Grassland species differentially regulate proline concentrations under future climate conditions: an integrated biochemical and modelling approach

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Summary

• Proline (Pro) is a versatile metabolite playing a role in the protection of plants against environmental stresses. To gain a deeper understanding of the regulation of Pro metabolism under predicted future climate conditions, including drought stress, elevated temperature and CO₂, we combined measurements in contrasting grassland species (two grasses and two legumes) at multiple organisational levels, that is, metabolite concentrations, enzyme activities and gene expression.
• Drought stress (D) activates Pro biosynthesis and represses its catabolism, and elevated temperature (DT) further elevated its content. Elevated CO₂ attenuated the DT effect on Pro accumulation.
• Computational pathway control analysis allowed a mechanistic understanding of the regulatory changes in Pro metabolism. This analysis indicates that the experimentally observed coregulation of multiple enzymes is more effective in modulating Pro concentrations than regulation of a single step. Pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) play a central role in grasses (Lotus corniculatus, Poa pratensis), and arginase (ARG), ornithine aminotransferase (OAT) and P5CR play a central role in legumes (Medicago lupulina, Lotus corniculatus).
• Different strategies in the regulation of Pro concentrations under stress conditions were observed. In grasses the glutamate pathway is activated predominantly, and in the legumes the ornithine pathway, possibly related to differences in N-nutritional status.

Introduction

Proline (Pro) is an essential proteinogenic amino acid, and is also known as a stress defence molecule. It is a compatible solute that adjusts cellular osmotic potential, protects membranes and proteins, stabilizes photosystem II and protects plants against oxidative damage (Szabados & Savouré, 2010). Pro metabolism has a regulatory function in cell homeostasis and survival (Phang, 1985; Liang et al., 2013). An increased rate of Pro biosynthesis can help to maintain higher NADP⁺: NADPH ratios and stabilize the redox balance (Hare & Cress, 1997; Szabados & Savouré, 2010). Other proposed functions of Pro include storage and transfer of energy (Abraham et al., 2003; Szabados & Savouré, 2010; Verslues & Sharma, 2010). Taken together, it is a remarkably multifunctional molecule involved in plant stress defence.

The earth’s current, anthropogenic climate change is manifested by increases in atmospheric CO₂ and temperature and more frequent heat waves and drought spells (IPCC, 2012). It is becoming increasingly clear that elevated CO₂ may modify the response of plants to environmental stresses, in particular by reducing stress impact and altering plant metabolism (Ainsworth & Rogers, 2007; Geissler et al., 2009; Li et al., 2014; Naudts et al., 2014; Zinta et al., 2014; Pandey et al., 2015). However, the molecular mechanisms underlying these effects are still largely unclear (Feng et al., 2014). In previous work we showed that elevated CO₂ alleviated photosynthesis inhibition under drought stress conditions, and reduced stress impact through reducing photorespiration and formation of hydrogen peroxide (H₂O₂; AbdElgawad et al., 2015).

Given the importance of Pro in plant stress, understanding the regulatory mechanisms of Pro accumulation is fundamental to our understanding of plant responses to global changes, and
potentially useful in making crops stress-tolerant. The glutamate (Glu) and ornithine (Orn) pathways can independently feed Pro synthesis (Fig. 1), and both can play a role in Pro accumulation during stress conditions (Hu et al., 1992; Roosens et al., 1999). In the Glu pathway, glutamine (Gln) is converted to Glu by glutamine synthetase (GS), reduced to glutamate-5-semialdehyde (GSA) by pyrroline-5-carboxylate synthetase (P5CS), which spontaneously converts to pyrroline-5-carboxylate (P5C) (Hu et al., 1992; Savoure et al., 1995). P5C is reduced to Pro by pyrroline-5-carboxylate reductase (P5CR) (Szoke et al., 1992; Verbruggen et al., 1993), a common enzyme in both pathways. In most plant species, P5CS is encoded by two genes (P5CS1 and P5CS2), whereas P5CR is encoded by a single isoform (Verbruggen et al., 1993; Armengaud et al., 2004). Yoshida et al. (1997); Huang et al. (2013), suggested that the P5CS-mediated reaction is a rate-controlling step in Pro synthesis and consistently it was reported as the predominant enzyme leading to Pro accumulation in drought-stressed plants (Kim & Nam, 2013). P5CR is an important enzyme for Pro synthesis and has a critical role in cycling Pro and P5C between cellular compartments (Phang, 1985; Miller et al., 2009). P5C produced in the mitochondria, can be transported into the cytosol and be re-reduced to Pro by cytosolic P5CR (Szabados & Savouré, 2010).

As an alternative pathway, Pro can be synthesized from Orn, which is generated from arginine by arginase (ARG), and transaminated to P5C by ornithine-δ-amino transferase (OAT) (Roosens et al., 1999; Yang et al., 2009). OAT is considered to control this pathway (Verbruggen & Hermans, 2008; Szabados & Savouré, 2010; Huang et al., 2013). Several studies have demonstrated a role of OAT in stress tolerance, and in regulating the plant cell redox homeostasis through modulating Pro metabolism (Delaney et al., 1993; Yamada et al., 2005).

Regulation of Pro content is also controlled by Pro catabolism. This occurs in mitochondria where Pro dehydrogenase (ProDH) generates P5C, which is converted by pyrroline-5-carboxylate dehydrogenase (P5CDH) to Glu. P5CS and OAT, ProDH is suppressed under stressful conditions, preventing Pro degradation (Kiyosue et al., 1996; Verbruggen et al., 1996). On the other hand, ProDH is induced upon recovery from stress (Kiyosue et al., 1996) or under normal growth conditions by applying high concentrations of Pro (Kishor et al., 2005).

In order to identify the reactions controlling Pro concentrations, and to clarify its physiological role, several studies have analyzed the effect of modifications in the Pro pathway in transgenic plants. For example, overexpression of P5CS or P5CR increased Pro content, which in turn stimulated plant growth under stress (Kishor et al., 1995; Hong et al., 2000; De Ronde et al., 2004; Hmida-Sayari et al., 2005). Higher Pro content and elevated stress tolerance were also achieved by antisense inhibition of ProDH transcription (Mani et al., 2002; Nanjo et al., 2003) and overexpression of OAT genes (Roosens et al., 2002; Kishor et al., 2005; Wu et al., 2005). Although genetic manipulations of enzyme activity are a powerful way to demonstrate the functioning of a pathway, they are limited to model species that can be genetically altered, or for which specific mutants are available. Computational modeling of metabolite data and enzyme activities possibly provides a more generally applicable, quantitatively measured in this study.

![Fig. 1 General overview of proline (Pro) metabolism. GDH, NADH-glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; GK, glutamate 5-kinase; P5C, α1-pyrroline-5-carboxylate; P5CS, P5C synthase; P5CDH, pyrroline-5-carboxylate dehydrogenase; P5CR, P5C reductase; ProDH, proline dehydrogenase; ARG, arginase; OAT, ornithine-δ-amino transferase. The interconversion between P5C and glutamate-5-semialdehyde (GSA) is spontaneous. Highlighted are parameters](image-url)
alternative way to elucidate Pro regulation in response to various conditions.

