Response to Referee #1

General comments: In this manuscript, Zhang et al. report new measurements of intra-plant variation in 15N, and discuss mechanisms that might be responsible for the observed patterns. Overall, the measurements provide a useful characterization of a previously little-studied species and ecosystem type, and the discussion provides a nice overview of some possible mechanisms. ***Response**: Thanks for a careful review and constructive comments.*

Comments: However, the attribution of particular mechanisms to the observed patterns is entirely speculative, and does not convincingly advance current understanding of controls on variation in 15N. Several suggestions to strengthen the manuscript are made below. ***Response**: It would be fantastic if a single paper can not only report a previously un-reported natural phenomenon but also provide a convincing explanation of the mechanism responsible for the observed phenomenon. Unfortunately most of the time science does not advance this way. Instead, scientists have to crawl along the path of research – discovery of phenomenon – proposal of hypothesis – research to test hypothesis – more research – more research …… finally convincing explanation. There is no short cut. In the revision, we have made sure we present our attribution of potential mechanisms as testable hypotheses for future research to tackle.*

Specific comments: [1] Background. It would be helpful for the introduction and discussion to include more information that is specific to the nitrogen cycle in the study systems. In particular, key questions to address are: What are the sources of nitrogen for the study species? How does _15N vary among those N sources? Critically, is Nitraria tangutorum a nitrogen-fixer? The clustering of the _15N values in this study close to 0 per mille, and the fairly high tissue nitrogen concentrations, both suggest that Nitraria either supports nitrogen-fixing symbionts itself, or is obtaining nitrogen from another nearby fixer that occurs in the community. This would have important implications for interpreting intra-plant variation in _15N. ***Response**: Nitrogen cycling in this remote desert region of China has rarely been studied. But it appears N. tangutorum is not a nitrogen fixer as we carefully examined the structures and morphology of excavated fine roots and did not observe any obvious nitrogen fixing features. There was a single conference report on the observation of the presence of endogenous nitrogen-fixing bacteria in N. schoberi. We have revised the site description to include these background information.*

Comment: [2] Statistical methods. The authors have used an approach based on fixed effect models which indicates that the interaction between tissue N and P concentrations is the strongest predictor of variation in _15N. However, it is not clear that the fixed effect approach is appropriate here, so this statistical result may not be reliable. Since the samples were collected in a hierarchical sampling design (i.e., different organs nested within the same nebka; different nebkas nested within the same site; multiple sites, etc.), it seems like analysis with mixed effect models would be a more appropriate way of testing for the best predictors of variation in _15N. Suggest either repeating the analysis with mixed effect models, or clearly justifying why fixed effect models were applied. ***Response**: Thanks for this interesting comment. We believe most regressional models in natural science should be fixed effects models because natural scientists are generally interested in causal effects. We believe the fixed effects model is the correct model to be used here. This is because we are only interested in the detection of the existence or absence of any potential correlation between the specific effect (nitrogen isotope composition) and independent variables (N and P contents) across plant organs and we are not interested in how any peculiarities of nebkas and locations might or might not affect the specific effect. The only purpose of including multiple nebkas and locations is to*

increase the power of statistical analyses (i.e., to avoid using data from a single nebka at a single location and getting a spurious correlation). Furthermore, we are not interested in making inferences outside the observed dataset. For all these reasons, the use of a fixed effects model with stepwise regression is the correct option. We have added this explanation in the revision.

Comment: [3] Interpretation of statistical results. In lines 355-356, the authors state, "To our knowledge, no previous studies have systematically evaluated relationships between intraplant variations in _15N and organ N or P contents." In fact, some previous work has addressed these relationships. One analysis that is particularly relevant is Kalcsits, Lee A., Hannah A. Buschhaus, and Robert D. Guy. 2014. "Nitrogen isotope discrimination as an integrated measure of nitrogen fluxes, assimilation and allocation in plants." Physiologia Plantarum 151: 293-304. Since this is a fairly recent paper and was not cited by the authors, they may not be aware of it. However, the theory developed by Kalcits et al. has the potential to be quite helpful as a foundation for interpreting the Nitraria results reported here. Suggest reviewing this reference, and incorporating it into the discussion.

***Response**: Thanks for pointing out to us the paper by Dr. Lee Kalcits and his collaborators. Indeed this paper and some other papers by Dr. Kalcits are very relevant to our study. We have revised our discussion in view of findings by Dr. Kalcits.*

Technical comments:

Comment: [a] In the methods, suggest including details of digestions used to prepare samples for ICP-OES analysis. ***Response**: Suggestion adopted.*

Comment: [b] Both in the methods and in the figure legends, suggest specifying whether these are molar or mass ratios (i.e., C/N, N/P, C/P). ***Response**: Suggestion adopted.*

Comment: [c] Figure 2, Difficult to focus on plotted data because ANOVA codes are so large. Suggest shrinking size of font used for ANOVA codes to improve readability. ***Response**: Suggestion adopted.*

Comment: [d] Figure 5, Seems redundant. Perhaps the information here could be somehow combined with Fig. 3. ***Response**: If Figure 5 is combined with Fig. 3, the resulted figure would be very complicated. It is probably better to keep them separated.*

Response to Referee #2

Comment: This paper from Zhang et al. is intituled "The interaction between nitrogen and phosphorus is a strong predictor of intra-plant variation in nitrogen isotope composition in a desert species". In this paper, the authors aim to demonstrate the origin of the intraplant variations in dïA¸d'15N by looking at its correlations with C,N,P concentration in different parts of the two desert plants in China. ***Response****: We thank this referee for taking the time to review our manuscript. It is NOT our aim 'to demonstrate the origin of the intraplant variations in dïA¸d'15N'. Our objective, which was understood well by the first referee, is to "report new measurements of intra-plant variation in 15N, and discuss mechanisms that might be responsible for the observed patterns (See Referee #1's review). In this revision, we make sure our objective is clearly stated to avoid misunderstanding.*

Comment: In general, this paper lacks details in background and analysis that makes it difficult to follow. In addition, the novelty of the paper stands at the comparison of dïA¸d'15N with other nutrients content and the analysis of a particular plant in China. The analysis of the data is not convincing and lack of deep analysis. Finally, the discussion is long and purely speculative when many of the speculation could have been supported by data eventually. Here are specific details that could help improve the manuscript: ***Response****: While we value this referee's effort to help improve the manuscript, we have hard time to understand some of the comments made by this referee. This is made worse by the fact that symbols do not show up properly in the review text (perhaps because the review was written in a non-English Word editing software). When we feel we have a sufficient understanding of a comment made or when a suggestion is specific enough for us to act, we revise our manuscript accordingly if we deem appropriate. If any misunderstanding occurs on our part, we ask this referee to let us know so that we can improve the manuscript further to address his or her concerns adequately.*

Comment: Introduction: This part is too long but somehow informative. Shortening the introduction incorporating specific details on fractionation factors and natural variations of ïA¸d'15N. The introduction is plagued by a lack of precision in the words used, especially with unnecessary adverbs and superlative. ***Response****: We try to remove any unnecessary words. Fractionation factors are now given. But we don't completely understand the first two sentences of this comment; each sentence seems to contain contradictory meanings. Please clarify.*

Comment:L.70: "plant photosynthesis, growth and metabolism and substrate supply for microbial activities" Why do you speak about microbial activity here, if you have no data to support it, why Photosynthesis if not measured? ***Response****: We are puzzled by this comment. This is in the very first paragraph of the whole paper and we are trying to place our particular study in a boarder context. Also we are not sure why the referee thinks we need to measure microbial activity and photosynthesis for this study and why the two questions are asked together. Please clarify and help us to understand your intention.*

Comment: L.73: "rarer N isotope 15" : : : "more abundant" why not using natural isotope composition. ***Response****: Suggestion adopted.*

Comment:L. 81: "types of mycorrhizal fungi" Are you planning to measure it? If so where are the

data? Do you have measured soil dïA¸d'15N? ***Response**: This study is a report on intraplant variations. Future studies should look at these issues.*

Comment:L.87: "relatively few studies" what does it mean? ***Response**: This phrase is revised to make it clearer.*

Comment: L.86-89: these 2 sentences seem in opposition. ***Response**: Thanks for pointing this out. These two sentences have been revised.*

Comment:L.100- 102: If both metabolisms are different could be useful to detail the discrimination factor and why is it so different. ***Response**: Agree. Modeling approaches could be very useful here.*

Comment:L.120: "This assumption led to the belief that organic N compounds: : :." It sounds like you are saying that science believes not that science is based on fact! Need to be rephrased. ***Response**: Suggestion adopted.*

Comment: L.129: "which has a large isotope effect" how much? ***Response**: Information is now added.*

Comment: Material and methods It is surprising that no analysis of the soil dïA¸d'15N was carried out. Statistical analyses: No detail on the analysis of slope of the regression was given. Arcsin analysis of slope sounds appropriate. Please explain how you obtain your p values in the correlations. ***Response**: The present study focuses on intraplant variations rather than the difference between the whole-plant d15N and the nitrogen sources. Although characterization of nitrogen sources is not critical for the present study, future studies that aim to providing a definitive explanation of the observed patterns should consider this aspect. The suggested statistical details about the regression are added in the revision.*

Comment: Results L.316-319: Since no data on the difference of dïA¸d'15N in soil at both sites was given, it is difficult to tell if this conclusion is not only associated with soil ïA¸d'15N variability. ***Response**: Good point. We point this out in the revision.*

Comment:L.340:"Since fine roots differ from other organs in that fine roots are the primary organs for nitrate reduction" Your data are actually showing the opposite since the ïA¸d'15N is way above the dïA¸d'15N of all organs. If fine roots were the main site of reduction of N then you should expect transport of amino acids to the leaves and a more homogeneous ïA¸d'15N between roots and leaves. In addition, if this means for the authors that NH4 is transported to the leaves, then there is still a 16-20 per mil fractionation by the NR that should be taken into account and should show the higher difference in dïA¸d'15N. dïA¸d'15N data should be presented relative to the substrate (soil NO3 or NH4) or relative to the origin (root). ***Response:** Sharp eyes! Thanks for catching the problem this sentence causes. Clearly our data do not support the literal meaning of it and we actually did not mean it. We have revised the sentence to "Since fine roots differ from other organs in that fine roots are the primary organs for nitrogen acquisition". Hopefully this revision clears up this referee's concerns.*

