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LETTER

N : P stoichiometry and protein : RNA ratios in vascular plants: an evaluation of the growth-rate hypothesis

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Abstract

The growth-rate hypothesis states that fast-growing organisms need relatively more phosphorus-rich RNA to support rapid rates of protein synthesis, and therefore predicts, within and among taxa, increases in RNA and phosphorus content (relative to protein and nitrogen content) with increased growth rate. Here, we present a test of this hypothesis in vascular plants. We determined nitrogen : phosphorus ratios and protein : RNA ratios in pines growing at different rates due to nutrient conditions. In general, when comparing leaves of the same species at low and high growth rates, the faster-growing plants had higher RNA content, higher %N and %P, and lower protein : RNA ratios, but not consistently lower N : P ratios. We found no link between growth rate and foliar N : P or protein : RNA when comparing multiple species of different inherent growth rates. We conclude that plants adjust the balance of protein and RNA to favour either speed or efficiency of protein synthesis, but this balance does not alone dictate leaf stoichiometry.

Keywords

Ecological stoichiometry, nitrogen : phosphorus ratio, *Pinus*, protein synthesis, ribosomal efficiency, RNA content.

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INTRODUCTION

The growth-rate hypothesis (GRH) (Elser *et al.* 1996; Sterner & Elser 2002) states that: (i) organisms require relatively more investment in phosphorus-rich ribosomes and rRNA to support the rapid protein synthesis associated with fast growth and (ii) the elemental stoichiometry of fast-growing individuals or taxa is therefore tipped toward phosphorus, such that fast-growing organisms exhibit lower tissue N : P and C : P ratios. The hypothesis links a key life history trait, growth rate, to the stoichiometry of element use by organisms.

Direct tests of the GRH in heterotrophic taxa have shown tight linkages among growth rate, RNA content, and body P content, especially when phosphorus supply is the factor limiting growth (Elser *et al.* 2003, 2006; Makino *et al.* 2003; Acharya *et al.* 2004; Makino & Cotner 2004; Kyle *et al.* 2006; Watts *et al.* 2006; Hessen *et al.* 2007).

In this paper, we ask whether the GRH can apply to photoautotrophs, specifically the foliage of vascular plants. Plant stoichiometry may be decoupled from physiological need by vacuolar storage of nutrients, which allows plants to take up N and P in excess of growth requirements when nutrients are abundant, and to sustain steady growth through periods of nutrient scarcity. Plants (including algae) vary considerably more in N : P ratios than do animals or bacteria (Güsewell 2004), and much of that variation reflects the relative supply of N and P in the environment. However, we also see evidence of homeostasis in the N : P of plants; for example, in a survey of wetland vegetation, plant N : P varied only two- to threefold when exposed to 10-fold variation in supply N : P (Güsewell & Koerselman 2002).

A true test of the GRH in plants would require determining a plant's 'optimal' stoichiometry – the N : P (or C : N or C : P) ratio that would reflect the plant's physiological requirements at a particular rate of growth, without the confounding effect of storage. Unfortunately, measurements of this quantity are rare (Ågren 2004, 2008). In the absence of data, an alternate approach has been to

examine very large plant databases for trends in N : P with growth rate, in the hopes that a clear signal would emerge amid the noise of widely varying nutrient supply rates. For example, a global dataset of plant functional traits suggested that N : P ratios decline in concert with increasing metabolic rates and faster leaf turnover times (Wright *et al.* 2004), although direct correlations between N : P and other traits associated with growth rate were weak (Wright *et al.* 2005). Nielsen *et al.* (1996) studied the N and P concentrations of 126 species of aquatic and terrestrial plants and concluded that growth rate increases exponentially with nitrogen concentration and linearly with phosphorus concentration, yielding lower N : P ratios at higher growth rates. Negative correlations between relative growth rate (RGR) and N : P ratio have also been observed in regional floras among herbaceous and woody species (Güsewell 2004).