Predicting the effect of future climate conditions on Pro metabolism is complicated by the multitude of environmental variables involved. For instance, Pro accumulated in *Arabidopsis* plants in response to drought, but it did not respond to combined drought and heat stress (Rizhsky et al., 2004). Similarly, drought and UV radiation increased Pro content in durum wheat, but not in the presence of elevated CO2 (Balouchi et al., 2009). Because the effects of combined perturbations of different environmental factors are not always additive, it is necessary to investigate their interaction and combined impact to understand the effect of future climate scenarios (Rizhsky et al., 2004; Miller et al., 2009). The extent of Pro accumulation also varies among plant species (Maggio et al., 2002; Reddy et al., 2004; Kishor et al., 2005). For instance, *Thellungiella halophila* accumulates two- to three-fold more Pro than observed in *Arabidopsis thaliana* under control and salt stress (Kant et al., 2006). It is therefore important to study changes in Pro synthesis in various species under future climate conditions, including agronomically and ecologically relevant crops.

In order to gain a deeper mechanistic understanding of the regulation of Pro metabolism under drought stress in future climate scenarios, we performed measurements at multiple organisational levels, and combined the experimental data with a pathway-control computational modelling approach. We compare responses and regulatory strategies in species from two agronomically important plant families – grasses and legumes.

Materials and Methods

Experimental setup and plant harvest

A mesocosm experiment was conducted at the Drie Eiken Campus of Antwerp University, Belgium. Temperate grassland species – two grasses (*Lolium perenne* L., *Poa pratensis* L.) and two N-fixing legumes (*Medicago lupulina* L., *Lotus corniculatus* L.) – were chosen, based on their general occurrence, CO2 sensitivity (*C3* species), small size and common soil requirements (all seeds from Herbiseed, Twyford, UK). Plants were grown for 4 months in 16 sunlit, temperature and CO2-controlled chambers. Each chamber contained two populations (nine individuals) of each species, grown in PVC tubes (19 cm diameter, 40 cm height) with sandy soil (96% sand, pH 7.6). The soil initially contained 1.3% carbon, 19 mg nitrate-nitrogen (N), 1.1 mg ammonium-N, 13 mg phosphorus (P) km⁻³ air dry soil (De Boeck et al., 2011), and was not additionally fertilized. Nodules were present (but not quantified) on the legume roots, indicating active N fixation. Previous work using identical substrates and species, showed that soil N content was hardly affected over the time of the experiment (Van den Berge et al., 2011).

As the primary aim of the study was to investigate the effect of CO2 on the water deficit stress and elevated temperature, and not the CO2 effect by itself, and because of space limitations, we opted for an incremental design of climate conditions maintaining sufficient (four) replicates. Climate conditions and treatments were: A, current climate, with ambient air temperature (*T*air) and CO2 and sufficient water; D, drought stress in ambient climate; DT, drought stress in a warming climate (*T*air + 3°C); and DTC, drought stress in a future climate (*T*air + 3°C, and elevated CO2 at 615 ± 81 ppm). The climate scenarios were chosen according to the IPCC-SRES B2-scenario prediction of moderate change for the year 2100 (Murray & Ebi, 2012). For more details about microclimate parameters/growth conditions, see AbdElgawad et al. (2014, 2015).

Drought stress was induced by withdrawal of irrigation, at 122 days after sowing, when plants of all species were already flowering. Averageground biomass (FW, 4 cm above soil surface) was harvested at the onset of visual stress symptoms (leaf discoloration, wilting and dehydration) in 50% of the population. At the same time, monitoring the reduction in photosynthesis rates and stomatal conductance demonstrated that plants experienced considerable and similar stress levels (AbdElgawad et al., 2014). Harvest time was therefore 1 wk in *M. lupulina* and *L. corniculatus*, 2 wk in *L. perenne* and 3 wk in *P. pratensis*. Plant material was frozen into liquid nitrogen, immediately after harvest, and stored at −80°C until analysis.

Amino acid measurements

Amino acids were extracted by homogenizing plant shoots (200 mg FW) in 1 ml of 80% (v/v) aqueous ethanol (MagNALyser; Roche, Vilvoorde, Belgium; 1 min, 7000 rpm), spiked with norvaline to estimate the loss of amino acids during extraction, and centrifugation at 20 000 g for 20 min. The supernatant was vacuum-evaporated, and the pellet resuspended in 1 ml of chloroform. The plant residue was re-extracted with 1 ml HPLC grade water using MagNALyser and the supernatant after centrifugation (20 000 g for 20 min) was mixed with the pellet suspended in chloroform. Then the extracts were centrifuged for 10 min at 20 000 g and the aqueous phase was filtered by Millipore microfilters (0.2-μm pore size) before assayng amino acid concentrations. Amino acids were measured by using a Waters Acquity UPLC-qtd system (Milford, MA, USA) equipped with a BEH amide 2.1 × 50 column (Sinha et al., 2013).

Enzyme activity assays

Enzyme activities were measured according to described procedures, without modifications. All measurements were scaled down for semi-high throughput analysis using a microplate reader (Synergy Mx; Biotek Instruments Inc., Winooski, VT, USA). Assays were optimized to obtain linear time and protein-concentration dependence. In short, the methods were as follows. For GS (EC: 6.3.1.2), PS5CS, PS5CR (EC: 1.5.1.2) and ProDH (EC: 1.5.99.8), tissue was extracted (100 mg ml⁻¹ 50 mM TrishCl, pH 7.4, 2% (w/v) polyvinylpyrrolidone, 4 mM DTT, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol and 2 mM PMSF) (Zhang et al., 1995; Temple et al., 1996; Lutts et al., 1999) in a MagNALyser (Roche, 3 × 20 s). GS activity was determined in Tris-acetate reaction buffer (Tris-acetate, 200 mM, pH 6.4), monitoring the accumulation of γ-glutamyl hydroxamate (*A*₅₀₀, Temple et al., 1996). PS5CS activity (assayed in 50 mM tris-HCl}
RNA extraction and quantitative (Q)-PCR

For total RNA extraction, _L. perenne_ and _M. lupulina_ shoot material was homogenized (MagNaLyser) and RNA was extracted from the homogenates using the RNeasy Plant Mini Kit (Qiagen). The RNA quantity and integrity was measured using a high resolution gel cartridge on a QIAxcel platform (Qiagen). A starting amount of 1 µg RNA was transcribed to first-strand cDNA (Maxima<sup>®</sup> First Strand cDNA Synthesis Kit; Fermentas, Hinxton, UK). mRNA expression in _L. perenne_ and _M. lupulina_ shoots exposed to D, DT and DTC was compared with that in ambient conditions by Q-PCR using the primers listed in Supporting Information Table S1. For the design of the primers, we used very highly conserved regions of the genes encoding these enzymes in multiple species (details in Methods S1). QPCR analyses were performed on an Mx3000P QPCR System (Agilent, Cedar Creek, TX, USA). A SuperMix-UDG (Invitrogen) was used to perform QPCR analysis (denaturation: 10 min at 95°C; amplification and quantification: 40 times, 40 s at 95°C, 20 s at 55°C, 30 s at 72°C). Melt curve analyses of the target genes and reference genes were performed, which resulted in single products with specific melting temperatures. In addition, ‘no-template’ controls (i.e. with water) were run to ensure no contamination of reagents and no primer-dimer formation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin 2 for _M. lupulina_ and elongation factor-4α (elf-4α) and ubiquitin C (UBC) for _L. perenne_; GAPDH and elf-4α were chosen for _M. lupulina_ and _L. perenne_, respectively, as the most stable genes across the samples and these were used as endogenous standards to calculate relative mRNA expression by the standard curve method. Standard curves were generated by serial dilution of a random mixture of control samples.

