Selection during crop diversification involves correlated evolution of the circadian clock and ecophysiological traits in *Brassica rapa*

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Summary

- Crop selection often leads to dramatic morphological diversification, in which allocation to the harvestable component increases. Shifts in allocation are predicted to impact (as well as rely on) physiological traits; yet, little is known about the evolution of gas exchange and related anatomical features during crop diversification.
- In *Brassica rapa*, we tested for physiological differentiation among three crop morphotypes (leaf, turnip, and oilseed) and for correlated evolution of circadian, gas exchange, and phenological traits. We also examined internal and surficial leaf anatomical features and biochemical limits to photosynthesis.
- Crop types differed in gas exchange; oilseed varieties had higher net carbon assimilation and stomatal conductance relative to vegetable types. Phylogenetically independent contrasts indicated correlated evolution between circadian traits and both gas exchange and biomass accumulation; shifts to shorter circadian period (closer to 24 h) between phylogenetic nodes are associated with higher stomatal conductance, lower photosynthetic rate (when CO₂ supply is factored out), and lower biomass accumulation. Crop type differences in gas exchange are also associated with stomatal density, epidermal thickness, numbers of palisade layers, and biochemical limits to photosynthesis.
- *Brassica* crop diversification involves correlated evolution of circadian and physiological traits, which is potentially relevant to understanding mechanistic targets for crop improvement.

Introduction

In his early study, ‘The variation of plants and animals under domestication’, Darwin recognized that studying domesticated plants provided insights into fundamental evolutionary processes (Darwin, 1868). In the process of domestication, a crop species becomes evolutionarily differentiated from a wild progenitor as humans transition from harvesting and stewardship of plants in wild populations to imposing selection on plants in an agricultural ecosystem. This is commonly distinguished from crop diversification, which occurs after domestication as humans select for secondary traits such as crop growth in diverse geographic regions, synchronized timing of life-history events, or enhanced traits, which is potentially relevant to understanding mechanistic targets for crop improvement.

Selection during crop diversification may lead to correlated evolution of multiple traits. Evolution of some traits (e.g. morphological ones) may be functionally dependent on other traits (e.g. physiological ones). During domestication or diversification, improvements in size or in relative allocation to the harvestable component are probably enabled by physiological evolution. For instance, sink strength appears to be greater in oilseed vs vegetable crops within a species, because oil per unit mass is more metabolically expensive to produce than vegetative organs; carbon assimilation is correspondingly higher in oilseed varieties (Evans, 1996; Ruuska et al., 2004; Goffman et al., 2005; Li et al., 2006). A small number of recent metabolomic studies also suggest correlated morphophysiological evolution (Del Carpio et al., 2014; Hennig et al., 2014). Nevertheless, despite the clear mechanistic links in contemporary populations and across species (Wright et al., 2004; Sack et al., 2013) and the potential relevance to crop improvement, examples of joint morphophysiological evolution during crop domestication or diversification are comparatively rare (Cook & Evans, 1983; Smartt, 1988). Simultaneously quantifying multiple morphological, physiological, and
Many developmental features could contribute to physiological diversification among crop types. One well-known regulatory control on physiological traits is the circadian clock. Circadian rhythms are biological oscillations that are c. 24 h in duration and coordinate the diurnal timing of many physiological and metabolic processes in plants. The circadian clock regulates eco-physiological traits (e.g. starch utilization and photosynthesis) in experimental genetic lines such as mutant genotypes that express discrete clock phenotypes that differ from the cognate wild-type (Dodd et al., 2005; Graf et al., 2010; Faure et al., 2012). Quantitative clock variation is also associated with net carbon assimilation and stomatal conductance in segregating progenies of experimental genetic lines such as mutant genotypes that express discrete clock phenotypes that differ from the cognate wild-type (Dodd et al., 2005; Graf et al., 2010; Faure et al., 2012). Quantitative clock variation is also associated with net carbon assimilation and stomatal conductance in segregating progenies of Brassica rapa (Edwards et al., 2009, 2011; Lou et al., 2011), potentially because clock cycles closer to 24 h enhance diurnal timing of gas exchange. During crop diversification, the circadian clock and gas exchange traits may evolve in a correlated manner if gas exchange responds to allele frequency changes at clock loci or if photosynthetic evolution alters sugar status and thereby affects clock function (Dalchau et al., 2011; Moghaddam & Van den Ende, 2013). More proximately (and quite possibly independent of the circadian clock), leaf anatomical features or photosynthetic biochemistry could contribute to crop variation in gas exchange (Kebede et al., 1994).

