The driving process for the evolutionary trajectory towards C_4 photosynthesis

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Compared to the plants with C_3 photosynthesis, the plants with C_4 photosynthesis have higher light, water, and nitrogen use efficiencies. Though only 3% of higher plants have C_4 photosynthesis, C_4 photosynthesis contribute 23% of terrestrial gross primary productivity. However, it remains elusive how the procedure for the evolutionary trajectory from C_3 towards C_4 photosynthesis is driven. Here, *Flaveria* genus, the model for C_4 evolutionary researches, was used to study the driving procedure through a combined analysis of eco-physiological, biochemical and anatomical data. From proto-Kranz to C_4-like, results showed that precipitation was negatively associated with the activity of C_4 metabolic core enzyme and C_4 flux, respectively. An increased temperature and precipitation which was concurrent with increased vein density were observed during the earlier evolutionary stage. Then, decreased precipitation and increased temperature accompanied the emergence of C_2 photosynthesis and further transition from C_2 to developing C_4 photosynthesis. There was a positive relationship between CO_2 compensation point, stomata density and precipitation, suggesting decreasing precipitation enhance photorespiration through closing stomata during evolution. As the driving force from proto-Kranz to C_4-like, drought is more important than heat during C_4 evolution. This study indicated that the stages during the evolution towards C_4 photosynthesis required different driving force and increased water availability should be needed in the initial phase.

Key words:
Evolution, C_4 photosynthesis, intermediate plants, precipitation, temperature, driving process

Introduction

Photosynthesis undergone evolution, which results in different photosynthetic type, and C_4 photosynthesis is one of these photosynthetic types(Sage 2004; Langdale 2011). Compared to C_3 relative, the plant with C_4 photosynthesis has higher water use efficiency, nitrogen use efficiency and light use efficiency (Vogan and Sage 2011; Taylor et al. 2010). Although only accompanying 3% plant species, C_4 photosynthesis contributes 23% of terrestrial gross primary productivity (Still et al. 2003; Sage et al. 2012). The evolution from C_3 to C_4 photosynthesis go through multiple stages (C_4 evolution) (Sage et al. 2012; Sage 2004; Schluter and Weber 2016; Brautigam and Gowik 2016). The first stage of C_4 evolution is preconditioning in which C_3 plants acquires new

cis-elements (Akyildiz et al. 2007).For this stage, gene duplication occurs (Akyildiz et al. 2007; Monson 2003), vein density increases (Marshall et al. 2007; Mckown and dengler 2007) and the number of plastids in the bundle sheath increases (Sage et al. 2013). The second stage is proto-Kranz. In this stage, plants still perform C_3 photosynthesis (Sage et al. 2013) while the number of plastids continue to increase (Stata et al. 2019; Sage et al. 2012) and chloroplast and mitochondria localize to the centripetal wall of bundle sheath cells. The third stage is C_2 photosynthesis (Type I) (Schulze et al. 2013; Sage et al. 2012; Edwards and KU 1987), which is characterize as the transfer of the location of glycine decarboxylase P subunit (GDCP) and photosynthetic apparatus from mesophyll cell to bundle sheath cell (Schulze et al. 2013; Stata et al. 2019; Sage et al. 2013). The released CO_2 from photorespiration can be moderately concentrated in the bundle sheath cell (Photorespiratory CO_2 pump) (Keerberg et al. 2014; Sage et al. 2012). The fourth stage is inducing and developing C_4 photosynthesis (Type II) (Schluter and Weber 2016; Sage et al. 2018; Nakamura et al. 2013; Mallmann et al. 2014). The establishment of C_2 photosynthesis could cause the imbalance of ammonia between mesophyll cell and bundle sheath cell, and the induction of C_4 metabolism is proposed as a mechanism to resolve this trouble (Mallmann et al. 2014). The fifth stage is functionally developed C_4 photosynthesis (C_4-like) (Stata et al. 2019). For this stage, though there are still some Rubisco in mesophyll (Taniguchi et al. 2021; Monson et al. 1987; Stata et al. 2019; Sage et al. 2018), various types of indicators at this stage are all close to plants with ultimate C_4 photosynthesis (Vogan and Sage 2011; Ku et al. 1991; Adachi et al. 2023). The final stage is the optimization (C_4 photosynthesis) (Sage et al. 2012), and mesophyll cell contain few Rubisco (Stata et al. 2019). These stages in the evolutionary transition between C_3 and C_4 photosynthesis are called intermediate stage, and the plants in these stages are intermediate plants (or intermediate species).

The evolution from C_3 towards C_4 photosynthesis is considered to be driven by enhancing photorespiration, which is promoted by the low atmosphere CO_2 concentration, drought, salt and heat (Sage et al. 2012; Brautigam and Gowik 2016; Sage 2004; Edwards and Smith 2010; Bromham and Bennett 2014). The low atmosphere CO_2 concentration, drought and salt might decrease the ratio of CO_2 to O_2 concentration, thereby enhancing photorespiration (Sage et al. 2012). Increasing temperature enhances photorespiration by reducing CO_2 relative to O_2 in the chloroplast stroma (Ogren 1984) and decreases the specificity of Rubisco for CO_2 relative to O_2 (Ogren 1984; Sage et al. 2012). Previous studies have shown that C_4 evolution are correlated with the decrease in atmosphere CO_2 concentration and the appearance of dry, salty and hot environment (Christin et al. 2008; Edwards and Smith 2010; Edwards and Still 2008; Bromham and Bennett 2014), which indicates they may be driving force for C_4 evolution. However, all these studies assume that the entire process of C_4 evolution were under a single driver force. Given C_4 evolution includes multiple phases and the evolution is complicate, it is possible that these phases of C_4 evolution might be under different driving force rather than the same driving force persistently along the evolutionary road towards C_4 photosynthesis (Heckmann et al. 2013; Sage et al. 2012).

Plants obtain fitness in each step of C_4 evolution (Heckmann et al. 2013; Sage et al. 2012). The increase in vein density is an important characteristics during the initial stage of C_4 evolution (Sage et al. 2012; Sedelnikova et al. 2018; Kumpers et al. 2017; Sage et al. 2014) (Mckown and dengler 2007; Marshall et al. 2007; Williams et al. 2013), which might help supply water to the mesophyll and maintain stomatal openning, since evaporation might be enhanced due to heat at

this time (Brautigam and Gowik 2016; Sage et al. 2018) (Sage et al. 2012). Therefore, available water content in the environment for this stage may be increased to ensure the fitness of plants (Brautigam and Gowik 2016). However, this contradicts with the notion that the origin of C_4 photosynthesis was promoted by dry environment (Sage et al. 2018; Edwards and Smith 2010; Sedelnikova et al. 2018). Since C_2 photosynthesis and C_4 photosynthesis could largely reduce photorespiration (Sage 2004; Schluter and Weber 2016; Sage et al. 2012), the formation of C_2 from C_3 photosynthesis and the transition from C_2 to developing C_4 photosynthesis may be driven by the conditions which could enhance photorespiration (Schluter and Weber 2016; Sage et al. 2018; Sage et al. 2012),but the driving forces during these transitions also remain undefined.

Flaveria genus is a widely used system to study C_4 evolution (Sage et al. 2012; Sage 2017; Schluter and Weber 2016; Mallmann et al. 2014; Sage et al. 2013; Vogan and Sage 2011; Nakamura et al. 2013), and the studies on *Flaveria* genus has laid the foundation for the pyramid model of C_4 evolution (Sage et al. 2013; Vogan and Sage 2011; Sage et al. 2012). The photosynthetic types of Flaveria species span entire evolutionary stage towards C_4 photosynthesis(Sage et al. 2012; Sage et al. 2014; Schluter and Weber 2016; R.K. Monson 1986). *Flaveria* is also the youngest genus among the known C_4 linage and it's species has the close relationship (Schulze et al. 2013). These make *Flaveria* genus an ideal model to study C_4 evolution (Christin et al. 2011; Sage et al. 2012). The ecology for the habitats of intermediate species and C_4 species could reflect the environmental conditions that drive C_4 evolution (Sage et al. 2012; Sage et al. 2018), therefore, the ecology for *Flaveria* habitats enable us to finely study the driving force in the evolutionary road towards C_4 photosynthesis. In this study, we collected the ecological, biochemical, anatomical and physiological data for different species of *Flaveria* genus to explore driving procedure for the evolutionary trajectory towards C_4 photosynthesis and how these different evolutionary phases help plants gain fitness.