Monte Carlo-driven metabolic control analysis

Because detailed reaction kinetics of the Pro biosynthesis pathways are not available, we developed a method that makes maximal use of the available information to understand pathway control. The method employs knowledge of the pathway topology, the (measured) metabolite concentrations, the pathway stoichiometry and a generic form of enzyme kinetics to calculate control coefficients (Table 1; see later Fig. 5; see later Methods S3). Such control coefficients are defined in metabolic control theory/analysis as the relative change in an output variable of a pathway operating at steady-state (e.g. metabolite concentration or flux), in response to a (small) relative change in a reaction rate (enzyme activity). Control coefficients represent perfect to relatively accurate predictions of pathway behaviour for infinitesimal and finite perturbations, respectively (Fell, 1997).

The Pro concentration [Pro] control coefficient is defined as:

\[
C_{i}^{[Pro]} = \frac{\partial \ln [\text{Pro}]}{\partial \ln v_i} = \frac{v_i \partial [\text{Pro}]}{[\text{Pro}] \partial v_i} \approx \frac{[\text{Pro}]_p - [\text{Pro}]}{[\text{Pro}]} \left( \frac{E_i - E_0}{E_i} \right)
\]

\( (v_i, \text{reaction rate}; E_0, \text{enzyme concentration of reaction i in the reference steady-state). The subscript p refers to the new steady-state after perturbing the enzyme activity (directly proportional to concentration) as indicated earlier. Control} \] coefficients can be derived by inversion of the Elasticity or E matrix, which depends on the pathway’s stoichiometric relations and elasticities.

Elasticities are derivatives describing the dependence of the enzyme rates on their substrates and products. These are ‘local’ (instead of pathway-level) properties that depend on the enzyme’s individual kinetic equations (including the kinetic constants). Our strategy to estimate elasticities was to use a generalized type of rate law (Liebermeister _et al._, 2010), for (rapid equilibrium) reversible enzyme mechanisms (cf. Methods S3). From this, the following expressions were derived for the corresponding elasticities:

\[
e^{e}_{i} = n_{i} \frac{1}{1 - \rho} - \frac{\alpha_{i} (1 + \alpha_{i})^{n_{i} - 1} \prod_{j \neq i} (1 + \alpha_{j})^{n_{j}}}{D}
\]

\[
e^{v}_{i} = -n_{i} \frac{\rho}{1 - \rho} + \frac{\pi_{i} (1 + \pi_{i})^{n_{i} - 1} \prod_{j \neq i} (1 + \pi_{j})^{n_{j}}}{D}
\]

\( (\alpha_{i}, \text{concentration of enzyme substrate i relative to the } K_{m} \text{ value for that metabolite; } \pi_{i}, \text{concentration of enzyme product j relative to the } K_{m} \text{ value for that product; } n_{i}, \text{stoichiometric coefficient of species i; } \rho = \Gamma_{m} \text{ mass-action ratio with } \Gamma = \prod_{i} n_{i} \alpha^{i} \Gamma_{i}, \text{mass-action ratio under biochemical reference conditions}; \text{correspondingly } K_{m}, \text{the equilibrium constant; } D, \text{denominator of the Liebermeister rate law without allosteric regulation (Methods S3)}. \)

For instance for the sensitivity of GOGAT with respect to Glu this would mean:
Table 1 Overview of the stoichiometric numbers of the pathway model reactions

|        | ARG | OAT | GDH | GOGAT | GS | P5CR | PRODH | PROCO | AKGPR | GLUPR | P5CDH |
|--------|-----|-----|-----|-------|----|------|-------|-------|-------|-------|-------|
| Orn    | 1   | −1  | 0   | 0     | 0  | 0    | 0     | 0     | 0     | 0     | 0     |
| akG    | 0   | −1  | 1   | −1    | 0  | 0    | 0     | 0     | 0     | 1     | 0     |
| Glu    | 0   | 1   | −1  | 2     | −1 | −1   | 0     | 0     | 0     | 0     | 1     |
| Gln    | 0   | 0   | 0   | −1    | 1  | 0    | 0     | 0     | 0     | 0     | 0     |
| P5c    | 0   | 1   | 0   | 0     | 0  | 1    | 1     | 0     | 0     | 0     | −1    |
| Pro    | 0   | 0   | 0   | 0     | 0  | −1   | −1    | −1    | 0     | 0     | 0     |

The rows specify the stoichiometric numbers of the pathway model variables (metabolites: Orn, Ornithine; akG, α-ketoglutarate; Glu, glutamate; Gln, glutamine; P5c, D1-pyrroline-5-carboxylate; Pro, proline) relative to the various reactions in the columns (ARG, arginase; OAT, ornithine α-aminotransferase; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; P5CS, pyrroline-5-carboxylate synthase; P5CR, pyrroline-5-carboxylate reductase; PRODH, proline dehydrogenase; PROCO, proline consumption; AKGPR, α-ketoglutarate production; GLUPR, glutamate production; P5CDH, pyrroline-5-carboxylate dehydrogenase; cf. Supporting Information Table S2).

\[ \epsilon_{\text{GOGAT}} = \frac{\rho_{\text{GOGAT}}}{1 - \rho_{\text{GOGAT}}} + \frac{\pi_{\text{Glu}}(1 + \pi_{\text{Glu}})(1 + \pi_{\text{NADP}})}{(1 + \alpha_{\text{deg}})(1 + \alpha_{\text{Glu}})(1 + \alpha_{\text{NADPH}}) + (1 + \pi_{\text{Glu}})^2(1 + \pi_{\text{NADP}}) - 1} \]

In order to determine the required elasticities, the \( \Gamma \) values were calculated with the measured or estimated metabolite and cofactor levels (details in Table S3) and the \( K_\text{eq} \) value of the corresponding reactions calculated based on tabulated or estimated values of free energies of formation (Methods S3). For the unknown \( \alpha \) and \( \pi \) values and the \( f_j \) ratios of the elasticity matrix, we performed a Monte Carlo sampling, randomly selecting values over realistic intervals (Methods S3). Each resulting \( E \) matrix was then inverted to derive the so-called control matrix \( C \), containing all relevant control coefficients (Reder, 1988). The ensemble of control matrices resulting from the Monte Carlo sampling was then used to plot the Pro concentration control distribution and calculate the reported median and 10 and 90 percentiles over 1000 Monte Carlo runs (a sufficient number for convergence of the statistics).

