Effect of carbon, nitrogen, and phosphorus resource on the reproduction of Fagaceae: allocation strategies and seasonal expression profiles of their transporter genes

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Effect of carbon, nitrogen, and phosphorus resource on the reproduction of Fagaceae: allocation strategies and seasonal expression profiles of their transporter genes

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Contents

Preface

Diversity in Fagaceae species

The Fagaceae family, includes 10 genera (Oaks, beeches, chest-nuts, stone oaks, and allies) and more than 1000 species and is widely distributed in the Northern hemisphere of temperate, subtropical and tropical regions (Kamiya et al., 2003; Satake & Kelly, 2021; Zhou et al., 2022). It has been reported that the genera of Fagaceae family are: *Fagus* L., *Castanea* L., *Castanopsis* Spach., *Chrysolepis* Hjelmquist, *Colombobalanus* (Lozano, Hdz-C. & Henao) Nixon & Crepet, *Formanodendron* (Camus) Nixon & Crepet, *Lithocarpus* Bl., *Quercus* L., and *Trigonobalanus* Forman. Fagaceae family species exhibit high trait diversity on vegetative and reproductive traits: leaf habit (evergreen or deciduous), flowering time, pollinator type (wind or animal), fruit maturation period (one-year or two-year fruiting after pollination), and fruit morphology (Manos et al., 2001; Satake & Kelly, 2021).

The significance of reproduction in life cycle of plant

Reproduction is the highest priority for all living things, involving a complex combination of processes, and whose variability between species has long puzzled evolutionary biologists (Barrett, 2010; Sreekala, 2017). And the basic reproductive strategies of flowering plants includes germination, vegetative growth, flowering, seed maturation and seed dispersal, and among these strategies and traits, flowering time and reproduction cost have emerged as key model traits (Andrés & Coupland, 2012; Laurie, 1997; Obeso, 2002).

Flowering time is a key component of the reproductive process, allowing plants to make optimal use of the available resources in their surrounding environment. Therefore, genes that control flowering time are the subject of research (Laurie, 1997). FLOWERING LOCUS T(FT)

gene is produced in annual or perennial plants as an integrator of the flowering pathway, and which is the most widely studied and effective gene for promoting early flowering in plants. It has been identified that the FT gene and its homologous genes exist in multiple plants(Andrés & Coupland, 2012; Turnbull, 2011; Xu et al., 2012). For reproduction cost, it has been considered that any of the various limiting resources might be appropriate as a currency of reproduction cost, and they are usually considered as carbon(C), nitrogen(N) and phosphorus(P) (Obeso, 2002). Specially, among Fagaceae family, some species show obvious masting phenomenon of synchronous mass flowering and fruiting in the interval year (Kelly, 1994). Between the masting year and non-masting year, the fruit production varies significantly (Koenig & Knops, 2005).

Endogenous carbohydrate, nitrogen and phosphorus have been implicated to affect reproduction events

External climatic factors and internal resource status have always been regarded as the main factors affecting reproduction of perennial plants (Miyazaki et al. 2014, Abe et al. 2016, Koenig et al. 2016, Pearse et al. 2016, Ascoli et al. 2017, Bogdziewicz et al. 2020, Bogdziewicz 2021), and internal resource status mainly focus on the carbon(C), nitrogen(N) and phosphorus(P). Nutrients are known to modify flowering time, and within a certain range, flowering is promoted with the increase of nitrogen concentration, but when nitrogen excess reaches a certain level, nitrogen addition will inhibit flowering(Lin & Tsay, 2017). It has been discovered nitrogen addition initiate flowering in *Fagus crenata* Blume. (Miyazaki et al. 2014; Miyazaki and Satake 2017) Sucrose is sensed by the plant directly, through the generation of hexoses and through sugar signals such as T6P (trehalose- 6-phosphate), And T6P pathway has been proved to be interacted with flowering pathways (Li et al., 2018; Wahl et al., 2013).

Carbohydrates, nitrogen, and phosphorus transport in trees

Phenological events and physiological processes of trees are accompanied by seasonal patterns of production, accumulation, and utilization of carbohydrates and nutrients. In wood species, nonstructural carbohydrates (NSC) mainly including soluble sugars (sucrose, glucose, and fructose) and starch are important energy substances of life strategies (Hoch et al., 2003; Magel et al., 2000). It has been found source-to-sink transport of carbohydrate is one of the major determinants of plant growth and relies on the efficient and controlled distribution of sucrose (and some other sugars such as raffinose and polyols) across plant organs through the phloem, and these processes required for sugar signaling that not only controls the flow of sugars to developing organs, but also influences gene expression and hormone signaling throughout the plant (Griffiths et al., 2016; Rolland & Sheen, 2005). Nitrogen and phosphorus transport from source leaves to sinks also take place in the phloem. And phloem loading takes place in the collection phloem of the leaf minor vein networks and phloem unloading happens in the release phloem of sink organs (Tegeder, 2014). For Fagaceae species, bud organ develops to form leaf and flower organs. Therefore, in this study, we focus on the transport of carbohydrate, nitrogen and phosphorus in leaf and bud organs.

Aim of this study

Although there have been some studies on the diversity of reproductive characteristics of Fagaceae species, the effect of carbon, nitrogen, and phosphorus resource on the reproduction of Fagaceae species has not been fully studied among different species. To further better understand the influence of carbon, nitrogen and phosphorus resources on the reproduction of Fagaceae species and the differences among their effects in the evolution of a wide variety of Fagaceae, by

combining molecular biological, physiological and ecological methods, we analysis and compared resource allocation strategies in the reproductive organs of several Fagaceae species and different seasonal expression patterns of carbohydrate, nitrogen, and phosphorus transporter genes in two evergreen Fagaceae species using transcriptome data, and also would like to try to explore the relationships between related three resource transporter genes with flowering phenology. The contents for every chapter are summarized as following:

Chapter 1: Resource allocation strategies in the reproductive organs of Fagaceae species

We calculated the cost of resource investment (three types of resource: C, N and P) in different reproductive organs of nine species respectively and measured representative defensive traits among seven species of Fagaceae, and our results highlighted the diversity of resource allocation strategies to reproduction and defensive traits of Fagaceae species. In terms of reproductive cost, we found that an important parameter in the Resource Budget model determining masting pattern, varied largely among three resource types (C, N, and P) and among species. We also found that there was a negative correlation between pericarp thickness and total phenolics concentrations among seven Fagaceae species, as which suggested by a possible trade-off between C-based physical and chemical defenses.

Chapter 2 Different seasonal expression patterns of carbohydrate, nitrogen, and phosphorus transporter genes in two evergreen trees and their relationships with flowering phenology

In this chapter, we combined a genome-wide field transcriptome analysis and relative gene expression analysis by using real-time quantitative PCR detecting method to compare and explore the seasonal changes of carbohydrates, nitrogen and phosphorus transporter between leaves and

buds of two evergreen masting species under the natural environment and their relationships with flowering phenology of evergreen trees. Our results showed transport of nitrate, amino acids and ammonium between leaves and buds was very frequent in spring and summer, while in winter related monosaccharide, nitrate, amino acid, and phosphorus transport were all more frequent and abundant than in other seasons. And we also found that the upregulation of FT genes in leaves was more likely related to nitrogen status in the two Fagaceae species.

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Chapter 1 Resource allocation strategies in the reproductive organs of Fagaceae species

The study in this chapter, done in collaboration with Prof. Akiko Satake, Dr. Qingmin Han, Prof. Kaoru Kitajima, Dr. Hiroko Kurokawa, Dr. Takuya Shimada, Ms. Tamaho Yamaryo, Dr. Daisuke Kabeya, and Tatsuro Kawasaki, and it has been accepted in *Ecological Research*. (In press)

Abstract

Allocation strategies of carbon [C], nitrogen [N], and phosphorus [P] are key to the reproductive processes of plants. Nutrient allocation to seeds depends on defensive traits of seeds and fruit because greater nutritional contents attract more seed predators. To compare resource allocation strategies for reproductive and defensive traits across species, we calculated the cost of resource investment in reproductive organs on nine species from four genera and defensive traits on seven species from two genera of Fagaceae. The results showed that no single element is the common resource currency across species, but that the reproductive strategy of each species is regulated by C, N, or P. Reproduction in *Fagus crenata* was limited by N but not by C, whereas in *Quercus serrata* it was co-limited by C and N. Among the seven Fagaceae species, there was a negative correlation between the thickness of the pericarp and the concentration of the total phenolics in the seed and pericarp, suggesting alternative strategies for developing defensive traits in ripening seeds with limited C-based resources. Overall, our results highlighted the diversity of resource allocation strategies to reproduction and defensive traits of Fagaceae species. To better understand the masting phenomenon at the population or community levels, comprehensive consideration of the diversity of resource allocation strategies among species is worth exploring in the future.

Introduction

Fagaceae exhibits high trait diversity among and within its ten genera in terms of leaf habit (evergreen or deciduous), fruit morphology, pollinator type (wind or animal), and fruit maturation period (one-year or two-year fruiting after pollination). The majority of Fagaceae species occur in a wide range of the Northern Hemisphere, with the center of diversity in Southeast Asia (Kremer et al., 2012; Manos et al., 2001; Satake & Kelly, 2021; Wilf et al., 2019). Fagaceae fruits are large dry nuts (acorns; Figure 1) that are rich in nutrients, which are often sought after by insects, birds,

rodents, and other animals (Chang & Zhang, 2014; Shimada, 2001). Therefore, the temporal fluctuations of acorn production can greatly impact the population dynamics of these seed eaters, and consequently the whole ecosystem(Koenig & Knops, 2005). Within Fagaceae, some species show strong "masting", i.e., synchronous mass flowering and fruiting once in several years with low or negligible reproduction in the interval (Kelly, 1994; Kelly & Sork, 2002; Koenig & Knops, 2005).

Many have hypothesized that fluctuation in reproductive outputs of perennial plants are linked to year-to-year variations in climate and other environmental factors, as well as internal resource status (Abe et al., 2016a; Ascoli et al., 2017; Bogdziewicz et al., 2020; Bogdziewicz, 2021; Koenig et al., 2016; Miyazaki et al., 2014; Pearse et al., 2016; Satake & Iwasa, 2002a). Reproduction requires a large resource investment. Hence availability of carbon [C], nitrogen [N] and phosphorus [P] can influence reproductive schedule (Crone et al., 2009; Han et al., 2014, 2017; Ichie et al., 2013; Kitayama et al., 2015). To support a large reproductive effort or event, theoretical models assume that some plants build up C allocation over the years prior to reproduction (Isagi et al., 1997). However, empirical studies show that the extent to which allocated C contribute to seed production differs widely among species, from little (Hoch et al., 2003; Ichie et al., 2013) to substantial (Han et al., 2016; Kabeya et al., 2021). Hence, C may not be the universal currency to regulate the reproductive strategy in perennial plants. Instead of C, N or P could be the resource that limits reproduction because empirical data strongly suggest that N is a limiting factor that affects mass flowering and fruiting in *Fagus crenata* (Abe et al., 2016; Han et al., 2014; Miyazaki et al., 2014). In some Fagaceae species, higher P concentrations in the canopy lead to greater fruit production (Fernández-Martínez et al., 2016).These studies suggest that the nutrient allocation

strategies differ among species, but comparative analyses of nutrient allocation to reproductive organs in different species is limited.

The allocation strategy of nutrients to seeds cannot be independent of the defensive traits of seeds and fruit, because greater nutritional contents attract many seed predators (Grubb et al., 1998). The damage level by insects may exceed 90% of the whole seed, which is a major factor that inhibits the production of healthy seeds (Xiao et al., 2003). Hence, part of the resources allocated to the reproductive organs are for defensive functions (Dalling et al., 2020; Wang et al., 2017; Xiao et al., 2003; Zhang et al., 2016). There are two types of defenses, C-based chemical defense and physical defense (Moles et al., 2013; Zalamea et al., 2018). While many studies have investigated resource allocation to physical and chemical defenses in seedlings or leaves (Hanley & Lamont, 2002; Moles et al., 2013), the relationship between resource allocation to chemical and physical defense in reproductive organs is unclear. Moreover, it remains elusive whether there is variation in resource allocation strategies among related species.

To compare resource allocation strategies to reproductive and defensive traits among species, here we calculated resource investment costs on reproductive organs in nine Fagaceae species and resource investment costs on defensive traits in seven Fagaceae species. To quantify reproductive costs, we adopted the framework of the resource budget (RB) model, initially proposed to explain the physiological mechanisms underlying masting by C storage (Crone et al., 2009; Han et al., 2017; Isagi et al., 1997; Satake & Iwasa, 2000, 2002a, 2002b). Based on the method proposed by Abe et al. (2016), we estimated the reproductive cost of C, N, and P to unravel which resource is a limiting factor for reproduction and how resource requirement differs among species. To quantify the costs of chemical and physical defense, we measured the quantity of total phenolic compounds and condensed tannins (Balasundram et al., 2006; Bocco et al., 1998) and the

thickness of the pericarp that envelopes a seed, respectively. Comparing the allocation of resources to reproductive and defensive traits among species may provide insights into the relationship between resource requirement and masting and the association between reproduction and defense.

Materials and methods

Study species and sample collection

Male flowers and acorns from nine species, including four genera in the Fagaceae family, were collected in 2017 and 2018 at one or more of three study sites in Japan: Tsukuba (an arboretum of the Forestry and Forest Products Research Institute, 36˚0'31.0"N, 140˚7'53.0"E , 24 m a.s.l), Fukuoka (the biodiversity reserve on the Ito campus of Kyushu University; 33˚35'47.5"N, 130˚12'50.0"E, 20 to 57 m a.s.l), and Mt. Naeba (36˚53'37.9"N, 138˚46'1.5"E; 900 m a.s.l.) (Figure 1, Table 1). Records from nearby meteorological stations (Japan Meteorological Agency) indicate that during 1976–2018, the mean annual precipitation and temperature were 1291 mm and 13.9 °C in Tsukuba, 1727 mm and 16.1 °C in Fukuoka, 2251 mm and 11.5 °C in Mt. Naeba. Male flower inflorescences were sampled at the timing of anther dehiscence in April or May. Acorn-burden twigs were taken from the upper parts of each tree crown using a 6 m telescopic pruner, and with the aid of ladders or an aerial work platform. Mature acorns were collected between September and November, depending on the maturation time of each species. All samples were immediately placed in a cool box, until acorns were separated into cupule, pericarp (including the seed coat fused to the pericarp), and seed (inner seed, excluding the seed coat) in the laboratory. All samples were stored at −80°C until lyophilization for 48 h. Dry mass was recorded for each organ. For chemical analyses, each sample was ground into a fine powder in a steel ball mill (MM400, Retsch, Haan, Germany).

To measure the thickness of pericarps and concentrations of the total phenolics and condensed tannins, mature acorns of seven Fagaceae species, which had fallen under the mother trees, were collected from various sites across Japan (Table 1). These are different samples from those used for the calculation of C, N, and P allocations.

To document the temporal change in pericarp thickness, developing acorns were collected approximately every two weeks from May to November for *Quercus glauca* and from May to October for *Lithocarpus edulis* at the Fukuoka study site. Ten intact acorns from each tree of 5–6 individuals of each species were collected. The selected acorns were cut into half and cross-sectional image data were taken at scale using a scanner (resolution: 300 dpi; CanoScaner LiDE400, Canon, Fukuoka City). The straight-line tool of ImageJ software was used to estimate the average pericarp thickness at four diagonal positions. Samples were then dried in a Forced Convection Oven (65 ˚C) for 1 to 2 days before storage and further analysis.

Ripe acorn samples were separated into cupule, pericarp, and seed to measure the contents of chemical defense in different organs. Because aborted acorns and young acorns of *Q. glauca* and *L. edulis* were too small to be able to separate the different organs, the whole acorns were used for measurement and analysis. All samples were lyophilized for 16 hours, and ground into fine powders as aforementioned for the further analysis of defense trait.

Resource contents in reproductive organs

The total N and C concentrations of powdered samples were measured after combustion with a CHN Analyser (Vario Max CN, Elementar, Hanau, Germany). The total P concentrations of powdered samples were determined after ignition (550 °C, 1 h) and extraction with 1 M H_2SO_4 with orthophosphate detection of neutralized extract by molybdate blue colorimetry at 880 nm

with a microplate reader. Measurement of P content was performed only for six species because the available sample quantity was insufficient for three species. We also calculated the depletion coefficient for three C, N, and P resources, for the six one-year fruiting species. The depletion coefficient was calculated according to the analysis by Abe et al. (2016). We used the following formula:

Depletion coefficient = RI_{af} /RI_{bf},

where RI_{bf} = [Resource Investment (RI) for reproductive organs before fertilization] = (RI to a male inflorescence × number of male inflorescences per female inflorescence) + (RI to a cupule × number of cupules per female inflorescence) + (RI to a pericarp × number of pericarps per female inflorescence); and RI_{af} = [RI for reproductive organs after fertilization] = RI to a seed × number of seeds per female inflorescence.

Physical and chemical defense

The methods used to measure the concentrations of condensed tannins and total phenolics of *Q. glauca* and *L. edulis* were modified according to Kurokawa & Nakashizuka, 2008. Condensed tannins and total phenolics were extracted from dried and powdered samples with 50% methanol. A proanthocyanidin assay was used to determine the condensed tannin content by using cyanidin chloride (FUJIFILM Wako chemicals, Oasaka, Japan), a commercially available anthocyanidin, as a standard (Julkunen-Titto 1985). The concentration of total phenolics was measured with the Folin-Ciocalteu method by using a commercially available tannic acid (FUJIFILM Wako chemicals, Oasaka, Japan), as a standard (Waterman and Mole 1994).

The procedures of chemical analyses for the other five Fagaceae species were slightly different from the above two species. Mature acorns of *Q. acutissima*, *Q. aliena*, *Q. gilva*, and *Q.*

serrata were collected in 2009, and those of *Q. salicina* were collected in 2011 from 3–5 individuals (Table 1). Five acorns were sampled from each individual tree. Acorns were separated into cupule, pericarp, seed coat, and seed. Then, samples were lyophilized and milled before extraction with 50% methanol. The method for measuring condensed tannins was the same as above (proanthocyanidin assay with cyanidin chloride as the standard). The concentration of total phenolics was measured using a modified Price-Butler method (Graham 1992; Hagerman 2011), with tannic acid as a standard. The contents of condensed tannin and total phenolics were measured separately for each organ (cupule, pericarp, seed coat, and seed). For the contents of the pericarp, we used the averaged values of the measurements for pericarp and seed coat according to their respective weight ratios. Although the methods for measuring total phenolics differed among the seven species as explained above, the measured values for the seven species were treated as a single dataset in the subsequent analyses because the standard material, tannic acid, was unified.