Results

1. Most of *Flaveria* species are distributed inland and a few species are distributed along the coast

The habitats of Flaveria species were mapped to the North and South America continents with the longitude and latitude of each *Flaveria* samples downloaded from Global Biodiversity Information Facility (GBIF) (Fig 1A, B). Species were ordered based on the amount of C_4 flux (Fig 1B, C, supplementary fig 1). We found most *Flaveria* species are located inland (Fig 1A, B). A few species (*F.bro*, *F.flo*, *F.lin*) are distributed along the coast (Fig 1B), which was consistent with the previous reports (Powell 1978). During evolution, intermediate species gradually spread to locations with higher latitude (Fig 1B). Compared with the intermediate species, the distribution of C_4 species is more widespread (Fig 1A, B, C), indicating that adaptive capacity of C_4 plants was enhanced (Fig1).

2. The temperature and precipitation of *Flaveria* growth habitats

The temperature and precipitation of the habitats for each *Flaveria* species were obtained from the worldclim database (Fick and Hijmans 2017). The results from physiological, anatomic, molecular and computational modeling experiments indicate the direction of C_4 evolution could be described by species's order towards C_4 photosynthesis in phylogenetic tree (Figure 2A) (Mckown and dengler 2007; Nakamura et al. 2013; Gowik et al. 2011; Sage et al. 2012; Sage et al. 2018; Schluter and Weber 2016). Along the evolution towards C_4 photosynthesis (Ordinal species

towards C_4 photosynthesis of the phylogenetic tree in Figure 2A), the annual mean temperature, mean temperature of warmest quarter, mean temperature of wettest quarter and max temperature of warmest month first increased and then decreased, though difference in some species habitats is not significant (Fig 2A, supplementary fig 2, supplementary fig 3, supplementary fig 4, supplementary file). Mean temperature of warmest quarter, mean temperature of wettest quarter and max temperature of warmest month increased significantly in the habitat of *F. rob* (C_3) and *F. son* (Type I) (Fig 2A, B, supplementary fig 2, supplementary fig 3, supplementary file), compared with those of *F. pri* (C_3). However, these environmental conditions significantly decreased in the habitat of *F. ang* (Type I), compared with *F. son* (Type I) (Fig 2A, supplementary fig2, supplementary fig3, supplementary file). For the mean temperature of wettest quarter, mean temperature of warmest quarter and max temperature of warmest month, compared with *F. son* (Type I), the habitat of species with C_2 photosynthesis (*F. ang*, *F. chl*, *F. pub*, *F. opp*, *F. ano*, *F. ram*) have a lower mean temperature, but the results of statistic test for some species is not significant or in margin (Fig 2A, supplementary fig 3D, supplementary file). The habitats of species *F. flo* (Type II) and *F. lin* (Type II) had significantly higher mean temperature compared to most of other intermediate species (Fig 2A, supplementary file). Max temperature of warmest month for *F. son* (Type II) habitats is extremely high (Fig 2d, supplementary file).

For the precipitation of wettest quarter, precipitation of warmest quarter and precipitation of wettest month, along C_4 evolution, they significantly increased in the *F. rob* (C_3) habitat, compared with those in *F. pri* habitat (Fig 2A, B, supplementary fig 3, supplementary file). Compared with *F. rob* (C_3) habitat, precipitation significantly decreased in the *F. ang* (Type I) habitat (Fig 2A, supplementary fig 3, supplementary file). Precipitation decreased in *F. son* (Type I) habitat, and though the difference was not statistically significant (Fig 2A, supplementary fig 3, supplementary file). Precipitation of warmest quarter was higher than precipitation of coldest quarter, and similar to precipitation of wettest quarter (Fig 2A, supplementary fig 2, supplementary figure 3, supplementary file). Expect *F. flo* (Type II) and *F. lin* (Type II), precipitation of the habitat of species with C_2 photosynthesis decreased persistently along C_4 evolution, though difference for the habitats of some species is not significant (Fig 2A, supplementary fig 3, supplementary file). In the initialization of C_4 evolution, the initial increase in precipitation and temperature (*F. pri* to *F. rob*) coincided with the initial appearance of the higher vein density (Fig 2A, B, C, supplementary fig 3, supplementary 4). Although the precipitation of the Type II species *F. flo* and *F. lin* habitat was high (Fig 2, supplementary fig 3, supplementary file), they inhabited the region including saline land (Table 1), probably experiencing strong osmotic stress.

3. The relationship between precipitation (or temperature) and C_4 metabolism related traits during the transition from proto-Kranz to C_4-like

For the clade A, the activities of C_4 metabolism related enzymes PEPC, PPDK and MDH showed a negative correlation with precipitation, and there showed a linear relationship between log enzyme activity and log precipitation (Fig 3 A, B, C, supplementary file). The negative relationship between C_4 flux and precipitation can be described as a linear function (Fig 3D). For the clade B, the relationship between log enzyme activity and log precipitation also can be described as a linear function (Fig 3 I, J, K, supplementary file). However, the linear relation between C_4 flux and precipitation in clade B is weaker (Fig 3L, supplementary file). The linear relation between log enzyme activity and log temperature is weaker than that between log enzyme

activity and log precipitation, regardless of clade A or clade B (Fig 3E, F, M, N, G, H, O, P, supplementary file).

4. The change in temperature and precipitation between the transition of stages.

The comparison of *F. rob* and *F. son* suggested that incipiently decreased precipitation along the evolution towards C_4 photosynthesis concurrently occurred with increased mean temperature (Fig 4A, F). Precipitation levels of the habitat of *F.ano* and *F.ram* were significantly lower than that of *F. ang*, and temperature values of the habitat of *F. ram* and *F. ano* were significantly higher than that of *F. ang* (Fig 4B, G, C, H). Precipitation of *F. pal* habitat was significantly lower than that of *F. ram*, and precipitation of *F. bro* habitat is significantly lower than that of *F. flo* (Fig 4D, E). However, temperature for the habitat of *F.pal* and *F. bro* showed the inconsistent change, temperature for the habitat of *F. pal* was similar to that of *F. ram*, and temperature of *F. bro* habitat was significantly lower than that of *F. flo* (Fig 4I, J). Furthermore, compared with their more ancient species, we observed that the decreased precipitation for the species habitat (*F. son*, *F.ram*, *F.flo*, *F.pal* and *F.bro*) concurrently appeared with the decreased stomatal density, decreased inhibition of O_2 and CO_2 compensation point, decreased maximum stomatal conductance (Supplementary Fig 5E, F, G, H). Although the precipitation of *F. flo* and *F. lin* were increased compared to other species with C_2 photosynthesis (Fig 2A, supplementary fig3, supplementary fig4), they also had the decreased stomatal density, maximum stomatal conductance (Supplementary fig 5), which might be related to their salty habitats (table1).

5. The relationship between precipitation, stomata and CO_2 compensation point.

Previous work found there is a difference in stomata density between species at different evolutionary stages in the *Flaveria* genus(Zhao et al. 2022). Furthermore, there was a positive and linear relationship between stomata density, CO_2 compensation point and precipitation during the transition between proto-Kranz and C_4-like species in *Flaveria* clade A and clade B (Fig 5A, B, C, D, E, and F). The linear relationship between stomata, CO_2 compensation point and precipitation in *Flaveria* clade B were weaker than that in clade A (Fig 5A, B, C, F, G, H, Supplementary file). The relation between temperature and stomata density or CO_2 compensation point was not apparent as the relationships between these two parameters and precipitation (Fig 5D, I, E, J).