Statistical analysis
Data were tested for homogeneity of variance and normality; transformations were not necessary. Results were analysed by one-way ANOVA, using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), and significant differences between the means were determined by using the Tukey test (\( P < 0.05 \)) (\( n = 4 \)).

Results
Pro synthesis through the Glu pathway
Drought stress alone (D) or in combination with elevated temperature (DT), significantly affected Gln (increase) and Glu (decrease) content in the grasses (\( P. \text{ pratensis} \) and \( L. \text{ perenne} \)), but not in the legumes (\( M. \text{ lupulina} \) and \( L. \text{ corniculatus} \)). Increasing CO\(_2\) (DTC) reduced the stress impact in the grass species, but had no effect in the legumes. Consistent with the changes in Gln and Glu concentrations, GS activity increased under stress treatment in \( L. \text{ perenne} \) and \( P. \text{ pratensis} \), but not in the legumes (Fig. 2b).

Glu is converted to Glu 5-semialdehyde by GK and P5CS (Fig. 1), which spontaneously converts to P5C. The activity of P5CS was induced by drought and elevated temperature, in the grass species but not in the legumes (Fig. 2d). In parallel, stress conditions increased the transcriptional level of the P5CS gene in \( L. \text{ perenne} \), but not in \( M. \text{ lupulina} \) (Fig. 2e) (transcription levels were not determined for \( P. \text{ pratensis} \) and \( L. \text{ corniculatus} \)). Elevated CO\(_2\) significantly reduced the stress effect in P5CS, at both the enzyme activity and the transcriptional levels.

Pro synthesis through the Orn pathway
Arg provides a second precursor that feeds into the Pro synthesis pathway via Orn. In contrast to Glu and Gln, the concentrations of Arg and Orn were considerably higher in the legumes compared with the grasses. Drought and warming had no significant effects on Arg or Orn concentrations in the grass species (Fig. 3a, c), whereas Arg significantly decreased and Orn concentrations significantly increased in the legumes. The lower concentrations of Arg and increased concentrations of Orn are consistent with increased ARG activity under stress conditions (Fig. 3b). Also ARG transcript levels (in \( M. \text{ lupulina} \)) increased under stress (Fig. 3e). Elevated CO\(_2\) reduced the stress effect on ARG activity and expression in \( M. \text{ lupulina} \) and \( L. \text{ corniculatus} \). OAT activity and expression levels also increased under stress conditions in the legumes, but not in the grass species, and this effect was reversed by elevated CO\(_2\) (Fig. 3f).

The P5C–Pro metabolism cycle
The Glu pathway and the Orn pathway converge via GSA on the P5C–Pro cycle. In the absence of stress, Pro concentrations were similar in all species (0.1–0.3 µmol g\(^{-1}\) FW, Fig. 4d). Drought
and elevated temperature generally caused pronounced increases in Pro content and concomitant decreases in its immediate precursor P5C. In most cases elevated CO₂ reduced the stress-induced Pro increase. Final Pro concentrations from P5C (Fig. 4a) are the net result of the activity of P5CR, P5CDH (not measured), ProDH, and Pro consumption. Changes in the Pro profiles correlated relatively well with changes in the P5CR activity in most species (Fig. 4b). Changes in P5CR activity also matched closely with changes in P5CR transcripts (data only for *L. perenne* and *M. lupulina*, Fig. 4e). Stress-induced P5CR
activity was reversed in elevated CO$_2$. ProDH activity, controlling Pro oxidation to P5C, showed opposite effects to P5CR in response to drought, elevated temperature and CO$_2$ (Fig. 4c). Its activity decreased under stress, and this effect was prevented under elevated CO$_2$. ProDH transcripts varied largely in parallel with the enzyme activity (Fig. 4f).

In summary, stress treatments strongly induced Pro accumulation and elevated CO$_2$ counters this effect. In the grass species,
Fig. 4 Δ1-pyrroline-5-carboxylate (P5C)–proline (Pro) cycle. Changes in transcript levels, enzymes activity and metabolic concentrations in four-grassland species, *Lolium perenne* (Lp), *Poa pratensis* (Pp), *Medicago lupulina* (Ml) and *Lotus corniculatus* (Lc). Plants were grown at: A, ambient CO₂ and temperature (T) and sufficient water; D, ambient CO₂ and T and drought stress; DT, ambient CO₂, elevated T and drought stress; and DTC, elevated CO₂ and T and drought stress. Panels show concentrations of (a) P5C and (d) Pro, (b, e) activity and expression level of P5C reductase (P5CR), and (c, f) proline dehydrogenase (ProDH). Different letters in the graphs represent significant differences between the four treatments (error bars, ± SE. Tukey test; \( P < 0.05; n = 4 \)). The inset shows the relative position of the results in the overall pathway.
increased Pro concentrations originate mainly through the Glu pathway, whereas in legumes the Orn pathway is responsible. Elevated CO₂ decreased the stress impact essentially at the activity level of all enzymes involved.

**Thermodynamics and metabolic control analysis**

Based on a newly developed computational method we derived estimates of control coefficients, which quantify the effect of regulating the activity of each of the enzymes in the pathway on the Pro concentration. This method uses pathway structure, thermodynamic information and generic reaction kinetics, combined with Monte Carlo sampling (Fig. 5; for a detailed description cf. Methods S3).

A crucial determinant of the control calculations is the distance from equilibrium of the reactions as expressed by their so-called disequilibrium ratio. Apart from the measured (and in some cases estimated, cf. Table S3) metabolite concentrations (or concentration ratios), this requires the reactions’ equilibrium constants ($K_{eq}$ values). For that purpose molar reaction free energies were calculated ($Δ_rG_0$; Tables 2, S4; cf. Methods S3) which specify the direction of the net (positive) flux. Their negative values indicated that nearly all reactions are spontaneous in the expected direction. Two exceptions are the ARG and ProDH reactions for which the disequilibrium ratio was adjusted (see Methods S3).

Our calculations yielded a set of (Pro) concentration control coefficient distributions (one per reaction step, cf. Figs S1, S2 for *L. perenne* and *M. lupulina*) reflecting how modulating the respective pathway enzymes potentially affects the Pro concentration (Fig. 6). The distributions are approximately mono-modal and we have represented them by their median values, $P_{10}$ and $P_{90}$ percentiles (Tables 2, S3). The Orn pathway has in general a strong potential for Pro upregulation ($P_{90}$ values up to 12 and 26%, respectively). Conversely, increasing GS activity is predicted to negatively affect Pro ($P_{10}$ as low as $-29$%). Although it is thermodynamically driven to produce Glu, GDH is predicted to negatively impact Pro concentrations ($P_{10}$ as low as $-19$%, Table 2; Fig. 7a), which likely originates from its competition with OAT for $α$-ketoglutarate ($α$-k-Gla). GOGAT and P5CDH again are less suitable for regulatory purposes with $-9.8$% as the most pronounced $P_{10}$ value, and median values close to zero (Table 2; Fig. 7b). The downstream-located P5CS and P5CR again are more likely to be Pro control sites with $P_{90}$ values of $c.25$%. The level of (negative) control exerted by ProDH appears to be limited ($P_{10}$ values not higher than $-9.7$%). Control calculations for a larger distance from equilibrium, for instance due to high NAD : NADH and Pro : P5CR ratios in the mitochondrial compartment yield roughly similar values. Importantly, our model suggests a negative effect of increasing (additional) Pro consumption (with median values near $-100$%, Fig. 7c) and a (broadly) positive effect of increasing $α$-k-Gla (median values up to $77$%) and Glu (median values up to $31$%) supplies on Pro concentration.

