Strong relationship between elemental stoichiometry and metabolome in plants

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Shifts in the elemental stoichiometry of organisms in response to their ontogeny and to changing environmental conditions should be related to metabolomic changes because elements operate mostly as parts of molecular compounds. Here we show this relationship in leaves of *Erica multiflora* throughout their seasonal development and in response to moderate experimental field conditions of drought and warming. The N/P ratio in leaves decreased in the metabolically active growing seasons, coinciding with an increase in the content of primary metabolites. These results support the growth-rate hypothesis that states that rapidly growing organisms present low N/P ratios because of the increase in allocation of P to RNA. The foliar N/K and P/K ratios were lower in summer and in the drought treatment, in accordance with the role of K in osmotic protection, and coincided with the increase of compounds related to the avoidance of water stress. These results provide strong evidence of the relationship between the changes in foliar C/N/P/K stoichiometry and the changes in the leaf's metabolome during plant growth and environmental stress. Thus these results represent a step in understanding the relationships between stoichiometry and an organism's lifestyle.

climate change | metabolomics | ecometabolomics | Mediterranean climate

The ratios of C/N/P concentrations in the environment and biomass have statistically significant relationships with traits of an organism's lifestyle and even seem to influence the structure and function of ecosystems (1, 2). The growth-rate hypothesis (GRH), one of the central paradigms of ecological stoichiometry (3, 4), proposes that growing organisms must increase their allocation of P to RNA to meet the elevated demands for the synthesis of proteins required for growth. Low ratios of environmental N/P and C/P favor species with very high rates of growth (5) and may induce shifts in species communities (6, 7). The GRH has strong experimental support in freshwater ecosystems (2), with a few exceptions related to the allocation of nutrients to functions other than growth (8–10). In terrestrial ecosystems, however, the direct application of the GRH frequently fails or is accomplished incompletely (2, 11). Apart from investing N and P in growth, terrestrial plants can invest important amounts of these nutrients to other functions, such as storage, defense, and mechanisms of stress avoidance. Therefore the phenotypic responses in these other basic organismal functions should be considered when assessing the relationships of C/N/P ratios with an organism's metabolome and lifestyle and with the structure and function of ecosystems (2, 12, 13). Our goal was to consider the first step of such relationships, (i.e., to link stoichiometry to the metabolome).

The metabolome is the entirety of small molecules present in an organism as the final expression of its genotype (14) and can be considered as the organism's chemical phenotype (12). Metabolomics has been applied recently to physiological and ecological studies to assess the physiological status and functions of organisms, including their energetic and oxidative states; functions of growth, defense, storage, and reproduction; and mechanisms of stress avoidance and health (15–18).

Global climate change and the marked ontogenic and seasonal variation throughout the year in most regions of the world should affect the elemental content, stoichiometry, and metabolome of organisms (3), but most metabolomic studies have not considered these effects (2, 19). We hypothesized that stoichiometric and metabolomic studies of plants in different ontogenetic stages or exposed to different environmental conditions should reveal an organism's flexibility in modulating its stoichiometry and metabolome to maintain optimal fitness under different conditions. We hypothesized that seasonal differences in the metabolome should be similar to the shifts that occur in individuals growing under varying conditions of temperature and water availability.

We conducted a stoichiometric and metabolomic study of the plant *Erica multiflora*, a Mediterranean shrub, during different ontogenetic periods and exposed to field conditions of moderate warming (0.9 ∘C) and drought (19% reduction of soil moisture). We thus tested our hypothesis that seasonal and climatic changes would force organisms to adjust both their C/N/P/K biomass stoichiometry and their metabolome in an interrelated way to maintain optimal performance under each specific condition. Elemental stoichiometry should determine an organism's capacity to build molecules and thus to shape metabolomic responses, so an organism's metabolomic adjustments should determine the C/N/P/K biomass stoichiometry, and the seasonal and climatic changes in environmental ratios of C/N/P/K availability should influence an organism's metabolomic responses.

Results

Seasonal Stoichiometric and Metabolomic Changes. The foliar concentrations of C, N, P, and K and their respective ratios (C/N, C/P, C/K, N/P, N/K, and K/P) changed with the seasons (mixed model analyses). The lowest N/P, C/P, and C/N ratios were found in spring, whereas summer leaves showed the highest K/P and the lowest N/K concentration ratios (Table S1). Almost all the elucidated polar and nonpolar compounds (Fig. 1) showed significant seasonal differences in concentration (mixed model analyses; $P < 0.05$) (Table S1). Spring leaves had the highest concentrations of polar metabolites, such as alanine, glutamine, asparagine, threonine, α-glucose, β-glucose, and sucrose. In contrast, spring leaves had the lowest concentrations of lipids and secondary metabolites, such as terpene compound 1 and derivatives of p-coumaric acid.