Comment: Finally, a lot of the correlations were made using the data for all organs and leaves. In

many cases, the 6 data points of the leaves affect the correlation. If leaves are removed from the data for this analysis, a different correlation could be found. Finally, since metabolisms of roots and leaves are likely to be different as suggested by the authors, at least in term of reduction of N sources, it could be interesting to present correlation by organs instead of pooling them. In figure 3 and 5, if leaves are removed from the graph, the correlation between d15N and P disappear highlighting the need to do the organ-specific analysis. ***Response:*** *We are puzzled by this referee's comment on the impact of leaf and fine root samples on the correlation between d15N and N and P contents. We have conducted analyses by excluding leaf samples (Figure 3 and 4) and fine root samples (Figure 5) and found that the correlation is still statistically significant, which seems contradictory to what this referee is stating here. Since our primary interest was in across-organ variations, our measurements were not designed to examine variations within the same organs (the number of independent samples would be too few for this purpose).*

Comment: Discussion Overall the discussion is very well written and clear. ***Response:*** *Thanks.*

Comment: It may lack a conceptual framework. Many of the explanation in the text stand on speculation more than the data presented. There is a clear disconnection between the interesting debate of the relationship between d15N and P and the data presented. An example of this discrepancy is the many recalls to the reader of the focus on leaves (L.404, L.419). ***Response:*** *We basically agree with this assessment. We have struggled to come up with a reasonable explanation for the observed patterns. Unfortunately, actual measurements on intra-plant variations in d15N and their relationships with organ nutrient contents are extremely rare. We believe this makes this present study valuable. We'd love to hear from this referee if he or she thinks there is a better conceptual framework than we propose here to explain the observed patterns.*

Comment: The utilization of unnecessary abbreviations clouds the main information. ***Response:*** *Thanks. The revision now minimizes the use of abbreviations.*

Comment: L. 361 – 363, why do you use EFO, IFO, and EIFT when you will be using it only 2-3 times? Simply use words, it is not much longer. ***Response:*** *Suggestion adopted.*

Comment: L.358-359: Is there a way to present this synthesis into a simple graph? ***Response:*** *Excellent suggestion! A diagram is added (see Figure 6).*

Comment:L.363: "External factors include different sources of nitrogen" Since you recognized it is an important factor, why no data were shown? ***Response:*** *This will be important for future studies when a convincing mechanistic explanation of the observed patterns is attempted. For the present study we are content with reporting a previously unreported phenomenon and developing testable hypotheses for future research.*

The interaction between nitrogen and phosphorous is a strong predictor of intra-plant variation in nitrogen isotope composition in a desert species

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Abstract

Understanding intra-plant variations in $\delta^{15}N$ is essential for fully utilizing the potential of $\delta^{15}N$ as an integrator of terrestrial nitrogen (N) cycle and as an indicator of relative limitation of N and phosphorous (P) on plant growth. Studying such variations can also yield insights into N metabolism by plant as a whole or by specific organs. However, few researchers have systematically evaluated intra-plant variations in $\delta^{15}N$ and their relationships with organ nutrient contents. We excavated whole plant architectures of *Nitraria tangutorum* Bobrov, a C_3 species of vital regional ecological importance, in two deserts in northwestern China. We systematically and simultaneously measured N isotope ratios and N and P contents of different parts of the excavated plants. We found that intra-plant variations in $\delta^{15}N$ of *N. tangutorum* were positively correlated with corresponding organ N and P contents. However, it was the N × P interaction, not N and P individually or their linear combination, that was the strongest predictor of intra-plant $\delta^{15}N$. Additionally, we showed that root $\delta^{15}N$ increased with depth into soil, a pattern similar to profiles of soil $\delta^{15}N$ reported by previous studies in different ecosystems. We hypothesized that the strong positive intra-plant $\delta^{15}N$ – N and P relationships are caused by three processes acting in conjunction: 1) N and P content-driven fractionating exchanges of ammonia between leaves and the atmosphere (volatilization) during photorespiration, 2) resorption and remobilization of N and P from senescing leaves, and 3) mixture of the re-translocated foliar N and P with existing pools in stems and roots. To test our hypothesis, future studies should investigate plant N volatilization and associated isotope fractionation and intra-plant variations in $\delta^{15}N$ in different species across ecosystems and climates.

Key words: Nitrogen isotope fractionation, volatilization, phosphorous, photorespiration, resorption and remobilization

INTRODUCTION

Nitrogen (N) is ~~frequently~~ the most limiting nutrient in many terrestrial ecosystems, especially those in temperate and boreal regions (Vitousek 1994). ~~Consequently, N and its cycle are fundamental to ecosystem structure and functioning.~~ As atmospheric CO_2 concentrations continue to increase due to anthropogenic fossil fuel emissions, the limiting effects of N on ecosystem productivity may become increasingly important (Luo et al. 2004; Thornton et al. 2007; Sun et al. 2014). Understanding the N cycle is essential to forecasting and predicting ecosystem dynamics in response to climate change. Plant N acquisition, transformation, and translocation are key steps in N cycling because they subsequently affect plant photosynthesis, growth ~~and metabolism~~, and substrate supply for microbial activities (Manzoni et al. 2010; Vitousek et al. 2010). Many physical, biological and chemical processes that control plant N acquisition, transformation, and translocation discriminate against ~~the heavier, rarer~~ N isotope 15 (^{15}N) in favor of ~~the lighter, more abundant~~ N isotope 14 (^{14}N). As a result, the variations in the relative abundance of ^{15}N to ^{14}N, quantified as $\delta^{15}N$, of plants contain rich information about these processes (Högberg 1997; Robinson 2001, Evans 2001, Dawson et al. 2002). For this reason, $\delta^{15}N$ is often considered an integrator of terrestrial N cycling and numerous studies have analyzed natural variations in plant $\delta^{15}N$ across disturbance and successional stages (e.g., Hobbie et al. 2000; Wang et al. 2007; Resco et al. 2011; Hyodo et al. 2013), climate and topoedaphic gradients (e.g., Austin and Sala 1999; Schulze et al. 1998; Martinelli et al. 1999; Amundson et al. 2003; Craine et al. 2005 & 2009; Bai et al. 2009), species (e.g., Cernusak et. 2009; Gubsch et al. 2011), and types of mycorrhizal fungi (Hobbie and Hobbie 2008; Hobbie and Högberg 2012). Other studies have used $\delta^{15}N$ as an indicator of relative N and phosphorus (P) availability and limitation on plant growth (McKee et al. 2002; Wigand et al. 2007; Inglett et al. 2007; Mayor et al. 2014). These studies have demonstrated the power of using natural variations in $\delta^{15}N$ to understand physical and biological processes controlling N cycling in terrestrial ecosystems.

Compared with the prolific studies on variations in $\delta^{15}N$ across ecological and climate gradients and species, ~~relatively~~ fewer studies have systematically evaluated intra-plant variations in $\delta^{15}N$. Studies that did examine intra-plant variations in $\delta^{15}N$ were often conducted in controlled environments. Most such studies focused on differences between roots and leaves~~However, large intra-plant variations in $\delta^{15}N$ have been reported and such variations~~. It has been~~Many such studies~~ found that leaves of a plant tended to be enriched in ^{15}N compared with the roots of the same plant

(Bergersen et al. 1988; Yoneyama and Kaneko 1989; Evans et al. 1996; Kolb and Evans 2002) and the difference can be as high as 7‰ which has the same magnitude as variations across ecological and climate gradients. ~~-~~ Such large intra-plant variations in $\delta^{15}N$, if not accounted for, may confound interpretation of large-scale patterns in variations of $\delta^{15}N$ (Evans 2001) and lead to misguided diagnoses of relative N and P limitation on plant growth. ~~-Studies that did examine intra-plant variations in $\delta^{15}N$ were often conducted in controlled environments. Many such studies found that leaves of a plant tended to be enriched in ^{15}N compared with the roots of the same plant (Bergersen et al. 1988; Yoneyama and Kaneko 1989; Evans et al. 1996; Kolb and Evans 2002) and the difference can be as high as 7‰ which has the same magnitude as variations across ecological and climate gradients.~~ Nevertheless, no foliar enrichment or mixed results have also been observed both in controlled experiments (Evans et al. 1996; Hobbie et al. 2008) and in natural environments (Dijkstra et al. 2003).

Several mechanisms have been proposed to explain intra-plant variations in $\delta^{15}N$ or lack thereof. The most commonly discussed mechanisms invoke the differences in the assimilation and transport of inorganic N of nitrate (NO_3^-) and ammonium (NH_4^+) within plants. Both the assimilation of NO_3^- and NH_4^+ discriminate against ^{15}N (Yoneyama et al. 2003; Karsh et al. 2012; Table 1 in Johnson and Berry 2013 lists values for various isotope effects) but fundamental differences exist in their metabolism in plants. NO_3^- is assimilated by nitrate reductase (NR) in a process that involves first the reduction of NO_3^- to nitrite and then to NH_4^+ and finally to amino acids. This process can take place in roots, stems, and leaves (Masclaux-Daubresse et al. 2010). Consequently organic N compounds originated from NO_3^- may come from assimilation events that take place in different parts of the plant (Evans et al. 1996; Evans 2001). The discrimination by NR in roots leads to an enriched pool of unassimilated NO_3^-, which is then transported to other parts of the plant via the transpiration stream of xylem. Thus leaves and shoots are expected to be enriched in ^{15}N as compared with roots when NO_3^- is the source of nitrogen. This enrichment has been found to be correlated with the transpiration efficiency of the N acquisition (Cernusak et al. 2009).

The impact of NH_4^+ assimilation on intra-plant variations in $\delta^{15}N$ was traditionally thought to be minimal but more recent studies indicate that this might not be the case. NH_4^+ is usually the most important source of N available to plant roots in natural terrestrial ecosystems (Schjoerring et al. 2002) although NO_3^- and NH_4^+ are often available together (Bijlsma et al. 2000). NH_4^+ is also

produced by plants as a central intermediate in a wide variety of metabolic processes such as NO_3^- assimilation, photorespiration, lignin biosynthesis, protein turnover, and degradation of transport amides (Joy 1988; Massad et al, 2010; Flechard et al. 2013). However, NH_4^+ is toxic to plants at high concentration (Britto and Kronzucker 2002). Because of this, early workers assumed that once absorbed by roots, it is immediately assimilated in roots by the glutamine synthetase/glutamate synthase (GS-GOGAT) pathway. This assumption led to a further hypothesis~~the belief~~ that organic N compounds derived from NH_4^+ ultimately result from a single assimilation event occurred in roots and therefore no intra-plant variation in $\delta^{15}N$ should occur for plants that have grown with NH_4^+ as the sole nitrogen source (e.g., Evans et al. 1996). However, later studies depict a much more complicated picture of assimilation and transport of NH_4^+ within plants. This complication involves two aspects. First, it has been found that a significant amount of NH_4^+ can be transported in the xylem from roots to shoots and the NH_4^+ pools in the apoplast in general and leaf tissues in particular can respond rapidly to the supply of NH_4^+ solution to the roots (Mattsson and Schjoerring 2002; Schjoerring et al. 2002). Second, a recent modeling study suggests that the photorespiration-induced exchange of ammonia (NH_3) between leaf and the atmosphere, which has a large isotope effect (the isotope effect for molecular diffusion of NH_3 is 1.0176 in still air and 1.0117 in leaf boundary layer; Farquhar et al. 1983), can substantially influence plant and atmospheric N isotopic compositions (Johnson and Berry 2013). This process has been mostly ignored in previous studies of terrestrial variations in $\delta^{15}N$. Thus it appears that the impact of NH_4^+ assimilation and transport as well as the NH_3 exchange with the atmosphere during photorespiration on intra-plant variations in $\delta^{15}N$ may have been underestimated.