Others have tried a modelling approach. Building on prior models for plankton growth that assume a dependence of growth rate on the fraction of N allocated to protein and the fraction of P allocated to RNA (Vrede *et al.* 2004), Niklas *et al.* (2005) used leaf N : P stoichiometry to predict the growth rate of vascular plant leaves between initial expansion and senescence. In accordance with the GRH, their model yielded increased leaf RGRs with decreasing N : P, and a sampling of information from 131 angiosperm species supported this prediction. Ågren (2004) modelled an autotroph-specific version of the GRH, recognizing that unlike heterotrophs, autotrophs acquire C separately from their N and P. He conceptualized C fixation as dependent on the rate of protein production, which is itself dependent on both the amount of N available for protein synthesis and the amount of P committed to ribosomes. In this model, if plants have their highest N : P at relatively low growth rates, then a (GRH-consistent) linear decline in N : P with increasing growth rate is expected for all growth rates likely to be observed in nature (Ågren 2004). However, a hump-shaped trend of N : P with growth rate is expected if N : P is relatively low at low growth rates, and trends in N : P should not be very sensitive to changes in growth rate. Element ratios from a freshwater alga and a tree seedling grown under conditions of simultaneous N and P limitation conformed to the hump-shaped model. Klausmeier *et al.* (2004) modelled optimal N : P ratios for different competitive strategies in phytoplankton and found that optimal allocation to ribosomes was highest for the maximal growth (ruderal/fast-grower) strategy, and lower under P-limitation than N-limitation; they concluded that high-resource conditions select for low N : P and exponential growth rates, while competitive equilibrium selects for high N : P ratios.

In this paper, we go beyond analyses of N : P ratios, focusing our attention on the variations in protein : RNA ratio that partially underlie N : P ratios. Protein : RNA ratios are directly applicable to the GRH as an indicator of the relative demand for P-rich 'assembly machinery' and the N-rich 'raw materials' collectively needed for protein synthesis (Klausmeier *et al.* 2004). We interpret the biochemical mechanism underlying the GRH thusly: given a particular amount of nitrogen with which to make proteins, a minimal amount of RNA is required for growth to proceed at all and a much greater investment in RNA is required for growth to be fast. Slow growth is therefore ribosome-use efficient, and fast growth is more consumptive of the resources necessary for ribosome construction, principally P. In an animal that has little capacity for storing nutrients consumed in excess of requirements, excess N or P is excreted and the N : P of tissue comes to reflect this balance between investment in P-rich protein assembly machinery and the available raw materials. In plants, the N or P not used for growth may be stored, obscuring the relationship between growth rate and stoichiometry. We aimed to discover if this relationship exists by directly measuring protein : RNA ratios and not just elemental stoichiometry.

There is a long history in animal physiology of using protein : RNA ratios as indices of growth rate, nutrient status or stress. Karpinets *et al.* (2006) tested the GRH in a variety of unicellular organisms using protein : RNA ratios as a proxy for protein synthesis efficiency and found that the ratios were lower when growth rates were higher, consistent with the GRH (although, interestingly, the only autotroph observed, an alga, did not conform to this pattern). In phytoplankton, Berdalet *et al.* 1994 showed that protein : RNA ratios increased dramatically as growth slowed under phosphorus limitation, for the photosynthetic marine dinoflagellate *Heterocapsa* sp. However, protein : RNA data from higher plants are lacking; we know of no other study that has characterized protein : RNA ratios in vascular plants varying in growth rate.

For this work, we first measured protein : RNA ratios and N : P stoichiometry in the leaves of pines growing rapidly in natural forests, comparing them with extremely slow-growing individuals from nearby nutrient-poor 'pygmy' forests. Next, we transplanted stunted pygmy forest individuals to a high-nutrient regime in greenhouse pots to see if decreases in the foliar protein : RNA and N : P ratio could be observed for an individual tree when its growth rate was increased. To measure growth rate more precisely and to minimize the effect of geographic variation in soil nutrient supply, we repeated these measurements on pines grown in the greenhouse under low and high rates of fertilization, but with N : P of nutrient supply held constant. Finally, we used the variation in inherent growth rate among the 14 greenhouse-grown species to look for interspecific differences in foliar protein : RNA and N : P.

METHODS

Tissue assays

For all experiments, the two youngest whorls of needles at each shoot tip were removed and immediately flash frozen in liquid nitrogen, then stored at −80° C. Frozen tissue was ground to a fine powder in liquid nitrogen, with aliquots dried at 55° C for N and P assays and to give a fresh weight–dry weight conversion, and frozen material used directly in RNA and protein assays. Nitrogen and phosphorus concentrations (%N and %P) were determined by Kjeldahl digest.

For RNA measurements, frozen tissue was double-extracted in an RNase-free buffer consisting of 2.0 M NaCl, 2% (w/v) polyvinylpyrrolidone, 25 mM EDTA, 100 mM sodium acetate, 3% (v/v) sodium dodecyl sulphate, 2% (v/v) β-mercaptoethanol and 2 mM aurintricarboxylic acid with alternate freezing and heating steps. Nucleic acids were precipitated onto silica particles in 6 volumes of 6 M NaI and 2.5 volumes of ethanol and the pellet washed in 70% ethanol. Nucleic acids were eluted in the presence of an RNase-inhibitor and later incubated with RNase-free DNase before RNA was quantified with the fluorescent dye Ribogreen (Molecular Probes, Eugene, OR, USA).