Crucifers provide some of the most dramatic examples of crop morphological diversification. Reproductive organs are highly altered in some members of Brassica oleracea, such as cauliflower and broccoli (Purugganan & Fuller, 2009). Vegetative features are also highly modified among varieties of both B. oleracea and B. rapa. Distinct B. rapa accessions have undergone selection for increased allocation to above-ground organs (i.e. leaves in cabbage, pak choi, and turnip green crops and seeds in oilseed types) as well as below-ground organs (i.e. enlarged root-like hypocotyl in turnips). Accompanying morphological evolution, we hypothesize that crop types will exhibit physiological diversification as a consequence of differences in overall size, sink strength, and proportionate allocation; the strength of morphophysiological correlations remains as an empirical gap in our understanding of evolution during domestication and diversification, and may inform targets for future crop improvement. Further, past studies provide evidence of genotypic variation in circadian clock period (Lou et al., 2011), but it remains unclear if circadian differences generalize to crop morphotype or if circadian phenotypes correlate with physiological traits over evolutionary time, which may again inform breeding for crop improvement.

Our overall aim was to test if the morphological variation arising from selection for different crop types is associated with differences in circadian, leaf gas exchange, and anatomical traits in different B. rapa accessions. With this study we accomplished the following specific aims: we first tested whether genotypic differences in circadian period generalize to crop type; we then explored if crop types differ in physiological (gas exchange) traits and, if so, whether gas exchange covaries phylogenetically with circadian period. Further, we tested the related gas exchange hypothesis in the crops: the rate of photosynthetic carbon assimilation ($A$) is limited by gas supply of CO$_2$ ($g$) and biochemical demand for CO$_2$; demand is, in turn, regulated by light-dependent and light-independent processes occurring in the mesophyll, which are either limited by light harvesting (ribulose-1,5-bisphosphate (RuBP) regeneration) or by sugar metabolism through CO$_2$ assimilation ($V_{\text{max}}$, maximum carboxylation capacity of Rubisco) and triose phosphate utilization. Finally, we examine leaf anatomical features (stomatal density and internal leaf features) that may contribute to physiological diversification. We explore how stomatal density and thickness of epidermis might contribute to CO$_2$ supply and resistance differences, and how palisade structure could affect light absorption, including electron transport and carbon fixation.

**Materials and Methods**

**Study system**

Brassica rapa L. (Brassicaceae) is an internationally important crop, and distinct subspecies are cultivated as oilseed crops (ssp. oleifera), leaf vegetable crops (ssp. chinensis and pekinensis), and root vegetable crops (subsp. rapa). Accessions used here reflect the global cultivation of the species; accession origin and germplasm identifiers are presented in Supporting Information Table S1.

**Circadian measures**

In all, 41 accessions were screened for circadian period (Table S1). Some accessions were screened previously by Lou et al. (2011). Here, previously published data, data for additional replicates of accessions within the original panel, and data on additional accessions are analyzed to test for circadian differences among accessions that generalize to crop type; within each accession, between eight and 15 replicates were screened. For details of the circadian screens, which used leaf movement as a proxy to estimate circadian period, see Lou et al. (2011) as well as reviews of this method (McClung, 2006; Greenham et al., 2015).

**Leaf gas exchange measurements**

In addition to differentiation of the circadian clock, we were interested in whether crop types differ in gas exchange, and whether there is evidence of correlated evolution between circadian and gas exchange traits. A subset of crop accessions (Table S2) was sampled for physiological and leaf anatomical traits. For gas exchange experiments, nine to 10 replicates of 21 representative genotypes were grown from seed in growth chambers (PGC-9/2 with Percival Advanced Intellus Environmental Controller, Percival Scientific, Perry, IN, USA). Seeds were planted into 6 × 6 × 9 cm plastic pots filled with Redi-Earth potting mix (Sunshine Redi-Earth Professional Growing Mix, Sun...
Gro Horticulture, Bellevue, WA, USA) and including a slow-release fertilizer (Scotts brand Osmacote Controlled Release Classic, NPK; Scotts, Marysville, OH, USA). Three seeds were planted in each pot, and then covered with vermiculite and saturated with water. Plants were placed in the growth chamber with following conditions: photoperiod of 15 : 9 h, 24 : 22°C, light : dark cycle, and photosynthetic photon flux density = 500 μmol photons m⁻² s⁻¹. Plants were thinned to one seedling per pot after cotyledons had expanded (c. after 5 d). At subjective dawn, plants were watered daily to retain consistently high soil moisture.

Gas exchange measurements were taken using a Li-Cor LI-6400 XT portable infrared gas analyzer with leaf chamber fluorometer (Open System Vers. 4.0, Li-Cor Inc., Lincoln, NE, USA). Measured traits were maximum photosynthetic rate (Amax) and stomata conductance (g). Measurements were taken from the third fully developed true leaf at least 1 h after subjective dawn under the following chamber conditions: photosynthetic photon flux density (PPFD) = 1500 μmol m⁻² s⁻¹, ref [CO₂] = 400 μmol m⁻² s⁻¹, Tleaf = 24°C, and vapor pressure deficit based on leaf temperature (VPDL, kPa) was kept between 1.3 and 1.7 kPa (Long & Bernacchi, 2003). Measurements were taken 3–5 min after all chamber conditions stabilized.