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Besides the mechanisms discussed above, other fractionating processes such as transamination, redistribution of relatively enriched or depleted metabolites, differential losses of N from plant organs, resorption and remobilization of N from senescing leaves have been suggested as potential causes of intra-plant variations in $\delta^{15}N$ (Evans 2001; Werner and Schmidt 2002; Cernusak et al. 2009; Tcherkez 2011; Gauthier et al. 2013). In particular, fractionations in metabolic reactions (e.g., amino acid syntheses) and transfers of isotopically different plant metabolites across plant organs have been highlighted as factors contributing to intra-plant heterogeneities in $\delta^{15}N$ (Gauthier et al. 2013).

Thus,~~From the literature review presented above, it is clear that~~ there is a strong need for systematical evaluation of $\delta^{15}N$ variation across different organs within the same plant. Such

evaluations will provide key guidance for using $\delta^{15}N$ as an integrator of terrestrial N cycling and as an indicator of relative nutrient limitation. They will also offer important insights into plant N metabolism. The present study represents a step in this direction. Our objective is to conduct the first measurement-based systematic evaluation of intra-plant variations in $\delta^{15}N$ and to shed light on potential mechanisms. We attempt to achieve this objective by comprehensively and simultaneously analyzing –variations in $\delta^{15}N$ with carbon (C), N and P contents in different plant organs with excavated whole architectures of a desert species grown in natural conditions.

The joint analysis of N and P is important for understanding variations in $\delta^{15}N$. These two elements are stoichiometrically coupled in plants (Gusewell 2004). ~~It is well known that~~ P availability affects plant photosynthesis and growth which may have implication for $\delta^{15}N$ variations in plants. For example, orthophosphate is a key reactant in photosynthetic carbon assimilation in chloroplasts and its supply directly affects the rates of carboxylation and photorespiration (Sivak and Walker 1986; Kondracka and Rychter 1997). Since fractionation occurs in foliar NH_3 exchange with the atmosphere during photorespiration (Johnson and Berry 2013), an effect of P on plant nitrogen isotope composition can be expected. Another consideration is that the relative availability of N vs. P to plants has consequences on N isotope fractionation. This is because of two factors. First, no fractionation can occur if all available N is assimilated, which may happen when N is limiting (Cernusak et al. 2009; Gauthier et al. 2013). Second, P availability affects when and where N limitation occurs (Vitousek et al. 2010). Indeed, previous studies have found that P availability is correlated with $\delta^{15}N$ in plant biomass. For example, Major et al. (2014) showed that long-term additions of both N and P reduced foliar $\delta^{15}N$ as compared with N or P addition alone in a lowland tropical rainforest. Studies such as this are the basis for the suggestion that $\delta^{15}N$ could be used as an indicator of ecosystem P limitation (McKee et al. 2002; Wigand et al. 2007; Inglett et al. 2007; Mayor et al. 2014).

The present study builds upon the earlier efforts reviewed above and fills a gap in systematic investigation of intra-plant variations in $\delta^{15}N$. We will demonstrate that the intra-plant variations in $\delta^{15}N$ in our study species *Nitraria tangutorum* Bobrov are closely related to organ N and P contents and their interaction. We will show that the intra-plant $\delta^{15}N$ – N and P relationships found in our study cannot be readily explained with mechanisms thought to be responsible for $\delta^{15}N$ variations across species, ecological and climate gradients. To stimulate future~~more~~ research in intra-plant $\delta^{15}N$

variations, we will propose a new, testable hypothesis that we believe most logically explains such variations.

MATERIALS AND METHODS

Nitraria tangutorum Bobrov and the study sites

We previously described in detail the biological and environmental characteristics of *N*. *tangutorum* and the study sites (Zhang et al. 2015). For convenience and completeness, some of the information presented in Zhang et al. (2015) is repeated here. *N*. *tangutorum* is a spiny C_3 shrub species in the *Nitraria* genus of the Zygophyllaceae family. It is endemic to northwestern deserts in China with a distribution including northeastern Tibet, Gansu, Qinghai, Xinjiang, western Inner Mongolia, western Ningxia, and northern Shaanxi. It is a pioneer species of high tolerance to a variety of stresses. *N*. *tangutorum* controls local landscape evolution, owing to its exceptional capability of fixing sands and building sand dunes known as nebkhas or coppice dunes around its extensive shoot and root systems (Baas and Nield 2007; Lang *et al*. 2013; Li *et al*. 2013). The phytogenic nebkha dunes formed by *N*. *tangutorum* prevent or slow down sand movement, making it often the most ecologically important species in its environment. The height of a *N*. *tangutorum* nebkha typically ranges from 1 to 3 m and some may reach 5 m. The base of a nebkha often has the shape of an ellipse with the major axis parallel to the local prevailing wind direction (Fig.1). The nebkha-building characteristic of *N*. *tangutorum* makes it relatively easy to excavate the whole plant including roots for isotope and nutrient analyses. Previously we studied intra-plant variations in carbon isotope composition of this species (Zhang et al. 2015). Wang et al. (2014) studied the variations of its foliar and root nitrogen and phosphorous contents in season and along aridity gradients. To our knowledge, this species has never been investigated for intra-plant variation in $\delta^{15}N$, whether in cultures or in natural environments.

Our field work was carried out in two desert locations. The first site was in Dengkou County, Inner Mongolia Autonomous Region, China. Dengkou County is at the junction between the Hetao Plain and Ulan Buh Desert of the Mongolian Plateau in the middle reaches of the Yellow River. The mean annual temperature is 8.84℃ and the mean annual precipitation is 147 mm with 77.5% of annual rainfall occurring from June to September (1983-2012 averages). The mean annual potential evaporation is 2381 mm (Li *et al*. 2013). The sampling was conducted within an experimental area

(40°24′ N, 106°43′ E) managed by the Experimental Center of Desert Forestry of the Chinese Academy of Forestry. The study site has sandy soil and gray-brown desert soil (Cambic Arenosols and Luvic Gypsisols in FAO taxonomy). The *N. tangutorum* nebkhas in the area are formed on clay soils deposited by the Yellow River. Although the plant community is dominated by *N. tangutorum*, xerophytic species such as semi-shrub *Artemisia ordosica*, perennial grass *Psammochloa villosa*, and annual species *Agriophyllum squarrosum* and *Corispermum mongolicum* can also be found.

The second study site was in Minqin County, Gansu Province, China. Minqin County is located in the lower reaches of Shiyang River, surrounded by the Badain Jaran Desert in the west and north and the Tengger Desert in the east. The mean annual temperature is 8.87℃ and the mean annual precipitation is 117 mm with 73.1% of annual rainfall occurring from June to September (1983-2012 averages). The mean annual potential evaporation is 2643 mm (Du *et al*. 2010). Thus the second study site is somewhat drier than the first site but with similar annual mean temperatures. The sampling was conducted within the Gansu Minqin Desert Ecosystem Research Station (38°34′ N, 102°58′ E). The soil at the Minqin site is similar to that at the Dengkou site with sandy soil in the nebkhas and gray-brown desert soil between nebkhas. The native vegetation is usually dominated by shrubs and semi-shrubs with species such as *N. tangutorum* and *Calligonum mongolicum*. Experimental plots used in this study contained semi-fixed nebkha dunes developed by the growth of *N. tangutorum*. Typically in dry years, *N. tangutorum* is the only species growing in the nebkhas although in wet years, annual species such as *Agriophyllum squarrosum* and *Corispermum mongolicum* can also be found. Because the Minqin site is drier than the DengKou site, the nebkhas at the Minqin site are generally smaller and less populated with plants than at the Dengkou site. The rooting depth is deeper at the Minqin site than at the Dengkou site (see Table 1 in Zhang et al. 2015).

Nitrogen cycling at these remote desert sites has rarely been studied. We are not aware of any previous report that indicates *N. tangutorum* or any co-existing species might be a nitrogen fixer. Throughout our investigation, we found no evidence of any nitrogen fixer existing at the two study sites. We did not investigate whether there might be any nitrogen-fixing symbionts in *N. tangutorum*. However, there was a single conference report on the observation of the presence of endogenous nitrogen-fixing bacteria in a related species *N. schoberi* (Li et al. 2015). Therefore it is possible that *N. tangutorum might also have nitrogen-fixing symbionts*. Nevertheless, soil is probably the primary source of nitrogen for *N. tangutorum* at our study sites. Since the present study focuses on intra-plant

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variations in $\delta^{15}N$, the nature of nitrogen source is not critically important for our study.

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Excavation of *Nitraria tangutorum* nebkhas

In August 2012, three nebkhas were excavated at each study site. The geometrical and biometrical characteristics of the six nebkhas were summarized in Table 1 of Zhang et al. (2015). At the Dengkou site, the three nebkhas were excavated in a sampling area of 40m×40m. At the Minqin site, nebkhas were generally much smaller. To ensure availability for analyses of sufficient biomass materials at this site, particularly the fine roots (see below), three sampling areas each with a dimension of 30m ×30m were established and three nebkhas from each sampling area were tentatively excavated. Two nebkhas from one sampling area and one from another were deemed to have sufficient amount of fine roots for analyses and were therefore excavated fully. The nebkhas were excavated by carefully teasing away the sands from the mounds to expose the root architecture of *N. tangutorum* with particular attention paid to preserving its fine roots and to distinguishing any roots from other plant species that may happen to grow in the same nebkhas. The roots of a *N. tangutorum* plant can be found inside the sand mounds as well as inside the clay layer that generally forms a plain on which the sand mounds rest. We therefore also excavated any roots inside the clay layer to a depth until no more roots could be found. Only biomass materials from *N. tangutorum* were harvested and any materials from all other species that may be present were excluded to ensure pure intra-plant analyses required by this study.

