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Running header:

Heterosis differs between Arabidopsis F1 hybrids

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Intraspecific Arabidopsis hybrids show different patterns of heterosis despite the close relatedness of the parental genomes

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Summary

Arabidopsis hybrids show different growth patterns in outperforming parents for biomass and yield, associated with differences in gene expression patterns, suggesting multiple routes for hybrid vigor.

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Abstract

Heterosis is important for agriculture, however, little is known about the mechanisms driving hybrid vigor. Ultimately, heterosis depends on the interactions of specific alleles and epialleles provided by the parents, which is why hybrids can exhibit different levels of heterosis even within the same species. We characterise the development of several intraspecific Arabidopsis F1 hybrids that show different levels of heterosis at maturity. We identify several phases of heterosis beginning during embryogenesis and culminating in a final phase of vegetative maturity and seed production. During each phase the hybrids show different levels and patterns of growth despite the close relatedness of the parents. For instance, during the vegetative phases the hybrids develop larger leaves than the parents to varied extents, and do so by exploiting increases in cell size and cell numbers in different ratios. Consistent with this we observed changes in the expression of genes known to regulate leaf size in developing rosettes of the hybrids, with the patterns of altered expression differing between combinations. The data demonstrate that heterosis is dependent upon changes in development throughout the growth cycle of the hybrid; with the traits of mature vegetative biomass and reproductive yield a cumulative outcomes of heterosis at different levels, tissues and times of development.

Introduction

Heterosis, or hybrid vigor, occurs in both plants and animals. In plants, F1 hybrid offspring exhibit enhanced yield compared to the parents and are extensively used in agriculture across a variety of crop species (for review, see Schnable and Springer, 2013). Heterosis is generated through interactions between the two parental genomes and epigenomes in the nucleus of the hybrid. The advantage of hybrid vigor is confined to the F1 with the level of phenotypic uniformity and of heterosis reduced in the F2 and subsequent generations.

Despite the obvious benefits of heterosis for crop production, the molecular mechanisms underlying F1 hybrid vigor and its subsequent decay remain elusive. Hybrid vigor is suggested to be dependent on heterozygosity between the parents and this is generally thought of as variation in gene sequences with increased heterozygosity correlated with a greater level of heterosis (for review, see Birchler, 2010). However, variation between the parent epigenome also appears to be a contributing factor. Changes to siRNA populations and DNA methylation patterns have been reported in various hybrid systems and contribute to altering the transcriptome of the F1 hybrid (for review, see Groszmann et al., 2013; Chen et al., 2013). The remodelling processes occur predominantly at regions that differ in their epigenetic states between the parents (for review, see Groszmann et al., 2011; Greaves et al., 2012). The epigenome diverges at a much faster rate than the genetic sequence (Schmitz et al., 2011), which could be especially important for heterosis in intraspecific hybrids where genetic variation may not be extensive.

Although instances of single-gene “heterosis” have been reported, in most cases QTLs provide evidence that heterosis is a polygenic trait (for review, see Schnable and Springer, 2013). Dominance, overdominance, epistasis, gene dosage and metabolic synthesis models are suggested modes of allelic interaction explaining the generation of heterosis in the F1 hybrid (for review, see Kaeppler 2012; Baranwal et al 2012; Goff 2011; Schnable and Springer, 2013).

The levels of growth and heterosis attained by a given hybrid combination depend on the interactions of the specific alleles and epialleles contributed by the parents. This is consistent with hybrid vigor being the cumulative outcome of different levels of heterosis in different tissues at different times of development (for review, see Schnable and Springer, 2013). Adding further to the complexity is that increases in some traits, such as seed yield, are likely to be the result of heterotic changes earlier in the developmental cycle. With the advent of large scale analytical methods to document the genome, epigenome, proteome

and metabolome, new opportunities have arisen to uncover the mechanisms generating heterosis. It is now feasible to carry out these various "omic" studies on many different tissue and cell types at different stages of development across a variety of hybrids.

Whole-genome transcriptome studies of hybrids in Arabidopsis, rice, maize and wheat have been conducted (for review, see Baranwal et al 2012; Schnable and Springer, 2013; Chen 2013) and although interpretation of these data is complex, indications are that hybrids have some combination of changes to energy production, metabolism, stress response, senescence and hormone signalling.

In this study we characterise a number of intraspecific Arabidopsis hybrids throughout their lifecycle. Despite the close relatedness of these Arabidopsis accessions, the parental combinations produce hybrids with different patterns of heterosis for vegetative biomass and seed yield. In some combinations, hybrid vigor was first apparent during embryo development indicating an early morphological genesis for heterosis. Following germination, all hybrids exhibit different patterns of rosette growth as well as morphological differences of the rosette leaves and changes in the architecture of the reproductive structures which affect fruit and seed yields. Consistent with their differences in leaf development, the hybrids were found to have altered expression levels of genes regulating leaf size through modulating cell size and cell number, with the expression profiles differing between hybrids. The results show that even between closely related hybrids, heterosis for biomass and yield can be achieved through multiple processes some of which are common to all hybrids and others which are specific to a hybrid combination.

Results

Hybrids with different levels of heterosis have different patterns of vegetative growth

Heterosis is assessed by comparing the trait value of the F1 hybrid against the performance of the parents. Agronomically important traits, such as vegetative biomass or seed yield, generally need to exceed the better-parent value (BPV) for the hybrid to be considered commercially beneficial. These mature traits are comprised of smaller component traits that may only exceed the average performance of the two parents (mid-parent value; MPV), but collectively result in BPV for important traits. Therefore the performance of a hybrid is often best evaluated using comparisons against both BPV and MPV. Traits exceeding MPV and BPV are categorised as exhibiting mid-parent heterosis (MPH) and better-parent heterosis (BPH), respectively.

The *Arabidopsis thaliana* accessions C24, Landsberg erecta (Ler) and Columbia (Col), show phenotypic differences in vegetative growth and reproductive yields. Crosses between these accessions produce F1 hybrid combinations that show heterosis at some point(s) during vegetative development and in traits associated with reproductive yield. The reciprocal combinations of each hybrid mostly show similar patterns of development and when appropriate were considered jointly in the assessment of the hybrids. The C24/Ler, C24/Col and Col/Ler hybrids (/ denotes both reciprocal combinations), show differences in growth vigor compared to the parents at various time-points throughout vegetative development (Fig. 1A,B; Supplemental Dataset S1). We recognised three phases of heterosis during vegetative development based on differences in the heterotic growth patterns among the hybrids (Fig. 1B). These vegetative phases are influenced by a preceding embryogenic phase of heterosis and affect the final phase of vegetative maturity and seed production.

The first vegetative phase of heterosis occurs in the period from germination to approximately 7-9 days after sowing (DAS; Phase 1) at which point the cotyledons are the largest aerial organ. The C24 and Col parents have an equivalent diameter across both cotyledons of the seedlings with Ler being smaller at this early stage (Supplemental Dataset S1). All hybrids had 16-32% increase over MPV in seedling diameter (Fig. 1B; Supplemental Dataset S1). The C24/Ler and C24/Col hybrids produce seedlings which are 11-12% larger than the better parent, while Col/Ler hybrids are slightly smaller and similar to BPV (Dataset S1). The early processes of germination, marked by primary root emergence through to splaying of the cotyledons, occur in some hybrid combinations faster than the MPV but not significantly faster than the more advanced parent (Supplemental Fig. S1). This indicates that the larger sizes of the hybrid seedlings in vegetative phase 1 result from increased post germination growth rates and not from disparity in emergence times.

The disparity in size between the C24, Col and Ler seedlings ends by 13 DAS, after which the parents show similar sized rosettes for the remainder of the vegetative growth phase (Supplemental Dataset S1). The hybrids involving C24, Ler and Col exhibit similar growth until ~9 DAS after which their growth rates diverge during the second vegetative phase of heterosis which ends at ~23 DAS (Fig. 1B). During this period, C24/Ler has the greatest levels of heterosis and largest rosette size of the three hybrids, being always larger than either parent (Fig. 1B, Supplemental Dataset S1). The growth rates of the C24/Col and Col/Ler hybrids were markedly less than C24/Ler until 16 DAS at which point the C24/Col rosette size increased (Fig. 1B, Supplemental Dataset S1).

Phase three of vegetative heterosis extends from ~24 to ~42 DAS when rosette sizes of both parents and hybrids (except for C24/Col) plateau prior to a reduction in size as senescence of the rosette proceeds (Fig. 1B; Supplemental Dataset S1). During this phase, both the C24/Ler and C24/Col hybrids show an almost linear trend of increased rosette diameter over MPV, at levels that exceed the better parent (Fig. 1B, Supplemental Dataset S1). Whereas the Col/Ler hybrid, which has the smaller maximum rosette size of the three hybrids, initially shows a steady rate of growth above MPV that then decreases to a level close to MPV (Fig. 1B, Supplemental Dataset S1).