A–Cᵣ response curves

A–Cᵣ response curves were used to estimate biochemical limits to photosynthesis, including the maximum rate of RuBP carboxylation (Vcmax) and the maximum rate of electron transport driving RuBP regeneration (Jmax). In further regard to Jmax, RuBP regeneration is controlled by the amount and activity of Calvin cycle enzymes as well as NADP and ATP pool sizes, while NADP and ATP are supplied by the light-dependent reactions and are thus limited by electron transport between the two photosystems.

Response curves were performed on the same leaf as the single time point measurement again using six Li-Cor 6400 XT portable photosynthesis systems within a 5 d period for the growth chamber experiment. Because of the time-consuming nature of A–Ci phenotyping (in which measurement of one replicate can take 1–2 h and measurements can only be taken during an approx. 2 h time window daily) and the fact that all replicates should be measured at a similar, preflowering stage, A–Ci curves were collected only on three to four replicates within each of four genotypes for each of the three crop types (n = 44). Chamber conditions were maintained at PPFD = 1500 μmol m⁻² s⁻¹, Tleaf = 25°C and VPDL between 1.3 and 1.7 kPa. Reference CO₂ concentration in the chamber was controlled with a CO₂ mixer across the series of 400, 500, 600, 800, 1000, 1250, 1500, 2000, 400, 300, 200, 100 and 50 μmol m⁻² s⁻¹ and measurements were recorded after equilibration to a steady state (5–7 min). The utility tool A/Cᵣ Response Curve Fitting 10.0 available at http://landflux.org/Tools.php, which is based on the equations of Farquhar et al. (1980) and an approach developed by Ethier & Livingston (2004), was used to estimate Vcmax and Jmax for each A–Ci curve.

Leaf morphological measures

Stomatal density was measured in a subset of plants on which gas exchange data were collected. The fourth leaf was chosen for stomatal measurements; this leaf was fully expanded and was developmentally close to the third leaf, which was used for gas exchange measurements. Each leaf was first preserved in formalin-aceto-alcohol (FAA) solution (ratios of ethanol, ethanoic acid, and formalin were, by volume, 18 : 1 : 1), following several ethanol washes over the course of 1 wk until the leaf tissue became transparent. An aqueous solution of toluidine blue O (0.05%) was used to stain the tissue for 30–60 s depending on the thickness of the leaves. Light micrographs were taken using an Olympus BH2 compound light microscope at a magnification of ×500. Within each leaf sample, stomata were counted visually in five to six areas chosen randomly. Five to six squares of dimensions 200 × 200 μM were used to count stomata visually. The range number of stomata per square was 10–35. The software program, Image J (Schneider et al., 2012), was used to calculate stomatal density, that is, the number of stomata counted within an image area. In total, measurements were taken from five to six images within each of six to eight replicates in each of 15 genotypes (equally representing the three crop types) (n = 639).

Leaf anatomical measures

Tissue samples were fixed in FAA and stored in 70% ethanol. Subsequently, leaves were dehydrated in a standard ethanol gradient. Ethanol was replaced with Histoclear, and the tissue was slowly infiltrated with paraffin at 60°C (Fowke & Rennie, 1995). Samples were embedded in paraffin, serially sectioned at 10 μM, and stained with toluidine blue O (Sakai, 1973). Light micrographs were captured using a Zeiss Primovert compound light microscope and Zeiss camera and AxioVision software (AxioVision Rel 4.9.1; Carl Zeiss Imaging Solutions). The transition from spongy mesophyll to palisade parenchyma was delineated by drawing a straight line through the center of multiple vascular bundles. All measurements were collected from a 1000 μM long area of tissue in Photoshop CS4. Internal leaf anatomy was likewise measured in a subset of samples including two to five replicates in each of nine genotypes (equally representing the three crop types) (n = 24), owing to the extensive time needed to clear, fix, embed, section, and analyze histological features.

Plant harvest, leaf area, nitrogen content

After 5 wk of growth, plants were carefully removed from pots, and roots and shoots were washed to remove soil. One leaf per plant was scanned using the Epson Perfection V500 software at a resolution of 150 dpi (Epson, Nagano, Japan). The software program IMAGEJ was used to calculate leaf area (Schneider et al., 2012). Plants were cut at soil level, and the single collected leaf, the remaining above-ground portion, and the below-ground portion were dried at 65°C for 72 h. DW was then measured. The oven-dried leaves were ground and analyzed for δ¹⁵N, δ¹³C, leaf
nitrogen concentration (wt% N), leaf carbon concentration (wt% C) and C:N ratio using an elemental analyzer (ECS 4010, Costech Analytical Technologies Inc., Valencia, CA, USA) coupled to a continuous-flow inlet isotope ratio mass spectrometer (CF-IRMS; Delta-plus XP, Thermo Scientific, Waltham, MA, USA). N data were collected on seven to eight replicates within each of 12 genotypes equally representing the three crop types.