In order to better understand how control by different reactions is related, we calculated correlation coefficients for all pairs of control coefficient (cc) distributions (Table S5). Sequential reactions in the principal branches such as the Orn pathway (e.g. cc of 0.64 and 0.63 for *L. perenne* and *M. lupulina* in the current climate, respectively), and P5CS-P5CR (cc of 0.60 and 0.49 in *L. perenne* and *M. lupulina*, respectively), are correlated, indicating that they share the control of that branch. Similarly, GS tends to have a more negative impact on Pro concentrations if P5CS has a more positive impact (cc of $-0.66$ and $-0.64$ for *L. perenne* and *M. lupulina*). Enzymes catalysing opposite

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**Fig. 5** Schematic representation of the Monte Carlo metabolic control analysis. The grey rectangles represent required input information, which is translated in a different form in the first steps of the procedure to yield an elasticity matrix. Matrix inversion is then used to produce a control matrix. The elasticity matrix is iteratively generated via a Monte Carlo sampling of saturation ratios and pathway flux ratios (for details see the Materials and Methods section).
Pro-consumption
Glu-supply 0.31 (1.0; 1.3) 0.38 (0.28; 1.4) 0.35 (0.22; 1.0) 0.32 (0.22; 0.8)

α-k-Gla-supply

Glu-supply

Pro-consumption

reaction such as P5CS-P5CDH (cc of −0.79 and −0.76 for L. perenne and M. lupulina), and P5CR-ProDH (cc of −0.69 and −0.76 for L. perenne and M. lupulina) tend to have positively correlated absolute values of their Pro control coefficients. Interestingly, control on the Pro consumption step negatively correlated with the ProDH (cc of −0.81 and −0.80 for L. perenne and M. lupulina; Table S5; Fig. 7d) and P5CR (cc of −0.63 and −0.52 for L. perenne and M. lupulina) steps. On the one hand, a more negative influence of Pro consumption is associated with a less negative influence of ProDH and P5CDH. On the other, for P5CR a more negative influence of Pro consumption is associated with a less positive influence of this step (cc of 0.68 and 0.71 for L. perenne and M. lupulina).

Taken together, our control calculations indicate the potential for regulatory changes of ARG, OAT, GS, P5CS and P5CR to significantly influence Pro concentration. The correlations indicate that coregulation through several of these steps is possible and would be more effective than regulation of a single step.

Discussion

In order to understand the regulation of Pro metabolism, under climate change scenarios, contrasting grassland plant species were subjected to drought and temperature stress and elevated atmospheric CO2, and Pro metabolism was analysed at the metabolic, enzyme activity and transcriptional levels. Because it is impossible to intuitively connect regulatory changes in metabolites and activities to the effective change of Pro concentrations, we developed a computational approach, which directly quantifies this causal relationship as control coefficients. The lack of kinetic information on the enzymes was circumvented in part by a Monte Carlo sampling over realistic parameter ranges, using a generic type of reversible enzyme kinetics. Furthermore, we made use of the available information about the metabolic concentrations by translating them into thermodynamic properties, which could be fed into kinetic expressions. This method allowed the calculation of control coefficients for all enzymes and precursors to the pathway, expressing their potential impact on Pro concentrations.

Drought and elevated temperature induce concerted changes in Pro metabolism

Individual effects of drought and high temperature on Pro have been studied and it has been shown that Pro accumulates under these stresses (Parida et al., 2008; Szabados & Savouré, 2010; Krasensky & Jonak, 2012). However, relatively little is known about how their combination alters Pro content. Drought significantly increased Pro content in all species we investigated, and additional temperature elevation generally increased the stress impact, as has been previously observed (De Ronde et al., 2004; Simon-Sarkadi et al., 2005). The temperature-induced changes indicate that even a relatively small increase in growth temperature, applied during the full life cycle, can significantly affect the response of Pro metabolism to drought.

Elevated CO2 reduced the stress impact on Pro concentrations, as has been also observed previously (Balouchi et al., 2009; Pérez-López et al., 2010). We found that this CO2 effect is the result of repressing Pro biosynthesis, as well as activating its degradation. Also our computational estimation of pathway control points to
A concerted regulation of anabolic and catabolic pathways. Moreover, these effects occur not only at the metabolite and enzyme activity levels, but also at the transcript level. The fact that elevated CO₂ causes a general reversal of multiple drought-induced regulatory changes, suggests that it impacts a common signal controlling the drought response across the whole pathway or upstream from changes in Pro metabolism.

An explanation for such an effect may lie in reduced H₂O₂ formation, as a result of suppression of photorespiration by elevated CO₂ (Ainsworth & Rogers, 2007; Jia et al., 2010; Salazar-Parra et al., 2012; Zinta et al., 2014; AbdElgawad et al., 2015). H₂O₂, induced by stress treatments, may act as a signal that activates stress-response pathways (Gill & Tuteja, 2010). Verslues et al. (2007) reported that increased H₂O₂ may induce Pro metabolism.
biosynthesis by affecting abscisic acid (ABA) concentrations. H₂O₂ treatment also activated Pro biosynthesis by increasing the activity and transcriptional levels of P5CS, ARG and OAT and decreasing the activity of ProDH in maize seedlings (Geissler et al., 2009; Yang et al., 2009). Reduced H₂O₂ formation could therefore be involved in the stress and CO₂ effects on Pro synthesis.

Elevated CO₂ also increases Pro catabolism (ProDH activity). Pro catabolism may be an important energy source (Kishor et al., 2005; Szabados & Savouré, 2010; Liang et al., 2013). Pro degradation provides reducing potential to the mitochondria for production of up to 30 ATP/Pro (Atkinson, 1977; Kishor et al., 2014). This process provides high-energy output to resume growth after stress (Verbruggen et al., 1996; Hare & Cress, 1997).

The role of the Glu or Orn pathways in Pro accumulation, differs between grasses and legumes, and is possibly affected by N status.

Despite the limitation that we have only studied four species, from two clades, and at a single developmental time point, our results quite clearly indicate that in the legumes the Orn pathway (ARG and OAT) is activated under drought stress, whereas in the grasses drought activates the Glu pathway (P5CS). From the literature it is also clear that the contribution of each pathway in the stress-induced Pro responses varies with species (Pardha Saradhi & Mohanty, 1997; Reddy et al., 2004; Kishor et al., 2005) and stress exposure (Xue et al., 2009; Yang et al., 2009). For example, no significant role in Pro accumulation was observed for the Orn pathway in Brassica napus and Vigna aconitifolia, whereas it was activated together with the Glu pathway in A. thaliana (Roosens et al., 1998) and M. truncatula (Armengaud et al., 2004).