Leaf proteins were extracted by heating pine tissue to 55° C in buffer consisting of 5% sodium dodecyl sulphate, 5% sucrose, and 5% β-mercaptoethanol and subjecting the supernatant to a Lowry-based assay before measuring absorbance with a spectrophotometer.

All leaf biochemistry measures are reported on a dry weight basis. Following terrestrial ecophysiology convention, N : P ratios are expressed on a mass basis.

Field and greenhouse growth conditions

We collected needles of *Pinus contorta* and *Pinus muricata* from the pygmy forest region of Mendocino County, California, USA, where uplifted marine terraces differ in soil age by > 300 000 years and exhibit well-characterized differences in soil fertility (Jenny *et al.* 1969; Yu *et al.* 1999) and net primary productivity (Westman & Whittaker 1975). We sampled stunted trees 1–3 m in height (n = 22) growing on the oldest, most weathered and nutrient-poor terraces (pygmy) and trees of normal stature (n = 22) growing on the youngest, most nutrient-rich terraces (normal). Phosphorus availability and net nitrogen mineralization rates in the pygmy forest soils are ≤ 10% that of the normal terrace soils (Yu *et al.* 1999), and the podzolized pygmy soils are underlain by an iron hardpan that restricts water availability in summer, resulting in low NPP (Westman & Whittaker 1975). Greenhouse experiments have documented significantly slower growth rates for individual trees in pygmy forest soils; McMillan (1956) showed that Mendocino-origin *P. muricata* seedlings potted in pygmy forest soils grew to only 1/6 the height of those grown in a naturally fertile, clay loam soil.

We also transplanted stunted *P. contorta* and *P. muricata* from pygmy forest sites to the greenhouse to measure tissue chemistry in the same individual at low and high growth rates. After an initial sampling of their needles, pygmy pines less than 25 cm tall and ranging in age from 5 to 12 years old were dug up and replanted in greenhouse pots (see conditions, below). Plants were fertilized with a half-strength Hoagland's solution weekly (N : P ratio 5 : 1 by mass), and 11 individuals survived and grew for a year. The change in growth rate was dramatic; stunted plants nearly tripled their average mainstem diameter, doubled in height and took on the appearance of healthy, normal saplings after transplantation.

To compare foliar chemistry at low and high growth rates with the stoichiometry of nutrient supply held constant, we grew seedlings of *Pinus banksiana*, *Pinus cembra*, *Pinus flexilis*, *Pinus halepensis*, *Pinus jeffreyi*, *Pinus lambertiana*, *Pinus muricata*, *Pinus patula*, *Pinus pinaster*, *Pinus pinea*, *Pinus radiata*, *Pinus sabiniana*, *Pinus sylvestris* and *Pinus torreyana* in pots of sand and vermiculite receiving either a low rate of nutrient supply (1 mg N and 0.2 mg P pot^{-1} $week^{-1}$) or a high rate of nutrient supply (50 mg N and 10 mg P pot^{-1} $week^{-1}$) for 12 weeks. Plants were given the balance of necessary micronutrients in a weekly watering with half-strength N- and P-free Hoagland's solution. Plants were grown from seed and transplanted into treatments when their second set of true leaves began to emerge. Ambient light in the greenhouse was supplemented with 1000-W sodium vapour and metal halide lamps (1 : 1) for a 14-h photoperiod. Average mid-day photosythentically active radiation measured at plant height was *c.* 1350 μmol m^{-2} s^{-1}. Daytime temperatures were set to 25° C and nighttime temperatures to 15° C. Soil moisture was monitored with a probe to ensure that water was not limiting to plant growth. Following terrestrial plant physiological convention, we defined RGR as growth increment per unit time per unit starting weight, and calculated it using two destructive harvests, one at 12 weeks of age and one at week 0 on an alternate set of seedlings. For the purposes of RGR calculations, data from harvested seedlings from each species/treatment combination (nine individuals) were averaged; for biochemical assays their needles were composited.

We used Student's t-test to analyse the pygmy and normal forest individuals (n = 44) and paired t-tests to compare high- and low-nutrient treatments by species in the greenhouse (n = 14 pairs) and to compare 'before' and 'after' pygmy individuals.