Data analysis

Statistical approach and data treatments We used ANOVA with post hoc Tukey tests to identify significant crop type and genotype within crop type effects (PROC GLM, SAS v.9.4., SAS Institute Inc., Cary, NC, USA). We observed a small number of outlier genotypes within crop types for circadian period. Notably, our assignment of ‘crop type’ and history of selection was based on germplasm annotation, but our observation of morphology and phenology suggested that some genotypic outliers might have been better assigned to a different crop type. For instance, in the group of oilseeds, the genotype WO-024 had an enlarged below-ground storage structure by midseason reminiscent of a turnip, and had a period phenotype that resembled other vegetable rather than oilseed types (Fig. S1). Likewise, several turnips had reduced vernalization requirements similar to oilseeds, and showed period lengths closer to the average oilseed genotype. We therefore tested for genotype (crop type) and crop type effects either using the germplasm designation to assign genotypes to a crop type or using phenotypic similarity to assign genotypes to crop type. Results were not affected by these differences in assignment, and we therefore present the more conservative results using the germplasm designation (Table S1).

We used linear regression to test for associations between quantitative clock variation and gas exchange traits, biomass accumulation, and flowering time. We used both unadjusted values of gas exchange in these analyses as well as \( A_{\text{max}} \) values adjusted for CO\textsubscript{2} supply limitation and \( g_s \) values adjusted for biochemical feedbacks on stomatal behavior. More specifically, we performed the genotypic regression of \( A_{\text{max}} = g_s \) to generate residuals and the variable \( rA_{\text{max}} \#. \( rA_{\text{max}} \) thus reflects variation in carbon assimilation after CO\textsubscript{2} supply limitation is considered. The variable \( rg_s \) reflects variation in stomatal behavior after biochemical demand is considered.

Phylogenetic analysis and phylogenetically independent contrasts To reconstruct a phylogeny, we used amplified fragment length polymorphism data from a previously published study (Zhao et al., 2005), which consisted of 527 aligned characters from 21 accessions representing three crop types (seven oilseeds, six leaf crops, and eight turnips; Table S1). Phylogeny reconstruction was carried out using a parsimony optimality criterion in PAUP\textsuperscript* 4.0b10 (Swofford, 2002). In all parsimony analyses, heuristic searches were conducted using 1000 random addition replicates and tree bisection and reconnection (TBR) branch swapping, saving 100 trees per replicate. All most parsimonious trees were stored, saving branch lengths, which were used for phylogenetically independent contrasts (PICs). Bootstrap analyses (1000 replicates) (Felsenstein, 1985) were used to assess branch support employing a heuristic search with TBR branch swapping, with 10 random additions per replicate, saving no more than 100 trees per replicate.

To calculate correlated changes in traits using a phylogenetic framework, we calculated PICs based on the most parsimonious trees resulting from phylogeny reconstruction. The PIC approach is a comparative one that assumes a Brownian motion model of evolution to test for evolutionary correlations of traits after explicitly accounting for phylogenetic relatedness. All PICs were carried out using the PDAP module in MESQUITE (Midford et al., 2005). The two most parsimonious trees with stored branch lengths (see the Results section) were used to compute contrasts. Before carrying out analyses, we checked diagnostic plots of the absolute values of the standardized PICs vs their standard deviations using the most parsimonious trees to determine whether the branch lengths of the phylogenetic tree adequately fitted the tip data (Garland et al., 1992; Diaz-Urriarte & Garland, 1996, 1998); because branch lengths were adequately standardized for all characters on both trees, these analyses are not discussed further. We used PICs to carry out two different types of analyses. First, we calculated an independent contrast between circadian period and each gas exchange and allocation trait as well as flowering time, all of which are continuous traits, to understand patterns of correlated evolution between these traits. Second, we used PICs to assess whether shifts in crop type (which is a categorical trait) were associated with significant shifts in gas exchange and allocation traits following Whitall & Hodges (2007). We calculated contrasts and then used a paired \( t \)-test to calculate whether contrasts resulting from a shift in crop type were significantly different from contrasts between nodes of the same crop type. We also repeated these analyses with all most-parsimonious trees resulting from phylogeny reconstruction and using trees that varied in rooting to assess how tree topology and rooting affected the results of the PICs. Because all results were consistent regardless of variation in topology and rooting, these analyses are not discussed further.

Results

Using ANOVA, we found significant effects of genotype (crop type) on circadian period (Tables S1, S3a). We further found a significant effect of crop type on circadian period duration (Fig. 1; Table S3a). In particular, we observed that oilseeds have circadian cycles c. 1 h shorter on average and closer to 24 h than those of leaf crops or turnips (Fig. 1).