We separated the whole plant biomass into groups of leaves, stems, in-sand roots, and below-plain roots. The in-sand roots, which were roots found inside the nebkha sands but above the plain formed by the underlying clay layer, were further separated into in-sand fine roots (diameter ≤ 2mm) and in-sand coarse roots (diameter > 2mm). The same root diameter threshold was used to separate the below-plain roots, which were found inside the clay layer under the nebkha sands. Furthermore, the below-plain fine and coarse roots were grouped in a 20cm depth increment from the plain surface. We did not separate the in-sand fine and coarse roots into layers because a nebkha has a cone shape on top, making a layer hard to define. Also we did not use a simple 'below-ground' group because 'ground' is not well defined in a nebkha-populated landscape and because there are large physical and chemical differences between sands and clay which may affect the isotope compositions of roots growing in them. Litter was rarely found on the nebkhas, presumably because

strong winds at the study sites can easily blow away any litter produced. However, woody debris from dead ramets was present inside the sand mounds and was collected during excavation. Thus for each nebkha, we differentiated the following categories of *N. tangutorum* biomass for intra-plant isotope analyses: leaves, stems, in-sand fine roots (ISFR), in-sand coarse roots (ISCR), below-plain fine roots (BPFR) in 20 cm depth increments, and below-plain coarse roots (BPCR) in 20cm increments, and woody debris (WD). Nutrient contents and nitrogen isotope compositions were measured separately for each category.

Measurements of nutrient contents and nitrogen isotope compositions with excavated biomass

All categories of *N. tangutorum* biomass (leaves, stems, ISFR, ISCR, BPFR in 20cm increments, BPCR in 20cm increments, and WD) from each excavated nebkha were dried to constant weight (60℃, 48 hours). The dry weight of biomass was determined with 0.01 g accuracy on an analytical scale. Dried materials were randomly sampled from each biomass category and ground to 80 mesh in Tyler Standard Sieve Series (0.177 mm opening). The resultant powder was separated into six duplicates. Three duplicates were analyzed for C, N and P contents and the remaining three for isotope compositions. The C, N and P contents were measured in the Environmental Chemistry Analysis Laboratory in the Institute of Geographic Sciences and Natural Resources Research, the Chinese Academy of Sciences, Beijing, China. Total sample carbon and N were measured with the vario MACRO cube (Elementar Company, Germany). The analytical precision was better than 0.5% Relative Standard Deviation (RSD). Total P was measured with the ICP-OES OPTIMA 5300DV (PE, USA). Wet digestions method was applied in the analysis (Webb and Adeloju 2013).-Sample preparation and assaying followed standard procedures per instrument instruction. The analytical precision was better than 2% RSD.

The nitrogen isotope compositions were analyzed at the Stable Isotope Ratio Mass Spectrometer Laboratory of the Chinese Academy of Forestry (SIRMSL, CAF), Beijing, China. The instrument used was a Delta V Advantage Mass Spectrometer (Thermo Fisher Scientific, Inc., USA) coupled with an elemental analyzer (FlashEA 1112; HT Instruments, Inc., USA) in the continuous flow mode. Isotope compositions were expressed using the delta notation (δ) in parts per thousand (‰): $\delta^{15}N$ (‰) = $[(R_{sample})/(R_{standard}) -1] \times 1000$, where R is the molar ratio of ^{15}N to ^{14}N. The measurement applied the IAEA-600 standard (Caffeine) relative to atmosphere N_2. The analytical precision was better than

0.2‰ based on replicate measurements of the reference standard.

Statistical analyses

Two-way ANOVA analyses (organ by site) were performed with SPSS (Ver.17.0). C, N, and P contents, $\delta^{15}N$, C/N ratios, N/P ratios, and C/P ratios were analyzed for differences between organs and between study sites. All ratios were mass-based. Tukey post-hoc tests were used to determine pairwise differences for significant effects ($P < 0.05$). Linear and multilinear regression analyses were used to determine the relationships between the organ $\delta^{15}N$ and nutrient contents. Due to the strong correlation between organ contents of different nutrients (Zhang et al. 2015) and therefore the potential presence of multicollinearity, we used stepwise regression to determine the most significant predictor(s) (including interaction) of intra-plant variations in $\delta^{15}N$. Both forward and backward methods were used in the stepwise regression with F-to-Enter and F-to-Remove set at 4.0 (P = 0.05) and 3.9 (P = 0.052), respectively. All P values for regression slope analyses were computed on a two-tailed basis.

Our regressional analyses were based on fixed effects models. Fixed effects models are appropriate for the present study because we attempt to explain variations in $\delta^{15}N$ as a function of non-random explanatory variables such as C, N and P contents and their ratios (mixed or random effects models are used more commonly in social sciences, Cameron and Trivedi 2005). We are only interested in the detection of existence or absence of any potential correlation between the specific effect (nitrogen isotope composition) and explanatory variables across plant organs but are not interested in how any peculiarities of nebkas and locations might or might not affect the specific effect. Nevertheless, we did analyze the data from the two study sites separately and came to similar concluisons. Therefore, to increase the statistic power of our analyses (i.e., to avoid using data from a single nebka at a single location and getting a spurious correlation), we pooled the data together but with sites and organs clearly marked in figures so that patterns for individual sites and organs can be seen clearly.

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RESULTS

Variations in $\delta^{15}N$ among plant organs and between study sites

For comparing $\delta^{15}N$ among plant organs and between sites (Fig. 2), we averaged the duplicate mean of each organ across the three nebkhas at each site. Results for comparisons of nutrient values were

already presented in Zhang et al. (2015) and thus not repeated here. There were considerable variations in $\delta^{15}N$ values among plant organs and between study sites. At both the Dengkou and Minqin sites, leaves had positive $\delta^{15}N$ and were enriched in ^{15}N compared with corresponding stems and roots at the same site. Also at both sites, the $\delta^{15}N$ value of fine root followed the same order: ISFR < 1FR < 2FR < 3FR < 4FR; i.e., it increased with depth into soil. Here 1FR, 2FR, 3FR, and 4FR refer to fine roots in 0 – 20 cm, 20 – 40 cm, 40 – 60 cm, and 60 to 80 cm soil depths, respectively. The same pattern was repeated for the $\delta^{15}N$ value of coarse root; the only exception was 2CR (coarse root at a soil depth of 20 to 40 cm) at the Dengkou site which dropped out of the general order. The $\delta^{15}N$ values of fine roots at the Dengkou site were consistently higher than the corresponding coarse roots both inside the nebkha sands and below the plain of the same site. In contrast at the Minqin site, the $\delta^{15}N$ values of fine roots were consistently less than the corresponding coarse roots except for the roots deep into the plain (40 - 80 cm) where the fine root was more enriched. At the Dengkou site, the stem had the lowest $\delta^{15}N$ while at the Minqin site, the ISFR had the lowest $\delta^{15}N$. At both sites, the $\delta^{15}N$ value in the woody debris was greater than the corresponding stem although the difference was not statistically significant. The foliar $\delta^{15}N$ at the Dengkou site was higher than at the Minqin site. In fact, in all biomass categories investigated, the $\delta^{15}N$ value at the Dengkou site was greater than its corresponding counterpart at the Minqin site. The $\delta^{15}N$ values of plant organs at the Dengkou site were mostly positive while at the Minqin site, the values were mostly negative. Unfortunately, these site differences cannot be attributed at the present study since we did not measure potential sources of nitrogen, particularly soil nitrogen, at the two study sites.

Intra-plant relationships between $\delta^{15}N$ and nutrient concentrations

Even though intra-plant and between-site variations in $\delta^{15}N$ were large, these variations were unified in relationships with organ nutrient contents (Fig. 3). The large intra-plant variations in $\delta^{15}N$ (~7‰ at the Dengkou site and 4‰ at the Minqin site) as well as in organ nitrogen and phosphorous contents facilitated regression analyses between these variables. Because the intra-plant relationships were not significantly different between the two study sites, we pooled the data from the two sites in regression analyses. We found that intra-plant variations in $\delta^{15}N$ were significantly correlated with the organ contents of carbon (Fig.3a, $R^2 = 0.25$, $P < 0.0001$), nitrogen (Fig. 3b, $R^2 = 0.44$, $P < 0.0001$)

and phosphorous (Fig. 3c, $R^2 = 0.40$, $P < 0.0001$) and with the organ ratios of carbon to nitrogen (Fig. 3d, $R^2 = 0.41$, $P < 0.0001$) and carbon to phosphorous (Fig. 3f, $R^2 = 0.25$, $P < 0.0001$). The correlations were positive with organ nitrogen and phosphorous contents but negative with the carbon content and the carbon to nitrogen and carbon to phosphorous ratios. No correlation with the organ nitrogen to phosphorous ratios was found (Fig. 3e).

Although intra-plant variations in $\delta^{15}N$ were significantly correlated with organ nitrogen and phosphorous contents, both forward and backward stepwise regressions consistently identified the interaction between nitrogen and phosphorous contents as the most significant predictor of intra-plant variation in $\delta^{15}N$ ($R^2 = 0.58$, $P < 0.0001$, Fig. 4). Adding organ nitrogen content or phosphorous content or both did not significantly improve the predictive ability of resultant equations.

Since fine roots differ from other organs in that fine roots are the primary organs for nitrogen acquisition~~nitrate reduction~~, we re-calculated the organ $\delta^{15}N$ – nutrient relationships by removing all fine roots from the analyses and found that all correlations became stronger (compare Fig. 5 with Fig. 3). In addition, because leaves had considerably higher $\delta^{15}N$ and nutrient contents than other organs, we similarly re-calculated the correlations by removing leaves from the analyses to avoid a foliar domination of the obtained relationships. After the leaves were removed, all correlations were still significant (data not shown, but can be seen from Figs 3, 4 and 5). Furthermore, the removal of either leaves or fine roots did not alter the finding that the N × P interaction was the strongest predictor of intra-plant variations in $\delta^{15}N$. Thus the intra-plant $\delta^{15}N$ - nutrient relationships appeared to be generic and independent of specific physiological or metabolic functions of particular plant organs.

DISCUSSION

Potential mechanisms for the observed intra-plant $\delta^{15}N$ – N and P relationships

This study appears to be the first to report that the strongest predictor of intra-plant variation in $\delta^{15}N$ is the interaction between organ N and P contents rather than N or P themselves or their linear combination. To our knowledge, no previous studies have ~~systematically~~ evaluated relationships between intra-plant variations in $\delta^{15}N$ and organ N or P contents through systematical measurement approaches although models have been developed to simulate root-shoot differences in $\delta^{15}N$ in responses to overall changes in nitrogen supply and demand (e.g., Kalcsits et al. 2014). What

mechanism(s) could be responsible for such close relationships? Clearly this question cannot be answered conclusively with data available in this study or existing data in other, published studies. Here we propose a hypothesis based on a synthesis of best available knowledge (Fig. 6). We hope our hypothesis will provide a starting point for follow-up research.

