Orn) were visualized by spraying with 1% (w/v) ninhydrin in a 100 ml acetone solution, followed by heating to 90°C for 5–7 min to ensure plateau intensity of the colored complex.

PA and amino acid bands were collected and immersed in 1 ml of scintillation fluid (PerkinElmer). Radioactivity counting was performed with a Tri-Carb2910TR-PerkinElmer scintillation counter, and expressed as CPM g^{−1} FW. The percentage of L-[U-¹⁴C]Args incorporation into each PA (i.e. Put, Spd and Spm) was calculated as the fraction of the sum of radioactivity present in all three PAs (100%). The analysis was performed with three biological replicates.

Activity of ADC and ODC

Enzyme activities of ADC and ODC were determined according to de Oliveira *et al.* (2017). Tissue samples were homogenized in an ice-cold mortar with liquid nitrogen, and 50 mg (FW) of tissue was transferred to 50 μ l of extraction buffer (50 mM Tris–HCl, pH 8.5, 0.5 mM pyridoxal-5-phosphate, 0.1 mM EDTA and 5 mM dithiothreitol). The solution was vortexed and centrifuged (13,000×g for 20 min at 4°C), and the supernatant used for ADC and ODC enzymatic assays. A reaction mixture containing 50 μ l of protein extract, 8.3 μ l of extraction buffer, 12 mM unlabeled L-Arg or L-Orn, and 25 nCi of either L-[U-¹⁴C]Arg (specific activity 274.0 mCi mmol^{−1}, PerkinElmer), or L-[1-¹⁴C]Orn (specific activity 57.1 mCi mmol^{−1}, PerkinElmer) was used. Blank samples contained only 50 μ l of extraction buffer. Reaction mixtures were incubated in glass tubes fitted with rubber stoppers and filter paper discs soaked in 2 N KOH. The material was maintained at 37°C and 120 r.p.m. (orbital shaker) for 90 min. The reaction was stopped by adding 200 μ l of perchloric acid, followed by further incubation for 15 min under the same conditions. Filter paper containing ¹⁴CO₂ was immersed in 1 ml of scintillation fluid (PerkinElmer). Radioactivity was then measured using a scintillation counter (Tri-Carb2910TR, PerkinElmer). The activities were expressed as pmol g^{−1} FW h^{−1} of CO₂ released.

Quantitative RT-PCR analysis

The ReliaPrepTM RNA Cell Miniprep System kit (Promega) was used for RNA extraction. cDNA synthesis, primer design and qRT-PCR analysis were performed according to Elbl *et al.* (2015). Gene-specific primers (Supplementary Table S4) used in the qRT-PCR assay were designed using the OligoAnalyzer 3.1 software (<http://www.idtdna.com/calc/analyser>) according to Minimum Information for Publication of qRT-PCR Experiments (MIQE) guidelines (Bustin *et al.* 2009). Quantification cycle (Cq) values from two technical replicates and primer efficiency were calculated using the LinRegPCR software (Ruijter *et al.* 2009). Target gene expression values were normalized against

geometric averages of the *AaEF-1 α* (elongation factor 1 α) and *AaEIF4B-L* (translational initiation factor 4B) reference genes (Elbl *et al.* 2015). Calculations of gene relative expression were based on average expression levels in the control samples, and are presented as log₂ fold changes.

Statistical analysis

Metabolites and gene expression data were analyzed by analysis of variance (ANOVA), followed by Tukey's test ($P < 0.01$) and log transformed when appropriate. Pairwise comparisons between the cell lines were analyzed by a Student's *t*-test ($P < 0.01$). Heatmap graphs were created using the heatmap.2 package. Statistical analyses were performed with the BioEstat (Version 5.0) software and 'R' (version 3.2.2, available in <http://cran.r-project.org/>). The number of replicates (*n*) for each experiment are given in the figure legends.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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