Analysis of gas exchange data showed that crop types also differ significantly in both maximum net photosynthesis rate (\( A_{\text{max}} \)) and stomatal conductance (\( g_s \)), such that oilseeds have higher values of both traits relative to leaf crops and turnips (Fig. 2a,b; Table S3b,c). A strong positive association was observed between \( A_{\text{max}} \) and \( g_s \) (Fig. 3a). No significant correlations were observed between unadjusted gas exchange values and circadian period lengths (data not shown). However, \( rA_{\text{max}} \) (which accounts for genotypic variation in stomatal conductance and CO\textsubscript{2} supply) and circadian period were positively correlated (Fig. 3b). \( rg \)
(which accounts for biochemical feedbacks on stomatal behavior) and circadian period were negatively correlated (Fig. 3c). Thus, circadian period is related to leaf gas exchange once gas supply or demand is statistically considered. Biomass was positively associated with circadian period (Fig. 3d), suggesting that the high \( r_{A_{\text{max}}} \) of the leaf crops and turnips (genotypes with long circadian period; Fig. 3b) is important to determining mass or simply that their preferential allocation of fixed carbon to vegetative organs rather than to metabolically expensive seed oil affects total biomass accumulation. No association was observed between circadian period and the phenological trait, flowering time (data not shown).

The significant association between circadian period and both gas exchange parameters and biomass (Fig. 3) can be explained either by inheritance of a combination of traits from a common ancestor (e.g. an oilseed ancestor that had both high gas exchange values and short 24 h circadian cycles) or by selection for correlated evolution of these two traits. Using PICs to assess correlated evolution while taking phylogenetic relationships into account, we observed significant correlations between circadian period and \( r_{A_{\text{max}}} \) \((r = 0.53, P = 0.02)\) and total biomass \((r = 0.47, P = 0.05)\) and a marginally significant correlation between period and \( r_{g_s} \) \((r = -0.45, P = 0.06)\), as illustrated in Fig. 3. These results suggest that observed correlations are significant not because phylogenetic groups have a common inherited relationship between these traits, but because evolution in circadian rhythms is accompanied by a corresponding response in the other traits regardless of phylogenetic relationships. In the second analysis, we used PICs to assess whether shifts in crop type (which is a categorical trait) between phylogenetic nodes were associated with shifts in gas exchange and allocation traits, but we did not find any significant association between crop diversification and evolution of these traits. This may reflect an absence of trait evolution attributable to independent crop diversification events, or simply that there is a limited number of discrete transitions between nodes (crops) on the phylogeny and therefore low power to detect traits associated with these transitions.

We were interested to further explore the source of genotypic differences in gas exchange parameters, \( A_{\text{max}} \) and \( g_s \). In order to explain limitations on photosynthetic rates, we measured the response of photosynthesis to changes in intercellular mole fractions of CO\(_2\) \((A–C_i)\) curves in a subset of genotypes using the Farquhar–von Caemmerer–Berry biochemical model of photosynthesis. Fig. 4(a) shows the \( A–C_i \) curve in three crop types of \( B.\ rapa \) and two parameters important to photosynthetic performance, including the initial slope (which denotes the carboxylation-limited region where CO\(_2\) supply limits the activity of Rubisco while RuBP is not limiting) as well as the plateau (which is the RuBP-limited region where CO\(_2\) no longer limits the carboxylation reaction, but availability of RuBP limits the activity of Rubisco). Carboxylation-limited and RuBP-limited regions appear qualitatively similar in turnips and leaf crops, while
oilseed replicates appear to differ from the vegetable crop genotypes in both features. Analysis of the curves indicates that \( V_{\text{cmax}} \), the maximum Rubisco carboxylation rate, is in fact significantly higher in oilseeds relative to leaf crops and tends to be higher in oilseeds relative to turnips (Fig. 4b; Table S3d). The values of \( J_{\text{max}} \), the maximum electron transport rate driving RuBP regeneration, and \( V_{\text{cmax}} \) are significantly correlated \((R^2 = 0.55, P < 0.001)\). Most of the points fall close to a straight line, and the slope of the fitted regression line equals 1.33, indicating that an increase in intracellular CO\(_2\) more favorably affects RuBP regeneration than carboxylation rate. Notably, the ratio of \( J_{\text{max}} \) : \( V_{\text{cmax}} \) is significantly higher in leaf crops than oilseed types or turnips (Fig. 4c; Table S3e). The high \( J_{\text{max}} \) : \( V_{\text{cmax}} \) ratio indicates more allocation to light harvesting (increased electron transport and RuBP regeneration) in leaf crops, with commensurately higher commitment to photosynthetic carbon metabolism (carboxylation) in oilseeds. A strong negative association is observed between leaf area and percentage of nitrogen (Fig. 4d), possibly indicating higher Rubisco concentrations in the smaller oilseed leaves.

Differences in CO\(_2\) supply could provide an additional explanation for higher carbon assimilation in oilseeds, that is, in addition to more efficient photosynthetic biochemistry (as indicated by \( V_{\text{cmax}} \) and \( J_{\text{max}} \) earlier), oilseeds may have higher CO\(_2\) supply as a result of leaf morphological features. The oilseed genotypes we sampled in fact have 40% more stomata than vegetable genotypes (Fig. 5a–c; Table S3f), and stomatal density is positively correlated with stomatal conductance, \( g_s \) \((r = 0.48, P < 0.001)\).

