Cytonuclear diversity underlying clock adaptation to warming climate in wild barley (Hordeum vulgare ssp. spontaneum)

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Abstract

In plants, the contribution of the plasmotype (mitochondria and chloroplast) in controlling of the circadian clock plasticity and possible consequences on cytonuclear genetic make-up has not been fully elucidated. Here, we investigated the cytonuclear genetics underlying thermal plasticity of clock rhythmicity and fitness traits in reciprocal hybrid (RH) and B1K diversity panels of wild barley (*Hordeum vulgare* ssp. *spontaneum*). Phenotypic analysis of the RH panel, showed higher abundance of plasmotype effects on chlorophyll fluorescence and its rhythmicity than plant phenology and growth. Performing a genome wide association study in the B1K panel found overlap with previously reported *drivers of clock* (*DOC*) loci yet due to intra-chromosomal linkage disequilibrium these loci encompass shorter intervals. Moreover, by incorporating long-range chloroplastic sequencing we identified significant inter-chromosomal linkage disequilibrium and epistatic interactions between previously *DOC3.2* and *5.1* loci and the chloroplastic *RpoC1* genes, indicating adaptive value for specific cytonuclear gene combinations. Finally, heterologous over-expression of two barley *RpoC1* alleles in *Arabidopsis* showed significantly differential plasticity under elevated temperatures. Our results unravel previously unknown cytonuclear interactions as well as alleles within the chloroplastic genome that control clock thermal plasticity.

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Running title: Cytonuclear control of clock and fitness in wild barley

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Summary

In plants, the contribution of the plasmotype (mitochondria and chloroplast) in controlling of the circadian clock plasticity and possible consequences on cytonuclear genetic make-up has not been fully elucidated. Here, we investigated the cytonuclear genetics underlying thermal plasticity of clock rhythmicity and fitness traits in reciprocal hybrid (RH) and B1K diversity panels of wild barley (*Hordeum vulgare* ssp. *spontaneum*). Phenotypic analysis of the RH panel, showed higher abundance of plasmotype effects on chlorophyll fluorescence and its rhythmicity than plant phenology and growth. Performing a genome wide association study in the B1K panel found overlap with previously reported *drivers of clock* (*DOC*) loci yet due to intra-chromosomal linkage disequilibrium these loci encompass shorter intervals. Moreover, by incorporating long-range chloroplastic sequencing we identified significant inter-chromosomal linkage disequilibrium and epistatic interactions between previously*DOC3.2* and *5.1* loci and the chloroplastic *RpoC1*genes, indicating adaptive value for specific cytonuclear gene combinations. Finally, heterologous over-expression of two barley*RpoC1* alleles in *Arabidopsis* showed significantly differential plasticity under elevated temperatures. Our results unravel previously unknown cytonuclear interactions as well as alleles within the chloroplastic genome that control clock thermal plasticity.

Introduction

Plants are composed of cells in which three different organelle genomes co-evolved to cope with a dynamic environment: the nuclear genome (nucleotype), and the chloroplast and mitochondrial genomes (plasmotype). Environmental constraints promote the selection of causal mutations in all of those genomes. At the same time, epistatic relationships between nucleotypic and plasmotypic loci, and co-evolution of adaptive gene complexes are able to promote adaptation to dynamic environment and further to shape genetic make-up owing to preference of specific gene complexes (Groen *et al.,* 2022). In recent years, several studies have shown that phenotypic effects are related to the genetic diversity of the plasmotype and its interactions with the nucleotype (Joseph *et al.* , 2013; Tang *et al.* , 2014; Roux*et al.* , 2016). An elegant use of the haploid-inducer line available in *Arabidopsis* (*GFP-tailswap*) (Ravi *et al.,* 2014), allowed the generation of a set of reciprocal and isogenic cybrids from several accessions that were phenotyped for metabolism and photosynthesis under different light conditions (Flood *et al.,*2020). Genetic analysis revealed that the nucleotype, plasmotype and their interaction accounted for 91.9%, 2.9% and 5.2% of genetic variation, respectively, thus highlighting the importance of interactions between nucleotype and plasmotype.

In crop plants and their wild relatives, few reports exist on the contribution of cytonuclear interactions (CNI) to a plant's phenotype and even less on its effects on its phenotypic plasticity. Examples, where the contribution of plasmotype to yield and grain quality has been demonstrated, exist in grasses (Frei *et al.* 2003; Sanetomo & Gebhardt, 2015). In cucumber, (Gordon & Staub, 2011) used reciprocal backcrosses between chilling-sensitive and chilling-tolerant lines to show that tolerance to reduced temperature is maternally inherited. Likely, these traits are the result of a local adaption of the original wild alleles, since for example in bread wheat (*Trictium aestivum*), cytoplasmic influence on fruit quality is affected by genotype-by-environment interactions (G x E) (Ekiz *et al.,* 1998). Nevertheless, many of these examinations of alloplasmic lines, which contained cytoplasm from distantly related wild relatives showed that effects on agronomic traits (rather than protein quality) are not frequent (Frei *et al.,* 2010). In maize, although cytoplasmic effects were

not significant between the direct and reciprocal populations, the interactions among the plasmotype and the nucleotype quantitative trait loci (QTL) were detected for both days to tassel and days to pollen shed (Tang *et al.,* 2014), further enforcing the increased explained variation between *Arabidopsis* cybrids when CNI are included (Flood *et al.,* 2020).