The predominant involvement of the Orn pathway may also be related to plant N status. Increased N input induced OAT expression, possibly via accumulation of Orn or Arg (Delauney et al., 1993). Increased OAT activity was also implicated in facilitating N recycling from Arg to Glu (Funck et al., 2008). In addition, leaf N content was not affected by the drought and CO₂ treatments in the grass species, but increased in the legumes (Abдельغاد et al., 2014). Therefore, the predominant activation of the Orn pathway in legumes under drought stress, may be closely related to their N-fixing ability. It would be of interest to further explore the relation between N content and Pro concentrations, by varying N fertilization in grasses and legumes.

Control analysis indicates potential mechanisms for Pro regulation.

Finding causal relations between changes in enzyme activities and changes in metabolite concentrations (such as Pro) is not

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**Fig. 7** Predicted control properties of (Lolium sp.) proline (Pro) pathway enzymes. (a) Histogram describing the control coefficient distribution for NADH-glutamate dehydrogenase (GDH). The negative reaction free energy (ΔrG) implies a net positive reaction flux in the sense of Pro production; however, the negative range of the control distribution (characterized by its 10th-percentile (P₁₀), median (m) and ninetieth percentile (P₉₀) values) indicates that GDH upregulation will reduce the Pro concentration. (b) Histogram describing the control coefficient distribution for pyrroline-5-carboxylate dehydrogenase (P5CDH). The negative reaction free energy (ΔrG) implies a net negative reaction flux in the sense of Pro breakdown, the control coefficient values indicates a small negative influence on the Pro concentration. (c) Histogram describing the control coefficient distribution for Pro consumption (ProCons) indicating a small negative influence on the Pro concentration. (d) Plot of proline dehydrogenase (ProDH) vs Pro consumption control coefficient values for all individual Monte Carlo simulation runs. The corresponding correlation coefficient (cc) shows a strong negative correlation with less negative values for Pro consumption control typically associated with a more pronounced negative control by ProDH.
straightforward, even for a linear pathway. Indeed, it has been demonstrated that enzymes of a pathway have different levels of control on the global pathway, determined by the location of the corresponding reaction in the pathway, how far it is from equilibrium and its kinetics. Control coefficients are a useful aid in quantifying the level of control (Kacser & Burns, 1979; Uys et al., 2007; Rohwer, 2012; Tang et al., 2012). To determine concentration control coefficients for all reaction steps, a complete and detailed kinetic model of the pathway is required (Fell, 1997). For Pro biosynthesis the structure of the pathway is well known (Fig. 1), but the kinetic equations of the respective enzymes are relatively poorly characterized (Verslues & Sharma, 2010). We have circumvented this limitation by applying metabolic control analysis (Kacser & Burns, 1979; reviewed in Morgan & Rhodes, 2002) in a new way using generic kinetics. This allows the prediction of which enzyme activity changes are most effective, and therefore more likely responsible, for the increase in Pro concentrations under drought and elevated temperature. However, the structural and thermodynamic constraints imposed by the network topology and reaction thermodynamics are not strong enough to explain the more subtle differences in the control between species and conditions of this study.

In general our analysis of correlations between control coefficients demonstrates that concerted changes of enzyme activities are more effective than single changes and are probably necessary considering that we observed up to 10-fold changes in Pro concentrations between conditions. This provides an explanation for the multitude of observed regulatory changes under stress conditions. Control coefficients calculated by Monte Carlo sampling of generic kinetic constants predict that for all studied species, the increased P5CR, and to a lesser extent decreased ProDH, were responsible for increased Pro concentrations. Furthermore, for the grasses the increased P5CS and for the legumes increased ARG and OAT activities, contributed to that effect. Because our findings are robust to variations in metabolite concentrations, modifying the activities of ARG, OAT, P5CS and P5CR can be predicted to be the most effective strategy to manipulate cellular Pro concentrations (Hmida-Sayari et al., 2005; Miller et al., 2009; You et al., 2012). Experimentally, this is supported by the observed regulation of ARG, OAT and P5CR for legumes, and P5CS and P5CR for grasses under different perturbations.

A powerful way to confirm the validity of a model lies in manipulating, genetically or pharmacologically, the activity of particular enzymes and analyse the metabolic effect. Notably, for Pro biosynthesis a significant number of experiments have been performed on plants with reduced or increased expression of Pro metabolism enzymes. For example P5CS has been altered in A. thaliana, but also in Glycine max, Medicago truncatula, Nicotiana tabacum and others. Also for ProDH, GS, OAT and P5CDH, lines with altered expression are available. A compilation of these studies (Table S6) demonstrates that the large majority of the experimental outcomes match the change predicted on the basis of our control analysis. These results therefore provide considerable independent experimental validation for the computational analysis. Regarding the discrepancies it should be noted that not all experimental results are mutually consistent (for instance for P5CS). In principle, a multitude of explanations can be postulated to explain aberrant control properties, for instance based on allosteric regulation (leading to feed-back or feed-forward relations; see, for instance, Fell & Snell, 1988; Hoffmeyr & Olivier, 2002) not modelled by our generic approach or compensatory expression and activity changes in response to the genetic perturbations. Basic additions or alterations to the model pathway such as adding an extra glutamine branch or modifying the kinetics of the branches could not resolve those issues.

An important aspect of the in vivo Pro pathway is, indeed, its connection to other metabolic pathways. Within the assumptions of our model a strong positive influence of αkGla and Glu supply and a strong negative influence of Pro consumption were identified. Kinetic parameter regimes with a less pronounced negative influence from Pro consumption tend to be characterized by significantly more control on some of the reactions upstream from proline like P5CR, ProDH and P5CDH. This inverse relationship could be instrumental in restoring Pro concentrations if the consumption rate is increased (for example increased protein synthesis under conditions of fast growth). To limit the number of unknown parameters, we have taken a minimalistic approach and selected the model variables in accordance with the available data. Extensions and refinements to the model are possible, such as integrating reactions of the urea cycle and other pathways of amino acid metabolism. Provided that the enzyme topology and metabolite concentrations are known, the method can in principle also be applied to other metabolic pathways.

Conclusion

It is clear that future climate conditions, such as drought and elevated temperature, are likely to negatively impact the growth of grasslands, and the associated feed and food production. By way of protection, Pro concentrations are strongly induced in all tested species, although through different mechanisms, and possibly directed by the plant N status. Ecological differences in the capacity of plants to assimilate N, may therefore affect Pro metabolism in adverse environmental conditions. Notably, elevated CO₂ nearly abolished the stress responses, by affecting both Pro biosynthesis and degradation. Computational analysis indicates that pathway regulation is obtained by simultaneous changes in multiple enzymes.