Multivariate analysis of variance (MANOVA) analysis showed a significant interaction between stoichiometric and metabolomic variables and seasons ($F_{3,13484} = 71.6, P < 0.0001$, Table S2). Thus, different distributions of global metabolomic and stoichiometric values were observed among seasons. Permutational MANOVA (PERMANOVA) analysis also showed significant global differ-

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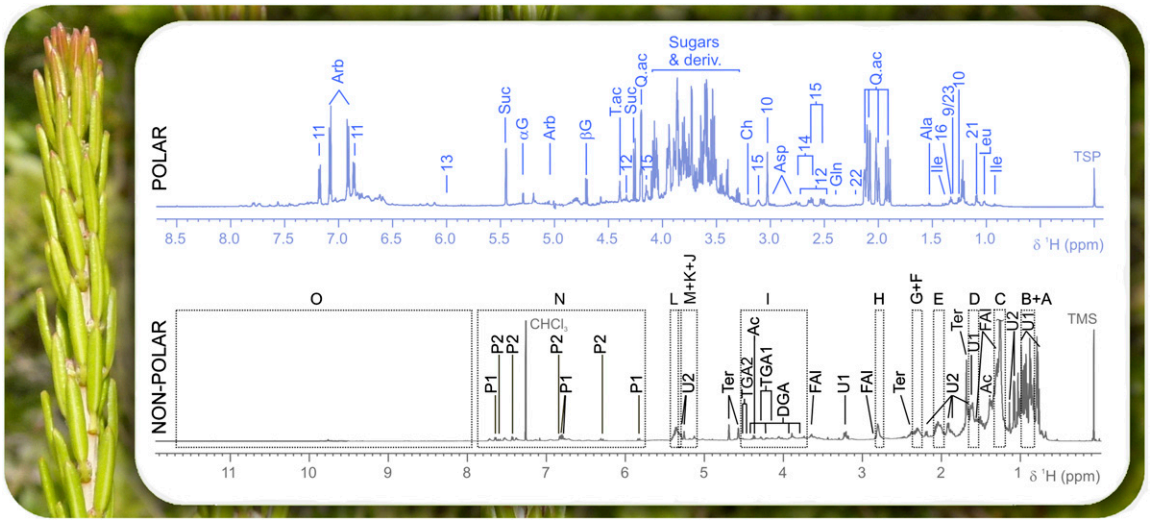

Fig. 1. Typical ^{1}H NMR spectra of polar (water–methanol) and nonpolar (chloroform) extracts of *E. multiflora* leaves. Assignments of signals to metabolites are indicated in blue in the polar profile. A number has been assigned to each metabolite and to overlapped signals: 1, α-glucose (αG); 2, β-glucose (βG); 3, sucrose (Suc); 4, alanine (Ala); 5, asparagine (Asp); 6, glutamine (Gln); 7, leucine (Leu); 8, isoleucine (Ile); 9, threonine (Thr); 10, 6-deoxypyranose; 11, 4-hydroxyphenylacetate; 12, malate; 13, maleate; 14, citrate; 15, 3-amino-4-hydroxybutyrate; 16, *N*-acetyl group; 17, quinic acid (Q.ac); 18, tartaric acid (T.ac); 19, arbutin (Arb); 20, choline (Ch); 21, 1,2-propanediol; 22, γ-hydroxybutyrate; 23, lactate. 30–55: Overlapped signals: 30, 11+15; 31, 5+11; 32, 5+11+13; 33, 6+21; 3, 12+unknown; 35, 15+22; 36, 16+19; 37, 3+16+19; 38, 6+16; 39, 1+2+3+16+18; 40, 1+2+3+5+16+18; 41, 1+2+3+16; 42, 11+14; 43, 9+14+22; 44, 13+14; 45, 1+2+9; 46, 1+2+3+18; 47, 1+2+3+18+19; 48, 1+2+3+18+19+20; 49, 1+2+3+9+18+21; 50, 1+2+3+unknown; 51, 1+2+18; 52, 1+2+9+18; 53, 1+2+6+18; 54, 2+20; 55, 2+3+18+19. Assignments of signals to metabolites are indicated in gray in the nonpolar profile. Letter codes have been assigned to each nonpolar region or metabolites. A, C, D, and F, fatty acid spectrum regions; B, linoleyl fatty acid region; E and L, unsaturated fatty acid regions; G, free fatty acids region; H, polyunsaturated fatty acids region; I, diacylglicerid and triacylglicerid region; J, triacylglicerid 2 region; K, triacylglicerid 1 region; M, 1,2 diacylglicerid region; N, polyphenols region; O, aldehydes group region; Ac, acetyl group; DGA, 1,2-diacylglicerid; FAI, fatty alcohols; P1, polyphenol derived 1 of p-coumaric acid; P2, polyphenol derived 2 of p-coumaric acid; Ter, terpene compound 1; TGA1, triacylglicerid 1; TGA2, triacylglicerid 2; U1, unknown compound 1; U2, unknown compound 2.