Circadian clock rhythms in plants are interwined with chloroplastic activities including photosynthetic parameters such as non-photochemical quenching (NPQ) and photosystem II (ΦPSII), whose values correlate with plant productivity (Kromdijk *et al.,* 2016). This insight led to the development of several high-throughput methods that measure the rhythmicity of the leaf chlorophyll fluorescence as an approximation to the period, phase and amplitude of the core clock (Gould *et al.* , 2009; Tindall *et al.* , 2015; Dakhiya *et al.* , 2017). The ability to measure hundreds of plants allowed for a comparison between species (Rees *et al.,* 2019), and to quantify the impact of temperature and soil composition on period and amplitude (Dakhiya*et al.* , 2017). Using the SensyPAM platform, which allows to infer photosynthetic rhythms based on repeated measurements of chlorophyll fluorescence (Bdolach *et al.,* 2019), we recently analyzed wild, landrace, and cultivar panels and showed a clear loss of thermal plasticity of photosynthetic rhythms under domestication. Notably, to corroborate more on the nature of the rhythms obtained by chlorophyll fluorescence (chlF) we also performed time-lapse transcription analysis. Core clock genes were compared to photosynthetic genes by RNAseq between wild vs cultivated genotypes (Prusty et al., 2021), or by qPCR for pooled genotypes based on a single QTL affecting period plasticity (Bdolach et al., 2019). In these experiments, unlike the match seen between the SensyPAM phenotype and photosynthetic genes (e.g.*PHOTOSYSTEM I SUBUNIT F* (*PsaF*), *LIGHT-HARVESTING Chl-BINDING* (*Lhcb5* : *CP26*)) under OT and HT conditions, none of the core genes (e.g., *TIMING OF CAB EXPRESSION 1*(*TOC1*), *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*)) showed a significant difference in their amplitude or period. These observations for the core clock genes support the existence of temperature-compensation mechanisms (Gould *et al.* , 2006b; Sorek & Levy, 2012; Ford et al., 2016) and indicate another layer of regulation between core clock and its outputs. Nevertheless, with SensyPAM analysis of interspecific populations we showed that some of the nuclear loci that control the photosynthetic rhythms were under selection during domestication. This could explain how modern crops lost the thermal plasticity of photosynthetic rhythms while maintaining a robust core clock (Prusty *et al.,* 2021). Furthermore, pleiotropic effects of these *drivers of clocks* (*DOCs*) loci on grain yield under stress indicate the adaptive value of clock plasticity. Nevertheless, this study did not consider the possible role of plasmotype diversity in modulating the effect of *DOCs* loci on circadian clock and fitness outputs, nor it examined the possibility that these effects on the clock plasticity may have been under selection also in the wild.

Here, we follow up on the photosynthetic rhythm analysis of a reciprocal wild barley bi-parental doubled haploid (DH) population segregating for both nucleotype and plasmotype (either "Ashkelon" (B1K-09-07) or "Mount Hermon" (B1K-50-04)) (Bdolach *et al.,* 2019). Photosynthetic rhythms measurements previously showed a significant difference of 2.2 h in the period between the carriers of the different plasmotypes (Bdolach *et al.,* 2019). Whole chloroplast genome sequencing of the two chloroplast identified several non-synonymous candidate polymorphism that could underlie these changes, including a N571K in the *rpoC1* , which is part of the plastid-encoded RNA polymerase (PEP) protein complex. This complex is composed of four subunit α, β, β' and β" encoded by *RpoA* , *RpoB* ,*RpoC1* and *RpoC2* respectively (Hajdukiewicz *et al.,*1997) and it requires sigma factor for promoter recognition and initiation of transcription. There are six sigma factors encoded by the*Arabidopsis* genome, and all these factors are regulated by the circadian clock therefore communicating circadian timing information from the nucleus to chloroplasts (Noordally *et al.,* 2013). Notably, previous studies identified the correlation of molecular evolution (i.e. dN/dS ratio) between genes encoding the plastid-encoded RNA polymerase (PEP) protein complex and nuclear genes (sig1-6) (Zhang*et al.,* 2015), which are ruled by the core clock genes (Belbin*et al.,* 2017). However, whether such selective forces acted on loci that regulate the core clock or its output rhythms remains unknown.

In the current study, we wished to, 1) extend the breadth of plasmotype diversity tested by examining reciprocal hybrids between 11 wild B1K that represent the different genetic clades (Hubner et al., 2009) and, 2) genetically characterize the B1K collection with a new SNP genotyping array and identify possible

DOC loci and, 3) to look into CNI and their possible consequences on genetic make-up by analyzing B1K collection and a derived reciprocal F2 population that segregates for one of the*DOC* loci, and finally, 4) test the functional consequence of variation in the *RpoC1* gene (Bdolach *et al.,* 2019) on photosynthetic rhythm plasticity by heterologous expression of two barley alleles in model plant *Arabidopsis* .

MATERIALS AND METHODS

2.1 Plant material

The source for the reciprocal hybrids (RH) population described in this study are barley accessions (*Hordeum vulgare* ssp.*spontaneum*) that we selected from the B1K collection in Israel to represent the different genetic clades (Hubner *et al.* , 2009). In addition, the HID386 is from the IPK collection (Maurer *et al.*2015). The wild accessions are from Yerucham (B1K-02-02), Michmoret (B1K-03-09), Ein Prat (B1K-04-04), Neomi (B1K-05-07), Ashkelon (B1K-09-07), Mount Arbel (B1K-29-13), Mount Harif (B1K-33-09), Jordan Canal (B1K-42-16), Mount Eitan (B1K-49-19), Mount Hermon (B1K-50-04) and Kisalon, Israel (HID386). The pairs of RH that differ in their plasmotypes were obtained by two-way intercrossing between these 11 wild accessions (from the B1K and HID386) and cultivar barley cv. Noga.

2.2 Growth and phenotyping.

We conducted the net house experiments in the Agricultural Research Organization - Volcani (ARO) Center (Bet Dagan, Israel) following similar sowing and transplanting as described previously for water limitation experiments by (Merchuk-Ovnat *et al.,* 2018) . Due to the sensitivity of wild barley to day-length conditions, we preferred to achieve mild higher temperature conditions by warming the nethouse rather than late sowing conducted for example for tomato (Bineau*et al.,* 2021). The thermal differences between ambient and high temperatures (AT and HT) is depicted in Figure **S1** , with a mean increase of 3.9 °C and 2.8 °C during day and night time and maximum delta of mean 7.5 °C between AT to HT.