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References
AbdElgawad H, Farfan-Vignolo ER, de Vogt D, Asard H. 2015. Elevated CO₂ mitigates drought and temperature-induced oxidative stress differently in grasses and legumes. Plant Science 231: 1–10.
AbdElgawad H, Peshev D, Zinta G, Van den Ende W, Janssens IA, Asard H. 2014. Climate extreme effects on the chemical composition of temperate grassland species under ambient and elevated CO₂: a comparison of fructan and non-fructan accumulators. PLoS ONE 9: e92044.
Abraham E, Rigo G, Szekely G, Nagy R, Konez C, Szabados L. 2003. Light dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroids in Arabidopsis. Plant Molecular Biology 51: 363–372.
Ainsworth EA, Rogers A. 2007. The response of photosynthesis and stomatal conductance to rising [CO₂]: mechanisms and environmental interactions. Plant, Cell & Environment 30: 258–270.
Armengaud P, Thiery L, Buhot N, Grenier-De March G, Savoure A. 2004. Cloning of ornithine delta-aminotransferase cDNA from Escherichia coli and regulation of proline biosynthesis. Biochemical Society Transactions 32: 18673–18678.
Atkinson DE. 1977. Cellular energy metabolism and its regulation. New York, NY, USA: Academic Press.
Baloussi H, Sanavy SM, Emam Y, Dolatabadian A. 2009. UV radiation, elevated CO₂ and water stress effect on growth and photosynthetic characteristics in durum wheat. Plant, Soil & Environment 55: 443–453.
Charest C, Ton Phan C. 1990. Cold acclimation of wheat (Triticum aestivum): properties of enzymes involved in proline metabolism. Physiologia Plantarum 105: 159–168.
De Boecq HJ, Dreesen FE, Janssens IA, Nips J. 2011. Whole-system responses of experimental plant communities to climate extremes imposed in different seasons. New Phytologist 189: 806–817.
De Ronde J, Cress W, Krüger G, Strasser R, Van Staden J. 2004. Photosynthetic response of transgenic soybean plants, containing an Arabidopsis P5CR gene, during heat and drought stress. Journal of Plant Physiology 161: 1211–1224.
Delauney AJ, Hu CA, Kishor PB, Verma DP. 1993. Cloning of ornithine delta-amino-transferase cDNA from Vigna aconitifolia by trans-complementation in Escherichia coli and regulation of proline biosynthesis. Journal of Biological Chemistry 268: 18673–18678.
Fell D. 1997. Understanding the control of metabolism. London, UK: Portland Press.
Fell DA, Snell K. 1988. Control analysis of mammalian serine biosynthesis. Feedback inhibition on the final step. Biochemical Journal 256: 97–101.
Feng GQ, Li Y, Cheng ZM. 2014. Nitric oxide synthesis and regulation of proline biosynthesis, accumulation and protection of plants from osmotic stress. Plant Physiology 122: 1129–1136.
Hu C, Deauney AJ, Verma D. 1992. A bifunctional enzyme (delta 1-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. Proceedings of the National Academy of Sciences, USA 89: 3934–3938.
Huang Z, Zhao L, Chen D, Liang M, Liu Z, Shao H, Long X. 2013. Salt stress encourages proline accumulation by regulating proline biosynthesis and degradation in Jerusalem artichoke plantlets. PlaS ONE 8: e62005.
IPCC. 2012. Field CB, Barros V, Stocker TF, Qin D, Dokken DJ, Ebi KL. Mastrandrea MD, Mach KJ, Plattner G-K, Allen SK et al., eds. Managing the risks of extreme events and disasters to advance climate change adaptation: a special report of Working Groups I and II of the Intergovernmental Panel on Climate Change. Summary for policymakers. Cambridge, UK: Cambridge University Press, 1–19.
Jia Y, Tang S, Wang R, Ju X, Ding Y, Tu S, Smith DL. 2010. Effects of elevated CO₂ on growth, photosynthesis, elemental composition, antioxidant level, and phytochelatin concentration in Loliisum muturifolium and Loliisum perenne under Cd stress. Journal of Hazardous Materials 180: 384–394.
Kacser H, Burns J. 1979. Molecular democracy: who shares the controls? Biochemical Society Transactions 7: 1149–1160.
Kant S, Kant P, Raveh E, Barak S. 2006. Evidence that differential gene expression between the halophyte, Thalassogella halophila, and Arabidopsis thaliana is responsible for higher levels of the compatible osmolyte proline and tight control of Na⁺ uptake in T. halophila. Plant, Cell & Environment 29: 1220–1234.
Kim GB, Nam YW. 2013. A novel Delta(1)-pyrroline-5-carboxylate synthetase gene of Medicago truncatula plays a predominant role in stress-induced proline accumulation during symbiotic nitrogen fixation. Journal of Plant Physiology 170: 291–302.
Kishor K, Polavarapu B, Sreenivasulu N. 2014. Is proline accumulation per se correlated with stress tolerance or is proline homeostasis a more critical issue? Plant, Cell & Environment 37: 300–311.
Kishor PK, Hong Z, Miao G-H, Hu C-AA, Verma DPS. 1995. Overexpression of [delta]-pyrroline-5-carboxylate synthetase increases proline production and confers osmoharance in transgenic plants. Plant Physiology 108: 1387–1394.
Kishor PK, Sangam S, Amrutha R, Laxmi PS, Naidu K, Rao K, Rao S, Reddy K, Theriappan P, Sreenivasulu N. 2005. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. Current Science 88: 424–438.
Kiyosue T, Yoshiha Y, Yamaguchi-Shinozaki K, Shinozaki K. 1996. A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in Arabidopsis. Planta Cell & Environment 18: 1323–1335.
Krasensky J, Jonak C. 2012. Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. Journal of Experimental Botany 63: 1593–1608.
Li X, Ahammed G, Zhang Y, Zhang G, Sun Z, Zhou J, Zhou Y, Xia X, Yu J, Shi K. 2014. Carbon dioxide enrichment alleviates heat stress by improving cellular redox homeostasis through an ABA-independent process in tomato plants. Plant Biology 17: 81–89.
Liang X, Zhang L, Natarajan SK, Becker DF. 2013. Proline mechanisms of stress survival. Antioxidants & Redox Signaling 19: 998–1011.
Liebermeister W, Uhlendorf J, Klipp E. 2010. Modular rate laws for enzymatic reactions: thermodynamics, elasticities and implementation. Bioinformatics 26: 1528–1534.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265–275.
Luttge S, Mayerus V, Kinet JM. 1999. NaCl effects on proline metabolism in rice (Oryza sativa) seedlings. Physiologia Plantarum 105: 450–458.
Maggio A, Miyazaki S, Veronese P, Fujita T, Ibeis JA, Damsz B, Narasimhan ML, Hasegawa PM, Joly Rj, Bressan RA. 2002. Does proline accumulation play an active role in stress-induced growth reduction? Plant Journal 31: 699–712.
Mani S, Van de Cotte B, Van Montagut M, Verbruggen N. 2002. Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in Arabidopsis. Plant Physiology 128: 73–83.
Miller G, Honig A, Stein H, Suzuki N, Mittler R, Zilberstein A. 2009. Unraveling delta-1-pyrroline-5-carboxylate-proline cycle in plants by uncoupled expression of proline oxidation enzymes. Journal of Biological Chemistry 284: 26482–26492.