Additional hybrids generated by crossing the Wassilewskija (Ws) accession with the other three accessions showed different patterns of heterosis during their lifecycle (Fig. 1C; Supplemental Dataset S1). The Ws/Ler hybrid was unique with rapid growth, exceeding both parents, until midway into vegetative phase 3 (~31 DAS), when it drops off. The Ws/Col hybrid does not show much hybrid vigor and has a growth pattern comparable to Col/Ler (Fig. 1C; Supplemental Dataset S1). The Ws/C24 rosette produces more vegetative growth than the other hybrids, with a strong linear growth pattern resembling that of the C24/Ler hybrid (Fig. 1C; Supplemental Dataset S1).

Since rosette size is positively correlated with flowering time in Arabidopsis, changes to flowering time in the hybrids could contribute to the increased growth of the rosettes, especially during phase 3. The late flowering phenotypes of the C24/Col and Ws/C24 hybrids are a consequence of their genotypes at the *FLC* and *FRI* loci (Sheldon et al., 2000). Consistent with a delayed transition to flowering, C24/Col shows elevated mRNA levels of the flowering time repressor *FLC* (Supplemental Fig. S2A). *FLC* levels are undetectable in the earlier flowering Col/Ler hybrid and at MPV for C24/Ler (Supplemental Fig. S2A). Differences in flowering times probably contribute to the increased rosette size of C24/Col and Ws/C24,however, other factors must be operating since at 42 DAS the Ws/C24 hybrid is larger than the C24/Col hybrid yet neither initiates flowering before 53 DAS (Fig. 1B-C; Supplemental Dataset S1).

The timing of the differentiation of successive leaves from the apical meristem is similar in parents and hybrids (Supplemental Table S2); indicating that the larger rosettes of the hybrid result from differences in growth processes of the lamina and petiole of each leaf and are not a result of accelerated leaf initiation timing. In addition, the larger leaves of the hybrids have a more rapid rate of growth as opposed to a prolonged duration of growth compared to the parents (Supplemental Fig. S2B).

Hybrids have increased photosynthetic cell size and/or cell number

The larger rosette sizes of the hybrids indicate that leaf growth is greater in the hybrids than in the parents. This increased leaf size must result from changes in the size of the lamina and/or length of the petiole due to increases in cell size, cell number or both. The first phase of vegetative heterosis begins with an increased diameter across the cotyledons. At 7 DAS, the hybrid and parent seedlings are developmentally similar with well formed cotyledons and two emerging initial leaves that are already larger in the hybrids than in the parents. All hybrids have an increased whole-seedling biomass at 7 DAS with BPH levels ranging from 23% (ColxWs) to 77% (WsxLer) (Supplemental Table S3). The cotyledons are larger in area (Fig. 2A) and generally thicker (Supplemental Table S3) in the hybrids compared to the parents. The anatomical organisation of the cotyledon, consisting of epidermal layers bounding the single palisade mesophyll layer and cells of the spongy mesophyll, remain unchanged in the hybrids. Cotyledon size differs among the hybrids, with WsxLer producing the largest cotyledons (BPH 75%) and the C24-maternally derived hybrids (C24xLer, C24xCol and C24xWs) also having large cotyledon areas (BPH 35%-41%; Fig. 2A). The smallest cotyledons of the hybrids were produced by ColxWs and were the only combination not to show BPH with a 12% increase over MPV (Fig. 2A). The contributions of cell number and cell size to the increased area of the hybrid cotyledons were assessed by scoring paradermal optical sections of cleared cotyledons (Fig. 2B; see materials and methods). The photosynthetic cells of the palisade mesophyll are increased in area by 8%-26% over BPV in the hybrids having C24 as a parent and in the WsxLer hybrid (Fig. 2C), while the estimated number of palisade mesophyll cells per cotyledon was greater than the parents in all hybrids, ranging from 25%-132% BPH (Fig. 2D). These data demonstrate that the larger cotyledons of the hybrids are due to an increase in cell number and in some cases cell size, with the extent and ratios of these two properties differing among the hybrids (Fig. 2C-E).

During phase 2 of vegetative heterosis, rosette growth of the hybrids diverged, indicating differences in leaf growth. These differences were prominent at 15 DAS when the two largest leaves on both parents and hybrids are the initial two leaves. Previous studies of the Col parental accession show that growth of the Arabidopsis leaf occurs initially through cell division followed by a phase of cell expansion (Donnelly et al., 1999, Beemster et al., 2005). Cell division rates of the first two leaves become minimal and localised to the most proximal region of the leaf blade at 12 DAS at which point leaf growth occurs through cell expansion with growth plateauing at ~21 DAS. Plant growth under our conditions was similar to the above studies, with leaves 1 and 2 first apparent at 6 DAS and leaf length plateauing at ~23 DAS for both parents and hybrids (Supplemental Fig. S2); at 15 DAS the first two leaves,

which are at >70% of their eventual length, are well into the cell expansion phase with little to no cell division occurring.

A comparison of the larger of the two leaves shows that all hybrids exhibit a greater leaf blade area than the parents, through increases in both length and width (Fig. 3A; Supplemental Table S4), indicating the hybrids have a significantly greater photosynthetic area than their parents. Most hybrids retain a leaf shape similar to those of the parents, except for Ws/Ler and Ws/Col in which the ratio of leaf length and width deviates from the MPV (Supplemental Table S4). The younger leaves of the 15 DAS rosette are also larger and result in a aerial fresh weight for the hybrids greater than BPV for all except Col/Ws (Supplemental Table S5). Among the hybrids, the leaf blades were most separated in C24/Ler and Ws/Ler due to their longer petioles (Fig. 3B), probably increasing the exposed photosynthetic area of the rosette and contributing to these hybrids having the greatest rosette diameters at this stage. The Col/Ler and Ws/Col have the shortest petioles and subsequently a more compact rosette (Fig. 3B). The palisade mesophyll cells in the leaf blade of the 15 DAS hybrids show increases in size and/or number compared to the parents, with the relative ratio differing between hybrids (Fig. 3C-E). Marked increases in cell size occur only in hybrids where C24 is a parent (Fig. 3C, F), whereas significant cell number increases occur in all hybrids except C24/Ws (Fig. 3D), with levels the greatest and exceeding BPV when Ler is a parent (Fig. 3F). These results suggest that the C24 parent provides allele(s) responsible for increasing cell size, whereas Ler allele(s) promote the greater increases in cell proliferation in the leaf lamina.

With the exception of cell size in WsxLer and cell number in C24xWs, the changes in cell number and cell size in the leaf blade of the hybrids resemble those occurring in the cotyledons. Increases in cell size contribute more to a larger mature cotyledon or leaf than does an equivalent increase in the number of palisade mesophyll cells (see materials and methods). This factor was taken into account (see materials and methods) in calculating the relative importance of increased cell size and cell number to the enhanced cotyledon and leaf size of the hybrids (Fig. 2E, 3E). We inhibited cell expansion expecting that the reduction in leaf/rosette growth would differ between the hybrids depending on their different proportion of cell size and cell number increases. Hybrid plants were treated with paclobutrazol which inhibits the biosynthesis of gibberellin, a hormone that promotes growth predominantly through cell expansion (Jiang et al., 2012). Paclobutrazol treatment caused reductions in rosette diameter in both the parents and hybrids (Fig. 3G). Of the three hybrids, paclobutrazol treatment most affected C24/Col, which showed the greatest reduction (53%) in rosette diameter making it the smallest of the three hybrids in the treated

group and decreasing its level of heterosis (31% to 12%), consistent with it being the most dependent on cell size for its increased leaf growth (Fig. 3G). The C24/Ler and Col/Ler hybrids were less impacted by a reduction in cell size with Col/Ler showing an increase in heterosis levels (14% to 30%) to above BPV (Fig. 3G), consistent with it being the least dependent on cell expansion for its greater leaf growth. These data support the cellular morphological profiles of the hybrids which show that the hybrids share some common components to their heterotic growth (i.e. increased cell numbers) but that there are also unique factors (i.e. increased cell size) associated with some hybrids.