The RH lines were grown during winter of 2019-2020 in a completely randomized block design. We began phenotyping by measuring Tiller height (TH) that is the length of the longest tiller from ground level to the beginning of the last fully expended leaf in the tiller. Tiller number (TN) is the number of tillers per plant and it was determined about one month after transplanting the plants. TH and TN ware measured once (_1) or twice (_2) with 14 days apart. We calculated TH rate by subtracting TH_2 with TH_1 and dividing with the number of days between these two measurements. We determined the number of days to flowering (DTF) based on the date when the first awns appear in the main tiller. During grain filling, we measured five spikes per plant for spike length (SL) and later to obtain SL coefficient of variation (SLCV). In addition, during grain filling we measured plant height (PH) from ground to the start of the tallest spike. We then cached the five and whole spikes of each plant in separate paper and nylon bags, respectively. Plants were left to dry for several weeks after irrigation was terminated. We harvested dry plants by cutting at soil level and placing them in the nylon bags. Weight of the nylon bag with the plant is the total dry matter (TDM). We collected dispersal units from bag and weighted them. We calculated average spike dry weight (ASDW) based on weighing the five spikes that we cached in the paper bag. We then summed the weight of spikes (dispersal units) in the plastic and paper bags to obtain spikes dry weight (SpDW). Vegetative dry weight (VDW) is the reduction of SpDW from TDM.

We measured circadian clock amplitude and period in high-throughput SensyPAM (SensyTIV, Aviel, Israel) custom-designed to allow Fluorescence measurements under optimal or high temperatures of 22^{o}C (OT) or 32^{o}C (HT) as previously described (Bdolach *et al.* , 2019).

2.3 Allele mining and design of a genotyping platform for wild barley (Hordeum vulgare ssp. spontaneum)

We collected all the available DNA sequencing data, including SSR (Hubner *et al.* , 2009), BOPA1 (Schmalenbach *et al.,* 2011), Exome-Seq (Looseley *et al.,* 2017), and Genotype-by-sequencing (Chang *et al.* , 2022). The workflow for designing and eventually selecting informative SNP for the genome wide association study (GWAS) analysis is depicted in Figure **S2** . This harvest identified 502K unique contigs. After filtering these SNPs with MAF>0.05, no heterozygosity, a total read coverage >20, and missing marker>0.2 in at least

80% of the samples, we were left with 38K markers. We then retrieved 100 bp sequence flanking each marker and carried out BLAST search against the reference genome MorexV1 IBSCV2 barley genome to assure their identity and selectively singleton unique markers. Finally, we filtered out the redundant markers and reached approximately 30K markers sent to LGC Genomics Ltd. (GmbH, Germany) to design the probe set. During the probe design, the low specificity probes those with an off-target hit were removed, and we ended with 22.7K SNPs on the SNP chip made by LGC (Queens Road, Teddington, Middlesex, TW11 0LY, UK).

2.4 Statistical analysis

The JMP version 14.0 statistical package (SAS Institute, Cary, NC, USA) was used for statistical analyses. Student's t-Tests between treatments, plasmotypes and alleles were conducted using the 'Fit Y by X' function. A factorial model was employed for the analysis of variance (ANOVA), using 'Fit model', with temperature or plasmotype treatment and allelic state as fixed effects. In the SensyPAM experiments we measured the F parameters every 2.5 hr for three days. The Fv/Fm and NPQlss for each time point where calculated for the circadian clock analysis in the BioDare (https://biodare2.ed.ac.uk). The Fv/Fm and NPQlss traits per se are the average of these three days measurements.

2.5 B1K accessions chloroplast DNA sequencing

From previous DNA shot-gun sequencing we realized that chloroplast isolation and sequencing were necessary to overcome possible confounding effects of the known transfers that occur from plastid to nuclear [nuclear plastid DNAs (NUPTs)] (Richly & Leister, 2004; Yoshida*et al.* , 2014; Greiner and Fridman, personal communication). Therefore, we isolated the chloroplast DNA and performed Nanopore amplicon sequencing.

Chloroplast DNA isolation

Ten grams fresh leaf tissue were taken and grinded in pestle mortar using liquid N2. Grinded powder was transferred in small beaker mixed with 100 ml isolation buffer (50 mM Tris-HCl pH 8.0, 0.35 M sucrose, 7 mM EDTA, 5 mM 2-mercaptoethanol, 0.1% BSA), kept in 4°C for 5-30 min followed by filtration using 8 layer of gauge. The filtrate centrifuged at 1000Xg for 15 min and pellet dissolved in 3 ml of isolation buffer. The dissolved solution were loaded on top of 45/20% sucrose gradient (50 mM Tris-HCl pH 8.0, 0.3 sorbitol, 7 mM EDTA, sucrose 20% / 45%). Tubes centrifuge at 2000Xg for 30 min. Carefully middle layer (sucrose gradient junction) transferred to new tube and filed with 3 times volume of isolation buffer followed by centrifugation at 3000Xg for 20 min. Pellet (isolated chloroplast) was used for DNA isolation using CTAB method.

B1K accessions PCR barcoding and Nanopore Sequencing

We PCR amplified the region 19409-24572 using chloroplastic DNA of B1K accessions with LongAmp Taq-polymerase 2X mix (M0287S). Two-step PCR performed to tag with 96 different barcode carrying the universal adapter sequence (Table **S1**). After the barcoding all samples pooled together and purified by precipitation. 1µg of barcoded pooled PCR product was taken for library preparation using kit as per manufacturer protocol (SQK-LSK109 kit, Nanopore). 5-50 fmol DNA loaded in Nanopore flow cells (R9.4.1) after priming. After 24 hr run data were collected and performed the basecalling and demultiplexing with Guppy 5.0. Further barcodes aligned to reference with BWA-MEM2 using (-x ont2d) criteria and BAM files are sorted by coordinate. BAM files used as input for variant calling using Medaka version 1.4.4. VCF files from every alignment merged using a custom R script demultiplexing.