Further, internal leaf anatomical features, such as a thinner adaxial epidermis (Fig. 6a; Table S3g), probably improve gas supply in oilseeds. Oilseeds also tend to have thicker mesophyll and more palisade layers (Fig. 6b,c; Table S3h). Thicker palisade mesophyll is one mechanism to achieve a high \( A_{\text{max}} \), because additional palisade cells provide more space for chloroplasts, light harvesting, and photosynthetic biochemistry. No crop type differences in abaxial epidermis were observed.

Discussion

In the present study, we examined hypotheses related to correlated morphophysiological evolution. To test for correlated trait evolution, we measured circadian rhythms, gas exchange, photosynthetic biochemistry proxies, and leaf morphological and anatomical features among three morphologically diverse crop types of \( B. \) rapa with past selection for different harvestable components (seed oil, tubers, and leaves). Differences in crop morphology that relate to size, sink strength, and/or allocation were associated with carbon fixation and stomatal conductance and with leaf histological features that affect these gas exchange traits, suggesting physiological evolution during crop diversification and thus filling an important empirical knowledge gap. As a novel component to crop diversification, the circadian clock also exhibited correlated evolution with gas exchange traits. This correlated evolution may reflect a response to selection during diversification, in which crop varieties (or possibly just individual genotypes) with greater sink strength, such as oilseeds, are selected for higher carbon fixation and a circadian period closer to 24 h enables higher photosynthesis. We further identified leaf-level biochemical features that contribute to genotypic differences in gas exchange. Later, we discuss the results in the light of previous studies examining phenotypic outputs of the clock and diversification of other crop species.

Circadian clock associations with physiological processes in crop morphotypes

Translational research has examined clock function and the \( Arabidopsis thaliana \) clock model in crop plants. In several cases,
orthologs of *A. thaliana* circadian genes with conserved function are found in crop species, including *Oryza* (Murakami et al., 2003), *Poplar* (Takata et al., 2009), and *B. rapa* (Murakami et al., 2003; Takata et al., 2009; Kim et al., 2012; Lou et al., 2012; Xie et al., 2015). Clock outputs documented in *A. thaliana* are also observed in crop species. For instance, in a single genotype of a legume, *Phaseolus vulgaris*, the circadian clock regulates photosynthesis and stomatal conductance (Fredeen et al., 1991; Hennessy & Field, 1991; Hennessey et al., 1993). Clock outputs documented in *A. thaliana* are also observed in crop species. For instance, in a single genotype of a legume, *Phaseolus vulgaris*, the circadian clock regulates photosynthesis and stomatal conductance (Fredeen et al., 1991; Hennessy & Field, 1991; Hennessey et al., 1993). Clock outputs documented in *A. thaliana* are also observed in crop species. For instance, in a single genotype of a legume, *Phaseolus vulgaris*, the circadian clock regulates photosynthesis and stomatal conductance (Fredeen et al., 1991; Hennessy & Field, 1991; Hennessey et al., 1993). Clock outputs documented in *A. thaliana* are also observed in crop species. For instance, in a single genotype of a legume, *Phaseolus vulgaris*, the circadian clock regulates photosynthesis and stomatal conductance (Fredeen et al., 1991; Hennessy & Field, 1991; Hennessey et al., 1993). Clock outputs documented in *A. thaliana* are also observed in crop species. For instance, in a single genotype of a legume, *Phaseolus vulgaris*, the circadian clock regulates photosynthesis and stomatal conductance (Fredeen et al., 1991; Hennessy & Field, 1991; Hennessey et al., 1993).

Here, we show that crop types of *B. rapa* differ significantly in circadian period. The mean period for the oilseed crop type was $c. 24$ h, while the vegetable types have longer circadian cycles of $25$ h, on average, for leaf crops and nearly $26$ h for turnips (Fig. 1). In combination with circadian clock differences, we observed a significant crop type effect on both $A_{\text{max}}$ and $g_s$ (Fig. 2), such that oilseeds have the highest photosynthetic rates while the leaf crops have the lowest. While clock period was not correlated with unadjusted values of gas exchange, we found that circadian period and stomatal conductance were negatively correlated after statistically factoring out differences in biochemical demand, as oilseeds with shorter circadian periods near $24$ h had higher $g_s$ (Fig. 3c). After statistically accounting for genotypic variation in CO$_2$ supply, circadian period and $rA_{\text{max}}$ were positively correlated; this suggests that long-period vegetable types have higher $rA_{\text{max}}$ than oilseeds when gas supply is factored out (Fig. 3b). Although any mechanistic connection to the clock remains to be explained, the higher adjusted values of $rA_{\text{max}}$ in