Discussion

Evolutionary conditions along the path towards C_4 photosynthesis

Heat and drought have long been thought to be associated with C_4 evolution (Sage et al. 2018; Taylor et al. 2012). Exploring environmental conditions of the habitats for C_4 and intermediate species could contribute to elucidate the drivers for C_4 evolution (Sage et al. 2018; Sage et al. 2012; Edwards and Smith 2010). In this work, we examined precipitation and temperature in diverse *Flaveria* species during different periods (Fig 2A, supplementary fig 2, 3, 4). We noticed that the quantity and trend of wettest quarter mostly coincided with those of warmest quarter (Fig 2A, supplementary fig 3), mean temperature of wettest quarter was overall higher than mean temperature of driest quarter (Supplementary fig 3D, Supplementary fig 2E) and precipitation of warmest quarter was overall higher than precipitation of coldest quarter (Supplementary fig 3C supplementary fig 2B). These results suggest that rain and heat were in the same season for the habitat of *Flaveria*, and sufficient water content is necessary to maintain high photosynthetic

activity for plants, which is in line with previous work that indicates *Flaveria* species perform high photosynthesis during summer with heat in Mexico (Powell 1978). Because there is no apparent change in Rubisco Sc/o (Kubien et al., 2008) and similar Ci/Ca between C_3 and type I intermediate species (Adachi et al., 2023), the higher photosynthetic activity would indicate also a higher photorespiratory flux in C_3 plants and initial intermediate species. Besides, under such conditions, photorespiration, long regarded as the driving factor of C_4 evolution, might further be enhanced by heat (Sage et al. 2012; Sage et al. 2018). Furthermore, any evolutionary selective pressure on photosynthesis should act in the periods with active photosynthesis (Sage et al. 2018), i.e. the driest and coldest season should not be considered for the study on the evolutionary trajectory towards C_4 photosynthesis (Supplementary fig 2). Therefore, the periods with rain and heat for *Flaveria* genus is valuable to explore the driving process of C_4 evolution (Fig 2A, supplementary fig 3).

We found that the initial stage of C_4 evolution was associated with high precipitation and temperature, and later stage was associated with decreased precipitation (Fig 2A, Fig 4, supplementary fig 3). Heat was previously considered to be a major factor that promoted the origin of C_4 photosynthesis (Sage et al. 2018). Here, our results suggest that in addition to increasing temperature, the increase in precipitation was also a vital factor to initiate the evolution towards C_4 photosynthesis (Fig 2, Supplementary fig 3). Previous study proposed it is possible that heat occurred with drought simultaneously to enhance photorespiration and then promote C_4 evolution (Sage et al. 2012). Here, data from this study indicates that this process indeed existed, and it was at the transition from C_3 to C_2 (*F. son*) and the transition from C_2 to developing C_4 photosynthesis (*F. ram*, *F. ano*) (Fig 4).

The evolutionary mechanism during the initial phase of C_4 evolution

The initial stage of C_4 evolution was featured as having increased vein density and increased number of chloroplasts in bundle sheath (Sage et al. 2012). The increased vein density can help maintain sufficient water content in leaf and avoid the reduction of stomata aperture, since the evaporation might be increased at this stage (Sage et al. 2012; Sage et al. 2018). In agreement with this, our data showed that, when at the stage of vein density increasing, there was no change in stomatal development, and temperature was indeed increased (Fig 2A,B,C,D, supplementary fig 5). The increased temperature could increase vapor pressure between leaf and air (supplementary fig 5B), hence enhanced the evaporation (Sage et al. 2018); while no change in the stomatal development indicates that water shortage in leaf did not occur (supplementary fig 5), since long-term water shortage could alter stomatal development (Franks et al. 2009; Taylor et al. 2012). Importantly, our data also show that precipitation was increased at this stage, which matched with the increased vein density and ensured sufficient water supply to the leaf (Fig 2A, B, C). Increased precipitation and keeping stomatal opening could not only maintain intercellular CO_2 concentration, but also could help alleviate the increase of leaf temperature to increase the acquirement of carbon. Therefore, the initial stage of C_4 evolution was promoted by high temperature and precipitation (Fig 2).

The increase in vein density could decrease volume of mesophyll in a leaf (Sedelnikova et al. 2018; Mckown and dengler 2007), which inevitably decreases the photosynthetic capacity of the leaf. In theory, this should create a strong evolutionary pressure to select for the increased photosynthetic capacity of BSC to offset the loss of photosynthetic capacity. The relative number

and size of chloroplast in bundle sheath/ area of BSC of *F. rob* was indeed potentially increased compared to those of *F.pri*. Besides, the light intensity of *F. rob* habitat was higher than that of *F. pri* (supplementary fig 5A). The size of bundle sheath in *F. rob* decreased compared with *F. pri* (Sage et al. 2013; Mckown and dengler 2007), the size of mesophyll cell decreased compared with *F. pri* (Mckown and dengler 2007), and thickness of leaf become less (Mckown and dengler 2007); all these enhanced the level of light that bundle sheath experiences, consistent with the notion that selection pressure for bundle sheath with higher photosynthetic capacity might exist at this stage.

Drought as a more important factor than heat for driving C_4 evolution

Some studies have proposed that drought is a primary driving force for C_4 evolution (Sage 2004; Zhou et al. 2018; Sage et al. 2018; Schluter and Weber 2016; Edwards and Smith 2010), and other studies suggested that heat is the critical environment factor driving C_4 evolution (Sage et al. 2018; Sage et al. 2012). In this study, we proposed that drought played a more important role than heat during C_4 evolution (Fig 3). From proto-Kranz to C_4-like, precipitation decline step by step, however, there is no clear trend of change for temperature (Fig 2). Regardless of clade A or Clade B, we found a negative association between core C_4 enzyme activity and precipitation (Fig 3) and positive association between CO_2 compensation point and precipitation (Fig 5). In particular, for clade A, there was a strong negative relationship between precipitation and activities of core C_4 enzymes (Fig3). However, the relationship wasn't apparent for temperature (Fig 3). These results indicated that the gradually enhancing C_2 photosynthesis and developing C_4 photosynthesis mainly associated to the decreasing precipitation (Fig 2A,B,C,D,E,F, Fig 3). Therefore, drought rather than heat, should be the major driver for the evolution from proto-Kranz to C4-like.

Different evolutionary drivers at the transition of the stages in C_4 evolution

The environmental drivers for the evolution towards C_4 photosynthesis has been intensively studied. For example, decreasing atmosphere CO_2 concentration has been thought as a driving force of C_4 evolution (Christin et al. 2008; Concentrations and Paleogene). Drought, salt and heat were also thought to as conditions promoting C_4 evolution (Sage et al. 2018; Sage 2004; Bromham and Bennett 2014) (Edwards and Still 2008; Edwards and Smith 2010; Pearcy and Ehleringer 2006; Sage et al. 2012). These studies assume that entire C_4 evolutionary process is promoted by a single ecological condition. However, here, our results suggested that environmental conditions promoted C_4 evolution in an orderly and stepwise manner (Fig 6), and different selection pressure drove the transition of the stages during the evolutionary way towards C_4 photosynthesis (Fig 6).