Morgan JA, Rhodes D. 2002. Mathematical modeling of plant metabolic pathways. Metabolic Engineering 4: 80–89.

Murray V, Ebi K. 2012. IPCC special report on managing the risks of extreme events and disasters to advance climate change adaptation (SREX). Journal of Epidemiology and Community Health 66: 759–760.

Nanjio T, Fujita M, Seki M, Kato T, Tabata S, Shinozaki K. 2003. Toxicity of free proline revealed in an Arabidopsis T-DNA-tagged mutant deficient in proline dehydrogenase. Plant and Cell Physiology 44: 541–548.

Naudts K, Van den Berge J, Farfan E, Rose P, Abdelhagaw H, Ceulemans R, Janssens I, Asard H, Nijs I. 2014. Future climate alleviates stress impact on grassland productivity through altered antioxidant capacity. Environmental and Experimental Botany 99: 150–158.

Nuzum CT, Snodgrass PJ. 1976. Multiple assays of the five urea-cycle enzymes in human liver homogenates. In: Grisolia S, Baguena R, Mayor F, eds. The urea cycle. New York, NY, USA: John Wiley & Sons, 325–349.

Pandey R, Zinta G, Abdelgawad H, Ahmad A, Jain V, Janssens I. 2015. Multiple assays of the five urea-cycle enzymes in soybean. New Phytologist 208: 1281–1301.

Pardha Saradhi P, Mohanty P. 1997. Involvement of proline in protecting osmolyte accumulation and cell wall elasticity to salt tolerance in barley cultivars. Journal of Plant Physiology 151: 1189–1202.

Reddy AR, Chaitanya KV, Vivekanandan M. 2004. Pyrroline-5-carboxylate reductase in root/nodule and leaf of soybean. Trends in Plant Science 9: 345–352.

Reder C. 1988. Physiological and molecular alterations in plants exposed to high [CO2] under phosphorus stress. Biotechnology Advances 6: 2375–2392.

Rohwer JM. 2012. Mathematical control analysis of developing oilseed rape (Brassica napus cv Westar) embryos shows that lipid assembly exerts significant control over oil accumulation. New Phytologist 196: 414–426.

Temple SJ, Kunjiibettu S, Roche D, Sengupta-Gopalan C. 1996. Total glutamine synthetase activity during soybean nodule development is controlled at the level of transcription and holoprotein turnover. Plant physiology 112: 1723–1733.

Uys L, Botia FC, Hofmeyr J-HS, Rohwer JM. 2007. Kinetic model of sucrose accumulation in maturing sugarcane culm tissue. Phytochemistry 68: 2375–2392.

Van den Berge J, Naudts K, Zachovali C, Janssens I, Ceulemans R, Nijs I. 2011. Altered response to nitrogen supply of mixed grassland communities in a future climate: a controlled environment microcosm study. Plant and Soil 345: 375–385.

Verbruggen N, Hermans C. 2008. Proline accumulation in plants: a review. Amino Acid 35: 753–759.

Verslues PE, Sharma S. 2010. Proline metabolism and its implications for plant–environment interaction. The Arabidopsis Book/ American Society of Plant Biologists 8: e0140.

Verbruggen N, Hua XJ, May M, Van Montagu M. 1996. Environmental and developmental signals modulate proline homeostasis: evidence for a negative transcriptional regulator. Proceedings of the National Academy of Sciences, USA 93: 8787–8791.

Verbruggen N, Villarroel R, Van Montagu M. 1993. Osmoregulation of a pyrroline-5-carboxylate reductase gene in Arabidopsis thaliana. Plant Physiology 103: 771–781.

Verslues PE, Kim YS, Zhu JK. 2007. Altered ABA, proline and hydrogen peroxide in an Arabidopsis glutamate-glyoxylate aminotransferase mutant. Plant Molecular Biology 64: 205–217.

Wu L, Fan Z, Gao L, Li Y, Chen Z-L, Qu L-J. 2005. Over-expression of the bacterial nbaA gene in rice enhances salt resistance and drought tolerance. Plant Science 169: 297–302.

Xue X, Liu A, Hua X. 2009. Proline accumulation and transcriptional regulation of proline biosynthesis and degradation in Brassica napus. BMB reports 42: 28–34.

Yamada M, Morishita H, Urano K, Shiozaki N, Yamaguchi-Shinozaki K, Shinozaki K. 2005. Effects of free proline accumulation in petunias during drought stress. Journal of Plant Physiology 162: 1095–1981.

Yang SL, Lan SS, Gong M. 2009. Hydrogen peroxide-induced proline and metabolic pathway of its accumulation in maize seedlings. Journal of Plant Physiology 166: 1694–1699.

Yoshida Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K. 2005. Dehydration-stress and osmotic stress responses in Arabidopsis thaliana. New Phytologist 166: 75–80.

Zglin M, Szabo A, Miao GH, Hong Z, Verma DP. 1992. Subcellular location of delta-1-pyrroline-5-carboxylate reductase in root/nodule and leaf of soybean. Plant Physiology 99: 1642–1649.

Zhang C-S, Lu Q, Verma DPS. 1995. Purification, characterization, and application of a novel dicyclic L-proline dehydrogenase from a hyperthermophilic archaean, Thermococcus profundus. Applied and Environmental Microbiology 67: 1470–1475.

Znanie M, Sragek L, Kocsy G, Varhegyi A, Galiba G, de Ronde J. 2005. Genetic manipulation of proline accumulation influences the concentrations of other amino acids in soybean subjected to simultaneous drought and heat stress. Journal of Agricultural and Food Chemistry 53: 7512–7517.
Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Proline concentration control distribution of the Lolium perenne Pro pathway metabolite data under current climate conditions.

Fig. S2 Proline concentration control distribution of the Medicago lupulina Pro pathway metabolite data under current climate conditions.

Table S1 Real-time PCR targets, GenBank identifier and primers for transcript analysis of reference genes and genes involved in proline metabolisms

Table S2 Overview of enzymes included in the computational model, with name, abbreviation, Enzyme Commission number and biochemical reaction scheme

Table S3 Free energies of formation and cellular metabolite concentrations used in the calculation of disequilibrium ratios

Table S4 Extended version of Table 2 with additional calculated values for Poa pratensis and Lotus corniculatus under ambient conditions

Table S5 Correlation analysis with correlation coefficients tabulated for the Lolium and Medicago control distributions (Figs S1 and S2, respectively)

Table S6 Comparison of changes in proline concentrations in plants with altered proline-biosynthesis enzymes, to changes expected on the basis of the proposed model

Methods S1 Primers design for Q-PCR.

Methods S2 Liebermeister kinetics based elasticity expressions used for metabolic control analysis.

Methods S3 Free energy calculations and Monte Carlo-based metabolic control analysis.

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