Statistical analysis

Differences in the total C, N, and P in each type of reproductive organ among species were assessed using the Kruskal–Wallis one-way analysis of variance (ANOVA) because the data were normally distributed (Shapiro test) but did not meet the homogeneity of variance test (Bartlett test). Games-Howell tests were used for post hoc pairwise comparisons. Differences in the C, N, and P concentrations among seeds, pericarps, and cupules within the same species were tested with a one-way ANOVA and post hoc Tukey HSD tests. A one-way ANOVA was also used to test for differences in the concentrations of total phenolics and condensed tannin among different developmental stages of acorns.

To investigate the bivariate relationships of the concentrations of the three elements (C, N, and P) and chemical vs. physical defenses, standardized major axis (SMA) was used with the 'lmodel2' package of R (Curran-Everett, 2013). R-squared showed the amount of variance explained by the model and an *F* test was used to test the overall significance of the equation. R software was used for statistical analysis (R version 3.6.1).

Results

C, N, and P allocations to reproductive organs

The contents of C, N, and P in four reproductive organs (male inflorescence, cupule, pericarp, and seed) per female inflorescence varied widely among species (Figure 2a–c). The total C, N, and P invested to the four reproductive organs were significantly higher in *Castanea crenata* than in other species except for *L. edulis* and *Q. acutissima* (Figure 2a–c; Games-Howell test, $p < 0.05$). Species pollinated by insects (*C. crenata* and *L. edulis*) had significantly higher amounts of C and N in male inflorescences compared to wind-pollinated species, except for *Q. aliena* (Figure 2a; Games-Howell test, $p < 0.05$).

Compared to the total C, N or P invested per female inflorescence, the C, N, and P concentrations of each reproductive organ were similar across species. There was small interspecific variation in C concentration across nine species and four different reproductive organs (Figure 2d). The concentrations of N and P in male flowers were significantly higher than cupules, pericarps or seeds in most species (Figure 2e, f). Only in *F. crenata,* were N concentrations in seeds as high as in male flowers (Figure 2e).

Depletion coefficients

The depletion coefficients differed among six species (Figure 3). Some species had values < 1 in all three resource types (*C. crenata* and *Q. glauca*), one species had values > 1 in all three resource types (*Quercus serrata*), while the other three species had values > 1 or < 1 depending on the resource type. The highest depletion coefficient value was observed for the N-based coefficient of *F. crenata* (Figure 3). The second largest was the C-based depletion coefficient in *Q. serrata*.

Resource allocation ratios

The N:C and P:C ratios in seeds and male inflorescences were significantly higher than those in pericarps and cupules (Figure 4). The N:C ratio in male inflorescences was the highest among the four reproductive organs (Figure 4a), and the P:C ratios in male inflorescences were not significantly different from those in seeds (Figure 4b). Among the four reproductive organs, N:C and P:C ratios in the male inflorescences showed the largest range across the six species (Figure 4).

In the several species we collected, there were significant negative correlations between concentrations of C and N in the pericarp, and no significant relationships in other reproductive organs (Figure S1). However, there was a significant positive correlation between the concentrations of N and P in male inflorescences, pericarps, and seed organs ($p < 0.05$; SMA regression; Figure S1, Table S2).

Chemical and physical defenses

The thickness of the pericarp was negatively correlated with the concentration of total phenolics in seeds and pericarps (Figure 5a; SMA regression; $R^2 = 0.760$, $p = 0.026$; $R^2 = 0.612$, $p = 0.038$). In contrast, there was no significant association between pericarp thickness and concentration of

condensed tannin in seeds or pericarps (Figure 5b). The thicknesses of the pericarps of acorns in *Q. glauca* and *L. edulis* increased gradually during the fruit development, but rates of the increase between the two species were very different (Figure 6). For *Q. glauca*, the average rate of increase in fruit thickness was 0.0364 (mm per month), while for *L. edulis*, it was 0.1854 (mm per month). For mature fruit, thickness of the pericarp in *L. edulis* was 3.39 times thicker than in *Q. glauca.* In *Q. glauca*, the concentration of total phenolics in seeds was significantly higher than in pericarps and cupules during seed maturation (October and November; Figure S2a), while the concentration of condensed tannin in seeds was very low (Figure S2c). Such a difference between the concentration of total phenolics and condensed tannin was not observed in *L. edulis* (Figure S2b, d).

Discussion

We compared resource allocation strategies to reproductive organs of species from four genera and defensive traits among species of two genera of Fagaceae. In terms of reproductive cost, we found that an important parameter in the RB model determining masting pattern, varied largely among three resource types (C, N, and P) and among species. We also found that there was a negative correlation between pericarp thickness and total phenolics among seven Fagaceae species, as suggested by a possible trade-off between C-based physical and chemical defenses.

Our results showed that the N-based depletion coefficient in *F. crenata* was the largest among the six species (Figure 3). This result is due to the particularly high N concentration of the seeds in *F. crenata* (Figure 2), resulting in a high demand for N during the seed development process in this species. According to the RB model, such a large N-based depletion coefficient leads to the evolution of significant year-to-year fluctuations in seed crop size (coefficient of

variation at the population level (CVp) = 1.04–1.79; Masaki et al. 2008) in *F. crenata*, as demonstrated by a previous study (Abe et al. 2016). In Q. serrata, depletion coefficients were estimated to be larger than one in all of the resource types (C, N, and P). These results suggested that moderate fluctuations of seed crop size in *Q. serrata* (CVp = 0.74–0.87; Fukumoto and Kajimura 2011) can be attributed to the joint allocation of C, N, P to flowers and fruit organs. In Q. glauca, the N- and P-based depletion coefficients were the smallest among the species studied, and the C-based depletion coefficient was also less than one. The CVp of Q. glauca was small (0.50; Supplementary data: data were provided by the Ministry of the Environment 2 Monitoring Sites 1000 Project), and this low CVp value was consistent with its low depletion coefficient (< 1) in all three resource types.

Overall, these results suggest that resource investment strategy can lead to the evolution of masting in Fagaceae species, but that the currency of each species is regulated by different resource types instead of a common resource. Because CVp values are available only for three species (*F. crenata*, *Q. serrata* and *Q. glauca*), accumulating seed crop data for the rest of the species is necessary to obtain firm support for the expected association between the magnitude of the resource depletion coefficient and the degree of masting.

We also found that the investment strategies of N and P in the reproductive organs were relatively similar among Fagaceae species. When compared to the depletion coefficient values of C, the values for P were closer to the values calculated on N. In male flowers, pericarps, and seeds, the concentration of N and P showed a significant positive correlation (Figure S1, Table S1). In addition, both the N:C and P:C ratios in male inflorescences and seeds, the organs that contribute to the next generation, were significantly higher than in pericarps and cupules (Figure 4). There were similar findings in a previous study by Hemborg and Karlsson (1998), which found that the

relative costs of N and P for reproduction in eight subarctic species were approximately the same, except that the costs of N and P biomass were lower than that of C.

In addition, our results clearly showed that among seven Fagaceae species, there was a significantly negative correlation between concentration of the total phenolics and the thickness of the pericarp, suggesting a trade-off between chemical and physical defense. Formation of physical defense is related to the high lignification of the pericarp, and the phenolic substances are a precursor substance of lignin (Caretto et al., 2015; Lattanzio et al., 2006; Mébarki et al., 2019). Therefore, the trade-off between physical and chemical defenses suggests that the total amount of C that could be invested into defense is limited. In this respect, C limitation due to fruiting has been detected as a temporary decrease in starch concentration in summer in coarse roots and stems of F. crenata (Kabeya et al 2021), and as shorter shoots produced in fruiting-trees than in non-fruiting trees of the same species (Han et al, 2016). Together with the different currencies in the depletion coefficients for different species, these results suggest alternative strategies in Fagaceae species to provide sufficient defense in ripening seeds with limited resources. Due to the different sampling regions used to measure physical and chemical defense traits of single species, direct comparisons of those two defense traits may contain limitations. To further investigate the association of resource allocation between reproductive and defensive traits, studying geographic variation in defensive traits under different seed predation pressures would be needed in the future.

In summary, our results highlighted the diversity of resource allocation strategies to reproduction and defensive traits of Fagaceae. To better understand the masting phenomenon at the population or community levels, comprehensive consideration of the diversity of resource allocation strategies among species is worth exploring in the future.

Acknowledgements

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Tables

Table 1 Comparison of reproductive traits of Fagaceae species

Species (Abbreviation)	Leaf habit	Pollination	Tree diameters at breast height (cm)	Fruit Maturity	Samples used for resource investment measurements		Samples used for defense measurements	
					Masting pattern[1] (citation)	Number of trees (Study Sites; Collection Year)	Number of trees (Study sites for physical defense traits)	Number of trees (Study sites for chemical defense traits)
Castanea crenata (Cc)	Deciduous	Animal	25.9–30.4	One year	Annual (personal observation)	3 (Tsukuba; 2017) 3 (Tsukuba; 2018)	None	None
Lithocarpus edulis (Le)	Evergreen	Animal	33.1–36.9	Two years	Annual (personal observation)	3 (Tsukuba; 2017) 3 (Tsukuba; 2018)	6 (Ito campus, Fukuoka; 33°35' 47.5"N, 130° 12' 50.0"E, 20-57m asl.)	6 (Ito campus, Fukuoka; 33°35'47.5"N, 130°12'50.0"E, 20-57m asl.)
Quercus acutissima (Qa)	Deciduous	Wind	22.7–40.8	Two years	Annual (Hashizume H, 1987)	3 (Tsukuba; 2017) 4 (Tsukubai; 2018)	5 (Ito campus, Fukuoka; 33°35' 47.5"N, 130°12' 50.0"E, 20-57m asl.)	5 (Shiga, Ohtsu, Shiga; 35°11.5'N 135°54.0' E, 235m asl.)
Quercus glauca (Qg)	Evergreen	Wind	22.9–39.1	One year	Annual (Hirayama et al., 2017)	3 (Tsukuba; 2017) 3 (Tsukubai; 2018)	5 (Ito campus, Fukuoka; 33°35' 47.5"N, 130° 12'50.0"E, 20-57m asl.)	5 (Ito campus, Fukuoka; 33°35' 47.5"N, 130° 12'50.0"E, 20-57m asl.)
Quercus phillyraeoides (Qp)	Evergreen	Wind	21.1–41.7	Two years	Annual (personal observation)	4 (Tsukuba; 2017)	5 (Ito campus, Fukuoka; 33°35'47.5"N, 130°12' 50.0"E, 20-57 asl.)	5 (Ito campus, Fukuoka; 33°35'47.5"N, 130°12' 50.0"E, 20-57 asl.)
Quercus serrata (Qs)	Deciduous	Wind	30.6–47.7	One year	Annual (Fukumoto & Kajimura, 2011)	5 (Tsukuba; 2017) 4 (Tsukuba; 2018)	5 (Ito campus, Fukuoka; 33°35'47.5"N, 130°12' 50.0"E, 20-57 asl.)	5 (Arashima, Toba, Mie; 34°28.3'N 136° 52.0' E, 60m asl.)
Quercus aliena (Qal)	Deciduous	Wind	64.5–93.8	One year	Annual (personal observation)	3 (Fukuoka; 2018)	None	None
Quercus gilva (Qgi)	Evergreen	Wind	67.4–83.0	One year	Annual (Tanouchi et al., 1994)	3 (Fukuoka; 2018)	5 (Ito campus, Fukuoka; 33°35'47.5"N, 130°12' 50.0"E, 20-57m asl.)	5 (Hirao, Yamashiro, Kizugawa, Kyoto; 34° 46.5' N 135°49.0' E, 33m asl.)
Quercus salicina (Qsl)	Evergreen	Wind	None	Two years	None	None	5 (Ito campus, Fukuoka; 33°35'47.5"N, 130°12' 50.0"E, 20-57m asl.)	5 (Shiga, Ohtsu, Shiga; 35°11.5'N 135°54.0' E, 235m asl.)
Fagus crenata (Fc)	Deciduous	Wind	23.6–47.1	One year	Intermittent (Masaki et al., 2008)	10 (Naeba; 2015)	None	None

[1]Masting pattern was classified into annual- and intermittent- fruiting from the literature if more than one sound seed per m^2 was observed in two consecutive years using the seed trap method.

Figures

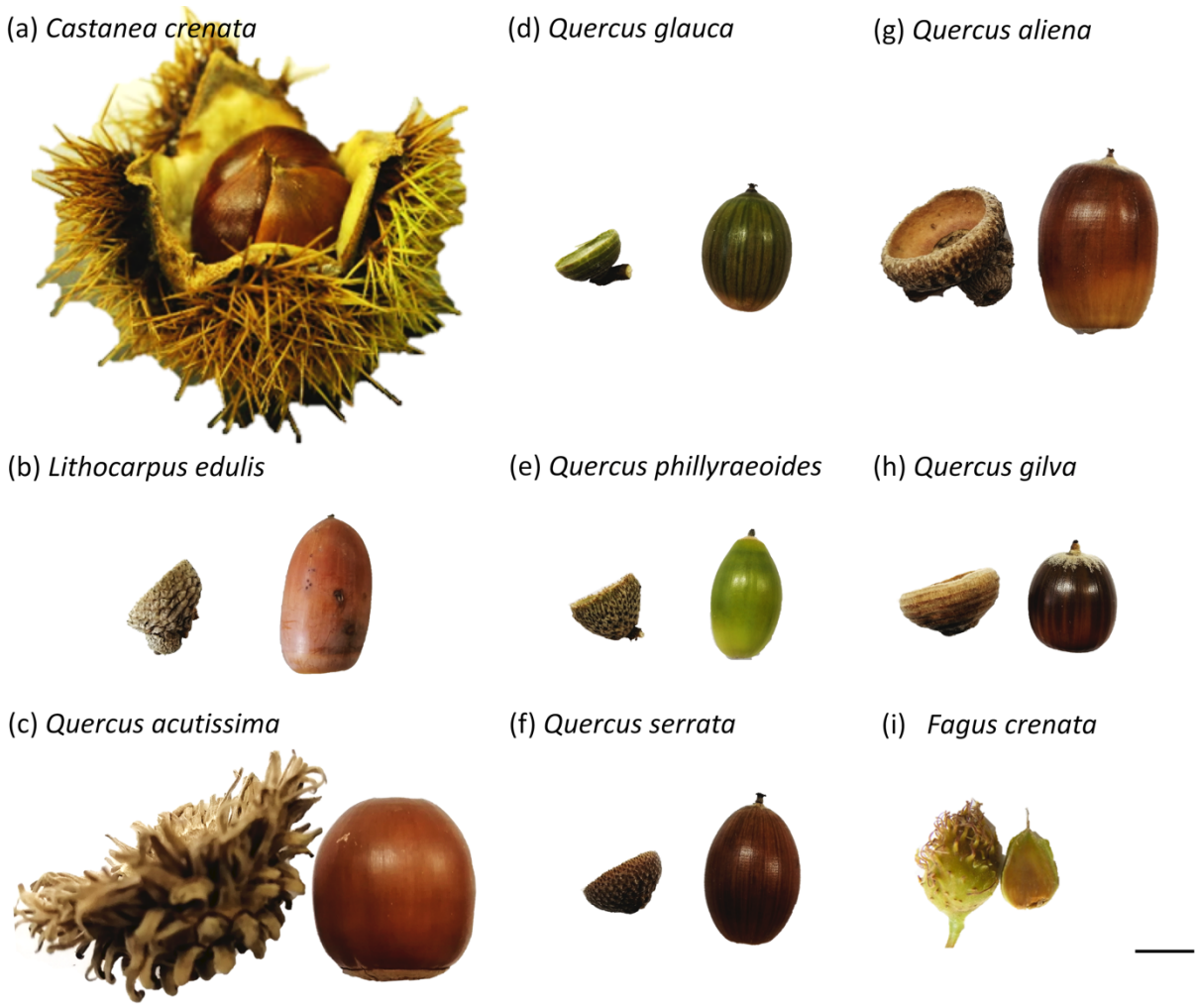

Figure 1. Fruit organs of Fagaceae species. Scale bar, 1cm.