ences in metabolomic and stoichiometric variables among seasons (pseudo-$F_{3,68} = 29.3$, $P = 0.01$, Table S2).

The seasonal principal component analysis (PCA) with all the stoichiometric and metabolomic data resulted in a first principal component (PC1) separating the foliar stoichiometry and metabolome in the different seasons (Fig. 2). The third principal component (PC3) separated the foliar stoichiometry and metabolome in summer from those in the other seasons (Fig. 2 and Table S3). Also, the PC3 was related directly to the effects of the climatic treatments, because drought-treated plants presented the highest foliar K/P and the lowest foliar N/K concentration ratios in autumn, winter, and summer.

In the additional PCA of only the metabolomic data (Fig. S1), the PC1 scores correlated significantly with the foliar concentrations of N and P and with the N/P ratio (Fig. 3). Discriminant analyses of those relationships also showed significant differences among seasons in all cases: PC1 vs. N (Wilks' $\lambda = 0.35$, $F_{(3,172)} = 108.6$, $P = 0.000$), PC1 vs. P (Wilks' $\lambda = 0.24$, $F_{(3,172)} = 183.6$, $P = 0.000$), and PC1 vs. N/P (Wilks' $\lambda = 0.61$, $F_{(3,172)} = 37.4$, $P = 0.000$).

Climatic Stoichiometric and Metabolomic Changes. In the experimental plots simulating climatic change, the leaves of drought-treated plants had the highest K/P and the lowest C/K and N/K concentration ratios, whereas leaves of the night-warmed plants showed the lowest C/P and N/P concentration ratios (Table S1). The metabolomic profiles of leaves in the water-deprived plots had the highest concentrations of polyphenolic compounds (region N of Fig. 1 and Fig. S2), quinic acid, tartaric acid, and choline, whereas the profiles of leaves in the night-warmed plots had the highest concentrations of fatty acids and compounds related to the amino acids and sugars of plant metabolism (RCAAS) (molecules 10–16 of Table S4).

The MANOVA analysis showed a significant interaction between stoichiometric and metabolomic variables and treatments ($F_{2,13484} = 5.14$, $P = 0.01$). Thus different distributions of global metabolomic and stoichiometry values were observed among climatic treatments. In the PERMANOVA analysis, the different climatic treatments also showed marginally significant global differences in metabolomic and stoichiometric variables (pseudo-$F_{2,68} = 1.90$, $P = 0.10$; Table S2).

The means of the PC3 scores for drought-treated plants in winter and autumn and the means for all treatments in summer presented similar values (Fig. 2), indicating a similar elemental stoichiometry and metabolome in individuals in summer and under drought. Additional PCAs, including only the variables that presented significant differences among climatic treatments within each season, were performed to identify the main patterns of those changes (Fig. 4 and Tables S5 and S6). In general, night-warmed plants were distinct from control and drought-treated plants in the coldest seasons (winter and autumn), whereas both night-warmed and drought-treated plants differed from control plants in the warmest seasons (summer and spring). Leaves of drought-treated plants tended to have more quinic acid, tartaric acid, and choline than control and night-warmed plants in all seasons, and they also had the highest K and K/P ratios and the lowest N/K and C/K ratios (Fig. 4 *A*–*C*). Night-warmed plants presented the highest foliar concentrations of RCAAS, such as malate, citrate, and 3-amino-4-hydroxybtuanoic acid, in all seasons. In autumn, drought-treated plants had higher foliar concentrations of polyphenolic compounds than did control and night-warmed plants, whereas control plants presented the highest concentrations of N and P and the lowest C/N, C/P, and N/P ratios (Fig. 4*D*). These trends also occurred in summer, when control leaves had the lowest C/*P* values (Fig. 4*C*). The highest concentrations of one terpene compound were seen in the leaves of drought-treated and night-warmed plants in autumn and summer (Fig. 4 *C* and *D*).