Fig. 4 Response of photosynthesis to internal CO$_2$ concentrations at $25^\circ$C and light saturation. (a) Representative CO$_2$ response curves of photosynthesis at $25^\circ$C for three crop types of *Brassica rapa*. (b) Differences in Rubisco carboxylation rate ($V_{\text{cmax}}$) ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$) between different crop types at $25^\circ$C. (c) Differences in the ratio of the maximum electron transport rate driving ribulose-1,5-bisphosphate (RuBP) regeneration ($V_{\text{max}}$) to the maximum RuBP carboxylation rate ($V_{\text{cmax}}$) at $25^\circ$C. Error bars indicate $\pm$ 1SE. Different letters indicate statistically significant differences among crop types ($P < 0.05$). (d) Association between percentage nitrogen per unit leaf mass as a function of leaf area. Each symbol represents a genotype; crop types are coded by color. The line represents the following relationship: $r = -0.44$ ($R^2 = 0.19$), $P = 0.0007$. 

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leaf crops relative to oilseeds may be explained, in part, by their higher $J_{\text{max}}/V_{\text{cmax}}$; leaf crops may be more biochemically efficient in light harvesting and electron transport, while oilseeds may allocate photosynthetic proteins preferentially to carbohydrate metabolism to support metabolically expensive oil production (see later). Differences in carbon allocation and associated feedbacks on photosynthesis (Farrar & Jones, 2000; Paul & Pellny, 2003) could also contribute to higher adjusted $rA_{\text{max}}$ in vegetable types. Long-period vegetable crops also showed higher amounts of total biomass accumulation than oilseeds in our study (Fig. 3d), suggesting that photosynthetic biochemistry of the former genotypes, allocation feedbacks on photosynthesis, and possibly allocation differences (e.g. to vegetative organs rather than metabolically expensive, low-mass seed oil) may outweigh their reduced CO$_2$ supply in determining vegetative growth.

The observed association between period and adjusted $rg$ is consistent with functional hypotheses regarding clock function, in which period lengths close to 24 h are predicted to increase gas
exchange by providing a match to the duration of daily cycles. The association between period and adjusted $A_{\text{max}}$ as well as vegetative biomass, in which cycles $> 24$ h correlate with increased values of these two traits, requires further mechanistic investigation; interestingly, vegetable crops of *B. oleracea* likewise exhibit longer period lengths than floral crops (A. Millar, pers. comm.). Regardless of the exact mechanistic relationships, the results provide a novel demonstration of correlated evolution between the clock and gas exchange, which is potentially relevant to understanding the developmental origin of crop differences in physiological traits as well as developmental targets for crop improvement.

Gas exchange, leaf biochemistry, and yield

Selection for oil production is associated with higher gas exchange rates in multiple oil crops. For instance, photosynthesis affects oil production in rapeseed, *B. napus*, by participating in lipid metabolism and serving as a source of NADPH and ATP (Ruuksa *et al.*, 2004; Goffman *et al.*, 2005; Li *et al.*, 2006). Cultivars of short-season soybean (*Glycine max*) show improvement of oil yield coupled with an increase in photosynthetic rates and stomatal conductance (Morrison *et al.*, 1999; Cober *et al.*, 2005). Oil production in *Helianthus annuus* is likewise positively correlated with leaf area, which is a proxy for photosynthetic rates in some species (Hervé *et al.*, 2001). Here, higher gas exchange is observed in oilseed relative to vegetable crops, and, specifically, high values of $g_{\text{m}}$ in oilseeds support high values of $A_{\text{max}}$ (Fig. 3a). In all of these species, high photosynthetic rates in oilseeds might be attributable to increased sink strength, because metabolically expensive oil production drives high carbohydrate demand.

The main biochemical processes that contribute to photosynthetic performance in *C_{3}* plants are RuBP carboxylation rates ($V_{\text{cmax}}$) and how rapidly sugars are thus produced, as well as RuBP regeneration (expressed as $j_{\text{m}}$) (Sage, 1994). The change in $j_{\text{m}} : V_{\text{cmax}}$ is correspondingly explained as a change in the allocation of photosynthetic proteins to light harvesting or to sugar production. Among *B. rapa* crop types, we observe that $V_{\text{cmax}}$ values are highest among oilseeds while $j_{\text{m}} : V_{\text{cmax}}$ ratios are higher among leaf crops than among either oilseeds or turnips. Combined with their high CO$_2$ supply, oilseeds of *B. rapa* seem to have more favorable allocation of these proteins, which leads to higher unadjusted values of $A_{\text{max}}$ (Fig 2b). When CO$_2$ supply is factored out, high $j_{\text{m}} : V_{\text{cmax}}$ values (in leaf crops) and possible feedback from allocation (in both leaf and root crops) may enhance adjusted values of $rA_{\text{max}}$ in vegetable relative to oilseed crops (Fig. 3b).