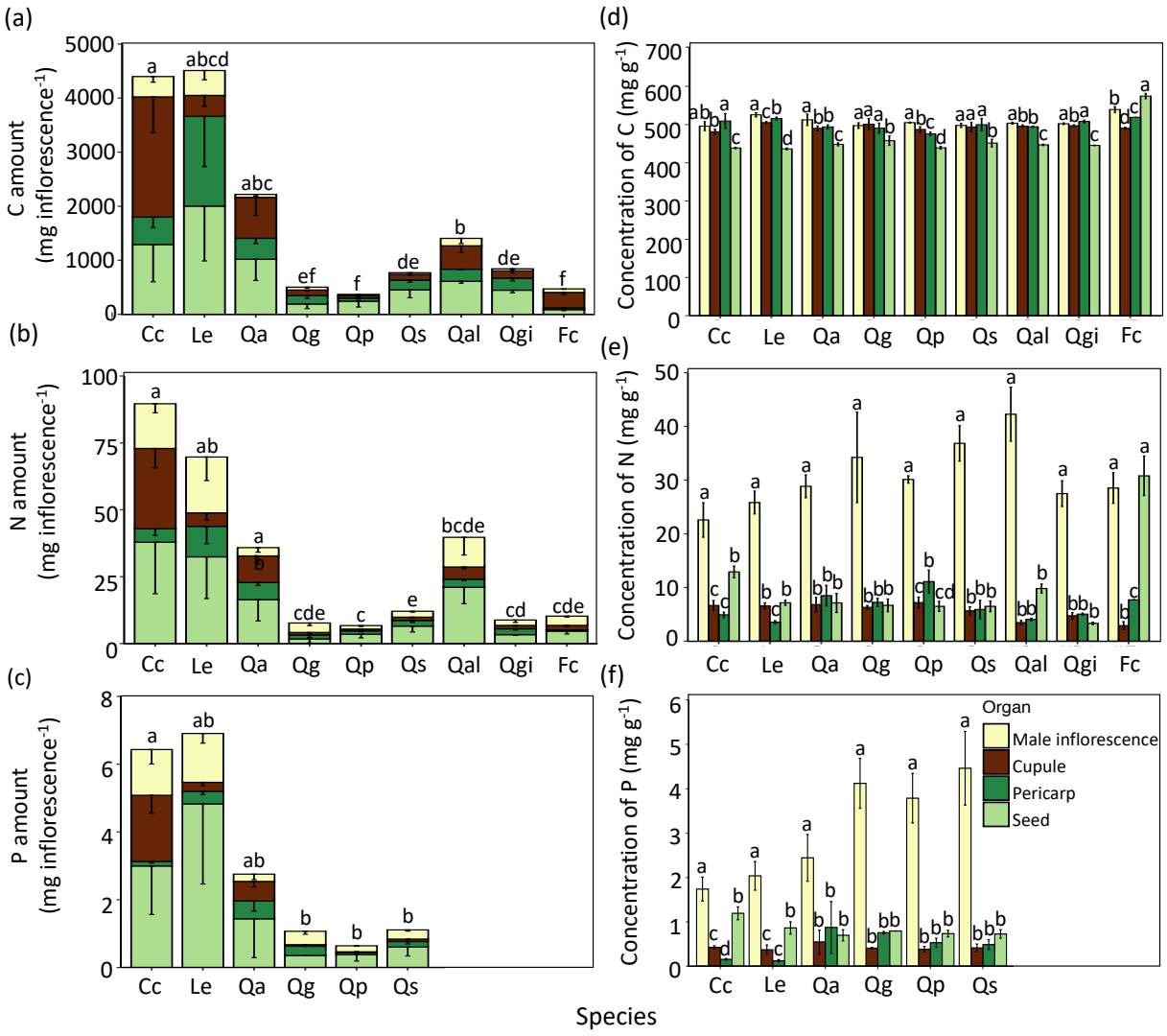

Figure 2. C, N, P resource in different reproductive organs among Fagaceae species. (a, b, c) Content of C, N, P resource in reproductive organs per one inflorescence (mean ± SE). **(d, e, f)** Concentrations of C, N, P resource in reproductive organs (mean ± SE). Average number of acorns produced per inflorescence for each species: *Castanea crenata*: 1.4; *Lithocarpus edulis*: 2.8; *Quercus acutissima*: 1.2; *Quercus glauca*: 2.3; *Quercus phillyraeoides*: 1.0; *Quercus serrata*: 1.1; *Quercus aliena*: 1.5; *Quercus gilva*: 1.2; *Fagus crenata*: 1.3. Different lowercase letters in the left panel present total reproductive cost (sum of resource allocation to four organs) differences among different species; Different lowercase letters in the right

panel present concentration differences among four reproductive organs in the same species. Abbreviation of species name is shown in Table 1.

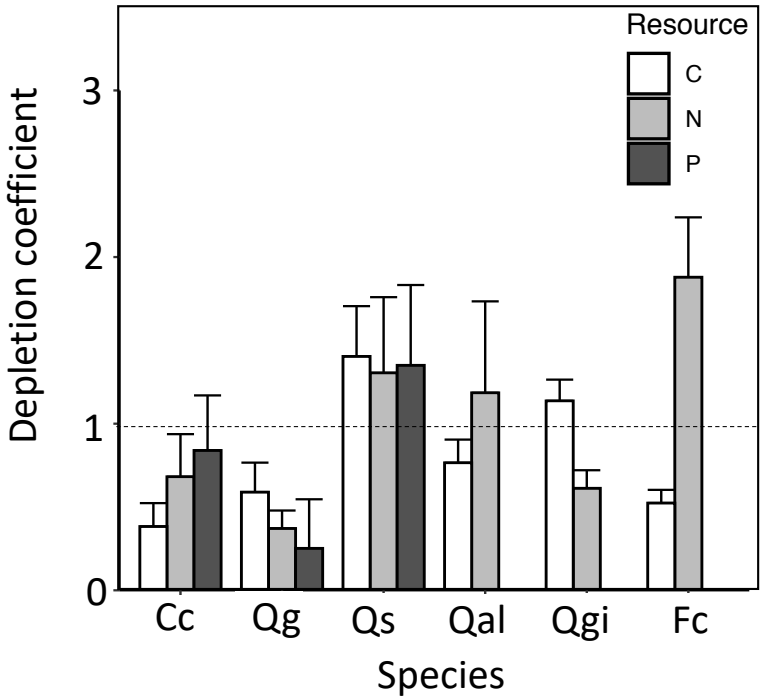

Figure 3. Depletion coefficient (mean ± SE) of three resource type among different Fagaceae species. The depletion coefficient values, the ratios of resource allocated to seeds to flowers, were calculated for C and N resource types of 6 one-year-fruiting species and P resource type of 3 one-year-fruiting species. Cc: *Castanea crenata*; Qg: *Quercus glauca*; Qs: *Quercus serrata*; Qal: *Quercus aliena*; Qgi: *Quercus gilva*; Fc: *Fagus crenata*.

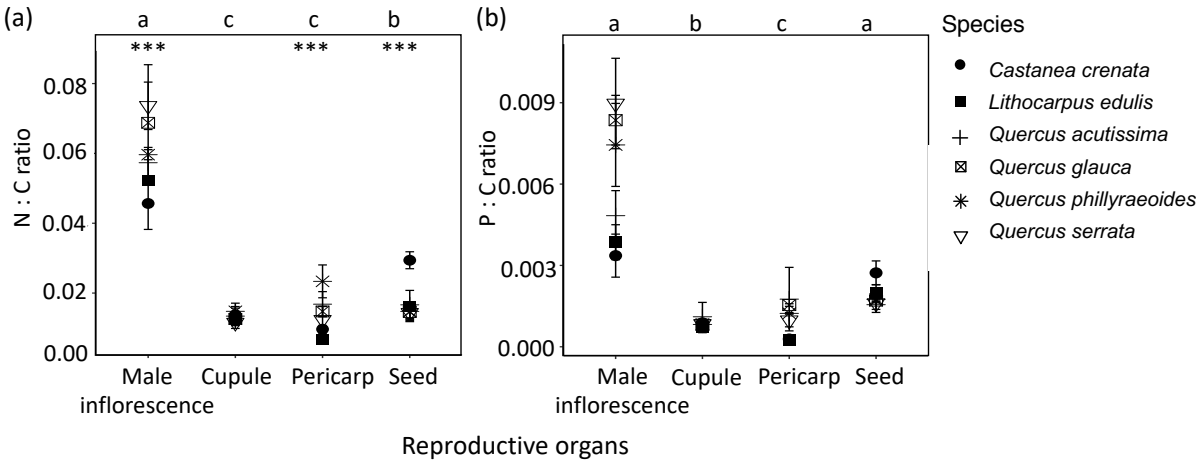

Figure 4. Difference of N:C and P:C ratios in reproductive organs among species. Vertical error bars represent standard error of the mean value. The different lowercase letters above the box indicate significant differences among different 4 organs at $p<0.05$. The black asterisks represent significant differences for a single organ among different species. ('***' represents statistically significant at 0.001 level, Tukey HSD test).

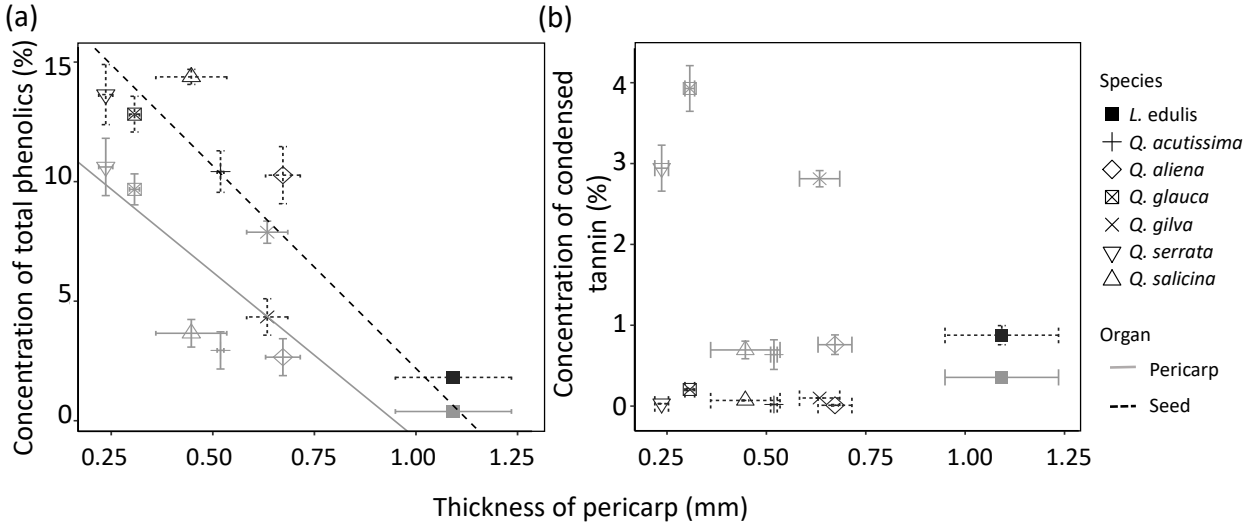

Figure 5. Bivariate standardized major axis (SMA) regression relationships of thicknesses of pericarp to (a) total phenolics and (b) condensed tannin concentrations in seed and pericarp among 7 Fagaceae species. Vertical error bars represent standard error of the concentrations of total phenolics or condensed tannin. Horizontal error bars represent standard error of the thickness of pericarps. The number of individuals for each species are shown in Table 1.

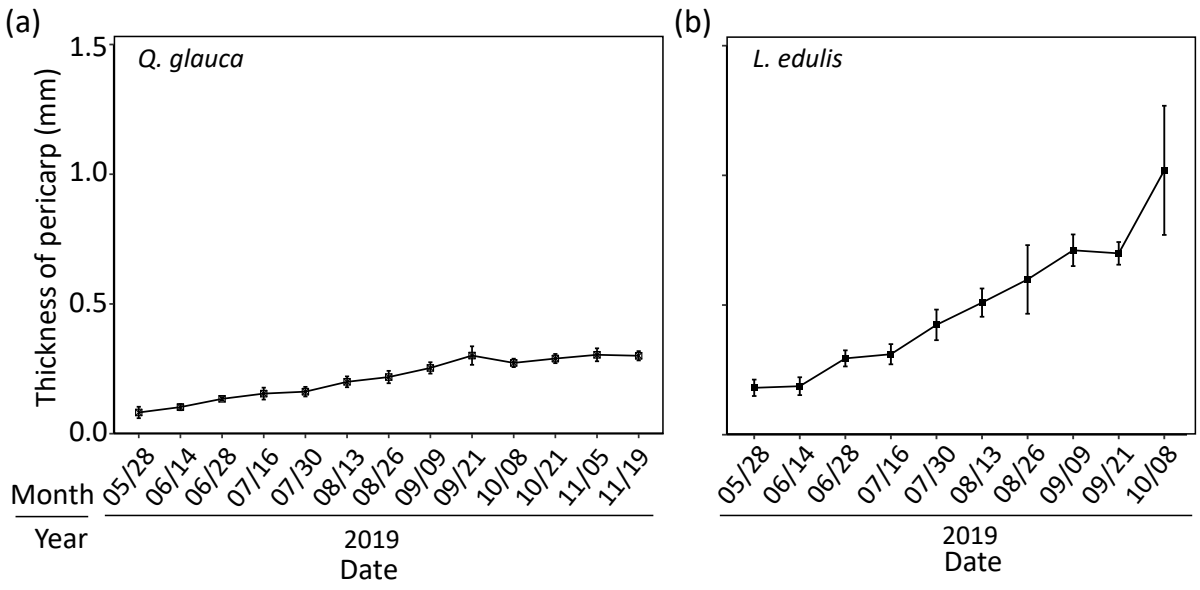

Figure 6. Thicknesses of pericarp of developing acorns in (a) *Quercus glauca* and (b) *Lithocarpus edulis*. (a) Mean values of thicknesses of pericarp at different developmental stages of acorns in *Q. glauca* (n=5). **(b)** Mean values of thicknesses of acorn pericarp at developmental stages of second year in *L. edulis* (n=6). n represents the number of individuals (Table 1).

Appendixes

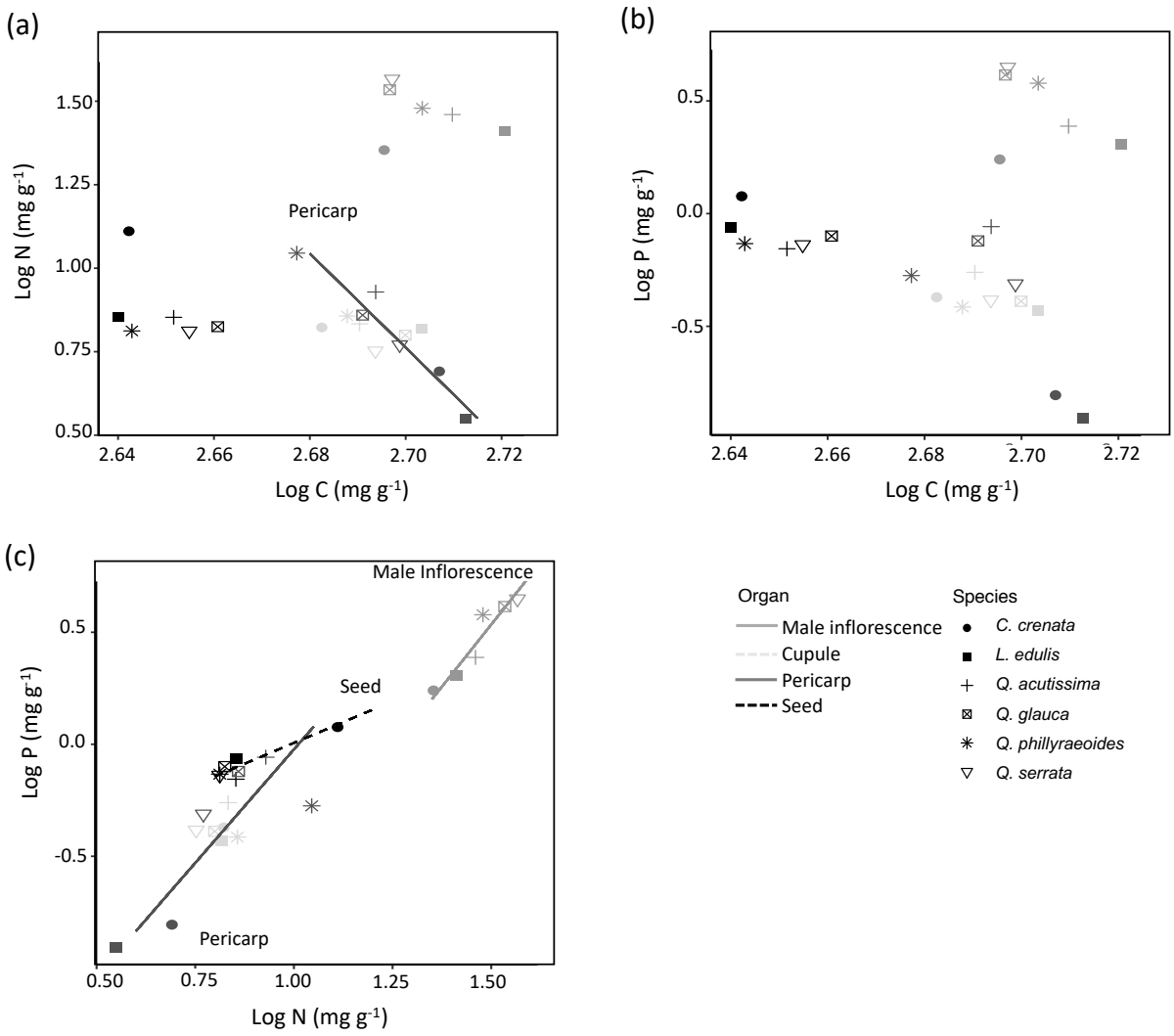

Figure. S1 Bivariate standardized major axis (SMA) regression relationships among Log_{10} scaled C N P concentrations in four reproductive organ types among 6 Fagaceae species. The SMA regression lines of different colors represent for relationships of log_{10} scaled concentrations of C vs. N **(a)**, C vs. P **(b)** and N vs. P **(c)** in four reproductive organs male inflorescence (gray), cupule (light gray), pericarp (deep gray) and seed (black) in figures.

Axes are $\log_{10}$-transformed data of C N P concentrations ($mg \cdot g^{-1}$), and results on traits pair X and Y of standardized major axis analyses are shown in **Table S1**.

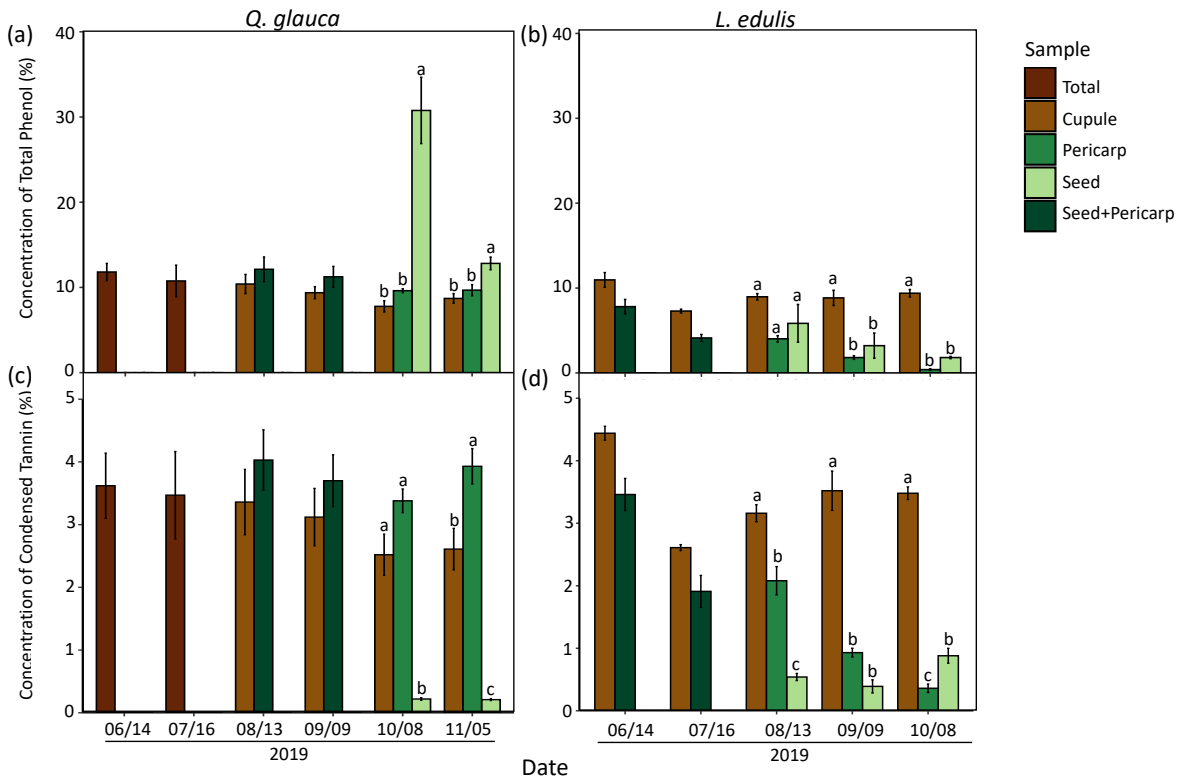

Figure S2. Concentrations of total phenol and condensed tannin in developing acorns of *Q. glauca* and *L. edulis*. (a) Concentrations of total phenol in developing acorns of *Q. glauca*. **(b)** Concentrations of total phenol in developing acorns of *L. edulis*. **(c)** Concentrations of condensed tannin in developing acorns of *Q. glauca*. **(d)** Concentrations of condensed tannin in developing acorns of *L. edulis*. Different lowercase letters present concentration differences among different reproductive organs at the same development stage.