RESULTS

Pygmy vs. normal pines

Pygmy pines exhibited characteristics consistent with nutrient stress; they had significantly lower mass-based concentrations of N, P, protein and RNA than did normal trees (Table 1). In accordance with the GRH, they also had higher N : P (Fig. 1) and higher protein : RNA ratios than the faster-growing normal trees (Fig. 2). Comparison of 'before' and 'after' pygmy trees gave a similar result. Well-fertilized, fast-growing 'after' individuals had significantly higher %N, %P and RNA content, but not protein content, compared with 'before' individuals sampled prior to transfer to the greenhouse (Table 1). Fast-growing 'afters' also had significantly lower N : P (Fig. 1) and lower protein : RNA ratios (Fig. 2) than slow-growing 'befores,' consistent with the GRH.

Multispecies experiment

The 14 pine species varied widely in maximal (fertilized) RGR. However, all species grew more slowly in the low-nutrient treatment than in the high-nutrient treatment, and on average, the well-fertilized plants grew nearly twice as fast as the low-nutrient ones (0.038 g g^{-1} d^{-1} vs. 0.021 g g^{-1} d^{-1}). Plants in the low-nutrient treatment had lower mass-based concentrations of N, P, protein and RNA, consistent with nutrient stress (Table 1). They also had higher protein : RNA ratios than plants in the high-nutrient treatment (Fig. 2), but not higher N : P ratios (Fig. 1).

Finally, although pines in general fall into the slow-growing end of the continuum of plant growth rates, we took advantage of the inherent differences in maximal growth rate among the 14 species to look for evidence consistent with the GRH. Using only data from the high-nutrient treatment, where differences among inherently slower-growing or faster-growing plants should be most apparent, we found no significant linear relationship between species' growth rate and protein : RNA ratio (Fig. 3), nor between growth rate and N : P ratio.

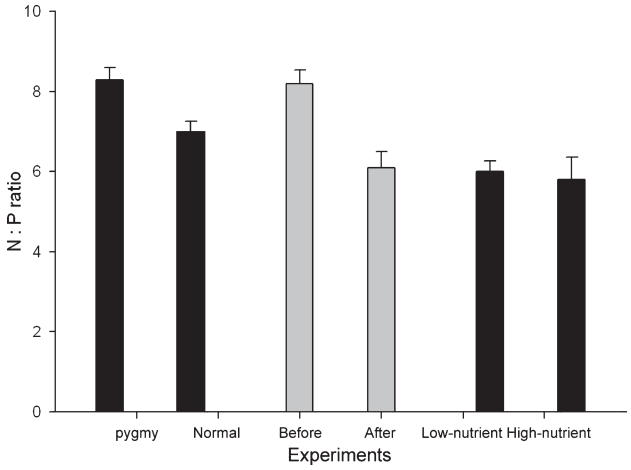

Figure 1 Mass-based nitrogen : phosphorus ratios compared between fast- and slow-growing trees in the three experiments. N : P ratios are significantly different ($P < 0.05$) in experimental pairs except between low-nutrient and high-nutrient treatments, where supply N : P was held constant. Error bar depicts the standard error of the mean.

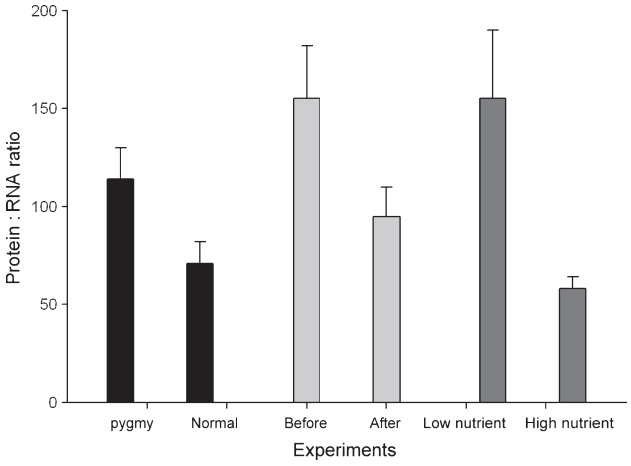

Figure 2 Protein : RNA ratios compared between fast- and slow-growing trees in the three experiments. Significantly lower ($P < 0.05$) protein : RNA ratios in faster-growing plants were observed in all three experimental pairs. Error bar depicts the standard error of the mean.