To facilitate our discussion, we group potential mechanisms into three categories: external factors only ~~(EFO)~~, internal factors only ~~(IFO)~~, and external and internal factors together ~~(EIFT)~~. Potential e~~E~~xternal factors include different sources of nitrogen (e.g., ~~–~~NO_3^-, NH_4^+, organic nitrogen, N_2 fixation). Because the patterns are observed across different organs of the same plants, rather than across different plants in different environments, an external-factors-only explanation ~~EFO~~ cannot be the responsible mechanism~~mechanism~~. An internal-factors-only ~~IFO~~ explanation requires a N and P allocation mechanism that allocates these two key nutrients roughly in proportion to $\delta^{15}N$ in plant organs and at the same time keeps the isotopic mass balance for the whole plant. Translocation of nitrogenous solutes (e.g., NO_3^-, NH_4^+, amino acids) is a mass flow process and mainly takes place from roots to shoots to leaves via xylem and from leaves to shoots to roots via phloem but lateral transfer between xylem and phloem may also occur (Simpson 1986; Bijlsma et al. 2000). $\delta^{15}N$ values probably originate mostly from fractionations in primary assimilation and exchange events and in subsequent metabolic reactions that create ^{15}N-enriched or depleted metabolites (Tcherkez 2011; Gauthier et al. 2013). The translocated nitrogenous compounds mix with existing structural (e.g., nitrogen in cell wall) or functional (e.g., nitrogen in photosynthesis, respiration, and storage that can readily mobilize and take part in metabolic reactions) nitrogen of an organ to give a bulk signal in $\delta^{15}N$. It is conceptually difficult to imagine how these numerous, loosely coupled processes internal to plants collude to produce coordinated variations in N, P and $\delta^{15}N$ across different plant organs. Therefore the probability that an internal-factors-only ~~IFO~~ mechanism causes the observed patterns is likely very small. In the following, we focus on the possibility that external and internal factors work together to create the observed intra-plant variations in $\delta^{15}N$~~EIFT~~.

Plants can emit a vast number of nitrogenous compounds to the atmosphere, which is known to affect atmospheric secondary aerosol formation and climate (Sintermann and Neftel 2015). These compounds are formed in metabolic processes such as the decarboxylation and transamination of amino acids (Bagni and Tassoni 2001; Dudareva et al. 2013). Emissions of these compounds from fruits and flowers are readily noticed without needing sensitive measurements. In addition to fruits

630 and flowers, leaves and stems can also emit nitrogenous compounds. Like many physical and
631 biochemical processes, it is probably not unreasonable to assume that fractionation occurs in the
632 emission of nitrogenous compounds from plants. Unfortunately no isotope fractionation
633 measurements have ever been made on the emission of most of these compounds. However,
634 considerable isotopic knowledge exists in the plant-atmosphere exchange of NH_3.
635 As shown in Fig. 6, NH_3 is a key link between plant metabolism and ambient air (Flechard et al.
636 2013). As discussed in the Introduction, living plant organs contain liquid pools of NH_4^+ maintained
637 by nitrogen translocation, nitrate and nitrite reduction, photorespiration, and other metabolic
638 processes. In these pools, the rapid protonation – deprotonation process sustains an equilibrium
639 between NH_4^+ and aqueous NH_3. In the apoplast, gaseous NH_3 is in equilibration with aqueous NH_3
640 across air-liquid interfaces (e.g., in the intercellular airspace), depending on the concentration ratio
641 (Γ) of NH_4^+ to H^+ and therefore apoplastic pH (Flechard et al. 2013; Johnson and Berry et al. 2013).
642 Isotope effects occur when the apoplast is able to exchange NH_3 with ambient atmosphere. The
643 fractionation has been estimated to be 17.6‰ for diffusion through still air and 11.7‰ through
644 boundary layer (Farquhar et al. 1983; Johnson and Berry 2013).
645 We are not aware of any reports that stems and aerial roots may emit or absorb NH_3. However, it
646 is a widely established fact that leaves exchange NH_3 with atmosphere through stomata (Farquhar et
647 al. 1980; Wetselaar and Farquhar 1980; Farquhar et al. 1983; Sharpe and Harper 1997; Johnson and
648 Berry 2013; Flechard et al. 2013; Sintermann and Neftel 2015). So for the momentum, let us focus
649 on leaves (Fig. 3 & 4). The exchange can be bi-directional, depending on the gradient in the
650 concentration of NH_3 across stomata. If the ambient concentration of NH_3 is above the stomatal
651 compensation point of NH_3 (χ, Farquhar et al. 1980), absorption occurs; otherwise, emission takes
652 place. χ is directly related to Γ which in turn is a function of leaf nitrogen content (Flechard et al.
653 2013). This is because nitrogen-containing proteins (enzymes) are critical to photorespiration which
654 releases NH_3 due to the decarboxylation of glycine in mitochondria (Keys 2006). If leaf nitrogen is
655 high and therefore photorespiration rate is high, the re-assimilation by glutamine synthetase may not
656 be fast enough to keep all NH_3 released from the mitochondria internal to the metabolic cycles,
657 leading to emission of NH_3 from leaves. This emission will result in ^{15}N-enriched pools in leaves.
658 Conversely, if leaf nitrogen is low and therefore photorespiration rate is low, ambient NH_3 may
659 diffuse into leaves, making leaf nitrogen pools more ^{15}N depleted. Thus a positive foliar $\delta^{15}N$ – N

660 relationship can be predicted. This prediction is supported by empirical evidence. For example,
661 Gauthier et al. (2013) showed that foliar nitrate content is positively correlated with foliar $\delta^{15}N$ in
662 *Brassica* napus L.

663 But how can we explain the positive $\delta^{15}N$ – P relationship and the even better $\delta^{15}N$ – N × P
664 relationship? Again, for the momentum, we focus on leaves. We believe the answer lies in the role
665 that phosphate (P_i) plays in photosynthesis and photorespiration. The photosynthetic reaction in
666 chloroplasts is described by $3CO_2 + 6H_2O + P_i \rightarrow$ triose phosphate + $3H_2O + 3O_2$. Chloroplasts
667 import P_i from and export triose phosphate to cytosol to sustain this reaction. P_i deficiency can limit
668 the maximum electron transport rate in thylakoid membranes (Sivak and Walker 1986) and therefore
669 photorespiration rate and NH_3 concentration in sub-stomatal cavities under full sunlight. Conversely,
670 increased P_i supply may boost photorespiration and NH_3 concentration. As a result, foliar $\delta^{15}N$
671 should be positively related to P, just as it should be positively related to N. Why can $\delta^{15}N$ be
672 predicted even better by N × P? This is because a stoichiometry is needed between N and P to keep
673 an efficient operation of the photosynthetic machinery (Gusewell 2004), i.e., an oversupply of N
674 cannot compensate for a deficiency in P and vice versa.

675 Our emphasis on photorespiration in the relationships of foliar $\delta^{15}N$ with N, P, and N × P should
676 be evaluated in the context of enormous importance of leaves in the nitrogen metabolism of the
677 whole plant. While roots are the primary gate for outside nitrogen to enter into the internal nitrogen
678 cycle, leaves are the 'theater' of nitrogen 'actions' within the plant. It is estimated that in C_3 species,
679 mesophyll chloroplasts may contain as much as 75% of total cellular nitrogen in a plant
680 (Hörtensteiner and Feller 2002). A major portion of leaf nitrogen is involved in photosynthetic
681 reactions; Rubisco alone, which catalyzes carboxylation and oxygenation, accounts for 15 to 30% of
682 total leaf nitrogen (Evans 1989). More importantly, the flux of NH_3, which is released by
683 photorespiration and subject to either re-assimilation into amino acids or emission into the
684 atmosphere, is five to ten times larger than the primary assimilation rate at roots (Keys 2006;
685 Masclaux-Daubresse et al. 2010). The fraction of emission to the atmosphere depends on a range of
686 biotic and abiotic factors. In measurements on two rice cultivars, Kumagai et al. (2011) reported that
687 12 and 21% respectively of leaf nitrogen were lost to the atmosphere due to release of NH_3 in
688 photorespiration. Thus it seems possible for the leaf-atmosphere exchange of NH_3 to fundamentally
689 affect the relationships of foliar $\delta^{15}N$ with N, P, and N × P as the model of Johnson and Berry (2013)

690 has suggested.

691 It is more challenging to include stems and roots in the equation. Clearly a photorespiration -
692 based mechanism alone is not sufficient to explain the observed overall relationships as they hold
693 across leaves, stems, and roots (Fig. 3 & 4). Assuming there are no N and P – mediated fractionating
694 processes that directly exchange nitrogenous compounds between stems (and roots) and the ambient
695 air, is it possible for the leaf-atmosphere exchanges of nitrogen isotopes to affect $\delta^{15}N$ values in
696 stems and roots such that $\delta^{15}N$ increases with N, P, and N × P across the whole plant as depicted in
697 Fig. 3 and 4?

698 We believe it is possible. Mature leaves export nitrogen and phosphorous to other organs of
699 plants (e.g., Aerts 1996; Killingbeck 1996; Jeschke et al. 1997; Hörtensteiner and Feller 2002;
700 Masclaux-Daubresse et al. 2010; Brant and Chen 2015). In particular, plants resorb and remobilize
701 essential nutrients to storage tissues in stems and roots during leaf senescence. In this process,
702 proteins, particularly those involved in photosynthesis, are degraded, providing an enormous source
703 of mobile nutrients. Resorption and remobilization of nutrients from senescing leaves are a vital
704 strategy for plant survival for multiple reasons. First, it requires energy to absorb and assimilate new
705 nutrients from soil solutions and thus recycling extant nutrients makes economic sense. Second,
706 nutrient availability in the soil may be low and the rate of absorption at the root-soil interface may
707 not be able to meet the instantaneous demand by new growth in the next spring. In a survey of
708 published values, Brant and Chen (2015) found that leaf nitrogen and phosphorus resorption
709 efficiencies are generally over 60% for a wide variety of plant species ranging from grasses and forbs
710 to deciduous and evergreen trees (see Table 1 in that paper). Franklin and Ågren (2002) showed that
711 a 70% leaf nitrogen resorption efficiency is needed to predict observed leaf area indices of several
712 plant communities. Because of methodological limitations, these estimates do not generally consider
713 volatilization losses to the atmosphere and thus are considered 'apparent remobilization'
714 (Masclaux-Daubresse et al. 2010). Nevertheless, there is little doubt that foliar nitrogen metabolism
715 can affect stem and root nitrogen status. The foliar nitrogen and phosphorus remobilized to storage
716 organs will support the growth of not only new leaves but also new tissues in stems and roots. Given
717 that large amounts of N and P participate in reactions in leaves and are processed through leaves, it is
718 reasonable to assume that the relationships of $\delta^{15}N$ with N, P, and N × P in the stems and roots may
719 bear a similarity to those of the leaves.

To summarize our fairly detailed reasoning above, the observed patterns in intra-plant variations in $\delta^{15}N$ appear to be most logically explained by the following three processes working together (Fig. 6):

- Leaf-atmosphere exchanges of nitrogenous compounds, particularly NH_3 released during photorespiration,
- Nitrogen and phosphorus resorption and remobilization from senescing leaves, and
- Mixture of resorbed and remobilized nitrogen and phosphorus with existing pools in stems and roots.