2.6 Marker designing and Genotyping of F2 population

For genotyping of *DOC3.2* locus in F2 population (derived from crosses between B1K-05-07 and B1K-50-04, we identified sequence diversity using Nanopore data covering region of *DOC3.2*(Chr3H_67433882-Chr3H_67434491). We found SNP marker Chr3H_67434330 have C to T in B1K-50-04 and B1K-05-07, respectively, that resides within the T*aqI* recognition. For genotyping of F2 population, we isolated leaf DNA using with CTAB method, PCR amplified using PM.22-0190 and PM.22-0191 primers (Table **S1**) followed by restriction digestion using T*aqI* restriction enzyme to distinguish between three genotypes.

2.7 Arabidopsis Plant material and Growth condition

Arabidopsis thaliana (L.) Heynh ecotypes Col-0, homozygous mutant and over expression (OE) lines used in this study. T-DNA insertion mutant of *rpoC1* (*cs835205*) was obtained from the*Arabidopsis* Biological Resource Centre (ABRC, Ohio State University, Columbus, OH, USA; https://abrc.osu.edu/). Seed sterilization and plants growth were performed according to Tiwari*et al.* , (2021). Homozygosity of *rpoC1* mutant confirmed by polymerase chain reaction (PCR) by using gene-specific right primer with a T-DNA left border specific primer (pDAP110/ pCSA110) listed in Table**S1** . For over-expression of *RpoC1* gene alleles from B1K-09-07 and B1K-50-04 in Col-0 background, coding sequence region (2046 bps without stop codon) were synthesized at Genewiz (www.genewiz.com) in pUC-GW-Amp vector. Further all the desired fragment were cloned in binary vector (pICH86966 Addgene) using Golden Gate assembly (Plant part: 1000000047 and toolkit: 1000000044, Addgene) (Figure **S3**).

RESULTS

3.1 Plasticity of life history traits in response to elevated temperature and their correspondence with plasmotype diversity

We wished to examine the effects of plasmotype diversity on barley phenotypic plasticity including growth, productivity and photosynthesis rhythmicity while growing plants under ambient vs. high temperatures. Based on considerations of maximal inclusions of genetic diversity we performed a set of crosses between 11 wild barley accessions and one cultivated variety to achieve reciprocal hybrids (RH) pairs with few genotypes missing to achieve diallel (see Methods).

The days to flowering (DTF) was almost identical under HT and AT (DTF=-109.9 ±5.6 and 111.73 ±4.97 days, respectively) (Figure**1a**). Unlike DTF, the vegetative dry weight of the plants were significantly affected by the heat, with change from mean of 11.3 gr to 16.4 gr between AT and HT, respectively (Figure **1b**). Similar to DTF, and unlike the significant effects of the thermal environment on the vegetative traits, the reproductive traits were less affected; For spike dry weight (SpDW), there was not a significant difference between environments (Figure **1c,** raw data are presented in Table**S2**) (7.13 ±2.2 and 7.35 ±3.16 gr, respectively).

The reciprocal nature of the hybrids allowed us to group the F1 genotypes into different plasmotype subpopulations and different male parent subpopulations (representing the nucleotype). One-way ANOVA for each of these two divisions of the hybrids indicated a larger percentage variation explained (PVE) by the nucleotype (male donors) in comparison to differences between plasmotype (female donors) for a few traits (Table **1**). For example, for the ASDW under HT the nucleotype factor explained 41% of trait variation (PVE=41%) vs. PVE=27% by the plasmotype. For most life history traits, however, we found higher variation explained by the plasmotype than by the nucleotype under both temperatures (AT and HT): for PH, PVE=39% vs. 30% and 33% vs. 21% under AT and HT, respectively. Higher variation explained by the plasmotype than nucleotype was true also for reproductive output, e.g., SpDW, which showed higher variance between plasmotypes under AT (PVE=35% vs. 21%) and to a lesser extent under HT (PVE=23% vs. 19% between plasmotype and nucleotype contributions).

3.2 Plasmotype effects on the plasticity circadian clock and ChlF traits in reciprocal hybrids

We also included a photosynthetic rhythmicity analysis to the RH under OT and HT using SensyPAM platform (See Methods). The average Fv/Fm was significantly higher under HT (0.93 ±0.01) in comparison to OT (0.92 ±0.01; Figure **1d**) and significantly different for the average NPQlss *per se* (0.66 ±0.1 vs. 0.43 ±0.08) under OT in comparison to HT (Figure **1e**). We calculated the amplitude and the period of the NPQlss rhythmicity and found that the former was significantly higher under HT (0.03 ±0.01) compared to OT (0.015 ±0.006) (Figure**1f**). This is compared to the period, which was significantly higher under OT (24.9 ±2.6 h) compared to HT (23.3 ±1.9 h; Figure**1g**). This clock plasticity is similar to the one described for the ASHER (Bdolach *et al.* , 2019) with acceleration of the rhythmicity under HT. Moreover, these results suggest that under HT, photosynthesis is more efficient than under OT.

In another way of comparing the contribution of the plasmotype variation to the phenotypic variation and plasticity we counted the number of reciprocal hybrids that significantly differ in the life history traits and Chl F traits (Figure **2**). Note that pairs of RH are sharing similar nucleotype (heterozygous) and differ only in their plasmotype identity therefore significant difference between the two types of non-segregating hybrids point to effect of the plasmotype on the trait. We clustered phenotypic traits measured in the net house as growth or reproductive and traits measured with SensyPAM as the clock or Chl F parameters. The percentage of different pairs of RH was highest for the Fv/Fm under OT (44.8%), and the lowest was zero hybrids for the difference of DTF under AT. If comparing the traits according to our clustering, we could see that the mean number of differing reciprocal hybrids is highest for Chl F (26.3%), second for clock traits (15%), and falling behind for growth and reproductive traits with 5.23% and 3.73%, respectively (Figure **2**).

Overall, these analyses implicate a significant contribution of the plasmotype diversity to the phenotypic plasticity, and moreover and to more extent, for the plasticity of the photosynthesis and its circadian rhythmicity.