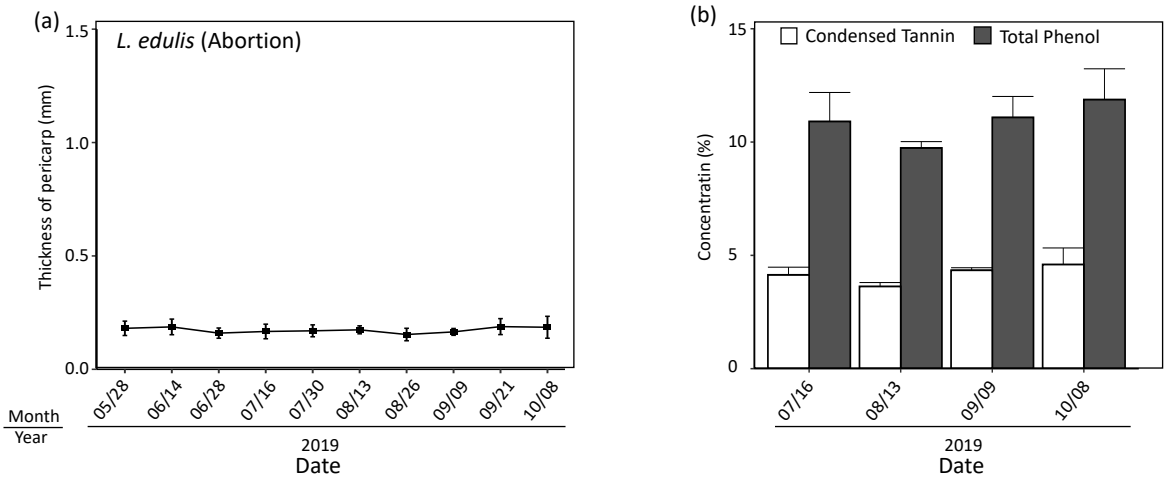

Figure S3. Thicknesses of pericarp of abortion acorns in *L. edulis* (a). Concentrations of total phenol and condensed tannin in abortion acorns of *L. edulis*(b).

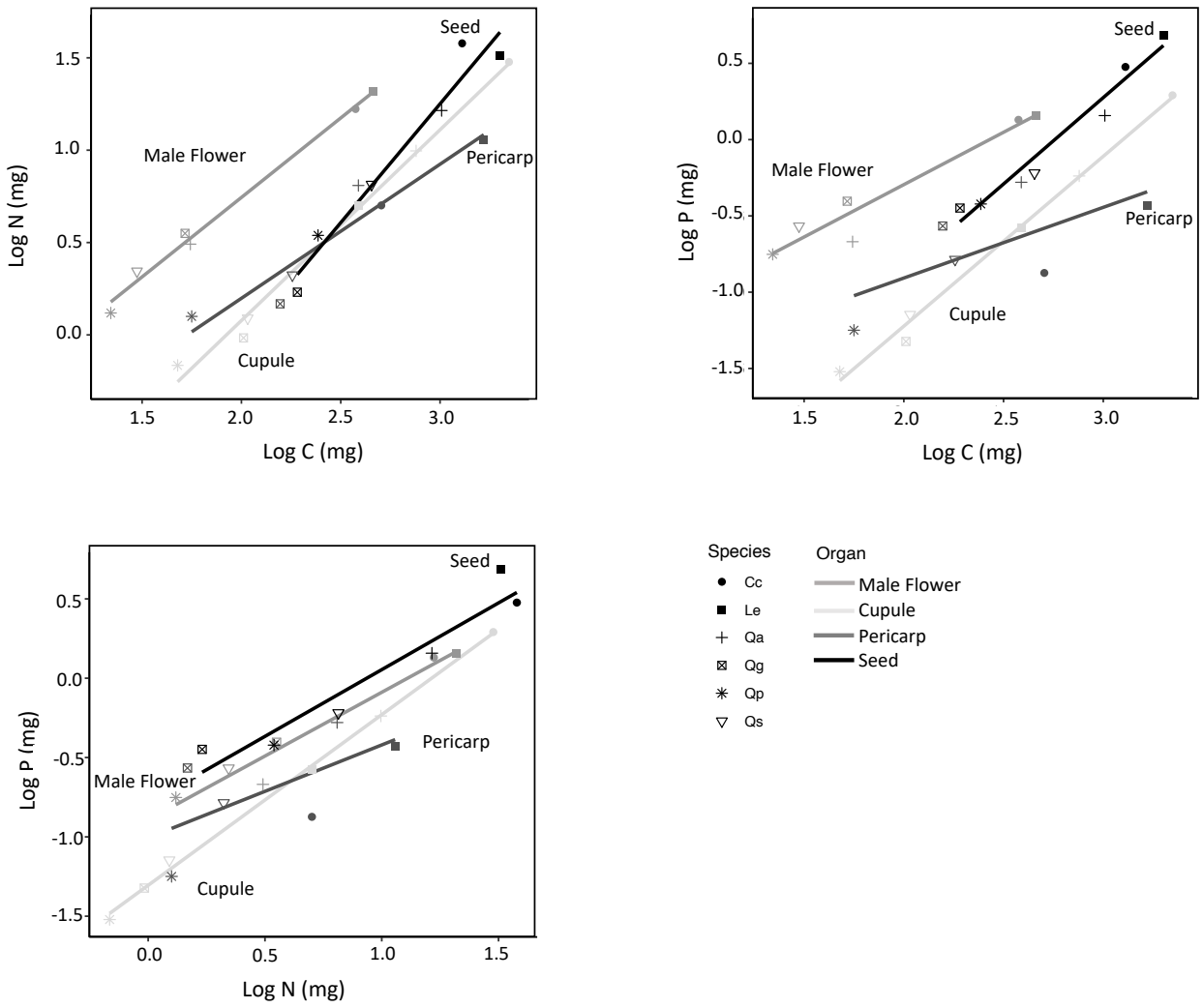

Figure S4. Bivariate standardized major axis (SMA) regression relationships among Log_{10} scaled C N P contents in four reproductive organ types among 6 Fagaceae species

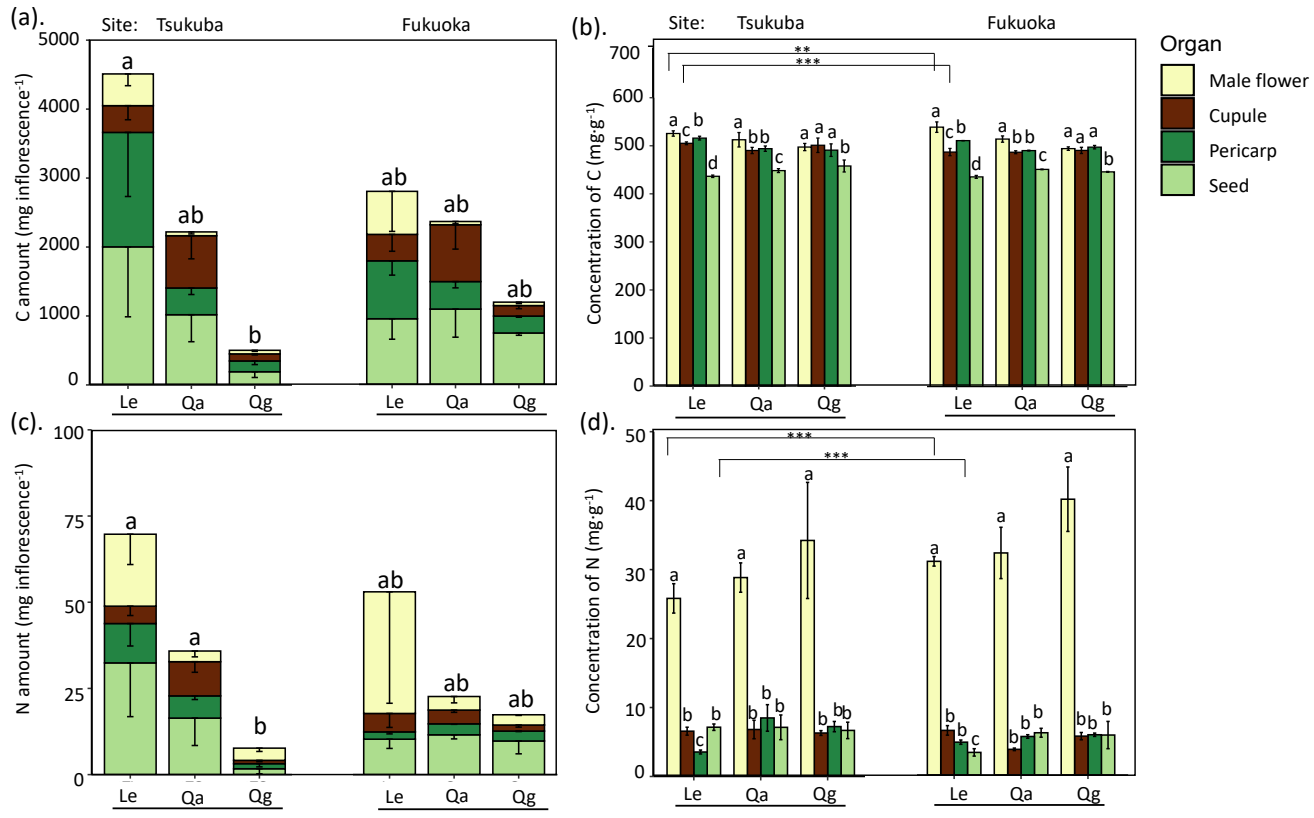

Figure S5. Reproductive costs and C, N, P resource concentrations in reproductive organs among 3 Fagaceae species in two regions. (a, c) Reproductive cost of C N P resource (mean ± SE) in different reproductive organs per one inflorescence (b, d,) Variations in element concentrations of C, N, P resource (mean ± SE) in reproductive organs. The different lowercase letters indicate significant differences among species at $p<0.05$. The black asterisks represent significant difference of same species at different region. ('**' represents statistically significant at 0.01 level, '***' represents statistically significant at 0.001 level, Tukey HSD test).

Table S1 Summary of standardized major axis (SMA) regression analysis for bivariate plots of C N P concentrations among reproductive organs (male inflorescence, cupule, pericarp, and seed) of different 6 Fagaceae species. (α_{SMA}=slope)

	Male inflorescence			
Trait	α_{SMA}	Intercept	R^2	*P*
C&N	-7.96	22.98	0.06	0.56
C&P	-17.66	48.21	0.17	0.41
N&P	2,22	-2.79	0.91	0.003**
	Cupule			
Trait	α_{SMA}	Inter-cept	R^2	*P*
C&N	-4.57	13.12	0.07	0.52
C&P	-7.70	20.37	0.12	0.50
N&P	1.69	-1.75	0.01	0.84

	Pericarp			
Trait	α_{SMA}	Inter-cept	R^2	*P*
C&N	-14.08	37.51	0.69	0.01*
C&P	-28.55	76.59	0.58	0.08
N&P	-2.04	2.03	0.68	0.04*
	Seed			
Trait	α_{SMA}	Inter-cept	R^2	*P*

C&N	-13.96	37.84	0.05	0.60
C&P	-10.38	27.42	0.22	0.35
N&P	-0.74	0.74	0.86	0.007**

Table S2 Data were provided by the Ministry of the Environment 2 Monitoring Sites 1000 Project: CV_p (Coefficient of Variation at the population level) data in seed crop size of Quercus glauca were caculated from 2005 to 2009.

Date	Species	number of individulas	Seed crop	Mean seed crop of every year
20051004	*Quercus glauca*	12	**0.91**	
20051004	*Quercus glauca*	22	**0.156**	
20051004	*Quercus glauca*	11	**0.4865**	
20051004	*Quercus glauca*	1	**0.108**	
20051107	*Quercus glauca*	2	**1.06**	
20051107	*Quercus glauca*	3	**0.45**	
20051205	*Quercus glauca*	4	**2.14**	
20051205	*Quercus glauca*	1	**0.6**	
20051205	*Quercus glauca*	1	**1.05**	
20051205	*Quercus glauca*	1	**0.35**	0.73105
20060407	*Quercus glauca*	1	**0.3582**	
20060904	*Quercus glauca*	4	**0.0207**	
20061107	*Quercus glauca*	1	**0.3721**	
20061107	*Quercus glauca*	25	**1.0356**	
20061107	*Quercus glauca*	1	**0.1316**	
20061204	*Quercus glauca*	14	**5.5017**	

20061204	*Quercus glauca*	6	**0.6092**	
20061204	*Quercus glauca*	1	**0.1188**	1.0184875
20070104	*Quercus glauca*	1	**0.0666**	
20070104	*Quercus glauca*	6	**0.0528**	
20070104	*Quercus glauca*	1	**0.0926**	
20070711	*Quercus glauca*	1	**0.2617**	
20070711	*Quercus glauca*	27	**0.6348**	
20070711	*Quercus glauca*	1	**0.024**	
20070711	*Quercus glauca*	150	**0.73**	
20070711	*Quercus glauca*	62	**0.501**	
20071112	*Quercus glauca*	2	**0.3482**	
20071112	*Quercus glauca*	34	**1.4372**	
20071112	*Quercus glauca*	1	**0.3514**	
20071210	*Quercus glauca*	1	**0.5037**	
20071210	*Quercus glauca*	2	**1.2374**	
20071210	*Quercus glauca*	4	**3.8363**	0.719835714
20080109	*Quercus glauca*	1	**0.7273**	
20080109	*Quercus glauca*	10	**0.9156**	
20080109	*Quercus glauca*	1	**0.6271**	
20080805	*Quercus glauca*	2	**0.0284**	
20081105	*Quercus glauca*	4	**0.9611**	
20081105	*Quercus glauca*	2	**0.096**	
20081105	*Quercus glauca*	1	**0.5377**	

20081105	*Quercus glauca*	3	**0.2256**	0.51485
20091014	*Quercus glauca*	1	**0.1646**	
20091014	*Quercus glauca*	1	**0.1501**	
20091014	*Quercus glauca*	1	**0.9688**	
20091106	*Quercus glauca*	7	**0.4289**	
20091203	*Quercus glauca*	8	**5.6882**	
20091203	*Quercus glauca*	4	**3.0893**	
20091203	*Quercus glauca*	1	**1.4789**	1.709828571

Year	**Seed Crop**
2005	0.73105
2006	1.0184875
2007	0.719835714
2008	0.51485
2009	1.709828571
CV_p of 5 years	0.497243624

Table S3 Comparison of reproductive traits in 7 Fagaceae species used for defense trait measurement

Species (Abbreviation)	Leaves	Pollination	Fruit Maturity	Individual; Study Sites
Lithocarpus edulis (Le)	Evergreen	Animal	Two years	6 individuals; Ito campus, Fukuoka Prefecture; 33˚35'47.5"N, 130˚ 12'50.0"E
Quercus acutissima (Qa)	Deciduous	Wind	Two years	5 individuals; Shiga, Ohtsu, Shiga Prefecture; 35°11.5'N 135°54.0' E, 235m asl.
Quercus aliena (Qal)	Deciduous	Wind	One year	5 individuals; Shiga, Ohtsu, Shiga Prefecture; 35°11.5'N 135°54.0' E, 235m asl.
Quercus glauca (Qg)	Evergreen	Wind	One year	5 individuals; Ito campus, Fukuoka Prefecture; 33˚35'47.5"N, 130˚ 12'50.0"E
Quercus gilva (Qgi)	Evergreen	Wind	One year	5 individuals; Hirao, Yamashiro, Kizugawa, Kyoto Prefecture; 34°46.5'N 135°49.0'E, 33m asl.
Quercus serrata (Qs)	Deciduous	Wind	One year	5 individuals; Arashima, Toba, Mie Prefecture"; 34°28.3'N 136°52.0' E, 60m asl.
Quercus salicina (Qsl)	Evergreen	Wind	Two years	5 individuals; Shiga, Ohtsu, Shiga Prefecture; 35°11.5'N 135°54.0' E, 235m asl.

Chapter 2 Different seasonal expression patterns of carbohydrate, nitrogen, and phosphorus transporter genes in two evergreen trees and their relationships with flowering phenology

The study in this chapter, done in collaboration with Prof. Akiko Satake (in preparation)

Abstract

A promising tool- genome-wide field transcriptome analysis is in a combination with relative gene expression analysis, by comparing and analysis the seasonal changes of carbohydrates, nitrogen and phosphorus transporter between leaves and buds of two evergreen masting species, to explore representative genes of these transporters between leaves and bud organs under the national environment and their relationships with leafing and flowering phenology of evergreen trees. How transporter genes are expressed in natural seasonal environments is rarely studied. We here investigate seasonal expression of transporter genes associated with nitrogen metabolism, sugar, and phosphorus transport about two years in natural conditions using two evergreen tree species, and which results showed transport of nitrate, amino acids and ammonium between leaves and buds was very frequent in spring and summer, while in winter related monosaccharide, nitrate, amino acid, and phosphorus transport were all more frequent and abundant than in other seasons. In these biological processes, we chose five candidate genes and a floral pathway integrator FT gene for the further verification of RT-qPCR experiment in leaves at different study sites underlying gene function. And we found that the upregulation of FT genes in leaves was more likely related to nitrogen status.