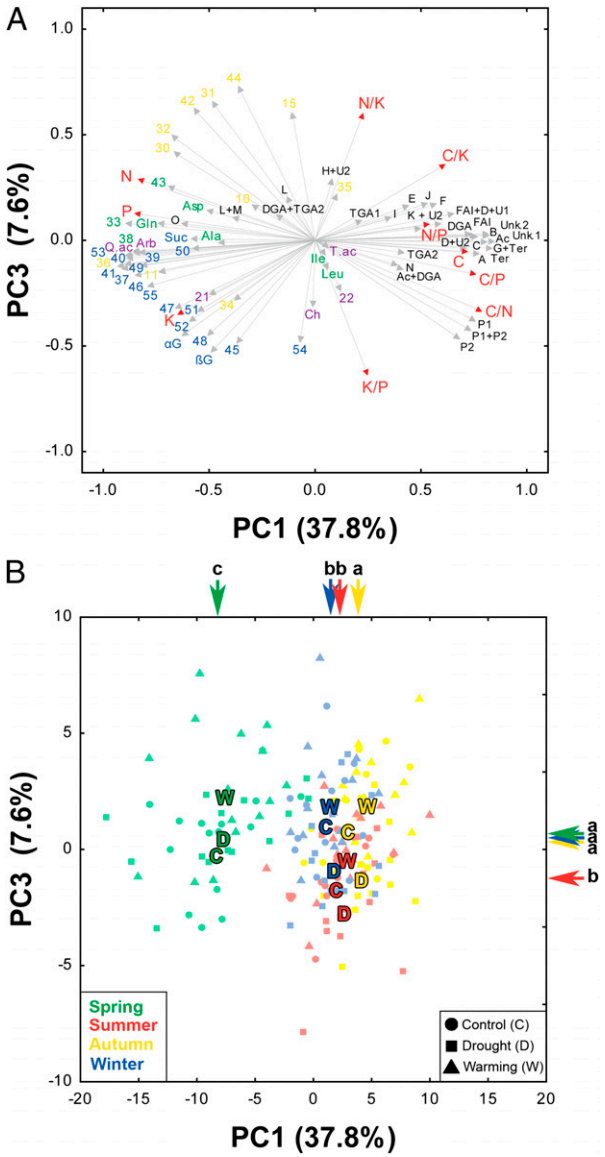

Fig. 2. Biplots of the third principal component (PC3) versus the first principal component (PC1) loadings and scores resulting from PCA conducted with the elemental stoichiometric and ^{1}H NMR metabolomic variables in *E. multiflora* leaves using PC1 and PC3 axes. (*A*) Panel of stoichiometric and metabolomic variables. C/N/P/K ratios are shown in red. Colors indicate different metabolic families: blue, sugars; green, amino acids; yellow, RCAAS; violet, secondary polar metabolites; black, nonpolar metabolites. Assignments are shown in Fig. 1. (*B*) Panel of samples categorized by season and climatic treatment. Seasons are indicated by different colors (red, summer; yellow, autumn; blue, winter; and green, spring). Treatment is indicated by geometric figures: circles, controls; squares, drought-treated plants; triangles, night-warmed plants. Arrows outside plots indicate the mean PC score for each season. The different letters of the cases plot represent the mean of PC1 and PC3 scores for each treatment within each season (C, control; D, drought treatment; W, nighttime warming). The statistically significant differences between seasons were detected by Bonferroni post hoc tests and are indicated by lowercase letters ($P < 0.05$).

Discussion

The ecometabolomic seasonal analyses showed the highest primary metabolic activity in spring (the growing season), when the concentrations of sugars and amino acids directly linked to growth were highest (Fig. 2 and Table S1). This increase in the concentration of primary metabolites coincided with an increase of N and P concentrations in leaves, with a proportionally higher increase in P than in N content leading to lower N/P and C/P content ratios. These results are in agreement with the GRH. The highest concentrations of sugars and amino acids and a lower N/P content ratio thus were associated with the high metabolic activity and rate of growth of the spring season and coincided with the lowest concentrations of nonpolar metabolites (Figs. 2 and 3 and Table S1). The favorable climatic conditions of spring, with high availability of soil water, enhance photosynthetic rates and the uptake of N and P. Higher levels of these elements allow more synthesis of amino acids and proteins (more N), which in turn requires more synthesis of RNA (more N and especially more P) (Fig. 3). These decreasing N/P ratios during the growing season also have been found in other terrestrial plants (20, 21) and in animals (22, 23). Moreover, under these favorable conditions for growth, the assimilated C is allocated more to growth and energy supply (more primary metabolism) than to antistress or defensive mechanisms (less secondary metabolism). In fact, C/N, C/P, and C/K ratios also were correlated positively with nonpolar metabolites, such as fatty acids, terpenoids, polyphenols, and other C-rich components (Fig. 2).