3.3 Genome wide association study (GWAS) for thermal plasticity of the circadian clock output

Next, we used the SensyPAM platform to investigate the genetic basis for the period and amplitude of circadian photosynthetic rhythms and their thermal plasticity (Table **S3**). To this end we selected 285 wild barley from the B1K collection (Hubner *et al.* , 2009) that originate from different geographic habitats. The rationale was to try to include single representative from each of the 255 micro-site of this hierarchical collection (51 sites including each 5 micro-sites with 4 representatives). We performed a GWAS using the traits values per se and by applying a QTL by Environment (Q x E) model for the OT and HT environments (Yamamoto & Matsunaga, 2021). Moreover, since the analysis of RH (this study) and the ASHER populations (Bdolach *et al.,*2019) indicated a significant effect of the plasmotype diversity on the photosynthetic rhythm plasticity, we included sequencing information from isolated chloroplast DNA for a portion of this panel (see Methods; Table **S4**).

We wanted to identify nuclear loci for which allelic diversity is associated with plasticity of photosynthetic rhythms. First, we increased the current coverage of B1K genomic analysis by harvesting genic and intergenic DNA variation from several sources (see Supporting Information and Methods). Prior to GWAS analysis, missing values were imputed with the "missForest" algorithm and filtered for markers with missing data >0.2, minor allele frequency < 0.03, monomorphic and multiallelic markers. The selection process led to a final set of 13,786 informative markers for GWAS analysis (Table**S5**). We wished to detect QTLs with persistent effects across the two environments (OT and HT) but also with Q × E effects, i.e., loci with specific effects to a certain environment (Malosetti *et al.*2013; Yamamoto & Matsunaga, 2021).

Briefly, the output of the analysis for each SNP included "Additive Main Effect" (*p.ame*), "All SNP effects" (*p.all*), "Interaction terms" (*p.int*) and a Wald score for each environment indicated the environment with the most significant effect of the locus on the trait (Table **S6** ; Figure **3**). In a previous study, we identified several *DOCs* loci that modulated the circadian clock output in the HEB-25 interspecific mapping population (Prusty *et al.,* 2021). Interestingly, some of these*DOC* loci are overlapping with signals in this current genome scan of the B1K, including some that contain genes reported to be involved in the circadian clock. Of note is the gene *GIGANTEA* (*GI*) that resides in the long arm of chromosome 3. Previously, we identified a large interval on chromosome 3, *DOC3.2* , associated with significant pleiotropic effects on the clock period and growth in the field. Nevertheless, *DOC3.2* , although harboring the barley ortholog of the *GI* gene (Fowler *et al.,* 1999), stretched in the previous HEB population analysis to a distance of 45.98 Mbp (Chr3H_35066186 – Chr3H_81047480). Here, in the GWAS in the B1K collection, we identified SNPs around the *GI* gene that were significantly associated with the amplitude of the clock and its plasticity (Figure **3a**). In fact, the current SNP arrangement point to a causal variation in 150 kb downstream to the *GI* gene. Phenotypic analysis showed that the mild non-significant difference in the amplitude between two allele under OT became more pronounced and significant under HT condition (Figure **S4**).

3.4 CNI and linkage disequilibrium between chloroplastic RpoC1 and DOC loci

One primary goal of this study was to explore the plasmotype variation's contribution to the circadian photosynthetic rhythm plasticity and growth together with the possibility of epistatic interaction with nuclear loci relevant to the clock output. Since earlier we identified variation within chloroplastic *rpoC1* as possible causal variation between B1K-09-07 and B1K-50-04 (Bdolach et al., 2019), we performed an additional long-range Nanopore sequencing of the *RpoC1* region with chloroplast DNA isolated from 75 B1K accessions (selected from the 285 GWAS panel).

Table **S7** includes the identified SNP within the panel of 75 B1K accessions. In *RpoC1* , there is one distinct SNP (position$RpoC1^{G1713T}$) that divides the sequenced B1Ks into 51 accessions that carry the $RpoC1^{1713T}$allele, while the other 24 accessions carry the$RpoC1^{1713G}$ allele. Notably, this SNP is a non-synonymous mutation of the *RpoC1* chloroplastic gene (changes N:AA**T** to K:AA**G** ; Table **S4**).

We tested the effects of two main QTLs, i.e. *DOC3.2* for the amplitude and *DOC5.1* for the period (Figure **4a, b**), in the complete and in the smaller panels (n=285 and 75, respectively), while considering *RpoC1* variation in the later (Table**S7**). For *DOC3.2* , we noticed segregation at chr3H_67267835 between A and G only for the 51 out of 75*RpoC1* -sequenced B1K that carry the$RpoC1^{1713T}$ allele (Figure **4c**). This is compared to the other 24 carriers of the$RpoC1^{1713G}$ where no segregation is found for chr3H_67267835. A strong CNI is also found between the *RpoC1* and*DOC5.1* (chr5H_648981054), a locus with significant effects on the period (Figure **4b**). While in the$RpoC1^{1713G}$ background there is mild difference in the period thermal plasticity between carriers of the two*DOC5.1* allele, a more significant and even opposite directionality is found for the nuclear locus in the background of$RpoC1^{1713T}$ (Figure **4b, d**).

3.5 CNI in F2 population for clock rhythmicity

To examine the QTLs found in the B1K related to circadian photosynthetic rhythms, including testing the CNI, we generated F2 population from reciprocal crosses between two wild B1K accessions, B1K-05-07 and B1K-50-04. Interestedly, the elevation of B1K-05-07 (Neomi) is -245 meter and the elevation of B1K-50-04 (Mt. Hermon) is 1469 meter above sea level. These crosses allow the analysis of the clock output phenotype linked with segregation in the nuclear locus and comparison of this relationship in two differing chloroplast genetic backgrounds (see raw data at Table **S8**). We phenotyped and genotyped the two F2 populations (B1K-05-07 and B1K-50-04 plasmotypes; n=84 and 113, respectively) for the chr3H_67434330 near the *GI* gene (Figure**S4**). In the total F2 plants, without considering the plasmotype identity, the *DOC3.2* allele of B1K-50-04 was associated with higher thermal plasticity for the period (accelerated clock in the transition to HT; delta=-1.34h) than B1K-05-07 allele (delta=-0.6h) (Figure **5a**). For the amplitude, there is no difference in plasticity between the two alleles (delta -0.006 and -0.008, respectively) and similar significant difference between temperatures (p=0.0003 and p=0.0006, respectively) (Figure **5b**).