Introduction:

Carbon(C), nitrogen(N) and phosphorus(P) are the most three important elements used to build living beings. They are required to form carbohydrates, proteins, nucleic acids, ATP, and many other compounds (Ågren et al., 2012; Marschner, 2011). The energy source carbohydrate is produced by photosynthesis in green organs and supply fixed carbohydrate to growing sink organs (Remi Lemoine et al., 2013). And for N and P, they can be absorbed by the root of plant in the soil,

and then transported to stems, branches and leaves, and stored or consumed by physiological metabolism (Bieleski, 1973; Chapin III & Kedrowski, 1983; Wang et al., 2018). And for the storage sites, C, N, and P are mainly stored in the leaves in evergreen species, and for deciduous trees, they are mainly stored in stems, barks, and roots (Chapin III & Kedrowski, 1983).

Sucrose is the predominant form of long-distance sugar transport from sink organs to source organs, and these processes required for sugar signaling that not only controls the flow of sugars to developing organs, but also influences gene expression and hormone signaling throughout the plant (Remi Lemoine et al., 2013; Rolland & Sheen, 2005). And for sucrose, it can be long transported through phloem, and then unloaded to the sink organs via symplastic or apoplastic pathways (Figure 1; Lalonde et al., 2004; Milne et al., 2017). And sucrose also can be transported passively by sucrose transporters (SUTs or SUCs) and SWEET proteins (Griffiths et al., 2016; R Lemoine, 2000). Other hexose sugars produced in sucrose metabolism, such as glucose (Glc), and fructose (Fru) can be transported by hexose transporters (STPs) (Figure 1; Büttner, 2010).

Nutrient transport based on seasonal nutrient cycling is a complex physiological trait that requires communication and coordination between source and sink tissues (Babst & Coleman, 2018). In evergreen (*Quercus ilex* subsp. ballota) oak species, branches had larger N and carbohydrate stores at the beginning of the growing season and that remobilized more stores to supply the earlier stages of shoot growth, grew more in spring. And according to this previous study, nutrient concentrations were the highest in young expanding leaves and stems, then became subsequently decreased or retranslocated as organs matured. N and P concentrations increased also in older cohorts of stems and leaves prior to bud burst, indicating the sequential allocation of

nutrients from other parts of the plant to old leaves and stems and then to new shoots (Palacio et al., 2018).

For trees, N and P partitioning from source leaves to sinks take place in the phloem. Phloem loading takes place in the collection phloem of the leaf minor vein networks and phloem unloading happens in the release phloem of sink organs (Mimura et al., 1996; Tegeder, 2014), and which process also needs the plasma membrane-localized transport proteins. For inorganic and organic N transporters, they have been identified and named as Nitrate (NO_3^-) Transporter 1 (NRT1) or Nitrate Transporter1/Peptide Transporter Family (NPF) and Nitrate Transporter 2 (NRT2); Ammonium (NH_4^+) Transporters (AMT); Amino Acid (AA) Transporters (AAP), and Lysine Histidine Transporter (LHT) (Figure 1; Neuhäuser et al., 2009; Tegeder & Ward, 2012; Wang et al., 2018; Yuan et al., 2007). And for P transporters, they are mainly included Phosphate Transporter (PHT) Gene Family (Figure 1; Fabiańska et al., 2019; Nussaume et al., 2011; Sun et al., 2017).

And flowering time has emerged as a model trait for investigating the evolutionary genetics of developmental plasticity (Andrés & Coupland, 2012). Generally, in deciduous trees floral induction occurs during summer, and flower buds develop just after floral induction; during fall, short days induce growth cessation and bud set, after which the tree enters dormancy and bud growth restarts with the onset of spring (Fumie et al., 2009). For example, *Fagus crenata*, a deciduous species, which flower initiation occurs in the year prior to anthesis, and the timing of floral initiation is likely to be early summer. Floral and leaf primordia develop in a bud during summer and autumn. After winter dormancy, buds break and flowers bloom between mid-April and mid-May. Flowers are self-incompatible and wind pollinated. Fertilized flowers develop seeds

that mature during summer and autumn and fall to the ground from September to early November (Hashizume 1983； Kon et al. 2005; Miyazaki et al., 2014).

Nitrogen is known to modify flowering time (Miyazaki et al., 2014; Satake et al., 2019; Teng et al., 2019; Yan et al., 2020). In a typical masting tree, *Fagus crenate*, it was found that nitrogen fertilization can result in higher flowering gene expression (Miyazaki et al., 2014), and it has been found there is a significant causality from *FcNPF1.2* (Nitrate Transporter1/Peptide Transporter 1.2; belong to the NPF family) to the molecular marker gene (*FcFT*) of floral induction. And there are few reports on the direct effect of phosphorus on flowering time at present. In previous research, it was found that in olive trees if the nutritional status of phosphorus is above the sufficiency threshold phosphorus does not affect flowering (Jiménez-Moreno & Fernández-Escobar, 2017). Nonstructural carbohydrates (NSCs) level has also been proved as a key determinant of floral initiation (Han & Kabeya, 2017). Sucrose is sensed by the plant directly, through the generation of hexoses and through sugar signals such as T6P (trehalose- 6-phosphate) which relay the sugar status of the plant into mechanisms that enable adaptation to different environmental conditions. And T6P pathway has been proved to be interacted with flowering pathways (Li et al., 2018; Vandesteene et al., 2010). It has been found that TPS homeolog is required for the timely initiation of flowering and T6P pathway affects flowering both in the leaves and at the shoot meristem (Wahl et al., 2013). The target of rapamycin (TOR), a sugar and nutrient sensor, has also been found that in *A. thaliana*, flower development was addressed only by Anderson et al. (2005), who described the raptor3g mutant as late flowering and sterile (Schepetilnikov & Ryabova, 2018). The conserved TOR (Target of Rapamycin) can be found in plants, which is central and crucial component regulating the perception and the responses to nutrients (sugars and amino acids) and energy levels in yeast and animal cells. AtTOR activity is

important throughout the entire plant life cycle and is mainly expressed in rapidly proliferating tissues such as meristematic regions and endosperm (Menand et al., 2002), suggesting it to be a central stimulator of growth and development. In previous studies on RAPTOR function in *A. thaliana*, flower development was addressed only by Anderson et al. (2005), who described the raptor3g mutant as late flowering and sterile. Further insights might be provided by studies on VPS34 and PTEN function in *A. thaliana*, as homologs of these proteins are linked to the regulation of TOR signaling in other species.

According to the previous studies, we raised questions: how nitrogen(N), carbohydrate(C) and phosphorus (P) transporter genes are expressed in source leaves and sink organs in natural seasonal environments? In *Fagus crenate*, nitrogen transporters were causally related to important flowering gene. Is there any causal relationship in other Fagaceae species, and is there any relationship between carbohydrate or phosphorus transporters and important flowering gene? To solve these questions, field transcriptome analysis and target gene expression level analysis were conducted to investigate seasonal expression of transporter genes associated with nitrogen, sugar, and phosphorus transport and their potential relationships with flowering phenology in two evergreen species *Quercus glauca* and *Lithercarpus edulis*.

Materials and Methods

Plant Material, study species and study site.

We collect plant materials from two evergreen species. *Quercus glauca*, is a ring-cupped evergreen oak, which is distributed in subtropic East Asia (Xu et al., 2014), and its new leaves expand in April or May. The mean longevity of the leaves is about 22 months (Ye et al., 2022). *Lithocarpus edulis*, an evergreen broad-leaved tree, which is widely allocated in the Kyushu area of Japan, and

the new leaves expand in May. The mean longevity of the leaves of *Lithocarpus edulis* is little longer than leaves in *Quercus glauca* (Hirose et al., 2005).

The study sites were Tsukuba (an arboretum of the Forestry and Forest Products Research Institute, 36˚0'31"N, 140˚7'53"E,) and Fukuoka (the biodiversity reserve on Ito campus of Kyushu University; 33˚35'47.5"N, 130˚ 12'50.0"E). The elevation of the biodiversity reserve of Ito campus and the arboretum of the Forestry and Forest Products Research Institute are from 20 to 57 m a.s.l and 24 m a.s.l respectively. Records from nearby meteorological stations (Japan Meteorological Agency) indicate that during 1976–2018, the mean annual precipitation and temperature were 1291 mm and 13.9 °C in Tsukuba, 1727 mm and 16.1 °C in Fukuoka.

A pair of a leaf and a bud were collected from different three current-year shoots per individual every month from April 2017 to March 2019. Samples were cut from the sun- exposed crown by using handle pruning shears from 11:30 to 12:30 AM in each sample day. Leaves samples were cut into squares containing main veins and big bud tissues were cut along the axis of symmetry immediately after harvest, and around 0.1-0.3g fresh weight of them were kept in a 2ml micro tube containing 1.5ml of RNA stabilizing reagent (RNAlater; Ambion, Austin, TX, USA) immediately after cutting. Samples were taken to the laboratory within 3hr after sampling and stored at 4°C overnight and then stored at −80°C until RNA extraction.

RNA Extraction

The extraction of total RNA for RNA sequencing and Real-time Quantitative PCR Detecting System (qRT-PCR) was performed with the modified method separately according to the previous study (Miyazaki et al., 2014; Satake et al., 2019). RNA was extracted from leaf and bud tissues separately from three different 0-year branches and mixed at each time point.

The modified SDS-LiCl method of gel electrophoresis, a nanodrop spectrophotometer, and Agilent RNA 6000 Nano kit on a 2100 Bioanalyzer (Agilent Technologies) were used to detect the RNA quality, concentration, and integrity.

RNA sequencing

We obtained transcriptome data from our samples to design DNA microarray probes. Transcriptome sequencing was conducted using the Illumina Hiseq2000 sequencer (Illumina, San Diego, CA, USA) for each sample. Illumina sequence adapters were removed from raw read sequences using cutadapt (Ver. 1.1). Low-quality bases (Q < 20) were trimmed from the tail of each read with Trimmomatic (Ver. 0.32). The resulting reads shorter than 50 bp were discarded. "De novo transcriptome assembly" was conducted using Trinity (Ver. 2.0.6). A total of 299 and 313 million 100-bp paired-end reads were obtained for each species. The resulting reads shorter than 50 bp were discarded. De novo transcriptome assembly was conducted using Trinity (Ver. 2.0.6). Read quality analysis was performed on the raw data using FastQC v0.11.7 (http://bioinformatics.babraham.ac.uk/projects/fastqc/). Quality trimming and adapter clipping were performed using Trimmomatic version 0.38 (Bolger, Lohse & Usadel, 2014), trimming trailing bases below the average quality 15, minimum length 36 and clipping Illumina adapters. The resulting reads shorter than 50 bp were discarded. De novo transcriptome assembly was conducted using Trinity (Ver. 2.0.6).

Probe design for DNA microarray

For custom microarray slides, we used the assembled sequences of the transcripts generated by NGS described above. We selected the assembled sequences for array design based on two steps.

We first extracted transcript sequences that showed high homology against Arabidopsis thaliana (%Identity >= 40%, qcovhsp >= 40%) by BLASTX searches for each species. For each extracted transcript sequence, top hit A. thaliana gene ID was selected. If multiple transcript sequences were annotated for the same *A. thaliana* gene ID, the transcript sequence showing the longest annotation was selected. As a result, we obtained 19,290 and 19,426 transcript sequences for *Q. glauca* and *L. edulis*, respectively. At the second step, we extracted transcript sequences that were eliminated from the homology selection but sequence homology to F. crenata transcript sequences used for DNA microarray (Satake et al. 2019) is high (%Identity >= 60%, qcovhsp >= 60%, e-value cut-off: 10−5) by BLASTX searches for each species. From the selection of step 2, we obtained 3,474 and 4,357 transcript sequences for *Q.glauca and L. edulis*, respectively. We pooled these transcript sequences for each species and designed the array using the e-array portal for array design hosted by Agilent (https://earray.chem.agilent.com/earray/) based on the total of 22,765 and 23,784 transcript sequences for *Q. glauca* and *L. edulis*, respectively. Two probes were designed for each transcript sequences. After removing probes with the same sequence, 42,121 and 42,436 probes were installed in the 8×60K array format.

Microarray analysis

One hundred nanograms of total RNA extracted from leaf and bud of each sample was amplified, labeled, and hybridized to a 60K Agilent 60-mer oligo microarray, in accordance with the manufacturer's instructions, for each sample for each time point based on the one-color method. Hybridized microarray slides were scanned by an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1). Among two probes designed for each transcript sequences, we selected the

probe with larger median. We also removed probes with low signal and low correlation between individuals using following three criteria: (1) no signal over all time points, (2) mean signal value over all time points is lower than 0.05, (3) mean of correlation between each pair of individuals is smaller than 0.2. Finally, we obtained time-series data of 15,451 and 15,182 independent probes for Q. glauca and L. edulis, respectively.

Prediction of orthologous genes

To identify orthologous genes across *Q. glauca* and *L. edulis*, we first used TransDecoder (http://transdecoder.sourceforge.net/) for detecting coding regions from the assembled contigs. To maximize sensitivity for capturing coding regions with functional significance, we scanned all coding regions detected by TransDecoder for the blastp or pfam searches. We used protein sequence database of green plants (Viridiplantae) for the homology searches with E-value < 1E-5. Among the assembled contigs of Q. glauca and L. edulis, TransDecorder identified 101,371 and 86,128 contigs containing candidate coding regions with homology to known proteins. The longest predicted protein sequences of candidate coding regions were used for subsequent analysis. The construction of groups of orthologous genes (orthogroups, referred to here as gene families including ortholog pair) was performed for 5 plant species: Q. glauca, L. edulis, two other oak species, Fagus crenata (75,926 sequences) and Quercus robur (25808 sequences from OAK GENOME SEQUENCING http://www.oakgenome.fr), and Arabidopsis thaliana (48,359 sequences from TAIR https://www.arabidopsis.org). The prediction of orthogroups was based on a blastp all-against-all comparison of the protein sequences (E-value < 10−5) of these species, followed by clustering with Ortholog-Finder (Horiike et al., 2016) using default parameters. We obtained 32,149 orthogroups in total. Next, we picked up pairs of orthologous microarray probe

for *Q. glauca* and *L. edulis* based on the predicted orthogroups. We considered a pair of the probes of which sequences belongs to an identical orthogroup to be ortholog gene. Some probes could not make orthologous pair because those belong to an orthogroup which lacks either of two species (*Q. glauca* and *L. edulis*). The probes which have multiple partners were excluded from the following analyses, because we could not conclusively identify the best orthologous pair among them. Sequences of such probes generally belong to a large orthogroup. We also excluded orthologous pairs of probes of which sequences belong to an orthogroup lacking *A. thaliana*, because we could not reliably assign their function.

Finally, 9,258 pairs of the probes were obtained which are predicted to be ortholog genes. GO terms of predicted proteins (orthogroups) were retrieved from annotation data of *A. thaliana*.

RT-qPCR analysis

Leaf samples collected during Aug–Sep in 2017–2018 at the two study sites were used for RT-qPCR analysis. RNA extraction and synthesis of cDNA were performed according to the method described by Miyazaki et al. (2014). Bio-Rad CFX connect real-time PCR detection system (CRX96 Touch) with SsoFast EvaGreen super mix was used. Primers used for RT-qPCR are listed in Table 2.

Seasonal expression analysis

Six clusters that we could assign as season specific for leaf and bud tissues and we designated as Spring, Summer, Autumn, and Winter. The season specificity of every gene was calculated using the season specificity score, which ranged from 0 to 1, indicating ubiquitous to specific expression, respectively ((Jokipii-Lukkari et al., 2018; Yanai et al., 2005).

The method is to calculate the mean expression value of one gene in one season, and then set the most expression value of season to 1, and then normalized other season's score from 0 to 1 as xi by the maximal component value, then use the equation in the follows. N is the number of Seasons (Supplemental Table S2). The index τ is defined as:

$$\tau = \frac{\sum_{i=1}^{N} (1 - x_i)}{N - 1}$$

rEDM Empirical Dynamic Model (EDM) by using convergent cross mapping (CCM) test

We explore the casual relationship between candidate genes and FT genes by using convergent cross mapping (CCM) test. CCM, seasonal-surrogate time series generation, and the best E value determination. (i) we determined the optimal embedding dimension using the time-series of the target species; (ii) tested its non-linearity; and (iii) u causality analyses in the direction of the target gene cross-mapping sugar or nitrogen signals. And the condition of causality happened: (i) the 95% lower confidence limit of the difference between ρ max and ρ min (ρmax – ρmin) is greater than zero; and (ii) the mean of ρ max is larger than the 95% upper confidence limit of ρsurr.

Statistical analysis

To perform all statistical analyses, we used R ver. 3.4.1 (the R project, http://www.r-project.org/).

Results

1. **Field transcriptome analysis of the seasonal changes of sugar, nitrogen and phosphorus transport genes in leaves and buds in two evergreen tree species**

In total, transcriptomes of 24 leaf and bud samples were obtained by RNA sequencing (RNA-Seq). We here investigate seasonal expression of transporter genes associated with nitrogen, sugar and phosphorus transport in leaves and buds about two years in natural conditions using two evergreen tree species, *Q. glauca and L. edulis*. We detected 28 nitrate, ammonium, and amino acid transporter genes, 13 genes related to sugar transporter and sugar sensor, and 7 phosphors transporter genes in *Q. glauca* and *L. edulis*.

We got 6 hierarchical cluster groups shown in the heatmap, and which revealed clear seasonal patterns. Moreover, different cluster groups showed similar or different seasonal expression between the two species (Figure 2). Then, we calculated season-specific scores for each gene in each cluster group in both species to assess whether it had significant seasonal expression.

Genes were highly expressed in spring or summer were classified into cluster 1. In this cluster, except for TPPA gene, which is related to sugar signaling, other genes belonging to this cluster are N-related genes (Table 1), such as genes encoded ammonium transporter-*AMT1.1*, amino acids transporters-*LHT9* and *AAP6*, hormone ABA and GA transporters-*NPF4.6* and *NPF3.1*. Moreover, the expression patterns of the genes in cluster 1 were very similar between *Q. glauca* and *L. edulis*, and the correlation coefficients of which were ranged from 0.38 to 0.85 (Table 1).