Besides the initialization of C_4 evolution, the evolution towards C_2 photosynthesis is also considered to be a crucial step during C_4 evolution (Sage et al. 2012; Schluter and Weber 2016). Previous studies have reported that *Flaveria* species with C_2 photosynthesis can represent a transition between C_3 and C_4 photosynthesis during the evolution (Heckmann et al. 2013; Mallmann et al. 2014; Schluter and Weber 2016; Sage et al. 2012), and *F. ram* and *F. ano* have enhanced C_4 cycle flux, C_4 cycle related enzyme activity and gene expression levels (Adachi et al. 2023; Mallmann et al. 2014; Vogan and Sage 2011; R.K. Monson 1986). According to the phylogenetic tree, *F. ang* is closer to *F.ram* as compared with *F.son* (Fig 2A), therefore, among all *Flaveria* species, *F.son* most likely represents the emergence of C_2 photosynthesis in *Flaveria*. Similarly, *F. ram* and *F. ano* most likely represent the emergence in clade A and clade B, respectively, and *F. pal* and *F. bro* most likely represent the emergence of a fully functional C_4

cycle for clade A and clade B, respectively (Fig 2A). Therefore, the increased temperature and decreased precipitation simultaneously further promoted the emergence of C_2 photosynthesis (Fig 4A, F). Increasing temperature and decreasing precipitation could enhance photorespiration and thus promote the formation of photorespiratory CO_2 pump which improve the fitness of these intermediate plants. The photorespiratory CO_2 pump could largely decrease the photorespiration (Sage et al. 2018; Sage et al. 2012; Schluter and Weber 2016; Ku et al. 1991), indicated by the large decrease in CO_2 compensation point and O_2 inhibition (Sage et al. 2013; Ku et al. 1991). Previous study proposed photorespiration promoted C_4 evolution by decreasing stomatal conductance under drought (Sage et al. 2012). As the partial support, we provided the evidence that there was a positive relationship between stomatal density, CO_2 compensation point and precipitation (Fig 5). These also suggested that the concurrent evolution for decreased stomata density and the emergence of C_2 and C_4 photosynthesis during C_4 evolution (Fig 5). The enhancement of C_2 photosynthesis has long been considered to cause the imbalance of nitrogen between mesophyll cell and bundle sheath cell, which induces C_4 cycle (Mallmann et al. 2014). In case of the evolutionary process from C_2 photosynthesis to developing C_4 photosynthesis, precipitation decreases and temperature increases (Fig 4B,C,G,H), which could drive this process. The formation of fully functional C_4 photosynthesis was driven by inconsistent conditions for Clade A and Clade B, and precipitation have played a dominated role in this step. There is no significant difference in temperature of the habitats, which suggested that the temperature was not a deciding factor controlling this transition.

In summary, this study analyzed the data of environmental ecology, biochemistry and anatomy for different species of *Flaveria* genus to reveal driving process of the evolution from C_3 towards C_4 photosynthesis, and results indicated plants suffered carbon limitation in the early phase of C_4 evolution and the primary driving force for this phase is carbon limitation (Fig 6). Later, plants suffered water limitation leading to decease in acquirement of carbon, therefore, the primary driving force for these phases of C_4 evolution is water limitation (Fig 6). For a long time, most of studies proposed that environmental condition causing the carbon restriction through enhancing photorespiration (drought, heat, salty and low atmosphere CO_2 concentration) drives the evolution towards C_4 photosynthesis(Christin et al. 2008; Edwards and Smith 2010; Schluter and Weber 2016; Sage 2004; Sage et al. 2012; Sage et al. 2018). Our study proposed these studies are not comprehensive and suggested that the steps of the evolution from C_3 to C_4 photosynthesis were driven by different environmental factors which sequentially promoted C_4 evolution, in contrast to the current notion that the entire process of C_4 evolution is driven by same environmental factor (Fig 6). The initial evolution was promoted by high temperature and precipitation, while later decreased precipitation further promoted the transition between C_2 and C_4 photosynthesis(Fig 6). More important, the increase in vein density and the activation of bundle sheath cell represent a starting point of the evolution from C_3 towards C_4 photosynthesis(Christin et al. 2012; Mckown and dengler 2007; Sage et al. 2012), therefore, high humidity and temperature together may be the most important condition facilitated the initial evolution towards C_4. Therefore, the origin of C_4 evolution is in the habitat with high humidity and temperature, rather than drought, heat, salty and low atmosphere CO_2 concentration (Bromham and Bennett 2014; Christin et al. 2008; Sage et al. 2018).

Methods

1. Obtain the ecological data of *Flaveria* species.

First, the geographical distribution of *Flaveria* was acquired from Global Biodiversity Information Facility (GBIF) database using the rgibf software package. Using ArcMAP software, we acquired from the Worldclim database the elevation data of the respective locations and 19 Bioclimatic factors, including Mean Diurnal Range, annual mean Temperature, Isothermality, Temperature Seasonality, Max Temperature of Warmest Month, Temperature Annual Range, Mean Temperature of Wettest Quarter, Mean Temperature of Warmest Quarter, Annual Precipitation, Precipitation of Wettest Month, Precipitation of Warmest Quarter, Precipitation of Wettest Quarter, Precipitation of Coldest Quarter, Min Temperature of Coldest Month, Precipitation of Driest Quarter, Mean Temperature of Coldest Quarter, Precipitation of Driest Month, Precipitation Seasonality (Coefficient of Variation), Precipitation of Coldest Quarter based on the geographical distribution information of the *Flaveria*. Specifically, we downloaded the data from the worldclim at first. Secondly, we prepared the file for the information of sampling location. Thirdly, we imported the layers file of climate factor. Fourthly, we imported the information of sampling location. Fifth, generating the new layers. Lastly, we exported the file that including sample and the data of geographical factors. All bioclimatic variables were averaged from 1970 to 2000. The elevation data came from SRTM elevation data. All data from the worldclim database have a maximum spatial resolution of 30 arc-seconds. The water vaper pressure and solar radiation were also extracted from the World clim database(Fick and Hijmans 2017) based on the geographical distribution information of *Flaveria*.

2. Obtain the anatomical and physiological data of *Flaveria* species.

The anatomical and physiological data for *Flaveria* was acquired from previous research. The data of vein data was from (Mckown and dengler 2007). The data of CO_2 compensation point and O_2 inhibition is from (Ku et al. 1991). The data related to stomata is from (Zhao et al. 2022). The C_4 metabolic enzyme activity data is from (Adachi et al. 2023) (supplementary table S7). The data in the graph was digitized with Origin 2018. The growth habitat was acquired from (Powell 1978; Kocacinar et al. 2008).

3. Map the *Flaveria* species

We mapped and marked the locations for the habitats of different *Flaveria* species to a world map using the software google earth Pro (version 7.3) based on the Latitude and longitude from Global Biodiversity Information Facility (GBIF). Coordinates were plotted as placemarks, with customized icons to distinguish between different species to facilitate the visualization and analysis of distribution patterns and geographical clusters. Google Earth Pro, version 7.3. (2020). [Software]. Google LLC. Available at: https://www.google.com/earth/.

4. Statistical analysis, regression analysis and Graph drawing.

The graph was plotted and the regression analysis was performed by using GraphPad prism 8. The statistic test was performed by GraphPad prism 8 and WPS office. The statistic test for the difference in precipitation or temperature between all *Flaveria* species was performed by one-way ANOVA and Tukey's multiple test (supplementary file). The rest of other statistic test was performed through Student's t test. We used the $\log_2$ value of the precipitation of wettest quarter

and $\log_2$ value of precipitation of C_4 metabolic enzyme activities, and linear regression between them were performed with GraphPad prism 8.