Genes that control leaf size show altered expression in 15 DAS hybrid seedlings

The increased rosette leaf size of the hybrids and the causal changes in cell size and cell number must be generated through alterations to the hybrid transcriptome. Hundreds to thousands of genes are differentially expressed in F1 Arabidopsis hybrids (Fujimoto et al., 2012; Meyer et al., 2012; Shen et al., 2012), many of which may have no direct impact on generating the increased vigor of the F1 plants. In this study we focused on genes that have been identified as increasing leaf size when mutated or ectopically expressed, together with a set of circadian clock genes that have been implicated in heterosis (Gonzalez et al., 2009; Breuninger and Lenhard 2010; Ni et al., 2009). 71 genes, associated with a broad range of functions including transcriptional regulation, hormonal regulation, and cell modifications, were examined for changes in expression in the F1 hybrids (Supplemental Table S6).

Expression values for each gene were obtained from an mRNA-seq dataset derived from aerial tissue of 15 DAS seedlings from C24, Ler, Col and their reciprocal hybrid combinations (see materials and methods). Genes of interest were those with expression levels in the hybrid that deviated from MPV and/or were different between the hybrids, as either pattern of differential expression may contribute towards the different growth patterns of the hybrids. Of the 71 genes, 26 fit these criteria (Fig. 4), with some of the genes expressed at levels different to MPV in all three hybrids, while others are different in only one or two of the hybrid combinations. For most of the 26 genes, their reported effect on cell size or cell number and the required change in expression needed to obtain a larger leaf, fit the altered expression and changes in cell size and cell number seen among the hybrids (Fig. 4). For instance, plants mutant for *OBP2*, a transcription factor that regulates indole glucosinolate biosynthesis, produce larger leaves through increased cell size (Skirycz et al., 2006). This gene is down-regulated compared to MPV in C24/Ler and C24/Col, the two hybrids that show enhanced cell size, while expression levels are at MPV and at the highest level in the Col/Ler hybrid in which cell size is not increased (Fig. 4). In only five cases does

the gene expression in the hybrids not fit the expected relationship between the known function of the gene and cellular morphological changes occurring in the hybrids (Fig. 4).

The 26 genes range widely in their placement within the regulatory cascade modulating cell size and cell number. At the lower levels of the hierarchy are genes such as *CYCD3;1* (*CYCLIN D3;1*) and *HEN3* (*HUA ENHANCER 3*), which are core members of the cell cycle and directly regulate cell proliferation. The expression of these two genes deviates from MPV in only the C24/Col hybrid, being down and up-regulated respectively. The function of these genes and pattern of differential expression is consistent with the C24/Col hybrid relying less on cell proliferation and more on cell expansion for achieving its larger leaf size (Fig. 4). qRT-PCR analysis of three additional cyclins (*A2;3*, *B1;1* and *D3;2*) show similar below-MPV expression patterns in the C24/Col hybrid (Supplemental Fig. S3). Expression levels of these genes are lowest in C24/Col and highest in Col/Ler (Supplemental Fig. S3), consistent with these two hybrids having the opposite cell size and cell number profiles for heterotic leaf growth. At the upper levels of the regulatory hierarchy are genes such as *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*), *LHY* (*LATE ELONGATED HYPOCOTYL1*), *TOC1* (*TIMING OF CAB EXPRESSION 1*) and *GI* (*GIGANTEA*), which as part of the circadian clock, regulate diurnal expression of ~30% of the transcriptome and have been implicated in heterosis (Chen, 2010). During the day/night cycles of the clock, *CCA1* and LHY have an oscillating pattern opposite to *TOC1* and *GI*. *CCA1* and *LHY* have their lowest expression levels during the day and highest levels at night (Chen, 2010). At ZT8 (Zeitgeber Time; ZT0 = dawn) *CCA1* is at MPV while the daytime-decreasing expression of *LHY* is below MPV in all hybrids with levels reduced to low-parent in C24/Ler and Col/Ler, and below low-parent in C24/Col (Fig. 4, Supplemental Fig. S4); whereas the daytime-increasing expression of *TOC1* and *GI* at ZT8 is above MPV and at high-parent in C24/Ler and Col/Ler and above high-parent in C24/Col (Fig. 4, Supplemental Fig. S4). The expression levels of these clock genes differ between the hybrids with the lowest levels of *LHY* expression and highest levels of *TOC1* and GI at ZT8 all occurring in C24/Col (Fig. 4, Supplemental Fig. S4). Differences in the expression levels of these clock genes from the parents and between hybrids are also observed at an earlier point in the day (ZT6) and an evening time point (ZT15; Supplemental Fig. S4). Together these data suggest that the oscillating patterns of these clock genes may differ between the parents and hybrids and also between the hybrids. For example, C24/Ler in comparison to C24/Col and Col/Ler, expresses *CCA1* at higher levels at ZT6, similar levels at ZT8, and then higher levels at ZT15 (Supplemental Fig. S4). This suggests C24/Ler has a delayed daytime repression but a more rapid evening increase of *CCA1* compared to the two other hybrids. C24/Ler also shows a delay in both the daytime

increase of *TOC1* at ZT6 and its evening suppression at ZT15 compared to C24/Col and Col/Ler (Supplemental Fig. S4).

Some of the differential expression of genes is unique to one hybrid. This suggests that the hybrids may achieve their increases in cell size and/or cell number through different pathways or via differences in the levels of change in a common pathway. For example, expression of *ARL* (ARGOS-LIKE) is different only in the C24/Ler hybrid where it is up-regulated compared to MPV and expressed at levels greater than in C24/Col and Col/Ler (Fig. 4, Supplemental Fig. S3). *ARL* regulates petiole length and leaf blade size as part of the brassinosteroid pathway (Hu et al., 2006), as do *EXO* (*EXORDIUM*) and *BEN1* (*BRI1-5 ENHANCED 1*) which are expressed at higher and lower levels respectively in C24/Ler compared to the other two hybrids (Fig. 4). Together, the higher *ARL*, higher *EXO* and lower *BEN1* expression levels of C24/Ler, are consistent with C24/Ler having the longer petioles (Fig. 4; 3B) and suggests the larger cells of the C24/Ler hybrid may be promoted more so through this pathway. Increases in cell number among the hybrids may also be achieved through different pathways, with genes such as *ARGOS* (*AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE*) up-regulated in C24/Ler and C24/Col compared to Col/Ler (Fig. 4, Supplemental Fig. S3), while other genes suggested to promote increased cell number, such as *SAMBA* and *CLE26* (*CLAVATA3/ESR 26*), appear to be higher in the Col/Ler hybrid (Fig. 4).

Heterosis occurs in embryogenesis but varies between reciprocal crosses

The 7 DAS hybrid seedlings have larger cotyledons than their parents as a product of increased cell size and cell number. As the cotyledons are an embryonic leaf structure it is possible that these changes first occur during embryo development. Seed size was found to strongly correlate with mature embryo size for both parents and F1 hybrids (R = 0.97; Supplemental Fig. S5), and was used as a proxy for assessing mature embryo heterosis across all of our hybrid combinations. 7 of the 12 reciprocal hybrid combinations produce mature embryos that are larger than MPV, indicating that heterosis can first arise during embryogenesis (Fig. 5A). Unlike post-germination vegetative growth where both reciprocal hybrid combinations show similar trends in their level of vigor, most hybrids differ in embryo size between the reciprocal combinations (Fig. 5A). Overall, the maternal parent is the major determinant of hybrid embryo size, with the paternal parent occasionally having an effect. C24 produces the largest embryos of the parental lines and supports BPH when present as the maternal parent (Figure 5A-B; C24xLer, C24xCol and C24xWs). However, C24 has the opposite effect as the paternal donor, producing the two smallest and only

hybrid embryos (LerxC24 and WsxC24) to show a decrease in size relative to the MPV (Fig. 5A-B). Ws as the maternal parent promotes BPH, except when it is crossed with a C24 male, with WsxLer having the greatest relative increase above MPV of all the combinations (Fig. 5A-B). Col and Ler as maternal parents have a minor influence on non-additive embryo growth (Fig. 5B; the small LerxC24 embryos can be attributed to the C24 paternal effect), although Ler is the most restrictive maternal parent on embryo size across both the parental and hybrid lines (Fig. 5A-B).

To characterise heterotic development during the embryogenic phase, the C24/Ler hybrid was examined given the large disparity in final embryo size between the reciprocal combinations (Fig. 5A). The differences between the Ler and C24 parents, is evident early in embryogenesis, with C24 producing embryos with a more rapid rate of development by 5 days post pollination (DPP; χ^2 $p < 0.01$; Fig. 5C) and becoming significantly larger than Ler by late heart stage at 7 DPP (Fig. 5D) and continuing to the mature embryo. In the C24xLer hybrid these traits are enhanced with heterosis first discernible at 5 DPP as a faster rate of development than the C24 maternal parent, which continues through 7 DPP (χ^2 $p < 0.01$; Fig. 5C). The C24xLer hybrid embryo becomes significantly larger than C24 from early torpedo stage as the embryo enters its growth phase (Fig. 5D) and by the bent cotyledon stage is 18% larger than MPV and 9% bigger than C24 (Fig. 5D), consistent with it maturing into a larger embryo (Fig. 5A). LerxC24 remained similar to its maternal smaller parent Ler (Fig. 5C-D), consistent with it being undersized compared to MPV at maturity (Fig. 5A).