The mean expression of all genes in cluster 2 did not show single seasonal expression (Fig. 2, b), but in winter, the genes expression of this cluster showed difference in two species. And according to the Season-specificity-score, the representative genes *AAP3*, *SWEET3* and *SWEET1* showed winter-specific expression in *Q. glauca*, while in *L. edulis*, *SWEET3* showed summer-specific expression, and *SWEET1* showed spring-specific expression.

Cluster3 contained only three genes whose gene expression levels were higher in spring and winter than in other seasons.

The gene expression patterns of the cluster 4 containing 7 genes were slightly different from those of the cluster 3, and their gene expression levels in spring and autumn were higher than those in other seasons. In this cluster, it was worth noting that the genes encoding nitrate transport proteins *NPF6.3* and *NPF5.1*. And *NPF5.1* in *Q. glauca* showed spring-specific expression, while spring was the season with the lowest expression in *L. edulis*. While *NPF6.3* showed spring-specific expression in *Q. glauca* and autumn-specific expression in *L. edulis*.

The difference between the cluster 5 and the cluster 6 was mainly manifested in that the genes contained in the cluster 5 showed a higher expression level in winter in *Q. glauca*, which pattern was the same as the genes in the cluster 6 of both species. In *L. edulis*, the expression level was higher in spring or autumn than in other seasons. Interestingly, among the 17 genes included in the sixth cluster, the *SUC4* gene encoding the sucrose transporter, the *STP13* and *STP7* genes encoding the monosaccharide transporter, the *PROT1*, *LHT1* and *ANT1* genes encoding the amino acid transporter, and the *PHT1;9* gene encoding phosphate transporter were expressed specifically in winter in one or two species.

2. Expression levels of important genes in leaf tissues among different study sites by using RT-qPCR method.

To better understanding the relationships of available carbohydrates or nutrient transport with leafing or flowering induction event, we performed RT-qPCR to examine the level of FT, and candidate genes expression in leaves throughout the year (UBQ10 was used as a reference gene). For candidate genes, we combined the season-specify-score in both species and the related gene

functions, two nitrate transporter gene *NPF1.2*&*NPF7.3*, two sugar and nutrient sensor *TOR*&*TPS7*, and one phosphate *PHT4;1* were selected.

As shown in the figure, the lowest expression level *QgNPF1.2* and *LeNPF1.2* were shown in April or May (Figure 3). For *QgNPF1.2*, the lowest expression of *QgNPF1.2* in Tsu and Kyu sites appeared in May. The leaves collected in these months were all just germinated young leaves. This result indicated that the expression level of *NPF1.2* was very low in germinating young leaves, and indirectly proved the function of this gene to transfer nitrate from source organs to sink organs. The lowest expression level of *LeNPF1.2* was in May. The highest expression level was found in leaves in January or August in *Q. glauca*, and in leaves in September, March (April), and July in *L. edulis*, indicating that the transport of nitrate was very abundant among these months.

For *Quercus glauca*, the change of *FT* expression in Tsukuba of all individuals was not obvious as the expression level of FT gene in Kyushu, and in which the expression showed obvious peaks in Winter and June. For *Lithercarpus edulis*, there were only one peak in March for all individuals at two study sites (Figure 4). The expression of TOR gene level in Tsukuba was also not obvious as which in Kyushu site. But it could be seen that the trend of *TOR* expression of two species at two sites are similar with the level change of FT gene.

3. Results of CCM analysis

And to futher explore the causal relationships among FT and candidate genes, the CCM method was used to test their relationships. The direction of arrow means cause. According to the results, expression of *NPF1.2* and *TOR* can cause the *FT* gene expression. And *TOR* as a sugar and nutrient sensor, it also has potential relationship with *NPF1.2*.

Discussion

Frequent transport of nitrate, amino acids and ammonium between leaves and buds in spring and summer

Spring involves the process of flowering and leafing, and young leaves grow vigorously. In spring and summer, the buds that have just spread their leaves gradually develop into mature leaves, and the flower organs gradually form. In *Q. glauca*, the emergence of new shoots and shedding of old leaves occur almost synchronously in spring (there is no detectable leaf shedding in summer) (Miyazawa et al., 2004). The leafing flush happened a little earlier in *Q. glauca* than that in *L. edulis*. The timelines of the occurrence of new leaves for these two species matched the up regulation of gene expression in cluster 1 of the heatmap (Figure 2). The genes encoding the transporters of ammonium, amino acids and nitrates were highly expressed in these two seasons. Leaves are generally considered to be the storage sites for nitrogen resources in the above-ground parts of evergreen trees (Chapin III & Kedrowski, 1983), and nitrogen is usually existed in the forms of nitrate, ammonium, amino acids, urea and protein (Tegeder & Masclaux-Daubresse, 2018; Y. Y. Wang et al., 2018), and stored in the form of protein with minor free amino acid (Li et al., 2019). In our study, the ammonium transporter *AMT1.1* were highly expressed in spring and summer, which indicates the transport of ammonium was very abundant and frequent in the two season. It has been proved that ammonium concentration in young leaves was significantly higher than that in mature leaves due to amino acid catabolism and photorespiration cycles (Masclaux et al., 2000).

We also found that the gene encoding the transport of ABA exhibited summer-specific expression in both species, which was related to the biological process response to high temperature (Kuromori et al., 2018). Exposure to high temperatures is often accompanied by water

stress, so leaf stomata act as valves to close under high temperature conditions to control water loss and carbon gain in plants. In this process, ABA is a key signal regulating stomatal movement (Wu et al., 2018; Zhou et al., 2010).

Related monosaccharide, nitrate, amino acid, and phosphorus transport were more frequent and abundant in Winter than in other seasons

Winter is mainly involved in the physiological process preparation for bud swelling, and the growth of bud was arrested (Sobral et al., 2020), with chilling damage because of low air temperature (Supplementary Table S3). Genes encoding the transporter of sucrose, monosaccharides, amino acids, and phosphoric acid all show abundant expression in leaf and bud during this season, however, the transport genes of inorganic nitrogen were not involved. Soluble sugars accumulated in the leaves of both species during winter (Han, unpublished data). Soluble sugars act as osmoprotectants and nutrients, and which can act upon with lipid bilayers to guard plant cells from cold stress (Tarkowski & Ende, 2015), phosphorus is required for lipid bilayers (Nunes-Nesi et al., 2010). And in our heatmap, the expression of winter 2017 genes in cluster 6 was significantly stronger than that of winter 2018, because the temperature in winter of 2017 was lower than the temperature of winter in 2018 (Supplementary Table 3). In other seasons, most stored carbohydrate and phosphorus can be consumed in situ, so transport between source and sink organs is not as frequent and abundant as in winter.

Upregulation of FT genes in leaves was likely related to upregulated of *TOR* and *NPF1.2* expression

FLOWERING LOCUS T(FT) gene is produced in annual or perennial plants as an integrator of the flowering pathway, which can induce flowering, or participate in some other developmental process, such as juvenile-to-adult transformation, control of plant structure, and fruit setting (Satake 2019; Sobral 2020). In the deciduous tree *Fagus crenate*, it has been found that the expression of *FcFT* in leaves showed peak around two weeks before the visible morphological changes of flowering in the buds in late July (Satake et al., 2019). While in *Q. glauca*, the expression of *FT* in leaves showed three peaks in Winter, June and August. But in *L. edulis*, the expression of *FT* in leaves only showed a peak in Winter. In *Q. suber*, it has been found that the *FT* gene expression showed two peaks in leaves throughout the year because of the separation of male and female flower organ development. And they concluded that the female flowers were most likely in spring, when the vegetative flush occurred, and male flowers may be induced in early summer (Sobral et al., 2020). Therefore, we speculated that in *Q. glauca*, the appearance of multiple peaks may be related to the induction of flowers of different sexes, and this speculation needs further research to confirm. And according our CCM results, the *FT* gene are related to the *TOR* and *NPF1.2* expression in two species.

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Tables

Table 1 transport and signal sensor genes list detected in the transcriptome data

	Gene name	Sub-strate	Refs	Related resource	Season-specificity-score in Qg	Confi-dence in Qg	Season-specificity-score in Le	Confi-dence in Le	Correlation coefficient
Cluster1	*NPF3.1*	GA, nitrate	(David et al., 2016)	N	0.16	Low	0.30	Medium	0.61
	NPF7.3	nitrate	(Meng et al., 2016)	N	0.50	Medium	0.72	High	0.45
	LHT9	amino acids	(Tegeder & Ward, 2012)	N	0.65	High	0.50	Medium	0.50
	AMT1.1	ammonium	(Yuan et al., 2007)	N	0.44	Medium	0.55	High	0.85
	NPF4.6	ABA	(Kanno et al., 2012)	N	0.69	High	0.77	High	0.38
	TPPA	T6P	(Vogel et al., 1998)	C	0.47	Medium	0.47	Medium	0.68
	APP6	amino acids	(Tegeder & Ward, 2012)	N	0.39	Medium	0.39	Medium	0.60
Cluster2	*AAP3*	amino acids	(Tegeder & Ward, 2012)	N	0.58	High	0.36	Medium	-0.41

	NPF7.1	Unknown	Unkonwn	N	0.26	Medium	0.13	Low	-0.44
	NPF1.2	nitrate	(Hsu & Tsay, 2013)	N	0.30	Medium	0.40	Medium	-0.15
	PHT2;1	phosphate	(Okumura et al., 1998)	P	0.09	Low	0.13	Low	0.32
	SWEET3	2-Deoxyglucose	(Xuan et al., 2013)	C	0.55	High	0.77	High	-0.48
	AT4G38250	amino acids , Auxin	(Fujiki et al., 2017)	N	0.23	Medium	0.33	Medium	-0.24
	SWEET1	Glucose, galactose	(Xuan et al., 2013)	C	0.58	High	0.50	Medium	-0.25
	NPF2.13	nitrate	(Fan et al., 2009)	N	0.37	Medium	0.421	Medium	-0.19
Cluster3	*NRT2.7*	nitrate	(Okamoto et al., 2003)	N	0.34	Medium	0.08	Low	-0.20
	PHT4;2	phosphate	(Irigoyen et al., 2011)	P	0.11	Low	0.11	Low	-0.11
	SUC3	sucrose	(Lalonde et al., 2004)	C	0.21	Medium	0.26	Medium	0.36

Cluster4	*HXK3*	glucose and G6P	Wyatt E，2010	C	0.48	Medium	0.48	Medium	-0.10
	Close ANT1	amino acids	Sugiyama et al. (2017)	N	0.20	Medium	0.24	Medium	0.51
	LHT4	amino acids	(Yao et al., 2020)	N	0.45	Medium	0.34	Medium	0.11
	NPF6.1	nitrate	Chen et al., 2010)	N	0.21	Medium	0.26	Medium	0.05
	SWEET 2	monosaccharide	(Jia et al., 2017)	C	0.43	Medium	0.20	Low	-0.10
	NPF6.3	nitrate	(Y. Y. Wang et al., 2018)	N	0.49	Medium	0.86	High	0.07
	NPF5.1	nitrate	(Y. Y. Wang et al., 2018)	N	0.61	High	0.30	Medium	0.81
Cluster5	*NPF8.1*	nitrate	(Y. Y. Wang et al., 2018)	N	0.64	High	0.64	High	-0.38
	PHT4; 5	phosphate	(Guo et al., 2008)	P	0.23	Medium	0.77	High	-0.37
	NPF8.3	nitrate	(Y. Y. Wang et al., 2018)	N	0.34	Medium	0.28	Medium	-0.47

	AMT2	ammonium	(Neuhäuser et al., 2009)	N	0.31	Medium	0.54	High	0.56
	AAP7	amino acids	(Yao et al., 2020)	N	0.12	Low	0.66	High	-0.21
	PHT4;3	phosphate	(Guo et al., 2008)	P	0.10	Low	0.59	High	-0.11
Cluster6	*STP14*	Galactose	(Büttner, 2010)	C	0.16	Low	0.32	Medium	0.56
	TPS7	T6P synthetase	(Vogel et al., 2001)	C	0.25	Medium	0.35	Medium	0.72
	SUC4	sucrose	(Lalonde et al., 2004)	C	0.87	High	0.12	Low	0.38
	PHT3;3	*phosphate*		P	0.75	High	0.75	High	0.96
	PROT1	gly betaine, proline and GABA	(Yao et al., 2020)	N	0.77	High	0.89	High	0.90
	STP13	glucose	(Büttner, 2010)	C	0.86	High	0.92	High	0.76

	NPF5.2	nitrate	(Y. Y. Wang et al., 2018)	N	0.40	Medium	0.42	Medium	0.76
	STP3	hexose	(Büttner, 2010)	C	0.22	Medium	0.37	Medium	0.70
	LHT1	amino acids	(Tegeder & Ward, 2012)	N	0.58	High	0.61	High	0.42
	ANT1	aromatic and neutral amino acids, IAA, and 2,4-D	(Yao et al., 2020)	N	0.37	Medium	0.52	High	0.43
	NPF2.11	nitrate	(Y. Y. Wang et al., 2018)	N	0.37	Medium	0.48	Medium	0.39
	PHT1;9	phosphate		P	0.53	Medium	0.68	High	0.79
	PHT4;1	phosphate	(Guo et al., 2008)	P	0.34	Medium	0.52	High	0.74
	STP7	pentoses l-arabinose	(Büttner, 2010)	C	0.89	High	0.68	High	0.75

		and d-xylose							
	NPF5.10	nitrate	(Y. Y. Wang et al., 2018)	N	0.53	Medium	0.45	Medium	0.81
	TOR		(Schepetilnikov & Ryabova, 2018)	C	0.28	Medium	0.29	Medium	0.71
	ATAVT3B	amino acids		N	0.14	Low	0.28	Medium	0.21

Table 2 Primers list using in the qRT-PCR experiment.

Species	Gene Name	Direction	Sequence (5' to 3')	Product size
Q. glauce	QgUBQ10	Forward	CTGGTAAGCAGTTGGAGGATGG	82
Q. glauce	QgUBQ10	Reverse	AGGCGAAGGACAAGGTGAAGAG	
L. edulis	LeUBQ10	Forward	GTCAAGGCGAAGATACAAGACA	161
L. edulis	LeUBQ10	Reverse	ATCTGCATTCCACCACGAAG	
Q. glauce	QgNPF1.2	Forward	CCGCCCAATTTAGCAGCAAT	85
Q. glauce	QgNPF1.2	Reverse	CCAAAGGACATGAGCCCGAA	
L. edulis	LeNPF1.2	Forward	AAACCATGCCGTCTCAACGA	108
L. edulis	LeNPF1.2	Reverse	TGCAAGTTCCCGCCTAACAC	
Q. glauce	QgNPF7.3	Forward	CATGTGATGGGTCAAGCTCCT	119
Q. glauce	QgNPF7.3	Reverse	TGGTGTCTGTGCGTTGAAGA	
L. edulis	LeNPF7.3	Forward	TGGAGCAAGGTGCTGTAATG	121
L. edulis	LeNPF7.3	Reverse	TCAAGAATTCGCCGGTAAAG	
Q. glauce	QgTOR	Forward	AGCCCTAGTGCAACTTGCTT	123
Q. glauce	QgTOR	Reverse	TTTCGTGTGGCTCCATCCTC	
L. edulis	LeTOR	Forward	CACCGCTCTAGTGGCAAGAT	156
L. edulis	LeTOR	Reverse	TGAACGGAAGTTGCCCTCAA	
Q. glauce	QgTPS7	Forward	GACCGATCTGACGAGGACAT	106
Q. glauce	QgTPS7-	Reverse	TTGGCTTCTGTCCAACAGTG	
L. edulis	LeTPS7	Forward	TGCTGAGTGTGTTGTGGTCA	163
L. edulis	LeTPS7	Reverse	AGGCGAACACCCAATGAACT	

Q. glauce	QgPHT4;1	Forward	ATGGGCAGACACAGTAGGTG	125
Q. glauce	QgPHT4;1	Reverse	AAGCGCGAACAACAAGTAGG	
L. edulis	LePHT4;1	Forward	ATGGGCAGACACAGTAGGTG	
L. edulis	LePHT4;1	Reverse	AAGCGCGAACAACAAGTAGG	
Q. glauce	QgFT	Forward	GGGAGGTCAATAATGGTTGTGAGC	138
Q. glauce	QgFT	Reverse	TGGGATCACTTGGACTTGGTGC	
L. edulis	LeFT	Forward	GGGAGGTCAATAATGGTTGTGAGC	138
L. edulis	LeFT	Reverse	TGGGATCACTTGGACTTGGTGC	

Table 3 CCM result in *Q. glauca*.