When we split the analysis between the two F2 populations, based on the plasmotype, we could see relatively mild differences between the two. Statistical testing using two factorial ANOVA of the interaction between the *DOC3.2* / chr3H_67434330 alleles and the plasmotypes for the period and the amplitude, we found significant interaction between the nucleotype and plasmotype loci only for the amplitude under OT (P=0.01) (Figure **5c, d** and Table **S9**). The "natural" combination of plamotype/*GI* of B1K-05-07/B1K-05-07 has the amplitude of 0.03 and the B1K-50-04/B1K-50-04 has the amplitude of 0.032, while the "unnatural" arrangement of B1K-05-07/B1K-50-04 and B1K-50-04/B1K-05-07 has lower amplitude of 0.025. These results imply that combination of the plasmotype and *DOC3.2* /chr3H_67434330 of the same origin (either B1K-05-07 or B1K-50-04) are associated with higher amplitude values. This could be viewed in the crisscross pattern where the mean amplitude values of two genotypic group is in opposite direction under OT (Figure **5d** , right-bottom panel).

3.6 Arabidopsis complementation with barley RpoC1 alleles confer differential clock output plasticity

Previously, the comparison between B1K-09-07 and B1K-50-04 chloroplast genomes identified a non-synonymous SNP at the *RpoC1* gene (position: 24530; N571K). We then speculated that this gene could

be responsible for the difference in photosynthetic rhythm parameters between the two subpopulations within ASHER (Bdolach *et al.,*2019). In the current study, some of the interactions we tested for the same nonsynonymous SNP in the wider B1K collection support an association to the clock plasticity (Figure **4**). Therefore, we first tested *rpoC1* mutant in the *Arabidopsis* background. We procured T-DNA insertion *rpoC1* mutant (*cs835205*) lines that showed integration in intron region of *RpoC1* gene (Figure**6a**). We analyzed the chloroplastic *rpoC1* mutant for clock phenotype and noticed that, *rpoC1* mutant showed plasticity in the period compared to wild type (WT) plants during temperature shift in short day entrainment (Table **S10**). Under OT, clock period in*rpoC1* mutant and WT varied between 20.71-27.44 hr and 20.8-29.08 hr, respectively, with coefficient of variance (CV) 9.28% (*rpoC1*) and 8.67% (WT). The mean period of the *rpoC1*mutant and WT plants under the two temperatures differed significantly with clock rhythm accelerated from period length of 24.28 hr under OT to 22.58 hr under HT in *rpoC1* mutant line as compared to relatively robust rhythmicity of the WT, i.e. 22.82 hr and 23.40 hr under OT and HT, respectively (Figure **6b** ; **S5a**).

Next, we wished to compare the possible consequences of the barley*RpoC1* variation on the clock output in the heterologous*Arabidopsis* system. We therefore over-expressed (OE) the chloroplastic *RpoC1* gene alleles from B1K-09-07 and B1K-50-04 barley in Col-0 *Arabidopsis* background. To direct translocation to the chloroplast we fused the barley chloroplastic *RpoC1* coding region with chloroplast transit peptide under the control of*CaMV35S* promoter (Figure **6c**). The homozygous OE lines were analysed for clock phenotype under OT and HT condition (Table**S11**). The *Arabidopsis* OE lines for B1K-09-07*RpoC1* showed, on average, a significant plasticity or acceleration of the period during temperature shift from 23.73 to 22.18 hr, similar to the *rpoC1* mutant line (*rpoC1* : OT-23.54 hr, HT-22.41 hr). This is compared to the B1K-50-04 allele OE lines that showed robustness in the period (OT-22.93 hr, HT-22.85 hr) during temperature shift similar to WT lines (OT-23.27 hr, HT-24.23 hr) (Figure**6d** ; **S5b**). Overall, in short day entrainment B1K-50-04 allelic OE lines and WT showed robustness while B1K-09-07 allelic OE lines and *rpoC1* mutant showed plasticity during temperature shift. Similar to mutant and WT lines, OE lines from both allele showed amplitude remain same and decelerated during temperature shift.

Discussion

4.1 Non-random association and interactions between drivers of clock (DOC) loci and chloroplastic RpoC1

Since its establishment, the Barley1K infrastructure has been an instrumental research tool to explore landscape genomics (Hubner*et al.* , 2009; Hübner *et al.* , 2013; Chang *et al.* , 2022), plant biotic and abiotic interactions (Sade *et al* ., 2012; Dakhiya *et al.* 2017; Alegria Terrazas *et al.* 2020; Dakhiya & Green 2023), and more recently as reference for the whole barley Pan genome (Jayakodi *et al.* 2020). Here, we extended the use of the B1K by 1) developing a more dedicated genotyping platform for the wild barley to allow GWAS and 2) start exploring the chloroplast variation and its possible relationship with fitness traits. At first glance, the results obtained in this study could not directly be translated to crop improvement mainly since adaptation strategies in the wild and agricultural set up might be different. However, the B1K resource bear genetic attributes that could assist in targeting specific genes and gene complexes mainly owing to the non-randomness and long years of recombination within and between nuclear and chloroplastic genomes, and the way natural selection works on these intra and inter-loci combinations. For the nuclear genome, it is clear that the linkage disequilibrium decay is enormously shorter than that one could find in interspecific populations (Morrell *et al.* 2005). *DOC3.2*may be a good example for this "gene discovery" use of the B1K panel for zooming-in on a causal diversity that otherwise would be delimited to 7.7 Mbp in using the interspecific population (Prusty *et al.,*2021). Here, a closer look on at the allelic diversity indicates the QTL for amplitude under HT, which was identified in the previous study, most probably corresponds to the signal identified at chr3H_67267835. This marker is flanked by the non-significant markers chr3H_66,838,086 and chr3H_68,838,244 (Tables S5 and S6), therefore limiting the source of phenotypic variation to approximately 2 Mbp for this QTL.