Nevertheless, we emphasize that this is a hypothesis only and it remains a research task to ascertain how N, P and N × P affect the divergence of $\delta^{15}N$ in different organs of plants.

Comparison with reported inter-plant relationships

It is interesting to compare the intra-plant relationships found here with the previously-reported correlations of foliar $\delta^{15}N$ with N across species, climate and ecological gradients. The positive intra-plant correlation between $\delta^{15}N$ and N content reported in the present study is reminiscent of the foliar correlations reported in those previous studies. Using a dataset that contained over 11000 plants worldwide, Craine et al. (2009) found that foliar $\delta^{15}N$ was positively correlated with foliar N. A subset of this dataset contained $\delta^{15}N$, N and P measurements. These authors subsequently analyzed this subset with a multilinear model that used N, P and their interaction as explanatory variables. It was not clear whether multicollinearity was controlled but they found that after controlling for variations in N, foliar $\delta^{15}N$ decreased with an increase in P and in N × P. We used the same model to fit our intra-plant dataset without consideration of multicollinearity and found that foliar $\delta^{15}N$ decreased with both N and P but increased with N × P. Thus controlling multicollinearity is important for ascertaining relationships between $\delta^{15}N$ and nutrient contents due to correlations between contents of different nutrients.

Positive foliar correlations of $\delta^{15}N$ with N have been reported in studies at smaller scales as well (e.g., Martinelli et al. 1999; Hobbie et al. 2000; Craine et al. 2005). In addition, Hobbie et al. (2008) reported a positive correlation for root tips. These positive correlations, which were all inter- rather than intra-plant in nature, are consistent with the reported experimental finding that an increase in

soil nitrogen availability tends to lead to an increase in $\delta^{15}N$ of non-N-fixing plants (Wigand et al. 2007; Hobbie et al. 2008; Mayor et al. 2014). A hypothesis based on plant-mycorrhizal interactions has been advanced to explain this positive relationship (Hobbie et al. 2000; Craine et al. 2009; Hobbie and Högberg 2012). Typically mycorrhizal fungi transfer isotopically depleted N to host plants. As soil N supply increases, the contribution from mycorrhizal symbionts to the total N budget of host plants may decrease, reducing the mycorrhizal dilution effect on the heavy isotope and resulting in a positive relationship of plant $\delta^{15}N$ with soil N supply. However, this explanation is only valid for $\delta^{15}N$ of the plant as a whole and cannot explain the positive relationship of intra-plant $\delta^{15}N$ with N and the interaction between N and P. In addition to the mycorrhizal hypothesis, a more general explanation for the N supply – plant $\delta^{15}N$ relationship involves the openness of the N cycle. This explanation hypothesizes that an increase in N supply promotes the openness of the N cycle and the increased openness results in higher losses of ^{14}N relative to ^{15}N from the system, leading to enrichment in ^{15}N in the remaining nitrogen pool. The openness typically refers to processes occurring in soil (e.g., N losses through denitrification via the release of N_2O and N_2 from soil which is a strong fractionating process, Mnich and Houlton 2016). Clearly a soil-central N openness explanation is also not valid for the intra-plant $\delta^{15}N$ - N × P relationship reported in this study.

Another possibility to consider concerns the situation when nitrate is the source of N for plants. If soil supply of nitrate is low, all nitrate absorbed by roots may be assimilated in the roots and no enriched nitrate pool is left for transport to other parts of the plant. As soil supply of nitrate increases, the proportion of the nitrate pool that is unassimilated by roots and thus is available for transport to other parts of the plant may not only increase in size but also become more enriched in ^{15}N (a system cannot discriminate if all substrates are assimilated; discrimination generally increases with substrate availability, Evans 2001). However, this possibility can only suggest that the difference in $\delta^{15}N$ between roots and the rest of the plant may increase with soil nitrate supply. It cannot account for the changes of $\delta^{15}N$ with organ N and P contents and their interaction within the plant.

We are not aware of any previous studies that systematically evaluated variations in root $\delta^{15}N$ with depth into soil. However, our finding that roots tend to become more enriched in ^{15}N deeper into soil is reminiscent of the general patterns of increasing soil $\delta^{15}N$ with depth as reported in previous studies (Hobbie and Ouimette 2009; Gubsch et al. 2011; Szpak 2014). Since the present study did not measure soil $\delta^{15}N$ profile it remains to be determined whether the profile of root $\delta^{15}N$ reflects that of

soil $\delta^{15}N$.

CONCLUSION

A systematical evaluation of nitrogen isotope composition in the desert plant species *Nitraria tangutorum* Bobrov reveals that the magnitude of intra-plant variations in $\delta^{15}N$ is close to the highest value reported in previous studies (7‰, Fig. 3 and also Evans 2001). These variations are positively correlated with corresponding organ N and P contents. However, it is the N × P interaction, not N and P individually or their linear combination, that is the strongest predictor of intra-plant $\delta^{15}N$. While the positive correlation of intra-plant $\delta^{15}N$ with organ N resembles the $\delta^{15}N$ – N relationships reported in previous studies on patterns across ecological and climate gradients and across species, explanations developed from these previous studies are not valid for the patterns reported in the present study. We also report that root $\delta^{15}N$ increases with depth into soil. This pattern in root $\delta^{15}N$ is similar to profiles of soil $\delta^{15}N$ reported in previous studies although the exact relationship between root and soil profiles in $\delta^{15}N$ is not clear. We hypothesize that the strong positive intra-plant $\delta^{15}N$ – N and P relationships are a result of three processes working together: 1) N and P-driven, fractionating ammonia exchanges between leaves and the atmosphere (volatilization) during photorespiration, 2) resorption and remobilization of N and P from senescing leaves, and 3) mixture of re-translocated foliar N and P with existing pools in stems and roots.

Knowledge of how plants acquire, transport and transform N is crucial for understanding how plants use this crucial resource for production, growth and reproduction and how the terrestrial N cycle operates. Intra-plant variations in $\delta^{15}N$ are an important outcome of the N cycle. The findings reported in the present study suggest that different mechanisms may operate at different scales to affect plant nitrogen isotope compositions and their relationships with nutrient availability. Alternatively, causes of variations in $\delta^{15}N$, whether they are intra-plant, inter-species, or cross ecological and climate gradients, may differ from previously thought. Our findings suggest that studies into intra-plant variations in $\delta^{15}N$ and their mechanisms can yield deep insights into the N cycle of ecosystem and plant nitrogen metabolism. Such studies have not been adequate in the past and are urgently needed.

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Figure captions

Figure 1. Pen drawings of typical nebkha formed by *Nitraria tangutorum* Bobrov at the Dengkou (a) and Minqin (b) study sites.

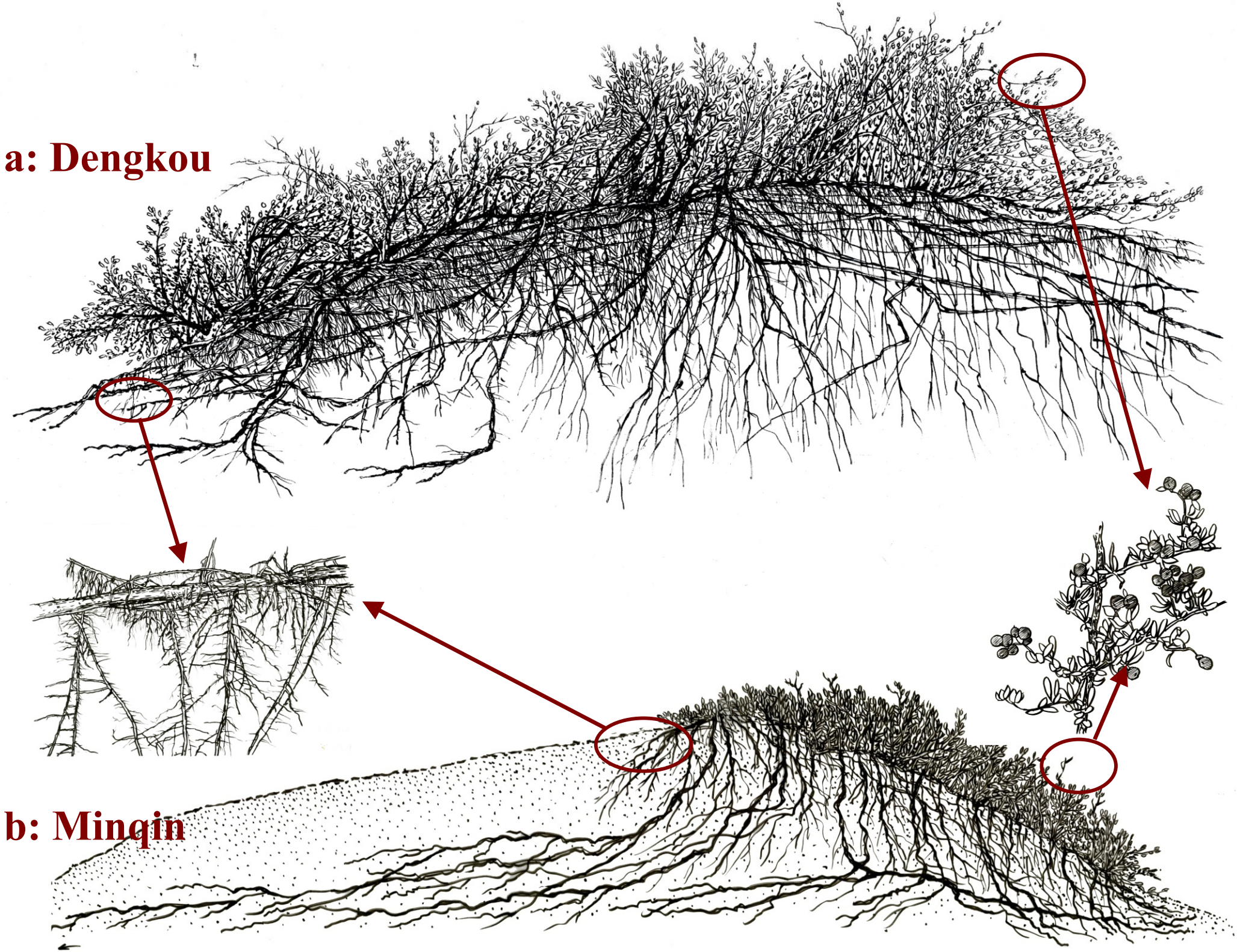
Figure 2. A comparison of $\delta^{15}N$ among different plant organs of *Nitraria tangutorum* Bobrov and between the Dengkou and Minqin study sites. The $\delta^{15}N$ value shown is averaged for each organ across the nebkhas excavated at the same site (Dengkou or Minqin). Upper-case letters denote ANOVA results within a study site (i.e., comparing $\delta^{15}N$ among different organs at the same site) and lower case letters between the two sites (i.e., comparing $\delta^{15}N$ of the same organ between the two sites). ISFR and ISCR stand for fine and coarse roots, respectively, in the sands of nebkhas. 1FR, 2FR, 3FR and 4FR stand for fine roots 0 - 20, 20 – 40, 40 – 60 and 60 – 80 cm depths, respectively, below the plains on which nebkhas rest. Similarly, 1CR, 2CR, 3CR and 4CR stand for coarse roots within these depth intervals. Fine and coarse roots are differentiated with a diameter threshold of 2mm. Woody debris (WD) from dead ramets is also included in the figure. No ANOVA results for 3FR and 3CR at the Dengkou site as there was only one nebkha having roots between 40 to 60 cm. No roots were found below 60 cm at the Dengkou site.