Gene name(lib)	Gene name(Tar) Trigger	Best em-bedding dimension (*E*)	Best non-linear parameter (q)	Causality	Mean of ρ_{max}	95% lower limit of ρ_{max-} ρ_{min}	95% upper limit of ρ_{surr}
FT	NPF1.2	2	0	+	0.3196729	0.1836526	0.2771349
NPF1.2	FT	2	0	+	0.1282746	0.07094274	0.1781161
FT	NPF7.3	2	4	-	0.06865353	0.04095621	0.1522108
NPF7.3	FT	2	0.75	-	0.1244139	0.1222789	0.1545625
FT	TOR	2	0.1	+	0.2783545	0.188465195	0.1746206
TOR	FT	2	1	-	-0.2441088	-0.1796721	0.1558962
FT	TPS7	2	4	-	0.124709	0.1293477	0.2950811
TPS7	FT	2	2	-	0.1940896	0.225321	0.2928576
FT	PHT4;1	2	0.1	-	0.03227643	0.2783051	0.1479348
PHT4;1	FT	2	0	-	-0.03011672	0.3282227	0.1496692
NPF1.2	TOR	2	0	+	0.3391999	0.2556327	0.272315
TOR	NPF1.2	2	4	-	-0.2712575	-0.1262151424	0.2746364
TOR	TPS7	2			-0.2847284	-0.156142	0.2064056
TPS7	TOR	2			-0.4671966	-0.3176305	0.216951
NPF7.3	TPS7	2	0		0.7672971	0.7598063	0.3855313
TPS7	NPF7.3	2	0		0.8273481	0.764427	0.3839933

Table 4 CCM results in *L edulis.*

Gene name	Gene name (Target) Trigger	Best em-bedding dimension (*E*)	Best non-linear parameter (q)	Causality	Mean of ρ_{max}	95% lower limit of $\rho_{max-\rho_{min}}$	95% upper limit of ρ_{surr}
FT	NPF1.2	2	0	+	0.4432116	0.3623041	0.2636154
NPF1.2	FT	2	8	+	0.2645872	0.2484150765	0.2296169
FT	NPF7.3	2	1.5	-	0.09752866	0.09841864	0.2145918
NPF7.3	FT	2	4	-	0.07978927	0.07188792	0.178327
FT	TOR	2	1.5	+	0.5649234	0.465702926	0.2181263
TOR	FT	2	0	+	0.4485371	0.3717698	0.2291764
FT	TPS7	2	0.1	+	0.4154143	0.3657991	0.2152942
TPS7	FT	2	0	-	0.2893632	0.256249177	0.2918412
FT	PHT4;1	2	1.5	+	0.3319984	0.2322424	0.3207063
PHT4;1	FT	2	8	-	0.3463823	0.3069696	0.3895942
NPF1.2	TOR	2	0		-0.2210552	-0.126058	0.2737315
TOR	NPF1.2	2	0.75		0.4660415	0.3462392	0.1898676
TOR	TPS7	2	0		-0.2847284	-0.1521117	0.2811308
TPS7	TOR	2	0		-0.4671966	-0.339786	0.3002785
NPF7.3	TPS7	2	8		-0.1121449	-0.05579062	0.2186945
TPS7	NPF7.3	2	0		0.1451041	0.1255365	0.2233771

Figures

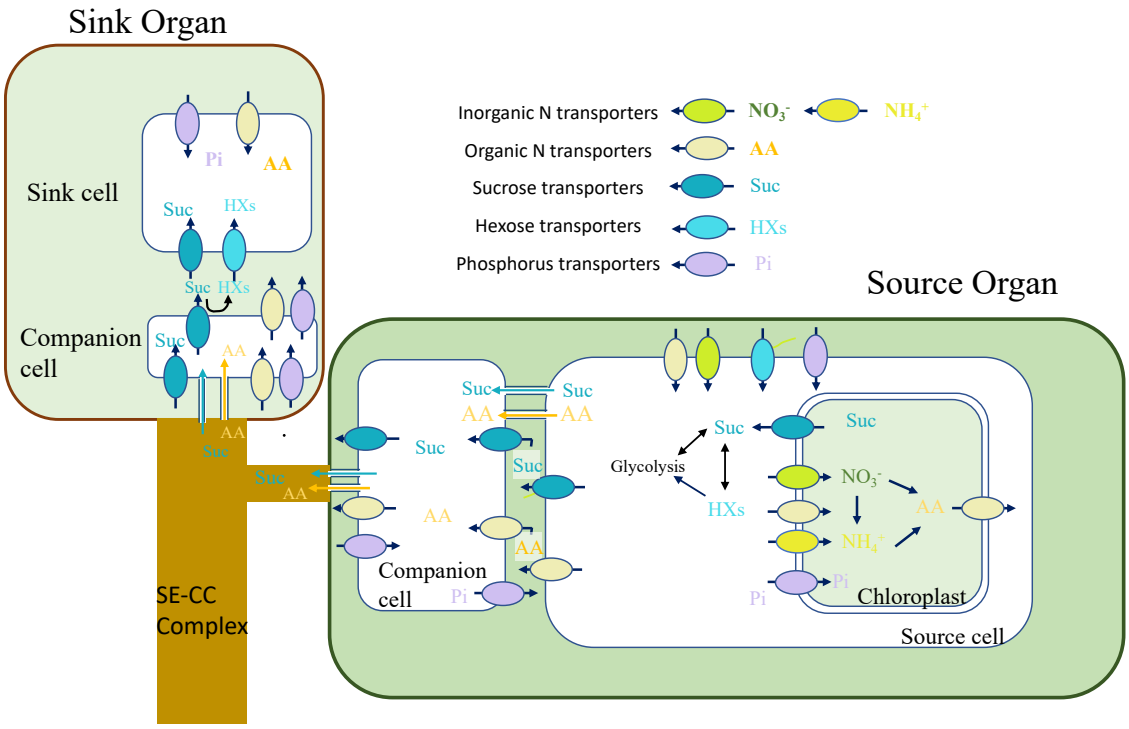


Figure 1. Model of inorganic and organic N, sugar, and phosphorus transport between bud (sink organ) and leaf (source organ). (Lalonde et al., 2004; Tegeder & Masclaux-Daubresse, 2018; Y. Y. Wang et al., 2018) Developing leaves and buds are the dominant N sinks during the vegetative phase**.**

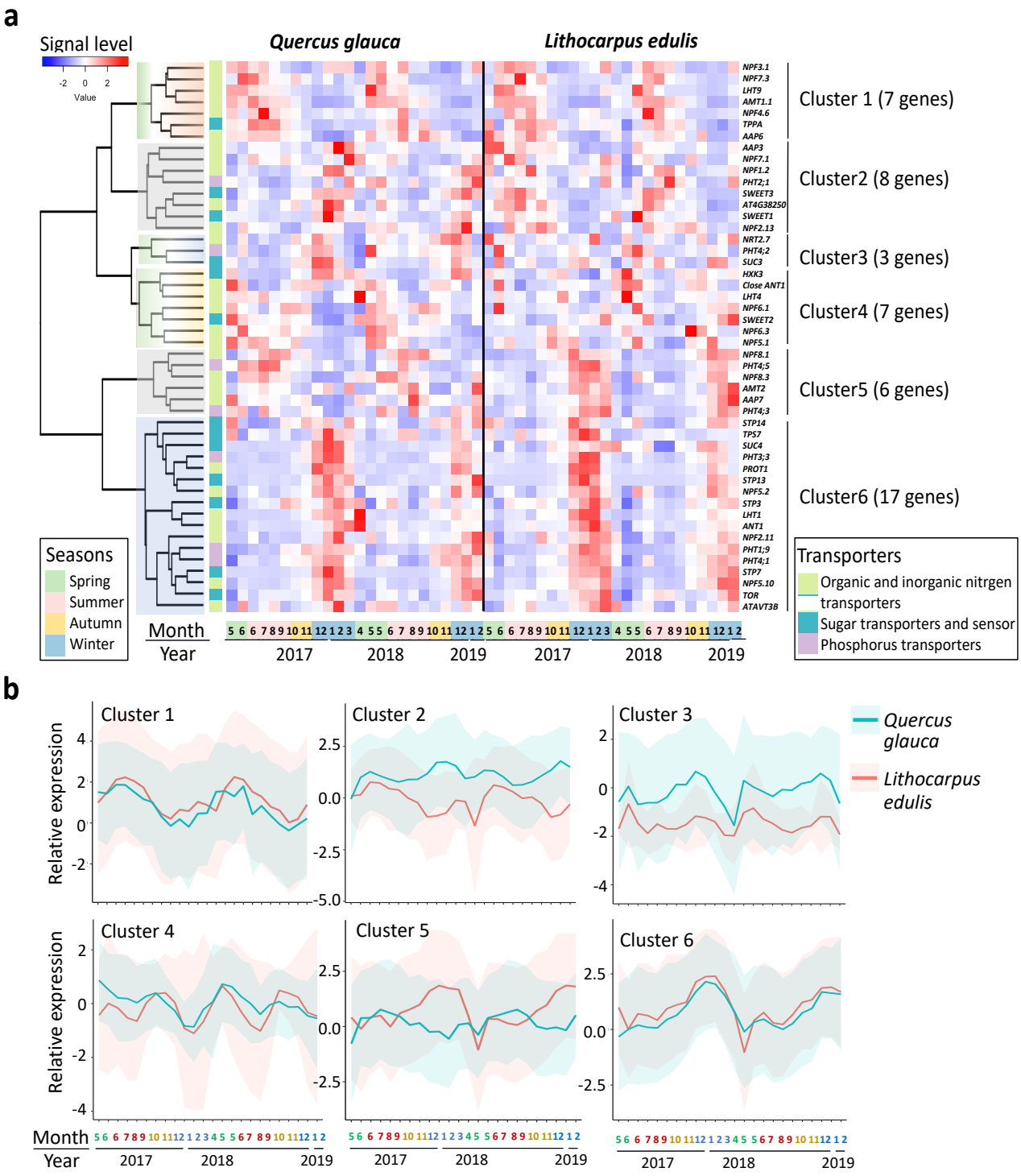

a
Signal level
-2 0 2
Value
Quercus glauca
Lithocarpus edulis
NPF3.1
NPF7.3
LHT9
AMT1.1
NPF4.6
TPPA
AAP6
AAP3
NPF7.1
NPF1.2
PHT2;1
SWEET3
AT4G38250
SWEET1
NPF2.13
NRT2.7
PHT4;2
SUC3
HXK3
Close ANT1
LHT4
NPF6.1
SWEET2
NPF6.3
NPF5.1
NPF8.1
PHT4;5
NPF8.3
AMT2
AAP7
PHT4;3
STP14
TPS7
SUC4
PHT3;3
PROT1
STP13
NPF5.2
STP3
LHT1
ANT1
NPF2.11
PHT1;9
PHT4;1
STP7
NPF5.10
TOR
ATAVT3B
Cluster 1 (7 genes)
Cluster2 (8 genes)
Cluster3 (3 genes)
Cluster4 (7 genes)
Cluster5 (6 genes)
Cluster6 (17 genes)
Seasons
Spring
Summer
Autumn
Winter
Month
Year
2017
2018
2019
Transporters
Organic and inorganic nitrgen transporters
Sugar transporters and sensor
Phosphorus transporters
b
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Relative expression
Quercus glauca
Lithocarpus edulis
Month
Year
2017
2018
2019

Figure 2. a. Hierarchical clustering and seasonal gene expression patterns of nitrogen, sugar and phosphorus transporters in leaf and bud tissues of *Q. glauca* and *L. edulis* species. **b**. Analysis of mean gene expression level in every cluster. Green and red lines represent the mean values of all gene expression level in each cluster of two species. Shaded area represents 95% confidence interval.

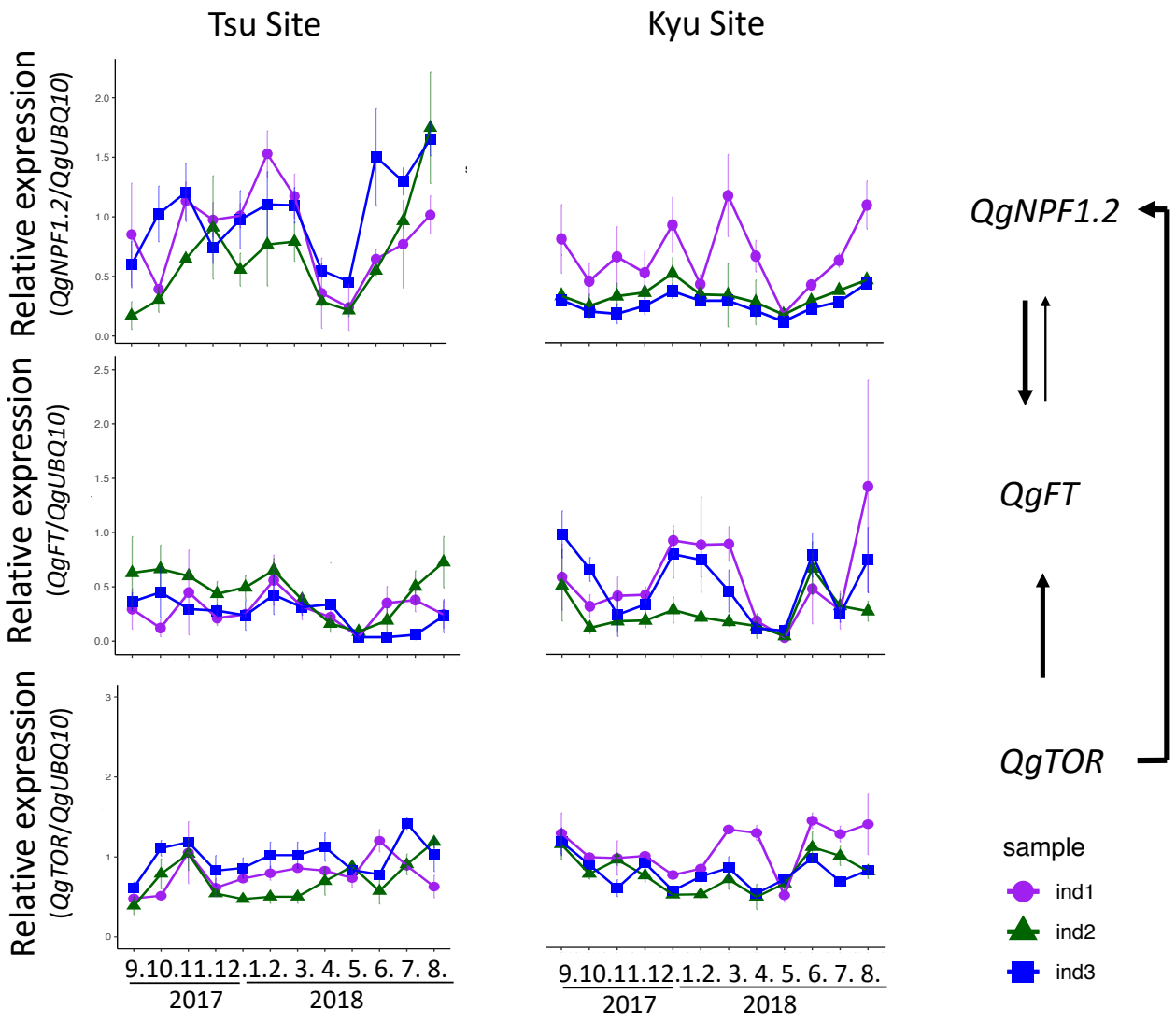


Figure 3. The relative expression level of NPF1.2，FT and TOR genes in *Q. glauca* with the UBQ10 as the house keeping gene by RT-qPCR at Tsu (Tsukuba) and Kyu (Kyushu) study sites. And their potential interrelationships using Convergent cross mapping (CCM) test.

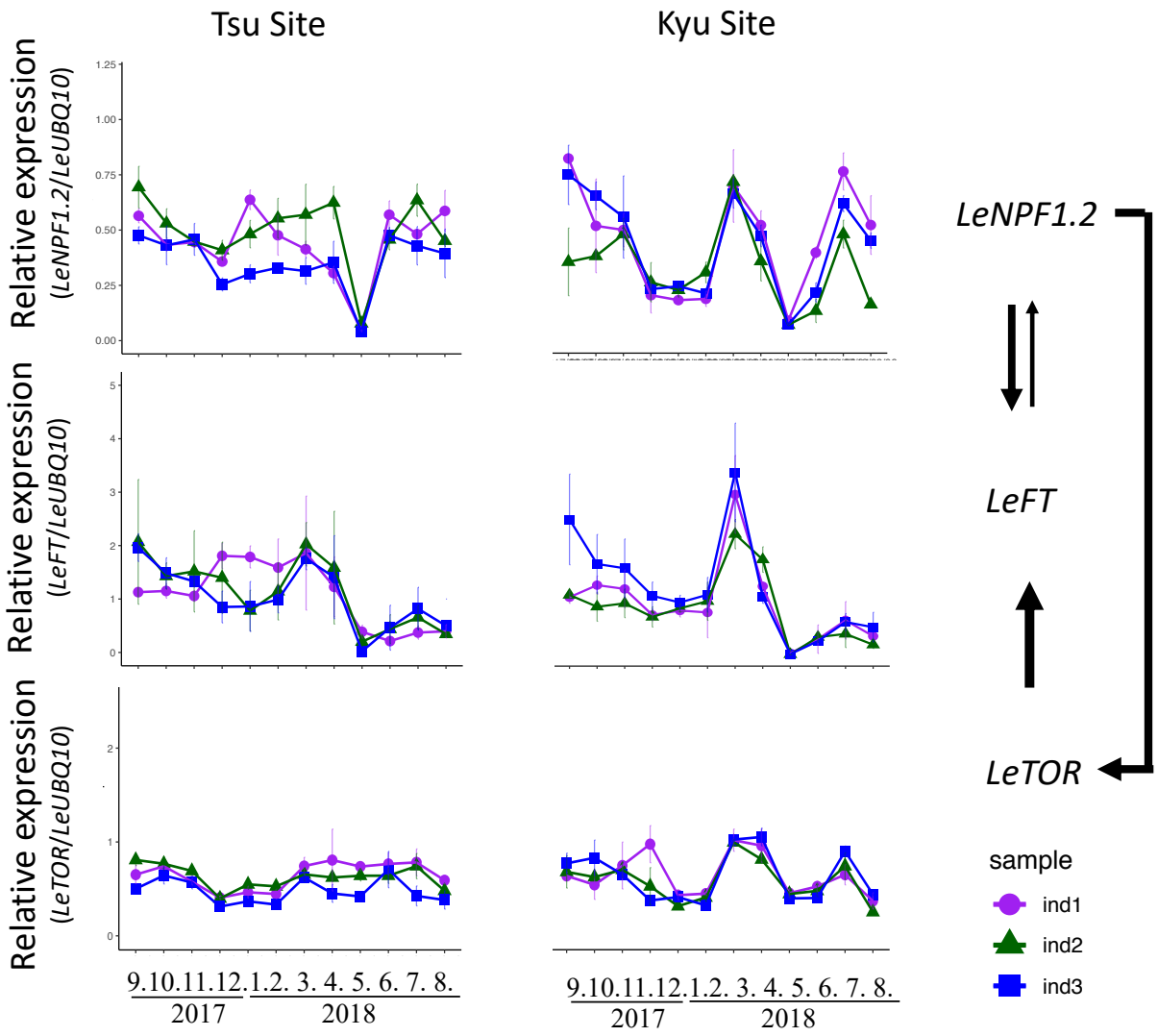


Figure 4. The relative expression level of NPF1.2， FT and TOR genes in *L. edulis* with the UBQ10 as the house keeping gene by RT-qPCR at Tsu (Tsukuba) and Kyu (Kyushu) study sites. And their potential interrelationships using Convergent cross mapping (CCM) test.

Appendixes

Table S1 Homology gene ID in Arabidopsis detected in transcriptome data of *Q. glauca* and *L. edulis*.