Table 1 Comparison of nutrient, protein and RNA content of fast- and slow-growing plants in three experiments

	%N	%P	Protein*	RNA†	RGR‡
Pygmy (slow)	1.11 ± 0.04	0.14 ± 0.01	54.8 ± 3.2	605 ± 57	n/a
Normal (fast)	1.57 ± 0.08	0.23 ± 0.02	67.1 ± 3.9	1663 ± 260	n/a
Before (slow)	1.20 ± 0.09	0.15 ± 0.02	75.3 ± 6.6	667 ± 126	n/a
After (fast)	1.67 ± 0.07	0.28 ± 0.02	88.3 ± 3.6	1170 ± 179	n/a
Low nutrient (slow)	1.29 ± 0.11	0.23 ± 0.02	38.5 ± 5.3	375 ± 76	0.021 ± 0.001
High nutrient (fast)	2.64 ± 0.15	0.46 ± 0.02	60.2 ± 5.1	1249 ± 200	0.038 ± 0.004

*mg g^{-1} dry weight; †μg g^{-1} dry weight; ‡g g^{-1} initial weight/day.
All comparisons between fast- and slow-growers in the same experiment are significant ($P < 0.05$) except protein content in the before–after experiment.

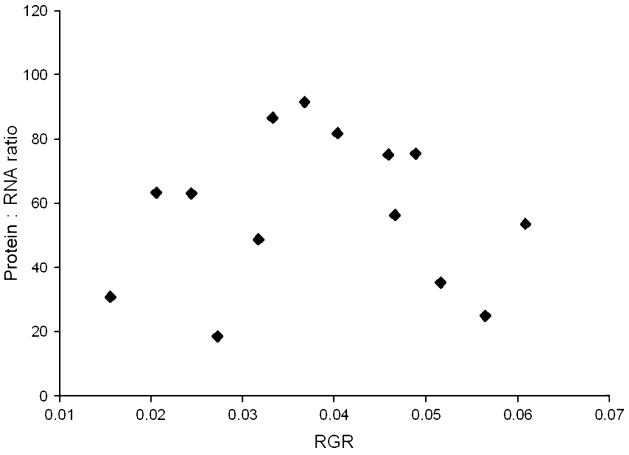

Figure 3 Relationship between maximal RGR and protein : RNA ratio for 14 species. Although species varied widely in inherent growth rate, no trend in protein : RNA was evident across taxa.

DISCUSSION

Under high-resource conditions conducive to fast growth, these pines adjust their biochemical composition by investing more heavily in RNA relative to the amount of protein they contain. There was a negative association in this study between growth rate and the protein : RNA ratio whenever we contrasted the same species or individuals at high and low growth rates. Across a number of related species, however, we did not see a monotonic negative association between inherent growth rate and protein : RNA ratio.

We found mixed evidence that leaf N : P ratios reflect the observed differences in protein : RNA. As predicted by the GRH, leaf N : P was higher in slow-growing plants than fast-growing plants in our field-based comparisons, but this was not true in the multispecies greenhouse experiments. When we controlled greenhouse N : P supply at 5 : 1 in the multispecies treatments, our plants closely reflected this stoichiometry regardless of growth rate, averaging an N : P of 5.8 in the high-nutrient treatment and 6.0 in the low-nutrient treatment.

Our results deviate from the predictions of the GRH in two major ways: N : P was not consistently lower in faster-growing plants; and the expected trend of lower protein : RNA and N : P ratios in fast-growers did not emerge when we made interspecific comparisons of 14 different pine species.

One possible explanation for the inconsistent N : P results is that N and P were simultaneously limiting in the field experiments, but only N was limiting in the multispecies experiments. In this scenario, the leaf N : P measured in the field experiments was reflective of protein : RNA allocation and not confounded by differences in storage, but in the multispecies greenhouse experiments, luxury uptake or storage of P obscured the stoichiometric differences that would be expected, given the protein : RNA ratios observed. This is plausible, as the supply N : P ratio of 5 : 1 in the multispecies experiments was relatively P-rich, and our seedlings (grown from commercially available seed) started out with high leaf P content at the time of transplant into the fertilization treatments (mean leaf P $0.54 \pm 0.05\%$). However, we cannot exclude the possibility that in all the experiments the leaf N : P was simply determined by the nutrient supply, including the greenhouse-grown pygmy 'afters,' whose average N : P of 6 : 1 is not far from the supply ratio of 5 : 1.