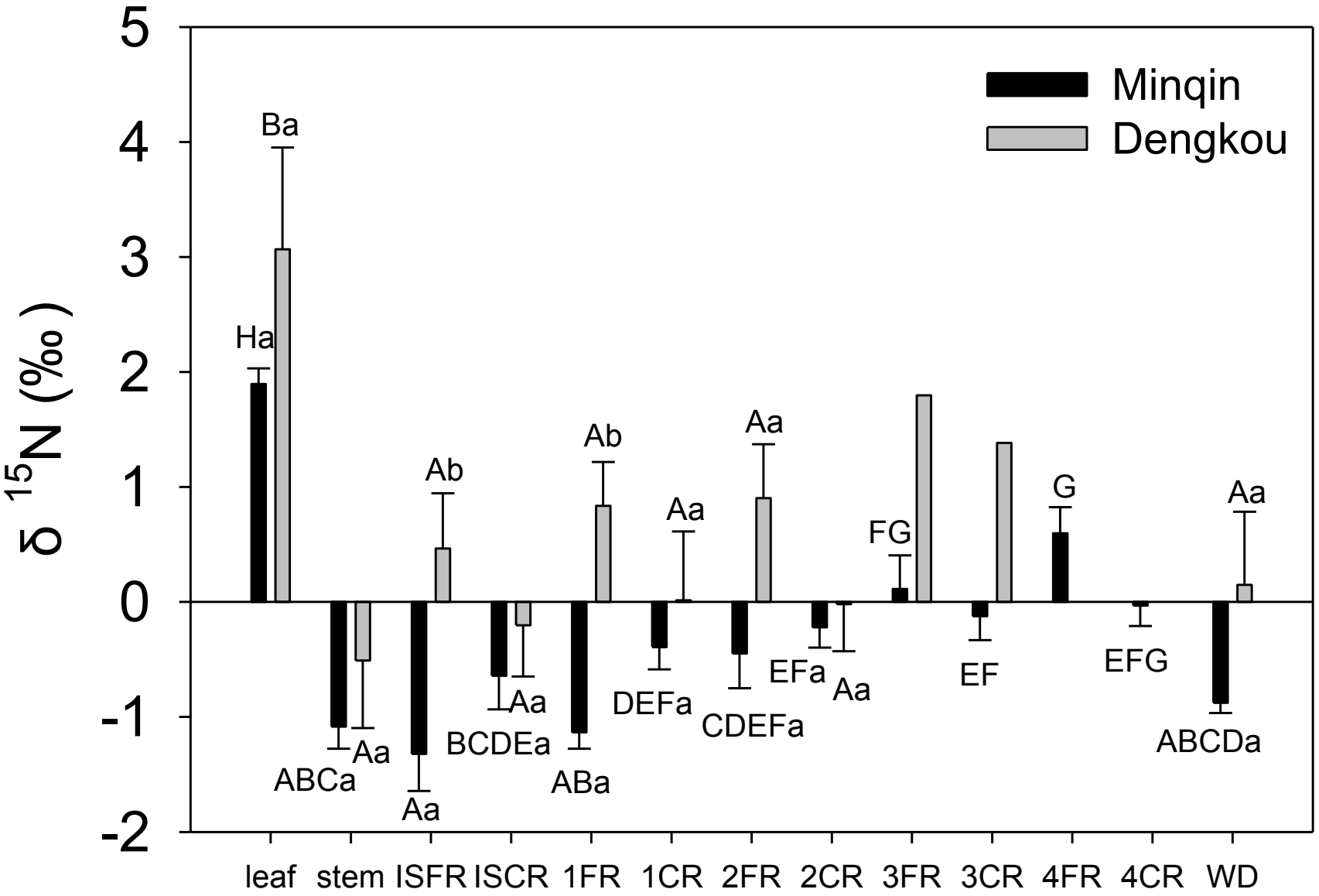
Figure 3. Changes of $\delta^{15}N$ as a function of organ contents of carbon (a), nitrogen (b) and phosphorous (c) and of organ ratios of carbon to nitrogen (d), nitrogen to phosphorous (e), and carbon to phosphorus (f). Filled and unfilled symbols represent organs at the Minqin and Dengkou site, respectively. Leaves are denoted by filled or unfilled triangles while other organs by filled or unfilled circles.

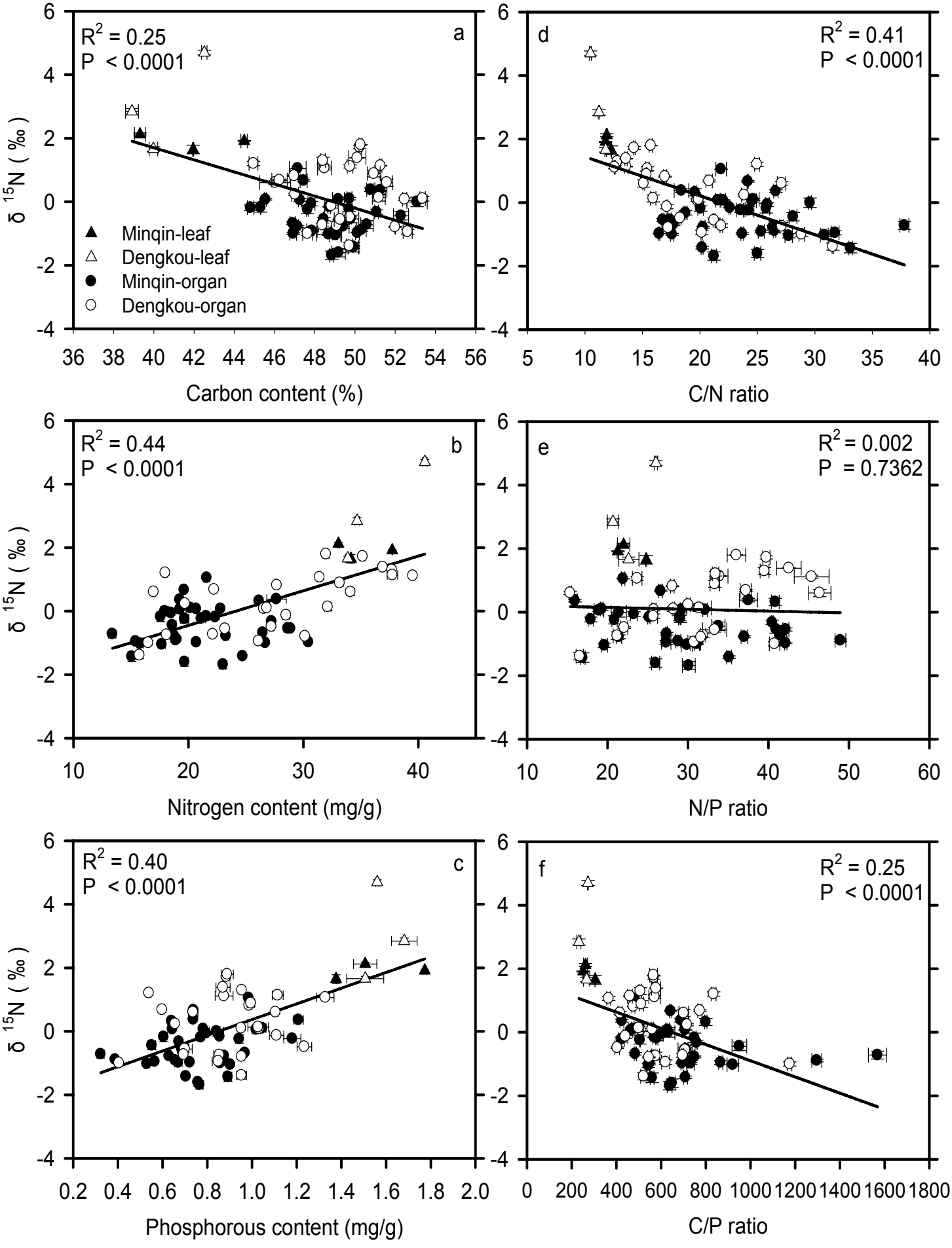
Figure 4. Changes of $\delta^{15}N$ as a function of the product of organ N × P contents. Filled and unfilled symbols represent organs at the Minqin and Dengkou site, respectively. Leaves are denoted by filled or unfilled triangles while other organs by filled or unfilled circles.