	Gene name	Homology gene ID in Arabidopsis
Nitrogen transporter	NPF1.2	AT1G52190
	NPF2.11	AT5G62680
	NPF2.13	AT1G69870
	NPF3.1	AT1G68570
	NPF4.6	AT1G69850
	NPF5.1	AT2G40460
	NPF5.2	AT5G46050
	NPF5.8	AT5G14940
	NPF5.10	AT1G22540
	NPF6.1	AT5G13400
	NPF6.3	AT1G12110
	NPF7.1	AT5G19640
	NPF7.3	AT1G32450
	NPF8.1	AT3G54140
	NPF8.3	AT2G02040
	NRT2.7	AT5G14570.1
	AMT1.1	AT4G13510.1

	AMT2	AT2G38290.1
	AAP2	AT5G09220
	AAP3	AT1G77380
	AAP6	AT5G49630
	AAP7	AT5G23810
	LHT1	AT5G40780
	LHT4	AT1G47670
	LHT9	AT1G25530
	AT4G38250	AT4G38250
	ANT1	AT3G11900
	close ANT1	AT1G80510
	ATAVT3B	AT2G42005
Sugar transporter and sensor	STP14	AT1G77210
	STP7	AT4G02050
	HXK3	AT1G47840
	STP1	AT1G11260
	KIN10	AT3G01090
	SUC4	AT1G09960
	STP3	AT5G61520
	SUC3	AT2G02860
	SUC2	AT1G22710
	STP13	AT5G26340
	TOR	AT1G50030

	TPPA	AT5G51460.2
Phosphours transporter	PHT3;3	AT2G17270
	PHT1;3	AT5G43360
	PHT4;2	AT2G38060
	PHT4;5	AT5G20380
	PHT4;1	AT2G29650
	PHT4;3	AT3G46980
	PHT2;1	AT3G26570

Table S2. Transport Genes annotated list in Table 1. The season specificity represented on a scale from 0 to 1, the seasonal expression specificity from ubiquitous to season specific, respectively.

GeneName	Species	Season-specificity-score	Spring	Summer	Autumn	Winter	Seasons-with-expression
NPF3.1	*Q. glauca*	0.15918755	35.850355	32.9562666	24.6057965	32.8682116	4
NPF7.3	*Q. glauca*	0.49000978	1.20616142	0.84694084	0.5732713	0.42517943	4
LHT9	*Q. glauca*	0.64896636	2.79949818	1.57385914	0.75753089	0.61676407	4
AMT1.1	*Q. glauca*	0.43697899	10.5207672	10.2711533	4.92938042	2.56970524	4
NPF4.6	*Q. glauca*	0.69464743	0.44416128	0.94689506	0.20796927	0.21527996	4
TPPA	*Q. glauca*	0.46809614	3.66168836	5.62283136	2.8660316	2.44469722	4
AAP6	*Q. glauca*	0.39124981	0.49917593	0.60232742	0.46005009	0.14077479	4
AAP3	*Q. glauca*	0.58363135	1.19454766	1.37775304	1.03018506	2.88405142	4
NPF7.1	*Q. glauca*	0.26406777	1.63073117	1.74505886	1.11973684	2.03620509	4

NPF1.2	*Q. glauca*	0.29986839	9.1030821	8.59189588	10.1142897	13.2400192	4
PHT2;1	*Q. glauca*	0.08964239	2.03684877	1.77623573	1.8279612	2.06550541	4
SWEET3	*Q. glauca*	0.55447628	1.84136949	1.81444592	3.2725977 6	5.18372192	4
AT4G38250	*Q. glauca*	0.22927038	10.8118606	10.4095291	10.9438711	13.9111737	4
SWEET1	*Q. glauca*	0.58229612	4.90446862	3.10146459	3.2726233 3	9.00044043	4
NPF2.13	*Q. glauca*	0.36647934	0.20488731	0.19577156	0.1589622	0.29445031	4
NRT2.7	*Q. glauca*	0.33868225	0.12258624	0.11435663	0.2095345 8	0.17876394	4
PHT4;2	*Q. glauca*	0.10986034	2.3202216	1.78740417	2.3739614 2	2.42717986	4
SUC3	*Q. glauca*	0.21133112	2.50536216	2.39652624	2.9749757 2	3.32918089	4
HXK3	*Q. glauca*	0.47605747	0.45502105	0.25965909	0.4175952 5	0.72035597	4
Close ANT1	*Q. glauca*	0.20247411	1.20657693	0.85019521	1.1032474 3	0.93338638	4
LHT4	*Q. glauca*	0.45173451	3.23417985	1.8837333	1.8913896 4	1.54444463	4
NPF6.1	*Q. glauca*	0.21416689	0.9022124	0.87372245	1.0002155 3	0.58207261	4

SWEET2	*Q. glauca*	0.43458636	5.702636	3.79266731	3.79211416	2.08826306	4
NPF6.3	*Q. glauca*	0.485061	0.70941936	0.504776	0.41098713	0.18015995	4
NPF5.1	*Q. glauca*	0.61308947	4.201996	2.58597065	1.57153895	0.7198799	4
NPF8.1	*Q. glauca*	0.63584705	1.34461503	4.88465025	2.71564179	1.27602253	4
PHT4;5	*Q. glauca*	0.22712513	15.7159852	18.768584	13.855051	13.946264	4
NPF8.3	*Q. glauca*	0.344368	0.54782956	0.60542453	0.34197595	0.30100158	4
AMT2	*Q. glauca*	0.31152315	0.76000314	1.21305297	0.58013017	1.23615208	4
AAP7	*Q. glauca*	0.12499719	0.68290039	0.66966596	0.62917667	0.49377665	4
PHT4;3	*Q. glauca*	0.10042146	0.29998462	0.2827807	0.25283129	0.27396718	4
STP14	*Q. glauca*	0.15508841	2.96476194	2.31052519	3.33564016	3.39717093	4
TPS7	*Q. glauca*	0.2482792	10.0037028	10.9459649	9.01255274	13.2860588	4
SUC4	*Q. glauca*	0.86769423	0.01874328	0.0250596	0.06759468	0.28065686	4
PHT3;3	*Q. glauca*	0.75257168	0.9817132	0.67489066	2.56170976	5.68287629	4

PROT1	*Q. glauca*	0.77416214	0.04806025	0.05461693	0.0675309	0.25122462	4
STP13	*Q. glauca*	0.86461929	2.10405429	3.87794338	5.36506984	27.9386606	4
NPF5.2	*Q. glauca*	0.40605711	3.1862561	3.31135858	3.83352785	5.79805607	4
STP3	*Q. glauca*	0.22381745	1.31127943	1.61663734	1.79095485	2.02652998	4
LHT1	*Q. glauca*	0.58113794	4.63907884	0.77746684	2.75492544	6.5029134	4
ANT1	*Q. glauca*	0.37207883	0.53113373	0.37122738	0.35959061	0.66990985	4
NPF2.11	*Q. glauca*	0.3753879	0.31465687	0.31743883	0.31872795	0.50742088	4
PHT1;9	*Q. glauca*	0.52548664	2.73285081	4.214383	9.79492177	11.7609303	4
PHT4;1	*Q. glauca*	0.340087	3.37199939	3.45680678	5.38840491	6.17112206	4
STP7	*Q. glauca*	0.88756725	0.79841877	0.54893929	2.00120835	9.92761303	4
NPF5.10	*Q. glauca*	0.53200765	5.26353827	5.40847353	7.15441161	12.6970903	4
TOR	*Q. glauca*	0.28011286	8.44910003	9.14669518	9.45149443	12.5238565	4
ATAVT3B	*Q. glauca*	0.1358275	1.37009354	1.25925122	1.24074253	1.4927912	4

NPF3.1	*L. edulis*	0.29937648	77.5343187	101.191109	55.5452556	79.6110382	4
NPF7.3	*L. edulis*	0.72013322	0.57907829	1.46342304	0.36530078	0.28431141	4
LHT9	*L. edulis*	0.45929377	0.99487072	0.79383117	0.39267707	0.42729015	4
AMT1.1	*L. edulis*	0.55384373	35.7136425	47.100659	18.920241	8.40887947	4
NPF4.6	*L. edulis*	0.69464743	0.53368992	2.36531895	0.33042988	0.75332984	4
TPPA	*L. edulis*	0.46809614	3.66168836	5.62283136	2.8660316	2.44469722	4
AAP6	*L. edulis*	0.39124981	0.49917593	0.60232742	0.46005009	0.14077479	4
AAP3	*L. edulis*	0.36218395	1.43074451	1.10088399	1.01707034	0.61970112	4
NPF7.1	*L. edulis*	0.13037916	0.12126982	0.12921954	0.11792029	0.09792591	4
NPF1.2	*L. edulis*	0.3968808	0.47814455	0.92381473	0.47063025	0.72273639	4
PHT2;1	*L. edulis*	0.1332431	1.60429664	1.84369222	1.49458474	1.6952175	4
SWEET3	*L. edulis*	0.77116776	1.08044616	2.58807642	0.51570404	0.18055574	4
AT4G38250	*L. edulis*	0.32642777	2.84450062	3.51914166	2.50331629	1.76337141	4

SWEET1	*L. edulis*	0.49731369	8.24903563	5.85001099	4.11182414	2.47819664	4
NPF2.13	*L. edulis*	0.42112256	2.70392158	6.64870976	6.01202948	2.83041327	4
NRT2.7	*L. edulis*	0.07746676	0.19525167	0.16501912	0.18104234	0.194317	4
PHT4;2	*L. edulis*	0.10986034	1.25502038	0.53721038	0.56349227	0.57828148	4
SUC3	*L. edulis*	0.26130976	0.45126582	0.4445553	0.35647462	0.56122927	4
HXK3	*L. edulis*	0.47605747	1.71652149	1.13124597	1.55270769	1.11997935	4
Close ANT1	*L. edulis*	0.2449068	0.87457635	0.60930124	0.73358296	0.63827576	4
LHT4	*L. edulis*	0.3412104	3.23417985	1.8837333	1.89138964	1.54444463	4
NPF6.1	*L. edulis*	0.25765127	2.00969349	1.32939959	1.48288549	1.66339517	4
SWEET2	*L. edulis*	0.19523509	3.29928612	3.05989064	3.06318068	3.90273704	4
NPF6.3	*L. edulis*	0.85666227	0.0969181	0.04511512	0.41710344	0.03732676	4
NPF5.1	*L. edulis*	0.29671291	1.28735587	0.82309884	1.54637868	1.15218976	4
NPF8.1	*L. edulis*	0.63584705	1.46869589	1.79010084	3.4471166	4.70875662	4

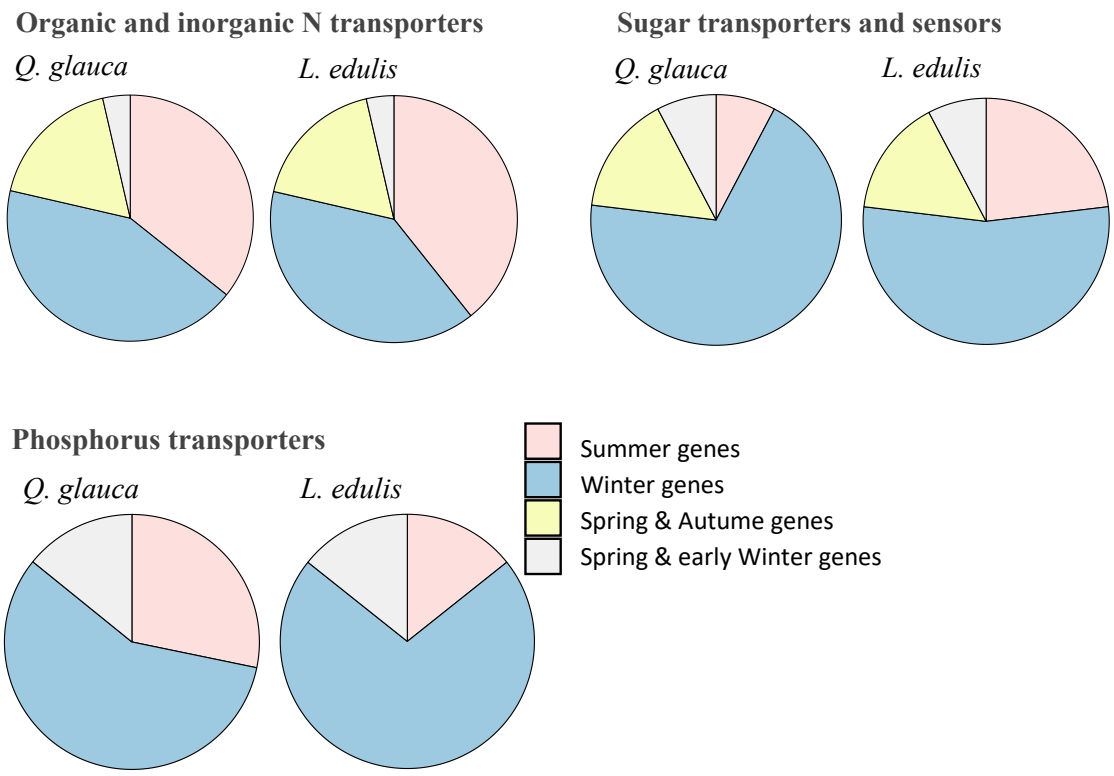
PHT4;5	*L. edulis*	0.76959796	2.72535189	2.35281125	4.41172462	13.7294612	4
NPF8.3	*L. edulis*	0.28414839	5.89395887	5.23167259	6.56571418	8.23790168	4
AMT2	*L. edulis*	0.54264729	4.98606413	6.48600537	6.42313749	13.0426012	4
AAP7	*L. edulis*	0.65744772	0.3121355	0.39876335	0.71354314	1.38610667	4
PHT4;3	*L. edulis*	0.5935683	0.0540464	0.06877564	0.08179702	0.1678175	4
STP14	*L. edulis*	0.31869965	2.03942903	1.01002714	1.64878256	2.2986625	4
TPS7	*L. edulis*	0.34823279	1.47395625	1.75736602	1.57823202	2.45975056	4
SUC4	*L. edulis*	0.12335245	0.72152273	0.62194467	0.76537242	0.80185772	4
PHT3;3	*L. edulis*	0.75257168	0.84113145	0.72782157	1.58838718	3.21569747	4
PROT1	*L. edulis*	0.88766224	0.03125705	0.01644538	0.04759694	0.28277629	4
STP13	*L. edulis*	0.91684313	3.13473018	3.3731043	15.395704	87.8000732	4
NPF5.2	*L. edulis*	0.41603656	4.33075879	4.03195629	5.50841466	7.9178072	4
STP3	*L. edulis*	0.37204007	0.80960392	1.22430616	1.24122353	1.73850456	4

LHT1	*L. edulis*	0.61143029	4.35513065	6.42275054	9.42678858	17.3325142	4
ANT1	*L. edulis*	0.51578089	0.59625404	0.94362909	0.94038602	1.7074014	4
NPF2.11	*L. edulis*	0.48182136	4.74317716	5.95970108	8.27727063	12.2095275	4
PHT1;9	*L. edulis*	0.67931417	0.81656537	0.58051867	1.6611147	3.17881082	4
PHT4;1	*L. edulis*	0.52399014	4.02739514	6.0158236	7.49179091	12.2791641	4
STP7	*L. edulis*	0.67745205	2.16159189	2.28439984	5.30670063	10.0788035	4
NPF5.10	*L. edulis*	0.45388636	3.63639779	4.70801616	6.43580989	9.02145805	4
TOR	*L. edulis*	0.28972654	12.0746935	15.988061	13.912262 6	19.6989937	4
ATAVT3B	*L. edulis*	0.28129981	0.32878305	0.35747289	0.32666769	0.46979424	4

Table S3 Monthly temperature change in Fukuoka between 2017 and 2019

Year	Month	Temperature				
		Average			Max	Min
					(℃)	(℃)
		Daily mean	Max	Min		
		(℃)	(℃)	(℃)		
2017	1	6.8	11.1	2.3	20.4	-3
	2	7.5	11.7	2.6	18.3	-1
	3	9.6	14.1	5.2	18.2	0.5
	4	15.7	20.4	10.7	27.3	2.7
	5	20.1	25.4	15.3	29.9	11.9
	6	22.3	27.2	17.9	32.3	13.5
	7	28.9	32.8	25.4	35.6	21.6
	8	28.8	33.2	25.1	38.9	21.8
	9	23.4	27.4	19.6	30.9	14
	10	19.2	22.8	15.9	28.9	8.1
	11	12.8	17.5	7.8	23.1	1.9
	12	7	10.5	2.8	16.4	-0.9
2018	1	5	8.6	1.1	17.1	-2.9
	2	5.4	9.6	0.7	16.1	-3.3
	3	10.8	16.3	5.5	22.6	0.4
	4	16	21.7	10.4	27.5	6.5
	5	20.1	24.9	15.5	31.8	8.4

	6	23	27.3	19.3	32.5	14
	7	28	32.2	24.6	36.8	20.4
	8	29.3	33.9	25.6	37.9	21.7
	9	24.2	28.1	21	33.8	15.7
	10	18.1	22.4	14	28.8	9.3
	11	13	18.4	8	23.1	0.5
	12	9.4	12.8	5.5	25.9	0.1
2019	1	7.3	11.3	2.6	15.1	-1.7
	2	8.5	12.8	3.8	21.9	-0.5



Appendix Figure S1. Components of seasonally expressed genes among different N C P transporter genes.

Chapter 3 Summary

In this thesis, molecular biological, physiological, and ecological methods were combined to explore influence of carbon, nitrogen, and phosphorus resources on the reproduction of Fagaceae species and the differences among their effects in the evolution of a wide variety of Fagaceae. Seasonal changes follow temperature and photoperiod changes, and in our Chapter 2 study we focus on the effects of gene expression under different temperature conditions. In the first chapter, we found that in *Quercus glauca*, carbon, nitrogen, and phosphorus have little effect on reproduction, so masting does not occur. And in Chapter 2, we found that sugar and nutrient signals affect important flowering gene and then affect their flowering time. It shows that the effects of available resource and total carbon, nitrogen and phosphorus resource on reproduction are different, which requires us to further subdivide the available carbon, nitrogen and phosphorus, and the different functions of carbon, nitrogen and phosphorus compounds with different divisions of labor. The research from molecular, physiological, and mathematical aspects will help us understand the effect of internal resource on the Fagaceae family and their evolutionary mechanism.