The growth of the embryonic cotyledons between the reciprocal combinations and between the hybrids resembles the patterns seen in cotyledons at 7 DAS (Fig. 5E, 2A). The C24xLer, C24xCol, C24xWs and WsxLer hybrids produce the largest embryonic cotyledons (Fig. 5E, Supplemental Fig. S6C-D). There was no obvious increase in cell size or cell number in the other hybrids (Supplemental Fig. S6C-D). The smaller size of the LerxC24 and WsxC24 embryos is due to reduced cell size (Fig. 5E, Supplemental Fig. S6C-D). These results indicate that the alterations to cell size and cell number of the 7 DAS cotyledons begin during the embryogenic phase. However, it also indicates that the majority of the cellular morphological changes in the 7 DAS cotyledons (i.e. increased cell size in the LerxC24, ColxC24 and WsxC24 and greater cell numbers in all hybrids) must be generated during the post-germination period. The extent of cell expansion and cell proliferation involved in post-germination cotyledon growth was estimated by comparing the embryogenic cotyledon and 7 DAS cotyledon data (Supplemental Table S7). The levels of post-germination cell expansion were increased above parental levels only in hybrids having C24 as a parent and in WsxLer (Supplemental Table S7), with the greatest levels occurring in the LerxC24 and WsxC24

hybrids, the two combinations with the below-MPV sized embryos. The levels of cell proliferation were greater than parental levels in all hybrids (Supplemental Table S7).

More cells and/or increased cell size in the hybrid embryonic cotyledons suggest a greater potential for vegetative growth, realised through post-germination cell division and expansion. This appears to be true among the hybrids, with mature embryo size (of the larger of the two reciprocal combinations) highly correlated with cotyledon area at 7 DAS (R = 0.84; Fig. 5F); and within reciprocal hybrids where the genetic backgrounds are identical yet the reciprocal combination with the larger embryo produces the bigger cotyledons by 7 DAS (Fig. 5G). Differences between reciprocal combinations are discernible as late as ~25 DAS in the C24/Ler and Ws/C24 hybrids (Dataset S1), which have the largest differences in embryo size between reciprocal combinations. This suggests that embryo heterosis may have positive affects late into rosette development.

Shoot architecture influences the level of fruit and seed yield in the hybrids

The rosette produces and mobilises resources for the development of the reproductive shoot structure (Fig. 6A). At the time of flowering and at their maximum size, the rosettes of C24/Ler and C24/Col are larger than BPV, while the smaller Col/Ler rosette is above MPV and at MPV, respectively (Fig. 1B, 6B; Supplemental Dataset S1). At the termination of the main shoot the three hybrids have larger shoot structures than their parental lines and differ in their architecture (Fig. 6A-D; Supplemental Table S8). Each hybrid has a main shoot taller than MPV (Fig. 6C), with their height exceeding BPV in C24/Ler and Col/Ler. The C24/Col hybrid develops a complex branched architecture (i.e. a greater number of lateral and secondary branches), significantly increasing the size of its shoot structure over the other two hybrids (Fig. 6D). The Col/Ler hybrid has branching equivalent to that of the low-parent, resulting in the least complex shoot structure of the three hybrids (Fig. 6D). Branching in the C24/Ler hybrid is at MPV and intermediate to C24/Col and Col/Ler (Fig. 6D).

As reproductive shoot development is largely fed by nutrients remobilised from the rosette (Bennett et al., 2012), it is reasonable to assume that the larger the vegetative biomass the greater the growth and output of the reproductive structure. Correlations between the three key structural traits of (i) mature rosette size, (ii) main shoot height and (iii) branching complexity reveal that mature rosette size does not correlate with plant height in the hybrids (Supplemental Table S9); however mature rosette size does positively correlate with branch number (r=0.76), while branch number negatively correlates with plant height (r=-0.69;

Supplemental Table S9). These patterns imply that on a gross structural level the larger rosette of the hybrids can support a greater reproductive structure but that resource sinks in the form of branches affect resource allocation for main shoot development.

Since resource levels and distribution are also important for yield, the association between rosette size (resource), branch numbers (sinks) and a number of yield traits was examined in the hybrids (Fig. 6E-J; Supplemental Table S8,S9). The large rosette size and intermediate branching complexity of the C24/Ler hybrid results in the highest resource:sink ratio of the three hybrids. Consistent with this, the C24/Ler hybrid is the best yielding hybrid with improved traits including BPH for number of fruit on the main shoot (23%), F2 seed size (1.3%), seed yield from the main shoot (28%) and total seed yield (38%). C24/Ler also shows BPV for fruit size and seeds per fruit (Fig. 6E-J; Supplemental Table S8).

Significant differences in yield were observed between the C24/Col and Col/Ler hybrids which exhibited the extremes of rosette size and branching complexity. Despite having by far the smallest mature rosette size, the reduced branching of the Col/Ler hybrid improves its resource:sink ratio which is consistent with it showing MPH for number of fruit on the main shoot and BPH for seeds per fruit (17%), fruit size (17%) and seed yield (25%) from the main shoot (Fig. 6E-G,I; Supplemental Table S8). However, the Col/Ler still only ranks as the second best yielding hybrid of the three with seed size only at MPV and total seed production not as great as in the C24/Ler hybrid (Fig. 6H,J; Supplemental Table S8).

The increased resource drain of the more branches supported by the C24/Col rosette, correlates with significant reductions in seeds per fruit and fruit size to levels below low-parent and MPV, respectively (Fig. 6F-G). A BPH increase in fruit number (32%) enabled the C24/Col hybrid to yield a modest BPH increase in seed production from the main shoot (21%) and MPV for total production, with seed size slightly larger than BPV (Fig. 6E,H-I). However, C24/Col produced the lowest yields, compared to MPV, of the three hybrids despite having an equivalently large rosette to that of C24/Ler and presumably greater amounts of available photosynthate compared to Col/Ler. Overall, the correlations of the various yield traits cluster into two main groups (Fig. 6K). Seed size and number of fruit on the main shoot positively associates with mature rosette size, while the resource drain of increased branch numbers negatively impacts fruit size, seeds per fruit and seed yield from the main shoot. Total seed production sits as an out-group in the relationship of these traits, consistent with it being the cumulative outcome of the other traits (Fig. 6K).

Discussion

The parental accessions C24, Ler, Col and Ws have similar genomes with relatively few genetic differences but more pronounced epigenetic variation likely associated with their different geographical origins (Cao et al., 2011; Schmitz et al., 2013; for review, see Schmitz and Ecker, 2012; Weigel, 2012). Together these properties result in the phenotypic differences in vegetative growth and reproductive yield observed between the parental accessions. The F1 hybrids between these accessions have unique combinations of these (epi)allelic variants and show different patterns of growth in discrete phases throughout their lifecycle.

Early in seedling development all six hybrid systems had sizeable increases in vegetative biomass exceeding the better parent. In the subsequent vegetative growth phases, only two of the hybrids, C24/Ler and Ws/C24, continued an almost linear trend of rosette growth greater than the better parent, with the heterotic growth in the other hybrids fluctuating during development. Two of the hybrids, Col/Ler and Ws/Col, despite showing early vigor finished with a mature vegetative size similar to MPV. Our data are consistent with reports that different combinations of Arabidopsis accessions produce hybrids with different levels of mature vegetative heterosis (Barth et al., 2003; Meyer et al., 2004; Moore and Lukens, 2011). In addition, the data demonstrate that heterotic phenotypes occur at many stages in the lifecycle of the plant, at levels that can vary between stages and hybrid combinations.

Delayed flowering time is considered to contribute to increasing vegetative growth (Aarssen and Clauss, 1992; Pigliucci and Schlichting, 1995; Pigliucci and Hayden, 2001). This may apply to late flowering hybrids such as C24/Col and Ws/C24 (Moore and Lukens, 2011), however, effects appear limited to late in development as increases in size prior to flowering (i.e. phases 1 and 2) are unperturbed by accelerating flowering time (Fujimoto et al., 2012). Factors other than delayed flowering time must also be contributing to late phase vegetative heterosis, as hybrids with similar prolonged vegetative phases (e.g. C24/Col, Ws/C24) can exhibit substantial differences in size (also seen in other hybrid combinations; Barth et al., 2003).