Summer leaves had higher concentrations of sugars that likely remained from the high accumulation during the spring and/or were the result of the increase in cellular osmotic potentials. On the other hand, summer leaves had the highest K/P and the lowest N/K and C/K ratios (Fig. 2 and Table S1). K is involved in the plant–water relationship (24) through plant osmotic control (24–26) and improvement in stomatal function (27). The present ecometabolomic study also demonstrates a shift in the metabolome of *E. multiflora* in response to the treatment representing moderate climatic change (Figs. 2 and 4 and Tables S1 and S5), which follows a very conservative projection for the forthcoming decades (28). Warming increased the level of fatty acids relative to the drought treatment and the control (Fig. 4), a result that agrees with other experimental studies of warming (29). RCAAS also tended to increase under the warming treatment compared with the control (Tables S1 and S5), in agreement with other studies in plants (30) and also in *Drosophila* (31). Interestingly, night-warmed plants showed low concentrations of P related directly to high C/P and N/P ratios (Fig. 4 and Tables S1 and S5). This response of P in plants exposed to warming is still unclear and warrants further study (19).

As expected in a water-limited Mediterranean ecosystem, the drought treatment had considerable effects on the foliar stoichiometry and metabolome because of the increased oxidative stress under drought conditions (32–35). In autumn and spring, the leaves of drought-treated plants had higher concentrations of compounds with antioxidant function, such as some polyphenolic compounds, quinic acid, and tartaric acid, than did the control plants (Fig. 4 and Tables S1 and S5). Quinic acid is a precursor of the shikimic acid pathway, a common metabolic pathway in the biosynthesis of aromatic amino acids such as tyrosine and phenylalanine (36) that are precursors of flavonoids (37). Their antioxidant capacity results from their high reactivity as H or electron donors (38) and from the role of some compounds (e.g., flavonoids) in altering the kinetics of peroxidation (39). In summer, the drought-treated leaves also had higher concentrations of choline, which is involved in osmotic protection (40). These results are supported by some studies that found high concentrations of aromatic amino acids in plants under drought stress (41, 42). These metabolomic differences were accompanied by an increase in K content, resulting in low C/K and N/K ratios and high K/P ratios that also were observed in summer (the drought season) (Figs. 2 and 4) and seem to be related to the improvement in the control of water use (25, 27). The means of the drought-treated plants of winter and autumn in Fig. 2 were located together with the means of all treatments in summer in the PC3 versus PC1 biplot, demonstrating a similar stoichiometry and metabolome in individuals grown in summer and in individuals grown under drought.

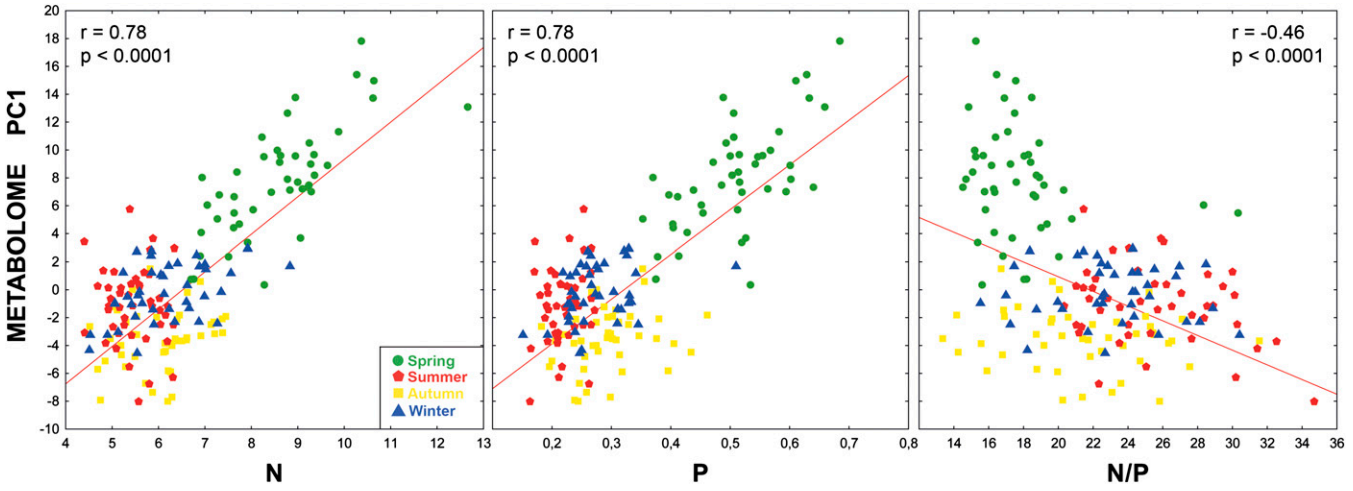

Fig. 3. Relationships of the PC1 scores of a PCA analysis conducted with only the metabolomic data (Fig. S1) with the foliar concentrations of N and P and N/P ratio. Seasons are represented by different colors: red, summer; yellow, autumn; blue, winter; and green, spring.