Patterns of carbohydrate production may be relevant to crop diversification for different targets of harvest. The product of carboxylation of RuBP consists of two molecules of 3-phosphoglycerate, which is the precursor to a range of carbohydrates that are stored and transported by other pathways. As described earlier, oilseeds probably require carbohydrates as a source of energy for oil production, which corresponds with our result of higher $V_{\text{cmax}}$ values in this crop type (Fig. 4b). Variation in $V_{\text{cmax}}$ can result from changes in Rubisco protein abundance or its activation status, or a combination of both (Quick *et al.*, 1991; Baker *et al.*, 1997). Lower $V_{\text{cmax}}$ rates are often explained by lower amounts of Rubisco (Jacob *et al.*, 1995; Nakano *et al.*, 1997; Tissue *et al.*, 1999) and sometimes by a low activation state (Sage *et al.*, 1989; Cook *et al.*, 1998). Usually, there is a positive correlation between light-saturating photosynthetic rate and N concentration, because N may reflect Rubisco abundance (Evans, 1989). Consistent with this pattern, we observed that the amount of N was significantly correlated with the leaf area; oilseeds have smaller leaves, more nitrogen per unit leaf mass, and potentially more Rubisco. Other processes that could explain crop differences in RuBP regeneration and electron transport and that warrant further investigation include regulation of enzymatic activity of photosynthetic pigment–protein complexes as well as enzyme kinetics, activation states, and activation rates that affect photosynthesis (Evans, 1987; Evans & Terashima, 1987; Bernacchi *et al.*, 2002).

Leaf anatomy and gas exchange

In several studies, leaf anatomy has been shown to affect gas exchange performance (Reich *et al.*, 1998; Niinemets, 1999; Wright *et al.*, 2004; Franks & Farquhar, 2007). The overall response of stomatal conductance to stomatal density is straightforward in the absence of other morphological changes, namely an increase in stomatal density directly increases stomatal conductance for a given set of environmental conditions (Woodward & Bazzaz, 1988; Franks & Beerling, 2009). Several cultivated species have increased stomatal density compared with the wild relatives (e.g. in the genera *Arachis*, *Beta*, *Gossypium*, and *Phaseolus*), although some domesticated species show a decrease in stomatal density (e.g. the genera *Avena*, *Cichorium*, and *Vicia*) (Milla *et al.*, 2013). We found that the increase in stomatal density in oilseeds relative to vegetable crops (Fig. 5) was positively associated with stomatal conductance. Leaf anatomical features can also affect several aspects of gas exchange. Specific mesophyll phenotypes, for instance, can affect light absorption and carbon fixation. Higher numbers of palisade mesophyll cell layers are associated with higher photosynthetic rates (Vogelmann & Evans, 2002; Terashima *et al.*, 2006). Fig. 6 shows an oilseed genotype (159 – WO-181) that has five palisade layers, while the vegetable turnip (189 – VT-091) has three layers, a phenotypic pattern consistent with our observation of higher $A_{\text{max}}$ in oilseeds than in turnips. The uppermost 20% of the leaf absorbs light on the leaf surface (Terashima & Saeki, 1983; Vogelmann *et al.*, 1989); thus, a thinner upper epidermis may be beneficial for increasing light absorption rates and CO$_2$ fixation. We observe thinner adaxial epidermis in oilseeds of *B. rapa*, which might contribute to their higher photosynthetic and stomatal conductance rates.

While past studies and the current study indicate an association between the circadian clock and gas exchange, there is no evidence in the literature that leaf morphological and anatomical traits are under circadian clock regulation. There are many annotated *A. thaliana* genes that control leaf anatomy and development (e.g. *SDD1*, *YABBY* genes, *PHB*, *PHV*, and
GTI). Using published *A. thaliana* transcriptome data (Covington et al., 2008), we examined whether *A. thaliana* genes that contribute to anatomical or morphological features are under circadian control, and found that no obvious candidate genes involved in anatomical patterning are regulated by the circadian clock.

Conclusions

Human selection during *B. rapa* diversification favored increases in specific harvestable components. Our results, including PICs, indicate that circadian period is correlated over evolutionary time with gas exchange and biomass accumulation in *B. rapa*. These results are consistent with the long-standing view that targeted modification of the circadian clock could enable further crop improvement. More generally, the results suggest the hypothesis that evolution at clock loci might partly contribute to increases in photosynthetic rates and conductance commonly observed in oilseeds of other crops (Hervé et al., 2001; Ruuska et al., 2004; Cober et al., 2005). The reversal in correlation between period and rA (or biomass) (Fig. 3b,d) compared with period and r0 (Fig. 3c) could contribute to the maintenance of clock variation within the clade of *B. rapa* crop genotypes. We further observed that enhanced photosynthesis is associated with leaf anatomical and morphological traits in a manner consistent with other crops, and suggest that further functional studies, including clock mutants in crop morphotypes, are needed to dissect the specific genetic mechanisms at work.

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Author contributions


References


**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Root morphology of different *Brassica* oilseed plant genotypes.

**Table S1** List of experimental *Brassica rapa* genotypes

**Table S2** Genotypic means for the gas exchange parameters in different genotypes of *Brassica rapa*

**Table S3** ANOVA tables

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