In the hybrids, all the leaves of the rosette show differences in their overall dimensions, shape and thickness, with the leaf blades generally larger than the parents. The larger leaves of the hybrids were associated with increases in size and/or number of the photosynthetic palisade mesophyll cells, with relative levels and ratios of change differing between the hybrids. Variation in the growth processes augmenting the leaf growth of the hybrids was reflected in the different expression levels of genes known to control leaf size.

In general, greater leaf cell numbers are associated more frequently with heterosis than are increases in cell expansion (for review, see Birchler, 2010; Blum, 2013). Accordingly, all but one of the hybrids had more cells in their leaves. However, increases in cell size were found to contribute proportionally more in generating a larger leaf (or cotyledon), with this feature promoted by the C24 parent.

The greater leaf area of the hybrids provides a greater capacity for photosynthesis and energy production important for heterotic growth and yield (for review, see Blum, 2013). This feature applies to hybrids in maize, cotton and Arabidopsis (Li et al., 2007; Zeng et al., 2012; Meyer et al., 2004; Fujimoto et al., 2012). In Arabidopsis hybrids, the greater capacity for photosynthesis is more prominent under high light intensities and appears common to hybrids having C24 as a parent (Meyer et al., 2004; Fujimoto et al., 2012). This feature coincides with our observations that C24 factors promote increased expansion of the photosynthetic palisade mesophyll leaf cells in the hybrids. These two traits appear developmentally related in that chloroplast number per cell is positively correlated with increased cell size (Pyke and Leech, 1991, Fujimoto et al., 2012) and artificially increasing cell size induces more chloroplast divisions (Pyke, 1997). Chloroplast targeted genes are increased in expression in the C24/Col hybrid, but not in the moderately heterotic Col/Ler hybrid that does not show increased cell size (Fujimoto et al., 2012). The increased expression of the chlorophyll gene *Lhcb* is associated with larger cells (Meehan et al., 1996) and impairment of chloroplasts via Norflurazon treatment causes a reduction in cell size (Meehan et al., 1996) and loss of heterosis in C24/Col hybrids (Fujimoto et al., 2012) but not in Col/Ler hybrids (Groszmann, unpublished).

The level of energy production required to support the greater growth of the hybrids may be provided by alterations to the circadian clock. The circadian clock regulates key processes such as day-time photosynthesis and night-time starch utilisation and its oscillating patterns vary among parental accessions (Dodd et al., 2005; Michael et al., 2003) with changes in these patterns implicated in heterosis (Ni et al., 2009). In 15 DAS hybrid seedlings we found key regulatory genes of the circadian clock show patterns of expression levels that differ between the hybrids and also from the parents. Similar changes in clock gene expression have been reported in Arabidopsis RILs, F1 hybrids and allopolyploids (Swarup et al., 1999; Michael et al., 2003; Ni et al., 2009; Miller et al., 2012; Shen et al., 2012) and are associated with changes in photosynthetic capacity and energy production (Ni et al., 2009, Miller et al., 2012). The circadian clock is under epigenetic control (Kurihara et al., 2008; Ni et al., 2009; Shen et al., 2012), suggesting that the differential expression of these clock regulators may be a consequence of the changes to the hybrid epigenome. These clock genes are not

differentially expressed in young hybrid seedlings (Meyer et al., 2012; Fujimoto et al., 2012), but are by 15 DAS (this study) and show greater differences by 25 DAS (Ni et al., 2009; Miller et al., 2012). Progressive changes in key regulatory mechanisms may contribute to the different patterns and levels of growth vigor in the hybrids over their life history and may be a common aspect of the hybrid F1 generation. Consistent with this, some of the altered epigenetic states affecting gene expression in the hybrids progressively develop throughout the course of the F1 generation (Greaves et al., 2014).

Heterosis during embryogenesis

We observed changes in growth during embryogenesis in the hybrids, resulting in some combinations developing larger mature F1 embryos. Embryo heterosis initially is seen as a faster rate of development and then an increase in size once the embryo enters the growth phase. Differences between reciprocal hybrids revealed parent-of-origin effects influence embryo heterosis. Such effects appear to determine levels of seed size heterosis in other Arabidopsis F1 hybrids (Alonso-Blanco et al., 1999; Meyer et al., 2004; House et al., 2010; Jong et al., 2011). The extremes of this effect occurred with C24 and Ler which, contrary to their roles as maternal parents, repress and promote embryo heterosis respectively as paternal parents.

Although both parents influence F1 hybrid embryo size, the maternal genotype is the major determinant, indicating that the maternally dominant tissues (endosperm and sporophytic seed coat) and other factors such as maternal nutrient supply, may contribute to embryo heterosis. The hybrids with increased F1 embryo size may be supported by greater nutrition supplied by the maternal parent and hybrid endosperm and possibly additional room to grow provided by a less restrictive seed coat growth. The opposite effects may restrict the potential for increased hybrid embryo size as seen when Ler is a maternal parent; a potential denoted by the marked increases in growth vigor once Ler maternal hybrids germinate.

In crop species, embryo heterosis has been reported in hybrids of rice (Akita, et al., 1990), faba bean (Dieckmann and Link, 2010) and maize (Meyer et al., 2007; Jahnke et al., 2010). In non-hybrid crops, larger embryo and seed size are desired traits selected by breeders as they often confer greater early seedling vigor and larger leaf area (Lopez-Castaneda et al., 1996; for review, see Richards, 2000). Consistent with this, the embryogenic phase of heterosis was found to have a positive effect on the post-germination growth vigor of the hybrids, including increased size of the photosynthetic cotyledons and initial set of leaves and potentially further into vegetative development.

Early vegetative phase of heterosis

Shortly after germination the young hybrid seedlings transition from heterotrophic to autotrophic growth and it is at this time that heterotic growth intensifies. The changes to cell size and cell number that determine the mature hybrid cotyledon size are most extensive during the early post-germinative period. In Arabidopsis and other higher plants, post embryonic growth of cotyledons occurs predominantly through light induced cell expansion, with minor contribution from cell division (Tsukaya et al.,1994; De Veylder et al., 2002; Stoynova-Bakalova et al.,2004). Surprisingly, all the hybrids showed substantial increases in post-germination cell proliferation in generating their larger cotyledons, whereas only hybrids having C24 as a parent and WsxLer had greater post-germination cell expansion. The patterns of change in the cotyledons are similar to those in the later developing rosette leaves of the hybrid. This suggests that some of the mechanisms determining later vegetative heterosis are set up early in hybrid development. Accordingly, the increases in photosynthetic capacity, metabolic rate and metabolite stores are already present at this early stage (Fujimoto et al., 2012; Meyer et al., 2012). These are influenced by the maternal parent (Meyer et al., 2012), which agrees with our assessment that the predominantly maternal influenced embryogenic phase of heterosis contributes to early seedling vigor.

Yield heterosis

Vegetative heterosis differed among the hybrids and was dependent on growth characteristics from embryogenesis right through to the mature rosette of the plant. As Arabidopsis, like other annual plants, enters into the reproductive stage, the resources accumulated in the rosette during the photosynthetic period are remobilised into the developing fruit and seeds (Bennett et al., 2012). The larger vegetative biomass was expected to result in an equivalent increase in fruit and seed yield. However, the relationship appears more complex with increases in fruit and seed yield relying on productive resource allocation into the reproductive structure. The better yielding hybrids, like C24/Ler, will be those with the most favourable resource to sink ratio produced by having a large rosette and simple branching structure. Pruning experiments in parental lines point to similar conclusions (Bennet et al., 2012), suggesting that manipulating the shoot architecture of hybrids, especially those having greatly enlarged vegetative structures, could improve yields.

Conclusion

Heterosis ultimately depends on the unique interactions of alleles and epialleles provided by the parents. This accounts for different hybrids exhibiting differing levels and patterns of heterotic growth, even within the same species. Increases in biomass and yield can be achieved through different patterns of heterotic growth driven by varied complements of altered gene expression. Various "omic" studies on the developmental stages highlighted here may identify key biological networks and their initial triggers which generate heterosis. Once identified, common networks and hybrid-specific exploits could be manipulated to 'capture' aspects of heterosis; not only in non-hybrid crops but also in hybrids to further enhance their vigor and yields.