Some studies have failed to corroborate or have not fully corroborated the GRH in terrestrial ecosystems. Matzek and Vitousek (11) found no link in trees between N/P and protein/RNA ratios. Terrestrial plants and animals invest large amounts of N and P in mechanical structures (wood and skeleton), reproduction, storage, defense, and mechanisms of stress avoidance, exceeding the amounts invested in growth and thus making the relationship between N/P and protein/RNA ratios less prominent. Our C/N/P/K analyses were based on whole-leaf contents (including structural elements), whereas the metabolomics were based only on the extractable aqueous and nonaqueous cellular components. The most abundant foliar structural compounds of plants (mainly cellulose and lignin), however, have no N and P. The global changes in foliar N and P thus can be compared directly with the shifts in foliar metabolism, because most of the N and P of leaves are extracted in the polar and nonpolar extracts. In any case, the link between metabolome and stoichiometry might have been even stronger had we analyzed only the N and P contained in the extractable fractions. This result, however, would not have been comparable with most studies of ecological stoichiometry in which all N and P contents of the tissues usually are analyzed.

In conclusion, the results show that the N/P and C/P content ratios decreased in the growing season, supporting the GRH, and that these changes were related to shifts in the metabolome of the plants, with high concentrations of sugars and amino acids. The results also show that the study of shifts in the stoichiometry of terrestrial plants should consider other elements, for example K and its elemental ratios C/K, N/K, or K/P, that may vary with metabolomic shifts in response to environmental changes such as drought. All these results support our hypothesis of a strong relationship between stoichiometry and the metabolome. By coupling stoichiometry and ecometabolomics, these results improve our understanding of developmentally and environmentally linked shifts in C/N/P/K contents and of how these contents can change to achieve an optimum allocation for growth and other functions, such as storage, defense, reproduction, or resistance to stress. This coupling enhances our understanding of the influence of stoichiometry on the lifestyle of organisms and on the structure, function, and evolution of ecosystems (2, 12, 13).

Materials and Methods

Study Site and Experimental Design. The study was conducted in Garraf Natural Park on the central coast of Catalonia (41°18′ N, 1°49′ E), which has a Mediterranean climate. Nine plots were established in March 1999: three as controls and six subjected to a treatment representing climatic change. Plants in three of the treatment plots were exposed to nocturnal warming, and three plots represented drought conditions. The warming treatment increased the temperature 0.9 °C on average during the night. The drought treatment reduced rainfall during spring and autumn so that soil moisture decreased an average of 19% in these plots (*SI Materials and Methods*). For details see refs. 33 and 43.

Sampling and Processing Leaves. Sampling was conducted once each season from summer 2009 to spring 2010. Five plants in each plot were chosen randomly as study objects. A homogeneous fraction of youngest-cohort, well-developed leaves from each individual in each season was frozen in situ in liquid nitrogen. The youngest leaves thus were spring leaves, and the oldest leaves were winter leaves. Frozen leaves were lyophilized and ground with a ball-mill grinder (*SI Materials and Methods*).

Chemical Analyses. Concentrations of C and N were determined by combustion coupled to gas chromatography with a CHNS-O Elemental Analyzer (EuroVector). For the analyses of P and K, samples first were digested in acid (44) in a high-pressure microwave and then were analyzed by optic emission spectrometry with inductively coupled plasma (Optima 2300RL ICP-OES; Perkin-Elmer) (*SI Materials and Methods*).

Plant-Extraction Procedures for NMR Analyses. Extracts with water–methanol (1:1) and chloroform were obtained. Briefly, 200 mg of powdered leaf material was introduced into a centrifuge tube. Six mL of water–methanol (1:1) and 6 mL of chloroform were added to each tube (45). Samples were vortexed for 20 s and then sonicated for 1 min at room temperature (46). All tubes were centrifuged at 1,000 × *g* for 30 min. Four mL of each fraction (aqueous and organic) were collected independently into jars. This procedure was repeated twice. Aqueous samples, previously redissolved in water (<15% methanol), were lyophilized. Organic samples were placed in a round-bottomed evaporation flask and dried in a rotary vacuum evaporator. Finally, 1 mL of KH_2PO_4-NaOD–buffered D_2O (pH 6.0) was added to the dried aqueous fractions, and 1 mL of chloroform-D was added to the dried organic fractions. All contents were transferred into Eppendorf tubes and centrifuged for 3 min at 6,000 rpm and for 2 min at 10,000 rpm. The supernatants were transferred into NMR sample tubes (*SI Materials and Methods*).