Table 1 The habitat of *Flaveria* species

Species (photosynthetic type)	Growth habitat	Geographic location
Flaveria pringlei (C3)	Rather arid and gypseous habitats, scattered localities, waste places, cultivated fields, roadsides	Southern Mexico
Flaveria cronquistii (C3)	Rocky limestone (possibly gypseous) areas	Southern Mexico
Flaveria robusta (C3)	Gypsum and slate, sandy clay, or open rocky slopes, weedy at roadsides	Western Mexico
Flaveria sonorensis (Type I)	Close to warm mineral springs	North-western Mexico
Flaveria angustifolia (Type I)	Sandy or loamy soils in open fields	Southern Mexico
Flaveria pubescens (Type I)	Scattered localities, gypsum soils	Central Mexico
Flaveria oppositifolia (Type I)	high gypsum plains or other areas with dilute gypsum, saline, or rarely loamy soil, usually in moist areas or in disturbed habitats at roadside	Central Mexico
Flaveria linearis (Type II)	Near the coast and on offshore islands	Western Florida, USA
Flaveria floridana (Type II)	Restricted to saline, white sand near the beaches of brackish marshes	Western Florida, USA
Flaveria ramosissima (Type II)	Sandy-clay bottoms, along dry washes, fields and roadsides, possibly in gypseous soils,	Southern Mexico
Flaveria anomala (Type II)	High gypsum plains, often in disturbed sites	Central Mexico
Flaveria palmeri (C_4-like)	Gypsum flats and knolls, or clay-loam soils probably with dilute gypsum, bolsons near lagunas, distributed areas near roads and fields	Northern Mexico
Flaveria brownie (C_4-like)	Saline, sandy, and marshy areas of the coastal flats and islands of the lower Texas Gulf Coast	North-eastern Mexico
Flaveria vaginata (C_4-like)	Arid and gypseous soils, scrub vegetation, cultivated fields	South-central Mexico
Flaveria bidentis (C_4)	A weed in moist places on waste ground, near rivers, streams, and barrancas, in fields, pastures, disturbed ground, near streets and sidewalks in towns, claygravel, or sand	Africa, Caribbean, South America, southern USA

Flaveria trinervia (C_4)	a variety of habitats, usually saline, gypseous, and disturbed, usually near ephemeral or permanent water sources	Africa, Caribbean, Central/South America, Mexico, USA
Flaveria chloraefolia (Type I)	Permanent or ephemeral saline marshy soils, springs, streams, roadsides	North Mexico

The information of habitat of *Flaveria* species was from (Powell 1978; Kocacinar et al. 2008)

Figure legends

Fig 1 Distribution of the *Flaveria* species

(A) The location of *Flaveria* species was mapped to the world map. The main habitat of *Flaveria* is Mexico which was displayed. (B) The location of *Flaveria* species except C_4 species was mapped to the world map. (C) and (D) The latitude and longitude of different *Flaveria* species. The latitude and longitude were acquired from GBIF. Different colors indicate different photosynthetic types.

Fig 2 The environment conditions of the habitats for *Flaveria* species

(A) The average temperatures of the warmest quarter for different *Flaveria* species (above panel). The precipitation of the wettest quarter for different *Flaveria* species (below panel). (B) The average temperatures of the wettest quarter for *F. rob* and *F. pri.* (C) The precipitation of wettest quarter for *F.rob* and *F.pri*. (D) The changes in vein density in the C_3 and intermediate species. Different colors indicate different photosynthetic types. Orange color represents C_3, grey color represents intermediate species, green color represents C_4-like, blue color represents C_4 species. Black lines represent median values.

Fig 3 The association between precipitation and temperature with C_4 enzyme activities or C_4 flux

Here the precipitation represents the precipitation of the wettest quarter. The temperature represents the average temperature of the wettest quarter. A-H, clade A. (A) The relationship between Log PEPC activity per Chl and Log precipitation. (B) The relationship between Log PPDK activity per Chl and Log precipitation. (C) The relationship between Log MDH activity per Chl and Log precipitation. (D) The relationship between C_4 flux and precipitation. (E) The relationship between Log PEPC activity per Chl and Log temperature. (F) The relationship between Log PPDK activity per Chl and Log temperature. (G) The relationship between Log MDH activity per Chl and Log temperature. (H) The relationship between C_4 flux and Log temperature. I-P, clade B. (I) The relationship between Log PEPC activity per Chl and log precipitation. (J) The relationship between Log PPDK activity per Chl and log precipitation. (K). The relationship between Log MDH activity per Chl and log precipitation. (L). The relationship between C_4 flux and precipitation. (M) The relationship between log PEPC activity per Chl and log temperature. (N) The relationship between log PPDK activity per Chl and log temperature. (O) The relationship between log MDH activity per Chl and log temperature. (P) The relationship between C_4 flux and temperature. The results of linear regression analysis are in supplementary file.

Fig 4 Precipitation and temperature during the transition from proto-Kranz to C_4-like

(A) Change in precipitation of wettest quarter during the transition from proto-Kranz (*F. rob*) to C_2 (*F. son*). (B) Change in precipitation of wettest quarter during the transition from C_2 (*F. son*) to developing C_4 (*F. ano*) in clade B. (C) Change in precipitation of wettest quarter during the transition from proto-Kranz (*F.son*) to developing C_4 (*F.ram*) in clade A. (D) Change in precipitation of wettest quarter during the transition from developing C_4 (*F.ram*) to functionally developed C_4 photosynthesis (*F.pal*) in clade A. (E) Change in precipitation of wettest quarter during the transition from developing C_4 (*F.flo*)to functionally developed C_4 photosynthesis (*F.bro*) in clade B. (F) Change in mean temperature of wettest quarter during the transition from proto-Kranz (*F.rob*) to C_2 (*F.son*). (G) Change in mean temperature of wettest quarter during the transition from C_2 (*F. son*) to developing C_4 (*F. ano*) in clade B. (H) Change in mean temperature of wettest quarter during the transition from C_2 (*F. son*) to developing C_4 (*F. ram*) in clade A. (I) Change in mean temperature of wettest quarter during the transition from developing C_4 (*F.ram*) to functionally developed C_4 photosynthesis (*F. pal*) in clade A. (J) Change in mean temperature of the wettest quarter during the transition from developing C_4 (*F. ano*) to functionally developed C_4 photosynthesis (*F. bro*) in clade B. The transition from C_3 to C_2 and the emergence of C_4 flux was promoted by increased temperature and decreased precipitation, indicated by the direction of arrow. That the arrow become broad indicates the enhancement of C_4 flux.

Fig 5 The association between stomatal density, CO_2 compensation point, precipitation and temperature

.A-E, clade A. (A) The relationship between stomatal density and precipitation. (B) The relationship between CO_2 compensation point and precipitation. (C) The relation between stomatal density and CO_2 compensation point. (D) The relationship between stomatal density and temperature. (E) The relationship between CO_2 compensation point and temperature. F-J, clade B. (F) The relationship between stomatal density and precipitation. (G) The relationship between CO_2 compensation point and precipitation. (H) The relationship between stomatal density and CO_2 compensation point. (I) The relationship between stomatal density and temperature. (J) The relationship between CO_2 compensation point and temperature. The results of linear regression analysis are in supplementary file.

Fig 6. Schematic diagram for the driver process of C_4 evolution.

The schematic diagram for the conditions driving the evolutionary process for C_4 photosynthesis. The raindrop number denotes the precipitation. The thermometer denotes the temperature. The arrows denote the direction of C_4 evolution. The cycle composed by white and black denotes the primary driving element that promote the corresponding stage of C_4 evolution. The primary driving reason in C_3 and proto-Kranz was carbon limitation, and the primary driving reason in the latter stage was water limitation. The black become narrow indicating the carbon limitation was increasing gradually, and the white become narrow indicating the water limitation was increasing gradually.

Supplementary fig 1. The C_4 flux for *Flaveria*.

The data of C_4 flux was from table 1 in (Vogan and Sage 2011)

Supplementary fig 2. Temperature and precipitation of habitat for *Flaveria* species in driest and coldest period.

(A) Precipitation of the driest quarter. (B) Precipitation of the coldest quarter. (C) Precipitation of the driest month. (D) Mean temperature of the driest quarter. (E) Mean temperature of coldest quarter. (F) Min temperature of coldest month. Orange, C_3; Purple, Type I; Yellow, Type II; Green, C_4-like; Blue, C_4. Black lines indicate median. The plants abbreviate genus written.

Supplementary fig 3. Temperature and precipitation of habitat for *Flaveria* species in wettest and warmest period.

(A) Max temperature of warmest month. (B) Precipitation of westest month. (C) Precipitation of warmest quarter. (D) Mean temperature of wettest quarter. Orange indicates C_3, purple indicates Type I, yellow indicates Type II, green indicates C_4-like, blue indicates C_4. Black lines indicate median. The plants abbreviate genus written.

Supplementary fig 4. Annual temperature and precipitation of habitats for *Flaveria* species

(A) Annual mean temperature. (B) Annual precipitation. Orange, C_3; Purple, Type I; Yellow, Type II; Green, C_4-like; Blue, C_4. Black lines indicate median. The plants abbreviate genus written.