Materials and Methods

Plant growth

Seeds were surfaced sterilised and sown on 150mm diameter plates containing Gamborg's B-5 Basal Media (Sigma; G5893-10L) balanced at pH 7.0 using KOH and supplemented with 0.6% wt/vol agar. Plates were sectioned into quarters consisting of 3 hybrid lines with the maternal parent in common sown alongside the maternal parent at a density of 6-8 plants per genotype. Plates were placed at 4^{o}C for two days to stratify the seeds and then transferred to a growth room (0 DAS) with conditions of 22^{o}C/18^{o}C (day/night) and 16hr/8hr photoperiod under Philips Cool Daylight TLD 58W/840 fluorescent tubes providing a photosynthetic photon flux density of 160-180 µmol m^{-2} s^{-1} as measured using a quantum meter (Model MQ-200 calibrated for electric light source, Apogee). At 18 DAS the seedlings were each transferred to 60mm^{2} by 70mm deep pots containing Debco Seed Raising Mix supplemented with 1g/L Osmocote Exact Mini® controlled release fertilizer pellets. 20 pots were placed in each tray at a density of 4 genotypes x 5 plants per tray, with positioning and combinations of genotypes randomised across the trays. Trays were placed in a larger growth room at 22^{o}C/18^{o}C (day/night) and 16hr/8hr photoperiod at 170-180 µmol m^{-2} s^{-1}. Clear plastic covers were placed on each tray for the first 3 days to assist in the continued healthy growth of the seedling after the transfer. The positioning of both plates and trays on the shelves were rotated and shifted daily to minimise possible positional effects on growth. Plants on soil were watered at a rate of 800ml per tray as needed (approximately every 3-4 days).

Hybrid crosses

Seed stocks: Both F1 hybrid and parental control seed were generated through hand pollination and restricting silique numbers on the mother plant as suggested by Meyer et al.,

(2004). *Crosses for examining hybrid embryo development at 5, 7 and 10 DPP*: For each sample (i.e. genotype and DPP) 6 siliques from 3 plants (i.e. two crosses per plant) were harvested and placed in a methanol : acetic acid : water (50%:10%:40%) fixative solution and stored at 4^{o}C. For consistency, developing seeds were harvested from the middle two-thirds of the silique to minimise possible apical basal bias caused by differences in pollen tube growth rates through ovary to ovule between genotypes. Developing seeds were mounted and cleared in a chloral hydrate solution (4g Chl: 2ml dH_2O: 1ml glycerol) and embryos visualised using a Zeiss Axioimager upright motorised microscope mounted with an Axiocam camera using differential interference contrast (DIC) optics and images captured using Axiovision software (http://microscopy.zeiss.com/). Embryo sizes were measured using ImageJ.

Expression analysis

RNA-seq: Tissue for RNA extraction was harvested from aerial tissues from 15 DAS seedlings from C24, Ler, Col and their reciprocal hybrid offspring. Two biological replicates were used for each sample with each replicate consisting of a pool of 15 seedlings. Total RNA was extracted using QIAGEN RNeasy Plant MiniKit^{TM} with on column DNA digestion using the QIAGEN RNAse free DNAse set^{TM}. Libraries were prepared and sequenced by a service provider using the illumina True-seq kit and sequenced on an illumina Hi-Seq as mRNA-seq pair ended 100nt runs. 65-70 million reads were sequenced per sample and mapped (>85% of sequenced reads) to TAIR10 reference genome using BioKanga align (http://code.google.com/p/biokanga/) using default settings and additionally implementing parameters -A5000 and -M5. Mapped reads were allocated to genomic features (as per TAIR10 annotations) using BioKanga maploci on default settings. Reads per genomic feature were standardised across libraries using the normalisation procedure in DESEQ (Anders and Huber, 2010; http://bioconductor.org/packages/2.13/bioc/html/DESeq.html). Negative binomial tests implementing the 'fitonly' variance dispersion model in DESEQ was used to determine significant differences in mRNA levels between samples (see Supplemental Table S6). *Quantitative RT-PCR*: RNA was extracted as above and cDNA synthesised using Superscript III reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed using SYBR green and Platinum Taq DNA polymerase (Invitrogen) as per manufacturer's instructions. Reactions were carried out in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Absolute transcript quantity was obtained using standards of Arabidopsis cDNA from each sample. Three biological replicates (each consisting of a pool of aerial tissue from 10 x 15 DAS seedlings) and between 2 to 4 technical replicates were performed for each sample and expression levels normalized against Ag4g26410 (Czechowski et al., 2005).

Rosette diameters, leaf dimensions and PM cell measurements

Rosette diameters: Rosette sizes (diameters) were obtained by imaging plants from an aerial perspective using a stage mounted Nikon DX3 camera using a Nikon 60mm lens. Rosette diameters were determined using ImageJ software (http://rsb.info.nih.gov/ij/).

Measurements of individual cotyledons and leaves: seedlings were harvested and submerged in a methanol : acetic acid : water (50%:10%:40%) fixative solution and stored at 4^{o}C. Samples were transferred to 50% ethanol and cotyledons or leaves dissected and ran through an ethanol:water series (50%,40%,30%,20%,10%,0%), cleared in a chloral hydrate solution (4g Chl: 2ml dH_2O: 1ml glycerol) and mounted for visualisation. Whole mount images of cotyledons and leaves were taken under a Leica MZFLIII dissector microscope mounted with an Axiocam camera and images captured using the Axiovision software. Length, width and area measurements were determined using ImageJ software. *PM cell measurements*: Images of palisade mesophyll cell were captured using a Zeiss Axioimager upright motorised microscope using DIC optics at x20 magnification mounted with an Axiocam camera and images captured using Axiovision software. All palisade mesophyll images were taken at a similar location on the cotyledons or leaves for all samples (distal from the mid-point of the leaf and either side of the mid-vein). Average cell size was determined by the number of cells in a 500µm^2 quadrant and total cell numbers estimated by extrapolating the average cell size across the area of the given cotyledon or leaf blade. Cotyledons and leaf blade thickness were measured using the vertical stage movement readings (µm sensitivity) of the software controlled Zeiss Axioimager upright motorised microscope. *Cell size and cell number contributions to increased leaf organ area over MPV*: Percentage increases over MPV for cell number and increases over MPV for cotyledon (or leaf) area were plotted for hybrids that showed no increases in PM cell size (i.e. Col/Ler, Ws/Col and LerxWs). A liner regression model was applied to this data whereby the co-efficient of X was considered the relative increases in cotyledon or leaf area due to increases in cell numbers. The linear regression equation was used to estimate the amount of cotyledon or leaf area in the C24/Ler, C24/Col, C24/Ws and WsxLer that was due to increased cell number, with the remaining area allocated as being due to increased cell size. The cell size portion of growth was plotted and a regression model applied to obtain an estimate of the contribution of cell size to increased area of the cotyledons or leaves. Increase in cell size over MPV contributes to increased cotyledon area 1.27x (leaf area it is 1.42x) more than the equivalent increase over MPV in cell number.

Paclobutrazol application

The experiment consisted of two replicate sowings each consisting of 5 plants per treatment for both parental and hybrids lines. Plants were grown on agar plates (as described above) for 13 DAS and transferred to a hydroponic system consisting of GroWool (Horticultural Systems, Girraween, NSW, Australia) placed on Osmocote Exact Mini® controlled release fertilizer pellets. At the time of transfer a single dose of $5x10^{-6}$M paclobutrazol was administered to the treated group. Rosette diameter was assayed at 29 DAS (as described above).

Yield data

All but total seed yield data was measured when the shoot apical meristem terminated. Siliques 5-15 on the main shoot were use for seed number and fruit length measurements. Total seed counts per main shoot were estimated by extrapolating the average number of seeds per silique across all fruit produced on the main shoot. Silique (or fruit) lengths were measured from the distal end of the gynophore to the base of the style (i.e. ovary region) using vernier callipers (Kincrome Tools). Dendogram of the relationship between reproductive traits was generated by using 'hclust' in R (http://www.R-project.org) inputting the pair-wise Pearson's correlation coefficients values between traits.

Seed size and embryo measurements

Seed measurements: Groups of separated seeds were imaged using the Leica MZFLIII dissector microscope and area measurements taken using ImageJ. *Embryo measurements*: mature seed were imbibed for 1 hour and embryos excised and mounted such that the embryos were fully spread revealing the entire structure. Embryos were imaged using Zeiss Axioimager and size (area) measured using ImageJ.

SUPPLEMENTAL DATA

Supplemental Figure S1. Timing of developmental events associated with germination and emergence in parental and hybrid lines.

Supplemental Figure S2. Leaf elongation rates in parental and hybrid lines.

Supplemental Figure S3. qRT-PCR expression analysis.

Supplemental Figure S4. Expression of circadian regulators CCA1, LHY, TOC1 and GI at three time points during the light period (ZT0 = dawn).