For the chloroplast genome per se, and for the allelic combinations with the nuclear genome, this study clearly shows the rich diversity repertoire found in this organelle within the wild (Table **S7**) and its relevance to

plant adaptation. Moreover, we could identify both linkage disequilibrium and CNI between *DOC* and the *RpoC1* alleles in the B1K panel, where in an extreme case of the *DOC3.2* this locus showed segregation only among carriers of the $RpoC1^{G1713T}$ (Figure **4c**). Non-random association of alleles in the nucleus and cytoplasmic organelles, or cyto-nuclear linkage disequilibrium, is both an important component of a number of evolutionary processes and a statistical indicator of others (Fields *et al.,* 2014). We also followed up on the GWAS to examine inheritance of these interactions, and, beyond validating the environment-specific effects of the *DOCs* , we could also get indication for preferred homogeneity of the CNI. For example, in the case of CNI for the *DOC5.1* combination of plasmotype and nuclear alleles from same origin (B1K-05-07 or B1K-50-04, the two parental lines of the F2) was associated with higher amplitude. In case of cereal evolution, it was hypothesized and tested *in silico* that in ancient hybridization, such as the one between the A and D-genomes of modern wheat there has been biased maintenance of maternal A-genome ancestry in nuclear genes encoding cytonuclear enzyme complexes (CECs) (Li *et al.,* 2019). It is tempting to hypothesize that we observe such scenario in the case of the *DOC* and *RpoC1* loci in the B1K. However, one way of testing the possible cause behind these linkage disequilibrium and CNI will require cloning of the underlying genes in the *DOC* loci and figuring out how they may interact with *RpoC1* gene product and what makes one allele different than other. Another way to test the implications of these CNI for breeding would require importing these combinations into a rather cultivated genetic background. This approach is now followed in a new interspecific cytonuclear multi-parent population (CMPP) that we are currently testing for clock and for agricultural performance (manuscript in preparation).

4.2 Validation of the $rpoC1^{G1713T}$ causality in Arabidopsis and implication for understanding PEP complex role in clock plasticity

Our heterologous expression of the B1K-50-04 and B1K-09-07 *RpoC1* alleles in the *Arabidopsis* chloroplast support the role of N571K substitution in manifesting heat responses and changes in the photosynthetic rhythmicity (Figure **6**). Zooming in on this significant and hitherto unknown relationship between PEP variation and clock thermal plasticity will require a more thorough analysis of more advanced and isogenic lines. In the PEP complex, one major functional group is comprised of PEP-associated proteins (PAPs) involved in DNA/RNA metabolism and gene expression regulation, while the second group is related to redox regulation and reactive oxygen species protection (Steiner *et al.* 2011). Moreover, the PEP is somehow coordinated with the nuclear encoding RNA polymerase (Pfannschmidt *et al.*,2015). Therefore, presumably non-synonymous variations (such as those between *RpoC2* alleles that we identified but not yet tested; Table **S7**) could be as effective as non-synonymous ones (between *RpoC1* alleles) in the functionality and variation we observed. It would be therefore required to look at different layers (transcriptome, proteome) between nearly isogenic and not necessarily knockout mutant lines to achieve relevant causal variation. Recent developments in plastid gene editing, also in cereals, may assist in generating and analyzing both types of mutations in barley and learn how they might modulate physiology and development of the plant under optimal and high temperatures. Recent experiments suggest that most recent developments of TALLEN-based allele editing tested in *Arabidopsis* (Nakazato *et al.,* 2021) could also be applied in barley (Fridman and Arimura, Personal communication) to allow such multi-layer analysis of isogenic mutants.

Conclusions

Naturally, occurring evolution and adaptation of wild populations reflect on the genetic make up their genomes, including the relatively neglected organelles. By combining an efficient clock phenomics tool and new genotyping platforms, we were able to show the importance of considering chloroplast diversity for gaining better understanding of plants behavior under warming temperatures and determine the genetic network underlying heat-conditioned effects on the circadian clock output. We also identified linkage disequilibrium between some of these DOC and the plasmotype loci therefore indicating selection on genes controlling clock output. Furthermore, heterologous complementation of barley *RpoC1* alleles in the model plant *Arabidopsis* indicate the significant role of the PEP complex in regulating rhythmicity of photosynthesis under changing environments and may suggest its adaptive role in a yet to be defined heat sensing mechanism.

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LIST OF AUTHOR CONTRIBUTIONS

E.B, L.D.T. and E.F. designed the experiments, collected, analyzed and interpreted data, and wrote the manuscript. M.R.P and A.F.D collected and designed the B1K SNP platform, and and E.Y. performed the QxE GWAS. E.B., L.D.T., M.R.P., S.B, E.Y., were involved in the data analyses, their interpretation and in writing the manuscript.

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Legends

Table 1. Contribution of the nucleotype and plasmotype diversity to phenotypic differences between reciprocal hybrids. Tiller height at two time points and the rate between them, Days to flowering (DTF), Average Spike dry weight (ASDW), Plant height (PH), Spike length (SL), spike length CV, Spikes dry weight (SpDW), Total dry matter (TDM) and Vegetative dry weight (VDW) in the nethouse under ambient temperature (AT) and high temperature (HT). For clock and Chl F traits: Amplitude, Period, delta Amplitude (dAMP), delta Period (dPeriod), Fv/Fm, F/Fmlss, NPQlss and Rfd in SensyPAN under optimal temperature of 22^{o}C (OT) or high temperature of 32^{o}C (HT) and the delta HT-OT.