Supplementary fig 5. The change trend for the evolution towards C_4 photosynthesis.

(A) Precipitation of wettest quarter. (B) Mean temperature of wettest quarter. (C) Vein density. (D) Distance between vein. (E) O_2 inhibition. (F) CO_2 compensation point. (G) G_{smax} (H) Stomatal density (SD). The plants abbreviate genus written.

Supplementary fig 6. The difference in solar radiation and water vaper pressure between *F. pri* and *F. rob*.

A, The difference in solar radiation between *F.pri* and F.rob. B, The difference in water vaper pressure between *F.pri* and *F.rob*.

Table 1 The habitat for *Flaveria* species

Supplementary file 1: The statistics test and regression analysis

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Author contribution

YYZ and ZGZ designed the study. YXZ and YYZ extracted the climate data and the latitude and longitude data, and YYZ analyzed these climate data. XQY and YYZ mapped and marked the coordinates of *Flaveria* genus location and do the visualization. YYZ extracted the physiological and anatomic data from the previous works. YYZ analyzed all these data, and did the statistic test and regression analysis. YYZ plotted the graphs. YYZ and XGZ wrote the paper.

Competing interests

The authors declare no competing financial interests.

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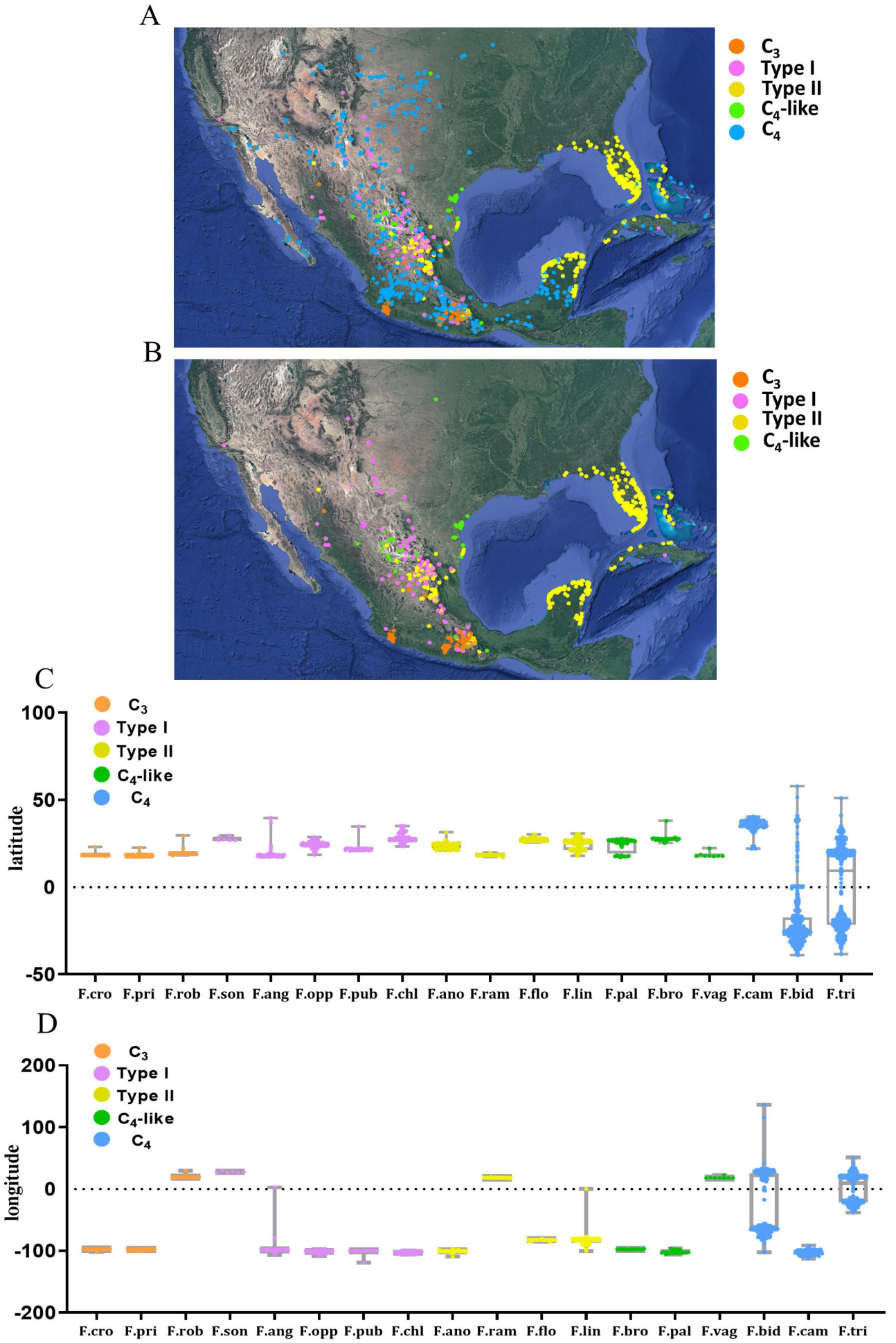
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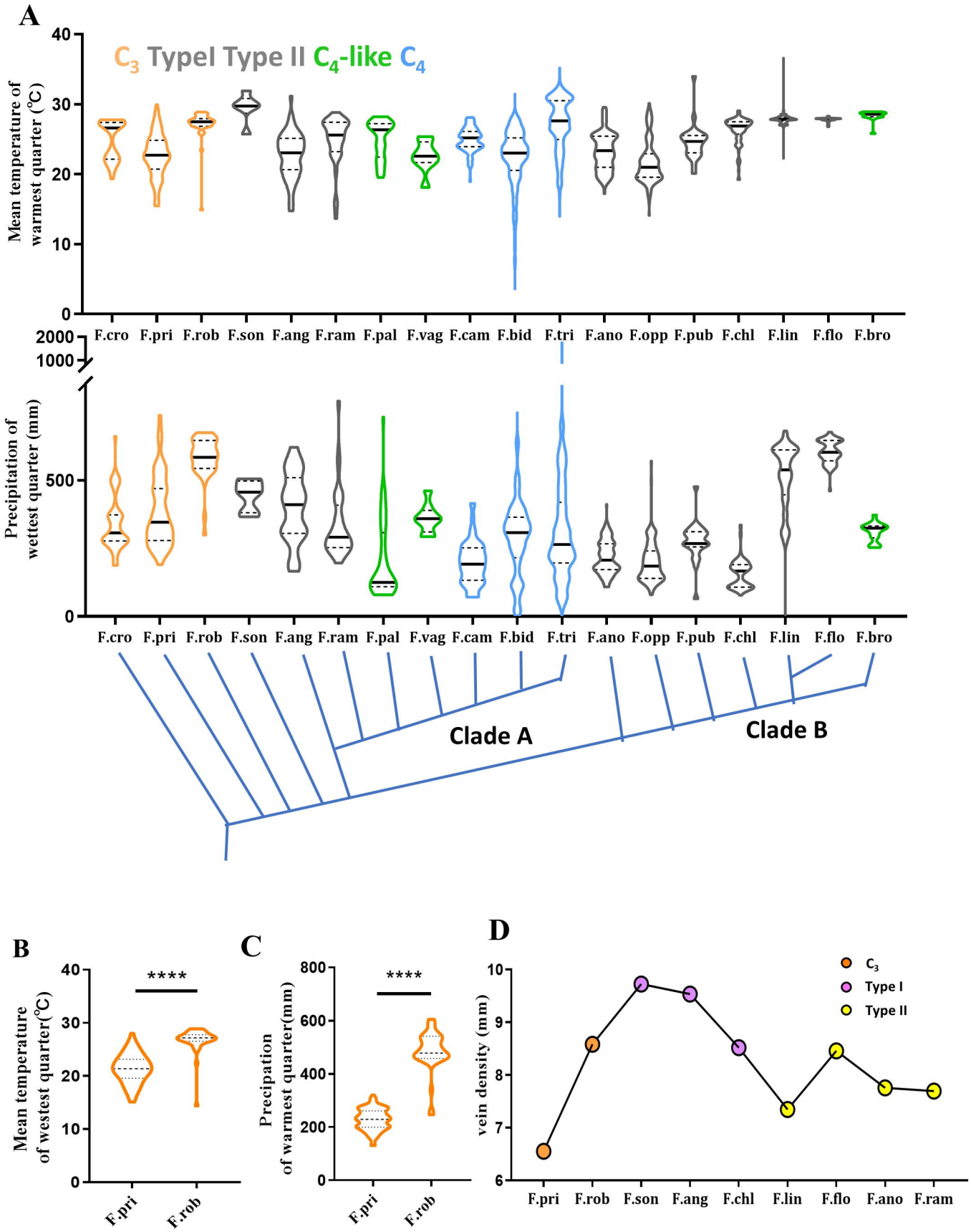