Supplemental Figure S5. Correlation between hybrid embryo size and seed size indicating that seed size can be used as a non-invasive proxy measurement for assessing embryo size and heterosis.

Supplemental Figure S6. Additional measurements assessing embryo heterosis.

Supplemental Table S1. Analysis of flowering time in the C24/Ler hybrid across several independent sowings.

Supplemental Table S2. A comparison of the differentiation rate of leaves from the apical meristem in parental and hybrids lines.

Supplemental Table S3. Cotyledon thickness and whole seedling FW of 7 DAS seedlings.

Supplemental Table S4. Additional leaf blade measurements.

Supplemental Table S5. Aerial fresh weights.

Supplemental Table S6. Comparison of mRNA levels between C24, Ler and Col and their hybrid offspring of 71 genes involved in determining leaf size through modulating cell size and cell number.

Supplemental Table S7. The extent of cell expansion and cell proliferation involved in post-germination cotyledon growth.

Supplemental Table S8. Reproductive trait measurements.

Supplemental Table S9. Pearson's correlation coefficients values for the pair-wise comparison between reproductive traits.

Supplemental Table S10. Primer sequences for quantitative RT-PCR reactions.

Supplemental Dataset 1. Rosette diameter measurements over the vegetative developmental period.

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Author responsibilities

M.G., W.J.P., and E.S.D., conceived and designed the experiments. M.G., R.G-B., I.K.G., L.W., A.K.H., performed the experiments. M.G., and R.G-B., analyzed the data. M.G., W.J.P., and E.S.D., wrote the article.

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Figure legends

Figure 1. Hybrids show differences in their vegetative growth patterns and levels of heterosis. (A) Representative hybrid plants showing increased vegetative growth compared to the parental lines at different time-points in development. (B-C) Levels of vegetative heterosis represented as the difference in rosette diameter between the hybrid and MPV. Error bars are S.E.M. N = 15-28 (see Supplemental Table S11). Significant deviations from MPV * Exceeds BPV, $^{+}$ Exceeds MPV (T-test $p < 0.05$). The three vegetative phases of heterosis marked below X-axis. Flowering-time indicated below X-axis labels, horizontal error bars are S.E.M. C24 flowering-time was variable between sowings but on average flowered at the same time as the C24/Ler hybrid (Supplemental Table S1).

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Figure 6. Measurement of reproductive architecture and yield of mature hybrid plants. (A) Examples of mature plants of C24, Ler and Col and their hybrid offspring. (B-D) The three

key structural traits of the mature plant. (E-J) Various yield traits. (K) A dendogram depicting the relationship between the major source component (rosette size) and the major sink component (branching complexity) have on various yield traits in the hybrids. There are two major clades identified in blue and orange. Traits in green are positively correlated with rosette size at maturity. Traits in red are negatively correlated with increasing number of branches. Total seed production sits as an out-group in this relationship of traits, consistent with it being the cumulative outcome of the other traits. Values above columns: percentages - level from MPV (red dashed line); red asterisk - significant difference from MPV; black asterisk above BPV or below LPV (T-test $p < 0.05$); Lettering - statistical comparisons among the hybrids (upper-case) and among the parents (lower-case) (T-test $p < 0.01$). All error bars are S.E.M. N = (B-E and I); 15-28, (F-G); 40-105, (H); 337-6926, (J); 5-6 (see Supplemental Table S11).

Supplemental data legends

Supplemental Figure S1. Timing of developmental events associated with germination and emergence in parental and hybrid lines. Error bar = S.E.M of the total process from radical emergence to splaying of cotyledons. DAS; Days After Sowing. DAPE; Days After Previous Event. Asterisk indicates event occurs more quickly in the hybrid than MPV (*) or BPV (**) (T-test $p < 0.05$).

Supplemental Figure S2. Leaf elongation rates in parental and hybrid lines. Leaves differentiate from the meristem and reach a maximum length at similar times between parents and hybrids. This indicates that the larger leaves of the hybrids are due to a more rapid rate of growth during the same time period. In both parents and hybrids, the elongation rate increases with each successive leaf as does the duration of growth consistent with each successive leaf being larger. Asterisk indicates an elongation rate in the hybrids exceeding MPV (*) or BPV (**) (T-test $p < 0.05$).

Supplemental Figure S3. qRT-PCR expression analysis. Expression vales represent the average expression level standardised to At2g24610, of 3 biological replicates consisting of aerial tissue from 10 pooled 15 DAS seedlings. Error bars = S.E.M.

Supplemental Figure S4. Expression of circadian regulators CCA1, LHY, TOC1 and GI at three time points during the light period (ZT0 = dawn). Expression levels at ZT8 are obtained from the RNA-seq datasets that are derived from two biological replicates

consisting of aerial tissues from 15 pooled 15 DAS seedlings. ZT6 and ZT15 are obtained through qRT-PCR that is from 3 biological replicates consisting of aerial tissue from 10 pooled 15 DAS seedlings. Error bars = S.E.M.

Supplemental Figure S5. Correlation between hybrid embryo size and seed size indicating that seed size can be used as a non-invasive proxy measurement for assessing embryo size and heterosis.

Supplemental Figure S6. Additional measurements assessing embryo heterosis. (A) Examples of the developmental stages of embryogenesis at 5, 7 and 10 DPP. Contrast of embryos has been digitally enhanced to aid visualisation (B) Embryo length at 5 DPP. (C) PM cell size in embryonic cotyledons. (D) Estimated PM cell number in embryonic cotyledons. For (C) and (D) values above columns: percentages denote level from MPV (red dashed line); red asterisk indicates different from MPV and black asterisk above BPV or below LPV (T-test $p < 0.05$); Lettering = differences between hybrids (upper-case) and between parents (lower-case; T-test $p < 0.05$).

Supplemental Table S1. Analysis of flowering time in the C24/Ler hybrid across several independent sowings.

Supplemental Table S2. A comparison of the differentiation rate of leaves from the apical meristem in parental and hybrids lines. Sample size: for 7 DAS; Two independent sowings of 10 and 15 plants for each category. For 15 DAS; 4 independent sowings of 15, 12, 10 and 10 plants for each category. For 21 DAS; 4 independent sowings of 15, 10, 10 and 15 plants for each category.

Supplemental Table S3. Cotyledon thickness and whole seedling fresh weight of 7 DAS seedlings. Sample size; 16-20 seedlings per line. * different from MPV, ** Above BPV; (T-test $p < 0.01$).

Supplemental Table S4. Additional leaf blade measurements from 15 DAS seedlings. Refer to Fig. 3 for sample sizes. * different from MPV, ** above BVP; (T-test $p < 0.05$)

Supplemental Table S5. Aerial fresh weights of 15 DAS seedlings. * different from MPV, ** above BVP; (T-test $p < 0.05$).

Supplemental Table S6. Comparison of mRNA levels between C24, Ler and Col and their hybrid offspring of 71 genes involved in determining leaf size through modulating cell size and cell number. Listed are the broad functional categories of the genes. Also listed for those genes with significance to the hybrids are the phenotypic effects on leaf organ development when expression levels of the gene are changed. FC = Fold-Change.

Supplemental Table S7. The extent of cell expansion and cell proliferation involved in post-germination cotyledon growth as estimated by the fold change in cell size and cell number between the embryogenic cotyledon and 7 DAS cotyledon data. Values in red are those exceeding BVP.