We anticipated the possibility that plant N : P ratios would not conform to the GRH because of the potential for nutrient storage. Another possible factor is that RNA constitutes too small a portion of total P to strongly influence leaf P content. RNA-P never exceeded 11% of leaf P in this study (based on RNA containing 9% phosphorus by mass). The heterotrophic taxa so far used to show evidence of the GRH are notable for the high proportion of their cellular P devoted to RNA, typically > 70% at maximal growth rates (Elser *et al.* 2003; Makino *et al.* 2003; Schade *et al.* 2003; Watts *et al.* 2006; Hessen *et al.* 2007). We are unsure if our finding that RNA is a small fraction of leaf P is generalizable, because many of our plants have probably accumulated luxury P and because the size of the RNA-P pool in plants is not well described in the literature. Few published estimates of plant RNA-P exist, and those that do are based not on direct extraction and quantification of RNA content, but on estimates of total nucleic acid content (RNA + DNA) from chemical fractionations of leaf P into presumed organic and inorganic pools. Variations of this method have put the portion of leaf P in total nucleic acids at 13–42% in barley (Chapin & Bieleski 1982); 16–38% in birch (Chapin & Kedrowski 1983); 34–40% in the alpine herb *Chionochloa* (Chapin *et al.* 1982); and about 15–25% in eucalyptus (Close & Beadle 2004). In the alga *Scenedesmus*, a physical/chemical fractionation method found that the largest fraction of algal P was polyphosphate, with a maximum of 30% of total P in RNA at the highest growth rate (Rhee 1973). We found no other estimates of RNA-P based on direct extraction and quantification. Our method may underestimate RNA allocation because the fluorescent dye does not pick up free nucleotides and has reduced sensitivity to short (< 500-bp) fragments (Jones *et al.* 1998). Niklas (2006) determined that allometric models of leaf growth rate conformed best to actual data when the RNA-P fraction was assumed to be between 5 and 15% – i.e. in the same range as our observations – but lamented the lack of reliable estimates of this fraction. Clearly, there is a need for better data on phosphorus allocation to RNA if investigation of the GRH in plants and phytoplankton is to continue.

Another result contradictory to the GRH is that protein : RNA ratios did not decrease monotonically with increasing growth rate, when the 14 different species grown in the greenhouse under high-nutrient conditions were compared. This is only puzzling if we expect that there is a deterministic relationship between the protein : RNA ratio and growth rate, whereby a particular protein : RNA ratio produces a particular rate of growth. Tests of the GRH in heterotrophic organisms have not supported such a deterministic view, even when there are strong intraspecific relationships between RNA content, body P content and growth rate (Elser *et al.* 2003, 2006; Kyle *et al.* 2006). In unicellular taxa, organisms differing as much as fivefold in maximum specific growth rate had nearly identical protein : RNA ratios (Karpinets *et al.* 2006). Additional reasons to doubt the existence of a single slope uniting the responses of many disparate species' growth rates and protein : RNA ratios are the fact that, in this study, RGR is a whole-body response incorporating the growth and differentiation of different kinds of tissues; and that proteins may contribute to functions other than overall growth in ways that differ among species. If a single relationship between growth rate and relative RNA allocation exists across taxa, it is subtle enough to evade detection when only a few species are analysed.

What can we conclude from this test of the GRH in plants? First, these organisms have flexibility in their investment in RNA to accomplish protein synthesis. In accordance with the findings of Karpinets *et al.* (2006) linking protein synthesis efficiency to slow growth, we conclude that under low-nutrient conditions, growth is slow but uses resources efficiently, with a minimal investment of ribosomes per unit protein synthesized; under high-resource conditions, where rapid growth is a better competitive strategy than efficiency, a higher investment in ribosomes per unit protein maximizes the speed of protein synthesis and therefore growth. Second, we conclude that a decrease in N : P with increasing growth rate should not necessarily be expected, because plants have other survival strategies besides growth (namely, storage and defence) that require investment in N and P.

The GRH is an exciting hypothesis because it links the smallest and largest levels of biological organization: from cellular biochemistry and physiology (allocation to RNA and protein for growth) to a whole-body trait (N : P ratio) with implications for ecological phenomena (e.g. nutrient cycling). However, in plants, evaluation of the GRH has tended to skip over the biochemicals and go straight to the N : P ratio, which we find to be an inadequate surrogate. Further evaluation of the GRH in plants should focus on: (i) linking growth rate to protein : RNA ratio; (ii) varying supply N : P to see if it affects protein : RNA in concert with foliar N : P; and (iii) quantifying the size of N and P pools devoted to growth, storage and defence.

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