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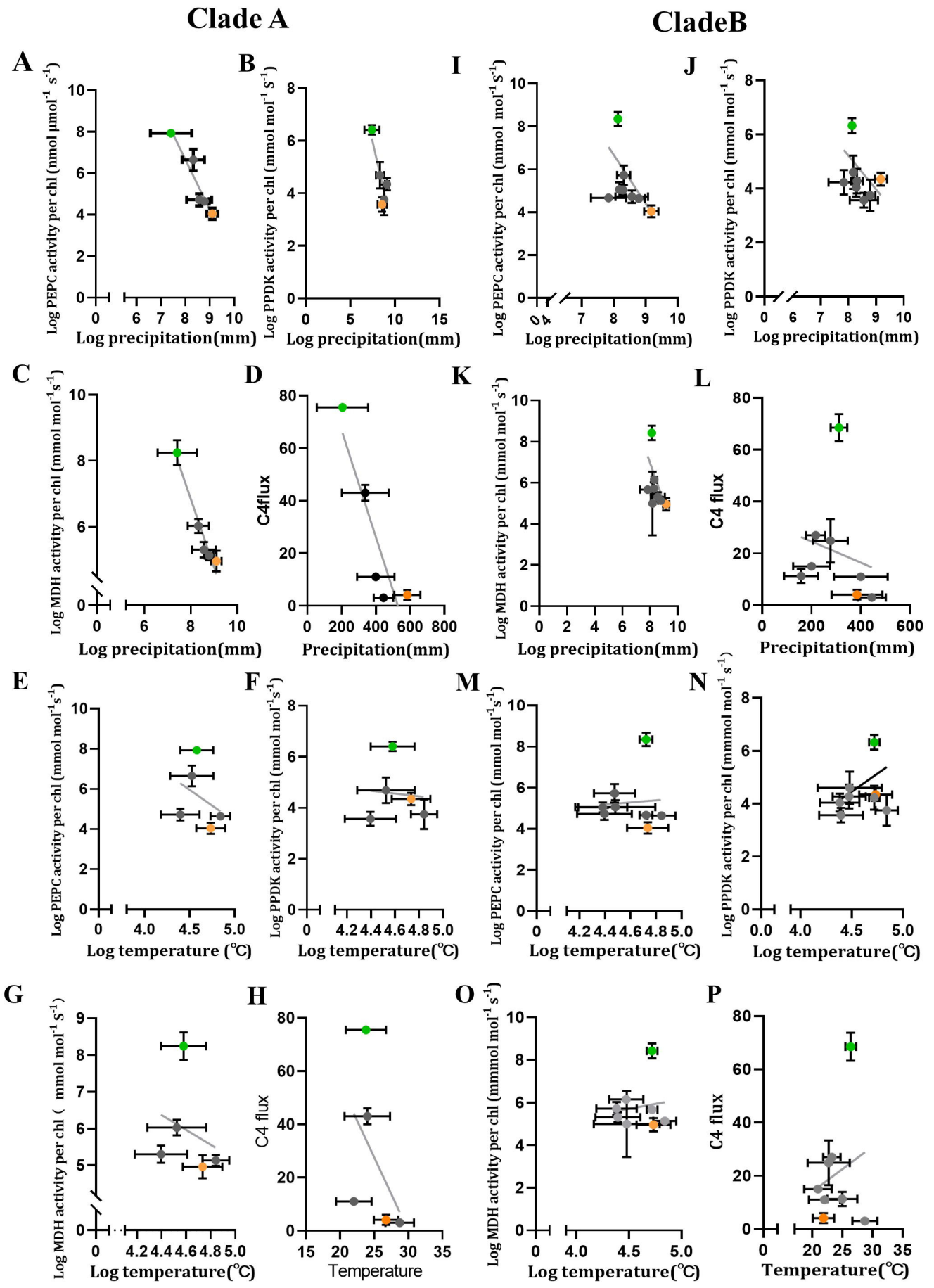
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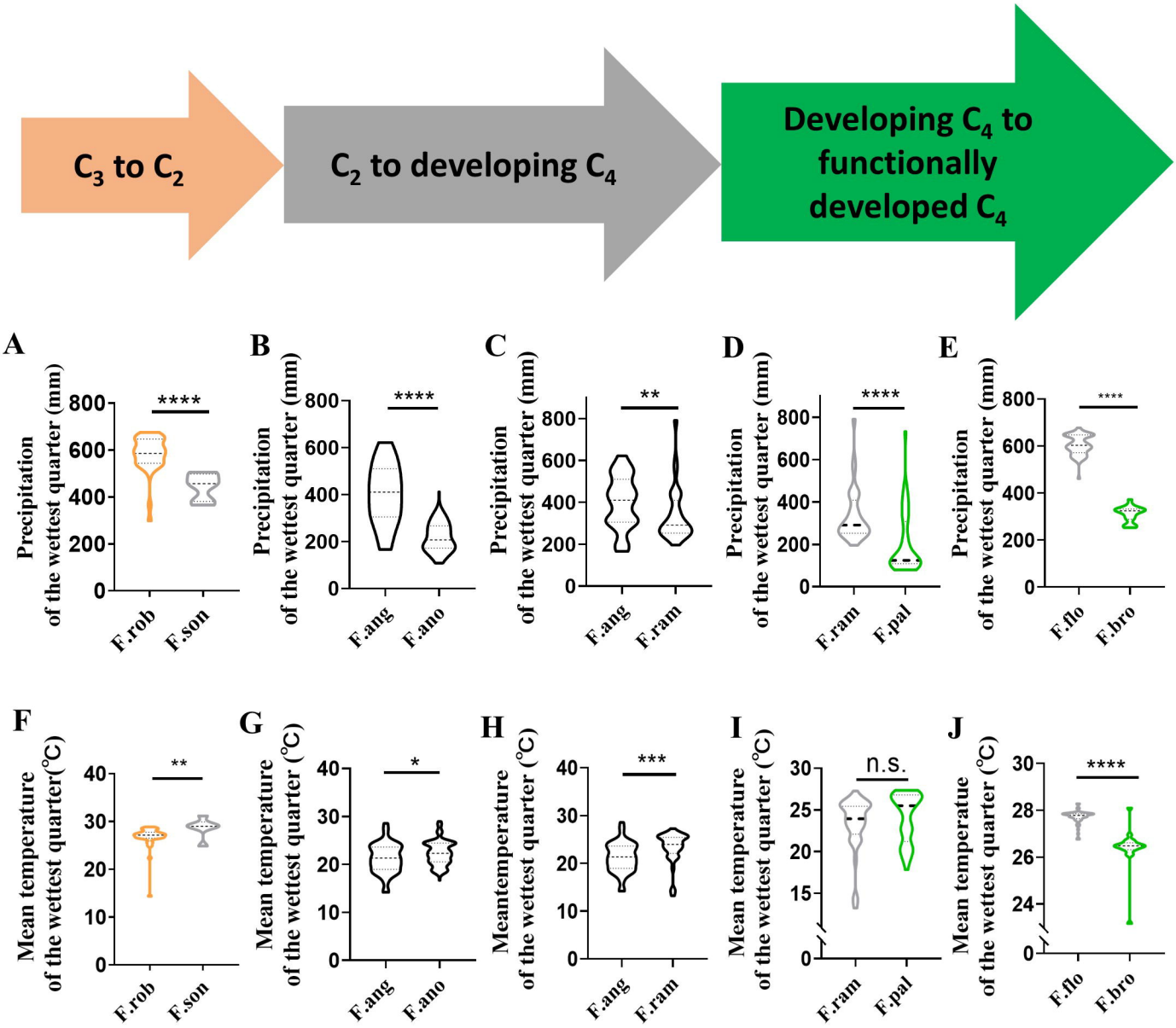
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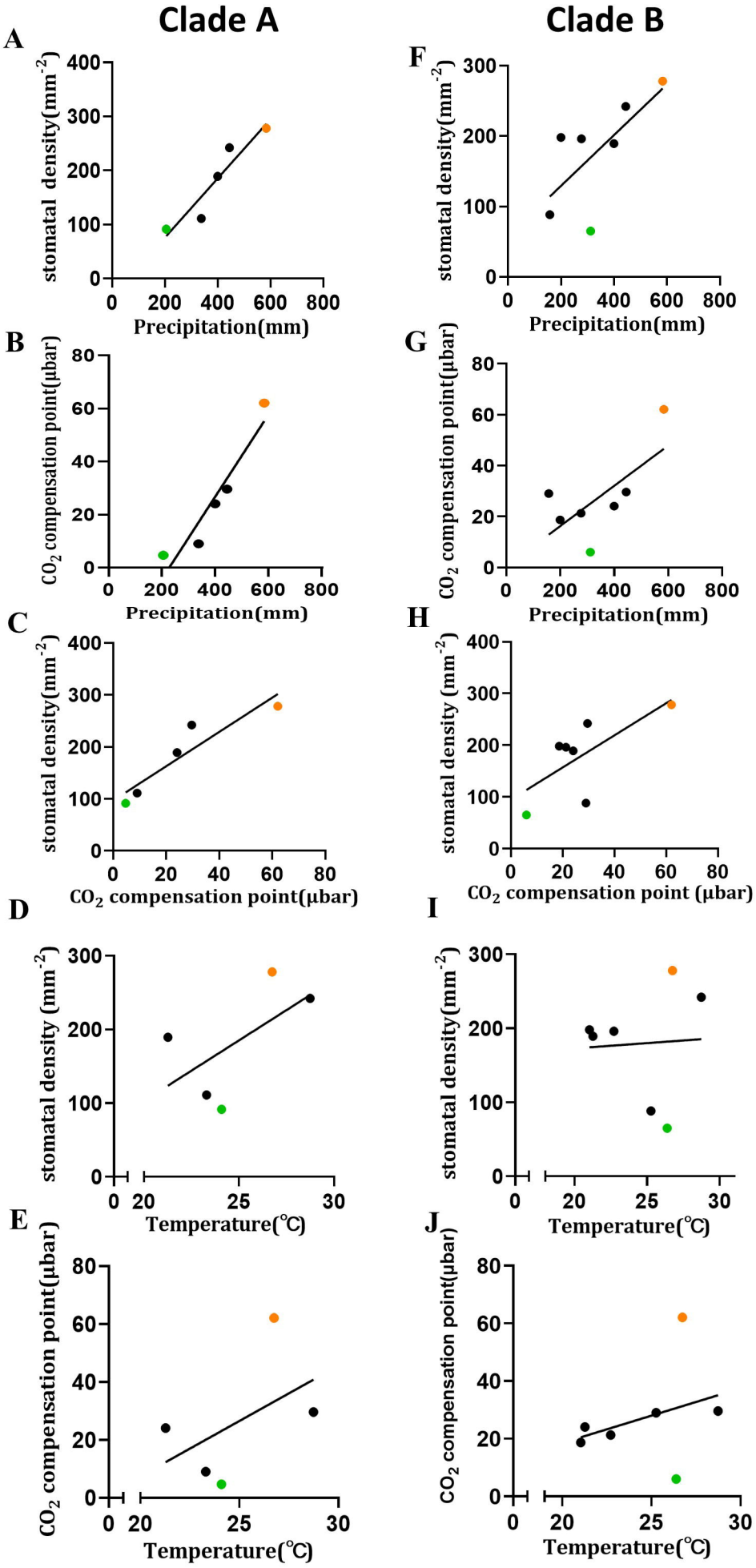
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Clade B
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stomatal density (mm-2)
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stomatal density (mm-2)
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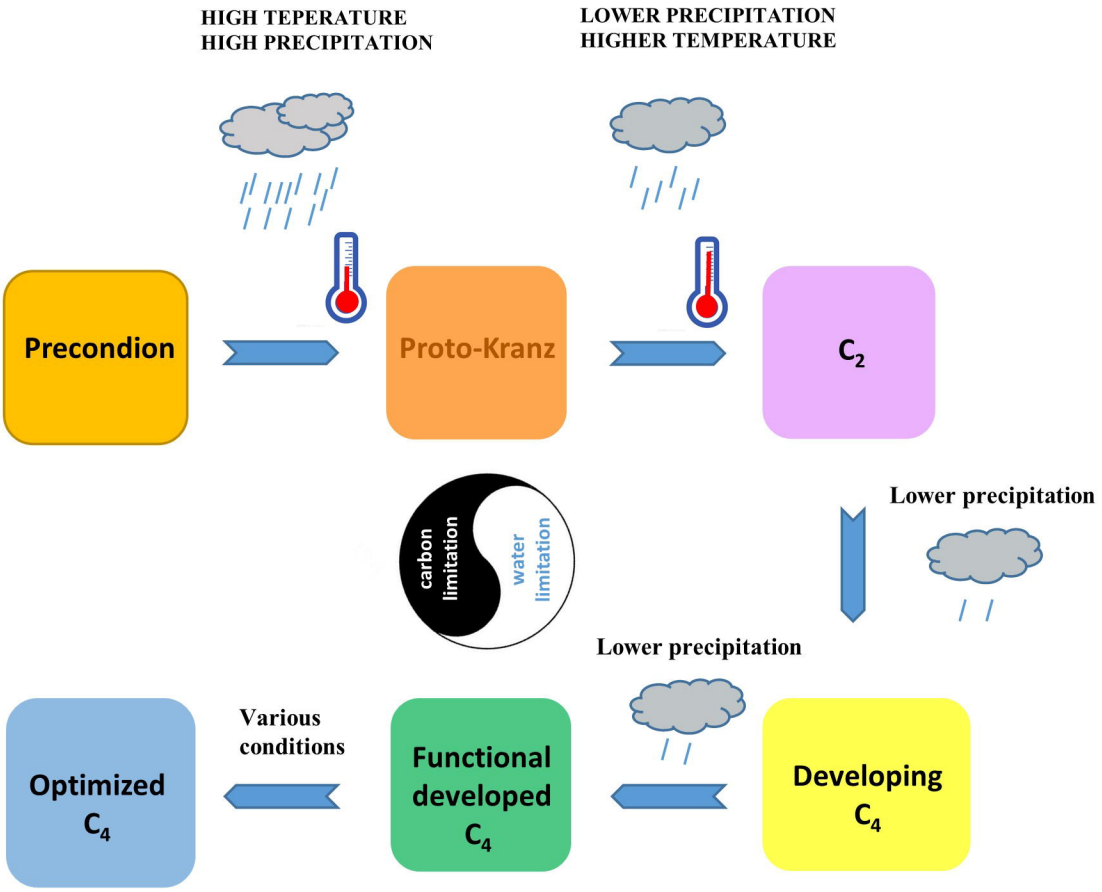
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Lower precipitation
carbon limitation
water limitation
Lower precipitation
Optimized C4
Various conditions
Functional developed C4
Developing C4