Figure 1. Life history traits, chlorophyll florescence and circadian clock rhythmicity in barley reciprocal hybrids lines grown under OT and HT . Mild increase in temperature has significant effect on plant performance in the field. Distribution and box plot of life history traits under ambient temperature (AT, black) and high temperature (HT, gray) in reciprocal hybrid lines **(a)** Days to flowering (DTF), (**b**) Vegetative dry weight, (**c**) Spikes dry weight (SpDW). Distribution and box plot of mean chlorophyll florescence traits and clock output rhythmicity: **(d)** Fv/Fm, (**e**) NPQlss, (**f**) Amplitude, and **(g)** Period, under optimal temperature (OT, black) and high temperature (HT, gray) in the reciprocal hybrid lines population. For each student's t-test, the p value is depicted as *: P<0.05, **: P<0.01 or ***: P<0.001.

Figure 2. Proportion of crosses with significant difference between reciprocal hybrids for phenotypic traits, under OT or AT and HT. Life history traits include: Tiller height (TH) and tiller number (TN) at two time points and the rate between them, Days to flowering (DTF), Average Spike dry weight (ASDW), Plant height (PH), Spike length (SL), spike length CV (SLCV), Spikes dry weight (SpDW), Total dry matter (TDM) and Vegetative dry weight (VDW) for plants in the nethouse under ambient temperature (AT) and high temperature (HT). For clock and Chl F traits: Amplitude, Period, delta Amplitude (dAMP), delta Period (dPeriod), Fv/Fm, F/Fmlss, NPQlss and Rfd in SensyPAM under optimal temperature of 22^{o}C (OT) or high temperature of 32^{o}C (HT) and the delta HT-OT. The mean for each type of traits is depicted: Growth or reproductive traits in the greenhouse experiment, and clock or Chl F in the SensyPAM.

Figure 3. Manhattan plots for the Q x E analysis for a) amplitude and b) period. The genome scan relates to three options of interactions between markers and environment following (Malosetti*et al.* 2013). The location of the previously identified*DOC* loci is indicated. Threshold FDR 0.05 is indicated by a red horizontal line following (Storey & Tibshirani 2003).

Figure 4. Cytonuclear interaction (CNI) in the B1K collection between the chloroplastic *rpoC1* gene and DOCs loci under OT and HT conditions Effects for **(a)** Amplitude for DOC3.2, and for**(b)** Period for DOC5.1 **(c)** CNI between DOC3.2 (chr3H_67267835) and rpoC1^{1713} allele for amplitude.**(d)** CNI between DOC5.1 (chr5H_648981054) and rpoC1^{1713} allele for period per se (left) and for its plasticity (right). Different letters indicate the one-way ANOVA test for significant differences.

Figure 5. B1K F2 population showing clock phenotype and effect of plasmotype in reciprocal lines. (a) Period, and**(b)** Amplitude, for mean chlorophyll florescence traits in B1K-05-07 and B1K-50-04 F2 population under OT (solid line) and HT (dotted line) conditions for GI gene. Role of plasmotype in clock,**(c)** Period, and **(d)** Amplitude, between B1K-05-07 (dotted line) and B1K-50-04 (solid line) in F2 population under OT and HT conditions. Different letters indicates the one-way ANOVA test for significant differences.

Figure 6. Differential clock plasticity in Arabidopsis is linked with disruption of *rpoC1* and with overexpression barley alleles. (a) Schematic diagram showing T-DNA insertion in *RpoC1* gene (AtCG00180) . Box showing the exon and line showing intron region. **(b)** Period phenotype of*rpoC1* and WT plants in OT and HT conditions. **(c)**Schematic diagram showing T-DNA of CaMV35S-Ch transit-RpoC1

construct of B1K-09-07 and B1K-50-04 alleles. (d) Period phenotype of *rpoC1* , OE-RpoC1 alleles and WT plants under OT and HT conditions. Different letters indicate the one-way ANOVA test for significant differences.

Supporting information

Table S1. List of primers used in this study.

Table S2. The field and SensyPAM phenotype of the reciprocal hybrids.

Table S3. SensyPAM phenotype of the B1K.

Table S4. The SNP diversity in 18Kb chloroplast region as determined by long-range Nanopore sequencing for selected B1K lines.

Table S5. Total 13,786 SNP informative SNP markers for GWAS analysis.

Table S6. The GWAS results for clock traits in B1K panel.

Table S7. The SNP variation of chloroplastic *RpoC1* and*RpoC2* region among 75 B1K accessions.

Table S8. The Sensypam clock phenotype and genotype at the chr3H_67267835 (150Kb from GIGANTEA) in two reciprocal segregating F2 populations

Table S9. ANOVA test for cytonuclear interactions underlying clock trait variation between chr3H_67267835 and plasmotype in reciprocal F2 populations.

Table S10. SensyPAM phenotype of the *rpoC1* mutant and WT plants.

Table S11. SensyPAM phenotype of the *rpoC1* mutant, OE-rpoC1-09, OE-rpoC1-50 and WT plants.

Figure S1. Daily temperature during the high and ambient temperature treatments (HT and AT, respectively) field experiments. We achieved high temperature treatment (HT) by covering half of the insect-proof with nylons and heating with electric heathers (3KW; Galon fans and pumps Ltd, Nehora, Israel). The second half of the nethouse remained with only net walls and ventilated with a large fan to take out the hot air for the ambient temperature treatment (AT).

Figure S2. Schematic diagram showing the workflow utilized for selecting informative SNPs for the GWAS analysis.

Figure S3. Schematic diagram showing the OE-RpoC1 cloning strategy. Different fragment were taken from diverse vector construct using B*saI* restriction enzymes in binary vector pICH86966 by golden gate assembly.

Figure S4. (a) Physical map of DOC3.2 loci and their coordinates. Significant marker and GI gene were shown in their respective positions. **(b)** Significant marker (Chr3H_67267835) in DOC3.2 loci showing the amplitude phenotype in short day (SD) and long day (LD) in changing environment.

Figure S5. Statistics analysis of *rpoC1* , OE lines and WT plants. **(a)** Each pair student's t-test **(b)** one way ANOVA test.

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`Table 1.docx` available at https://authorea.com/users/561760/articles/625014-cytonuclear-diversity-underlying-clock-adaptation-to-warming-climate-in-wild-barley-hordeum-vulgare-ssp-spontaneum