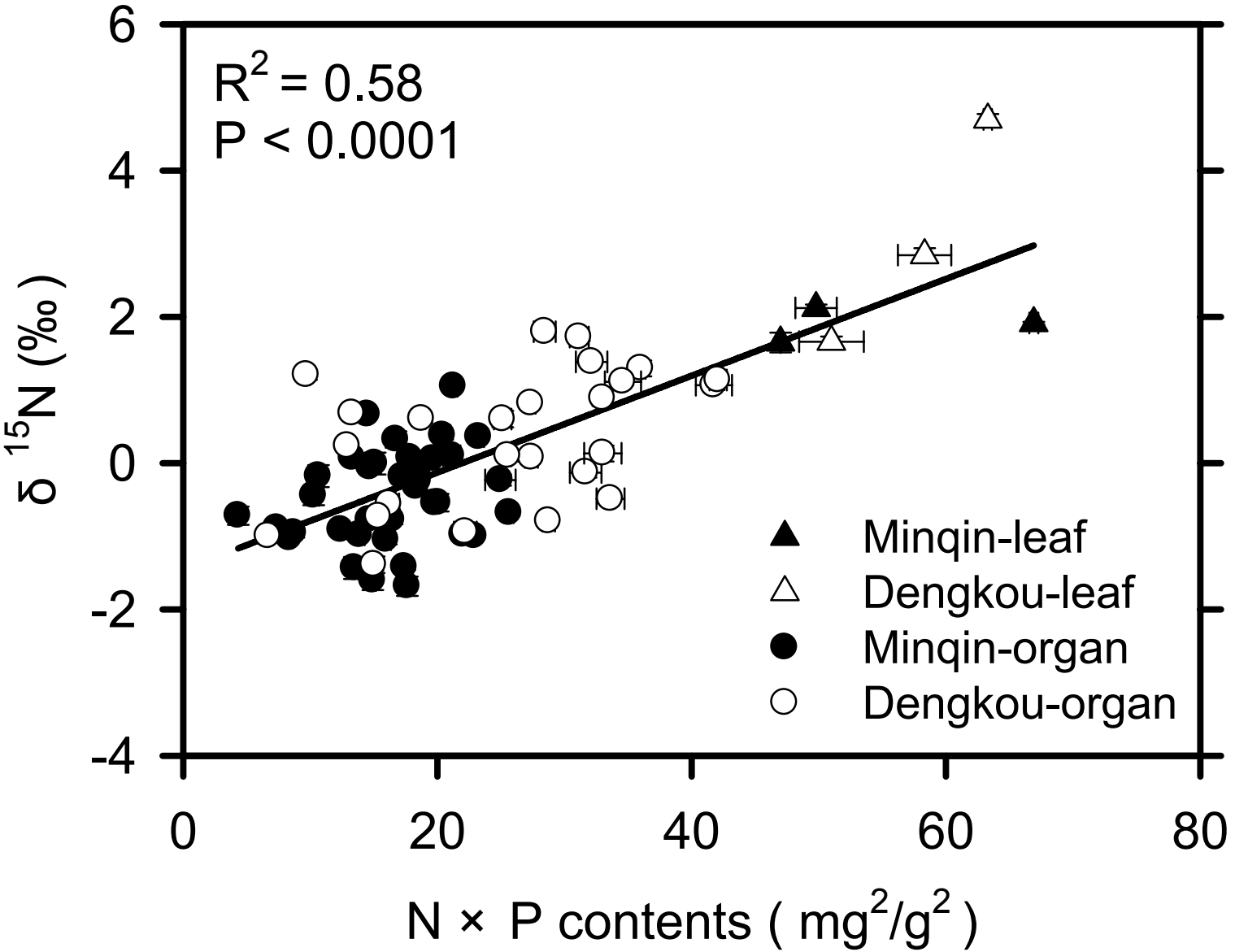
Figure 5. The same as Fig. 3 except that data of fine roots are not included.

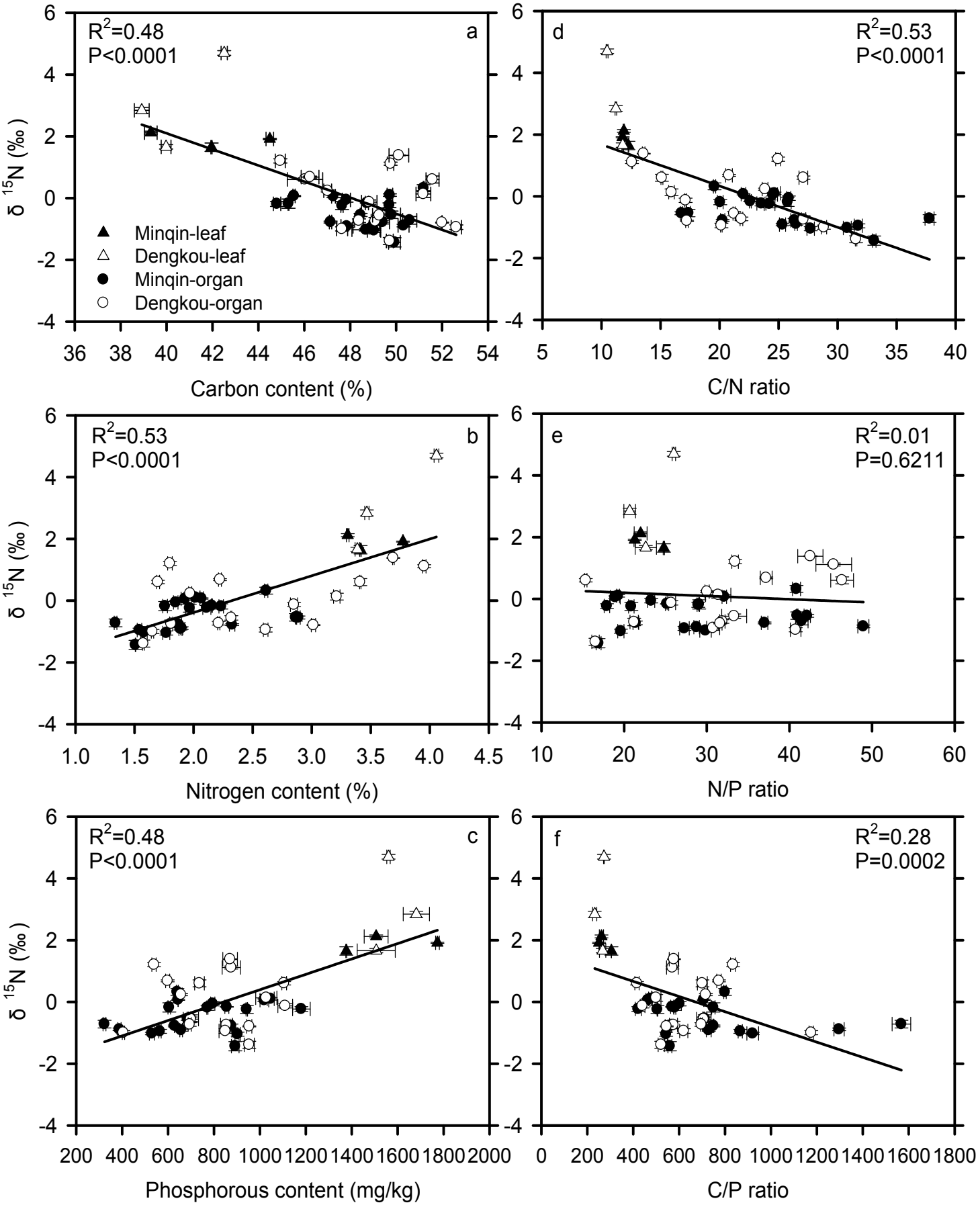
Figure 6. intra-plant nitrogen cycling and flux exchanges with external environments.

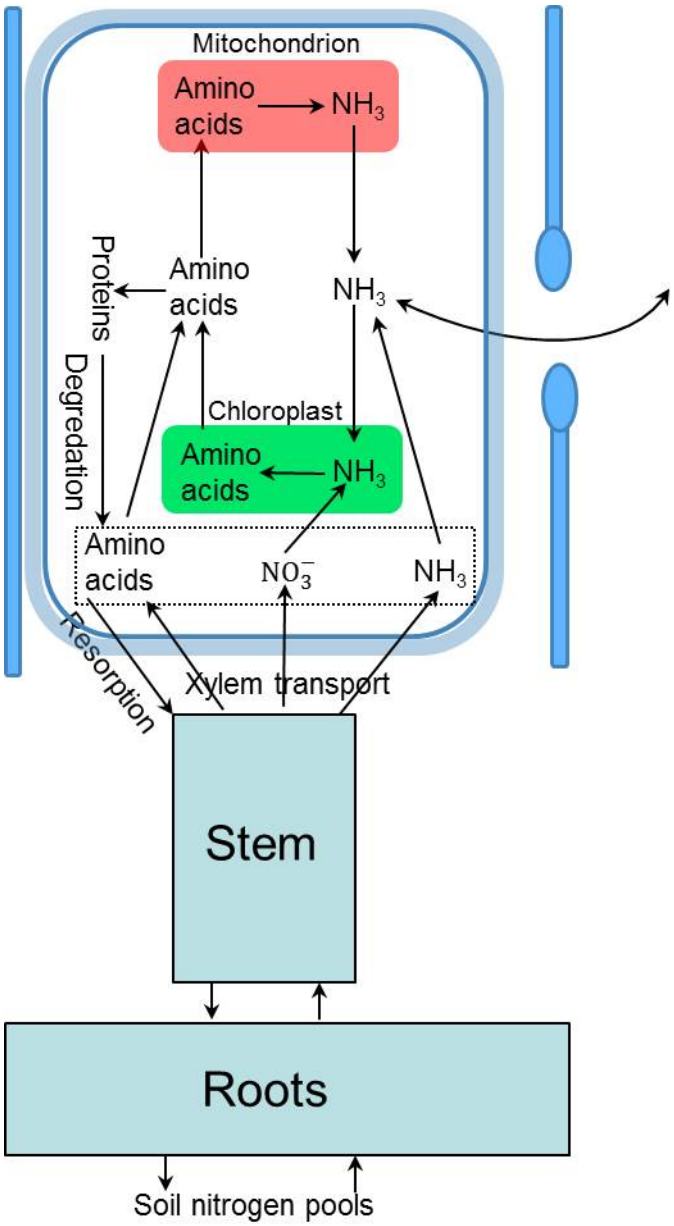

a: Dengkou
b: Minqin


δ 15N (‰)
5
4
3
2
1
0
-1
-2
Minqin
Dengkou
Ha
Ba
ABCa
Aa
Aa
Ab
BCDEa
Aa
ABa
Ab
DEFa
Aa
CDEFa
Aa
EFa
Aa
FG
EF
G
EFG
ABCDa
Aa
leaf
stem
ISFR
ISCR
1FR
1CR
2FR
2CR
3FR
3CR
4FR
4CR
WD


a
R^2 = 0.25
P < 0.0001
Minqin-leaf
Dengkou-leaf
Minqin-organ
Dengkou-organ
δ ^{15}N (‰)
Carbon content (%)
d
R^2 = 0.41
P < 0.0001
C/N ratio
b
R^2 = 0.44
P < 0.0001
Nitrogen content (mg/g)
e
R^2 = 0.002
P = 0.7362
N/P ratio
c
R^2 = 0.40
P < 0.0001
Phosphorous content (mg/g)
f
R^2 = 0.25
P < 0.0001
C/P ratio


R2 = 0.58
P < 0.0001
δ 15N (‰)
6
4
2
0
-2
-4
0
20
40
60
80
N × P contents (mg2/g2)
Minqin-leaf
Dengkou-leaf
Minqin-organ
Dengkou-organ


a
R2=0.48
P<0.0001
Minqin-leaf
Dengkou-leaf
Minqin-organ
Dengkou-organ
δ 15N (‰)
Carbon content (%)
d
R2=0.53
P<0.0001
C/N ratio
b
R2=0.53
P<0.0001
Nitrogen content (%)
e
R2=0.01
P=0.6211
N/P ratio
c
R2=0.48
P<0.0001
Phosphorous content (mg/kg)
f
R2=0.28
P=0.0002
C/P ratio


Mitochondrion
Amino acids
NH3
Proteins
Amino acids
NH3
Degredation
Chloroplast
Amino acids
NH3
Amino acids
NO3−
NH3
Resorption
Xylem transport
Stem
Roots
Soil nitrogen pools