NMR Experiments. Samples were scanned through high-resolution 1D ^{1}H NMR spectroscopy generating polar and nonpolar metabolic profiles (spectra) (Fig. 1, Figs. S2 and S3, and Table S4) using a Bruker Avance 600 spectrometer (Bruker Biospin) at a field strength of 14.1 T (^{1}H frequency, 600.13 MHz). The probe temperature was set at 298.0 K. Sample handling, automation, and acquisition were controlled using TopSpin 2.1 software (Bruker Biospin). For both kinds of samples, a standard ^{1}H 90° pulse sequence was used, and the residual water resonance was suppressed in the samples extracted with water–methanol. Following the introduction of the probe, samples were allowed to equilibrate for 1 min. Each spectrum was acquired as 32k data points over a spectral width

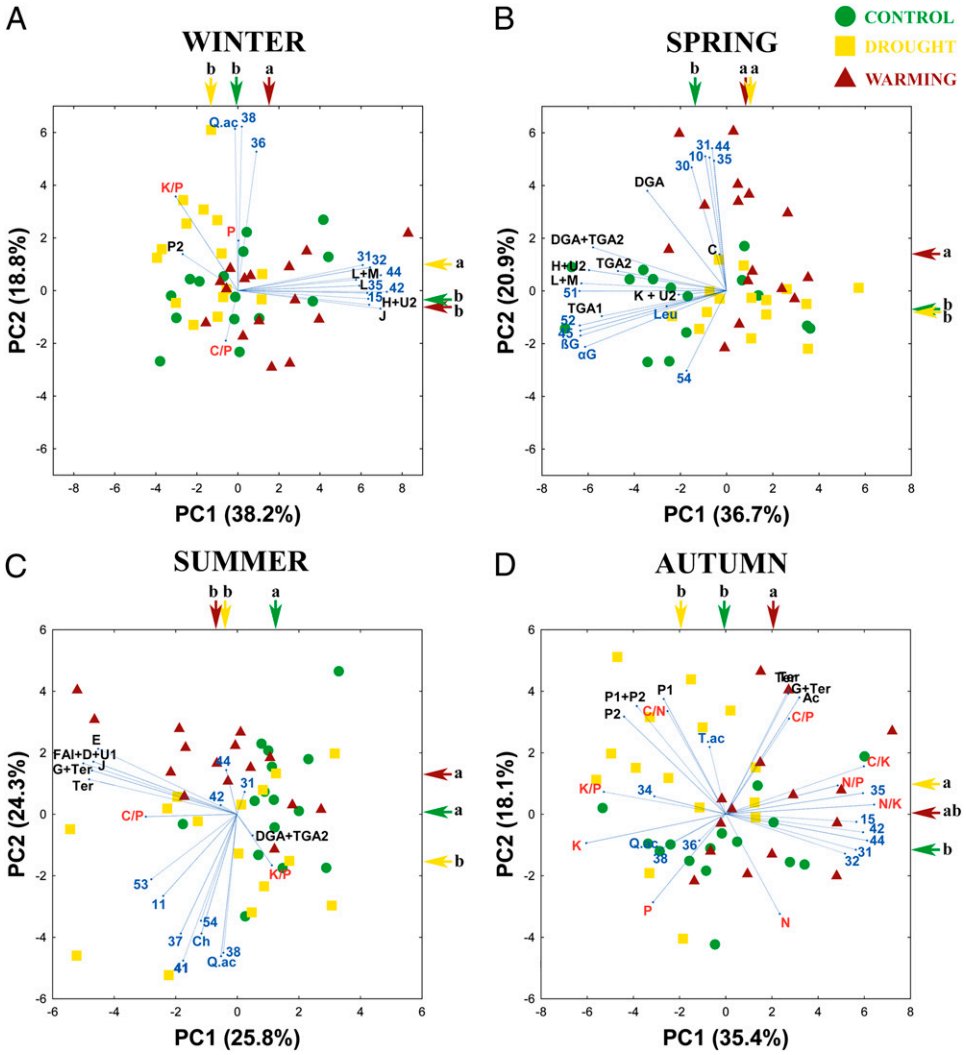

Fig. 4. Plots of the PCAs conducted with the ^{1}H NMR metabolomic and stoichiometric variables that presented different responses to climatic treatments in each season of the study, (*A*) Winter, (*B*) Spring, (*C*) Summer, and (*D*) Autumn. C/N/P/K ratios are represented in red. Organic and water-soluble fractions are indicated by color (black, nonpolar; blue, polar). Variable labels are as described in Fig. 1. Treatment is indicated by color: green, control; yellow, drought; red, nighttime warming). Arrows outside the plots indicate the mean PC score for each treatment. The statistically significant differences were tested by Bonferroni post hoc tests and are indicated by lowercase letters ($P < 0.05$).

of 16 ppm, as the sum of 128 transients and with a relaxation delay of 2 s. The total experimental time was *ca*. 8 min per sample (*SI Materials and Methods*).

1D and 2D NMR experiments to identify the metabolites were performed in a Bruker Avance 500 spectrometer (Bruker Biospin) equipped with a high-sensitivity, cryogenically cooled, triple-resonance, TCI probehead at a field strength of 11.7 T (^{1}H frequency, 500.13 MHz). The probe temperature was set at 298.0 K. The software used was TopSpin 1.3 from Bruker Biospin. ^{1}H (500.13 Hz) and ^{13}C (125.76 MHz) NMR experiments were performed on the control samples. The 1D ^{1}H selective total correlation spectroscopy (TOCSY) experiments, as well as 2D experiments, such as ^{1}H-^{1}H–correlated spectroscopy, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C heteronuclear single-quantum correlation, and ^{1}H-^{13}C heteronuclear multiple-bond correlation, allowed the identification of the metabolites. All 1D and 2D experiments were performed directly in the samples prepared for the metabolomic study and used standard Bruker pulse sequences and routine conditions (*SI Materials and Methods*).

Data Analysis. 1D ^{1}H NMR spectra were used for statistical analyses. All ^{1}H NMR spectra were treated by TopSpin 1.3 (Bruker Biospin). Bucketing was conducted with AMIX (Bruker Biospin) to obtain the integral values for spectral peaks (for the bucketing details see *SI Materials and Methods*). All ^{1}H NMR signals corresponding to the same molecular compound or molecular family or with the same molecules overlapped were summed to reduce the final number of variables. The overlapped signals were given different code numbers (Fig. 1).

We tested the normality of each variable in each season by Kolmogorov–Smirnov tests. All variables followed normal distributions. The differences in the ^{1}H NMR spectral peaks and in elemental stoichiometric variables among seasons and/or climatic treatments (control, drought, and night-warming) were analyzed using mixed models with individuals and plots as random independent variables and with individuals nested within plots and with climatic treatments and seasons as fixed independent categorical variables (Table S1). We thereafter performed a mixed model for repeated measures of the stoichiometric and metabolomic variables. The MANOVA model included individual plants and plots as random factors and climatic treatment, season, and stoichiometric and metabolomic concentrations (under an unstructured correlation structure) and their interactions as fixed effects. When an interaction between effects was detected, Bonferroni's multiple comparisons were performed. SSPS 19.0 (SPSS Inc.) was used to conduct the mixed model. To test for the differences among seasons and climatic treatments in nutrient concentrations, stoichiometry, and the metabolome, and to accommodate random effects and interaction terms better, we also conducted PERMANOVAs (47) using the Euclidean distance, with season (spring, summer, autumn, winter), and treatment (control. drought, warming) as fixed factors and block and individuals nested in block as random factors. When PERMANOVA analyses were significant, we subsequently ran univariate permutational ANOVAs on the concentrations and ratios of nutrients and metabolites using the Euclidean distance. These univariate analyses allowed us to detect the variables causing

the differences in nutrient and metabolomic composition among seasons and treatments. All these PERMANOVA analyses were conducted with the software PERMANOVA+ for PRIMER v.6 (47).

Multivariate ordination analyses (PCAs based on correlations) also were performed to detect patterns of sample ordination in the metabolomic and stoichiometric variables. Additional PCAs for each season, including only the variables that presented differences among climatic treatments, were performed to identify the main patterns of those treatment-induced changes (Fig. 4). Finally, discriminant analyses were conducted to identify the capacity of the PC1 axes of Fig. 2 and the N, P, and N/P variables to separate plants of different seasons. Statistica v8.0 (Statsoft) was used to perform ANOVAs, post hoc tests, PCAs, Kolmogorov–Smirnov tests, and discriminant analyses.

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