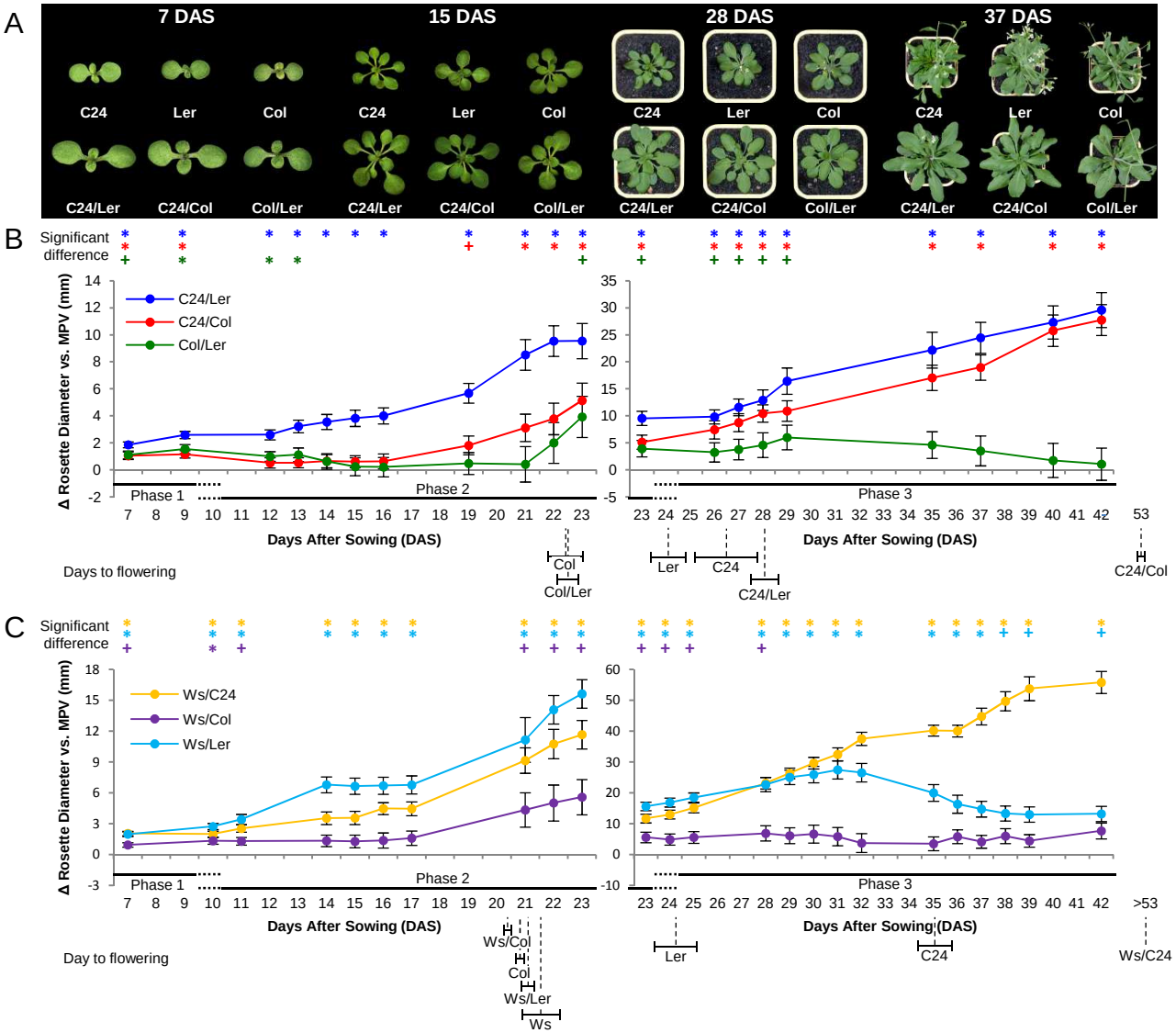
Supplemental Table S8. Reproductive trait measurements. * different from MPV, ** above BVP or below LPV; (T-test $p < 0.05$).

Supplemental Table S9. Pearson's correlation coefficients values for the pair-wise comparison between reproductive traits.

Supplemental Table S10. Primer sequences for quantitative RT-PCR reactions. HK; House Keeping

Supplemental Table S11. Detailed sample sizes for figures in main text

Supplementary dataset 1. Rosette diameter measurements over the vegetative developmental period. **Tables 1A-1C.** C24, Ler, Col and their hybrid offspring measurements. **Tables 1D-1F.** C24, Ler, Col and their hybrid offspring with Ws measurements. * different from MPV, ** above BVP or below LPV; † difference between the reciprocal hybrid combinations (T-test $p < 0.05$). **Figure 1.** Plots of rosette growth relative to MPV for the combined reciprocal hybrids along with the respective parental lines. **Figure 2.** Plots of rosette growth relative to MPV for the individual reciprocal hybrids along with the respective parental lines.



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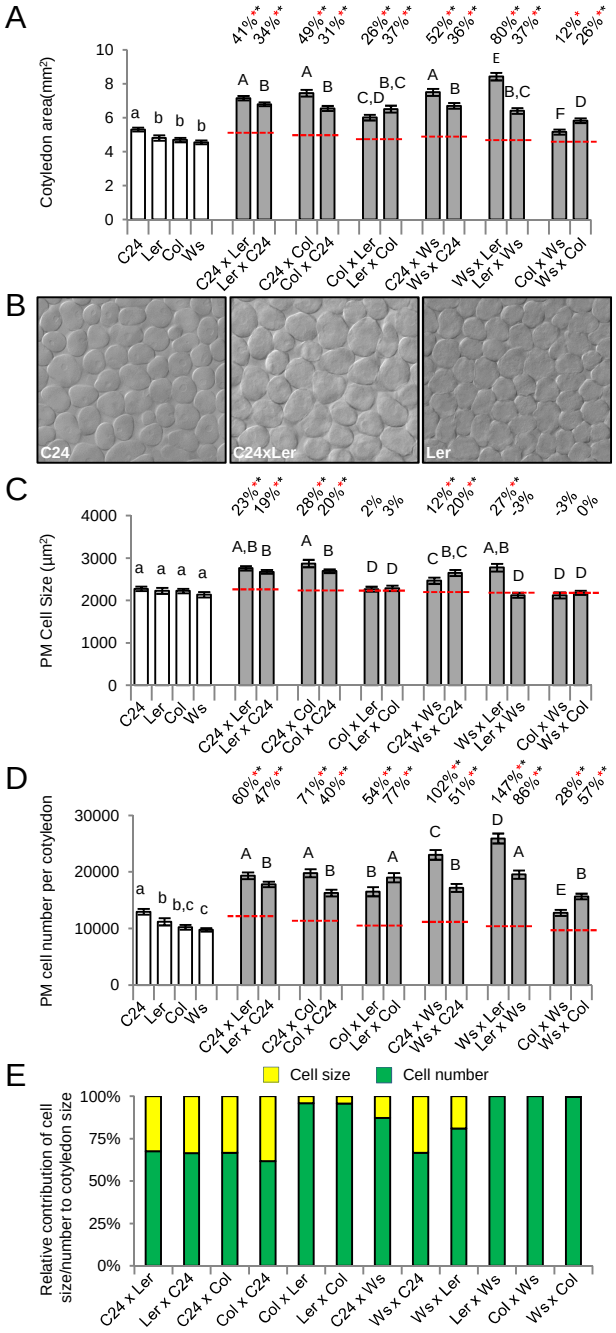

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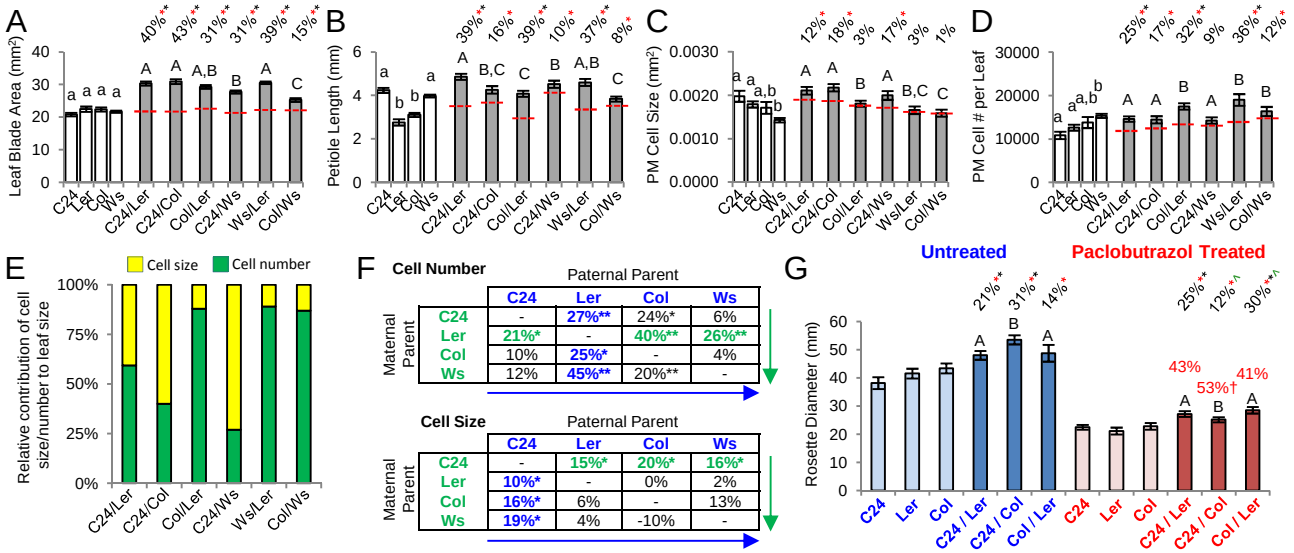

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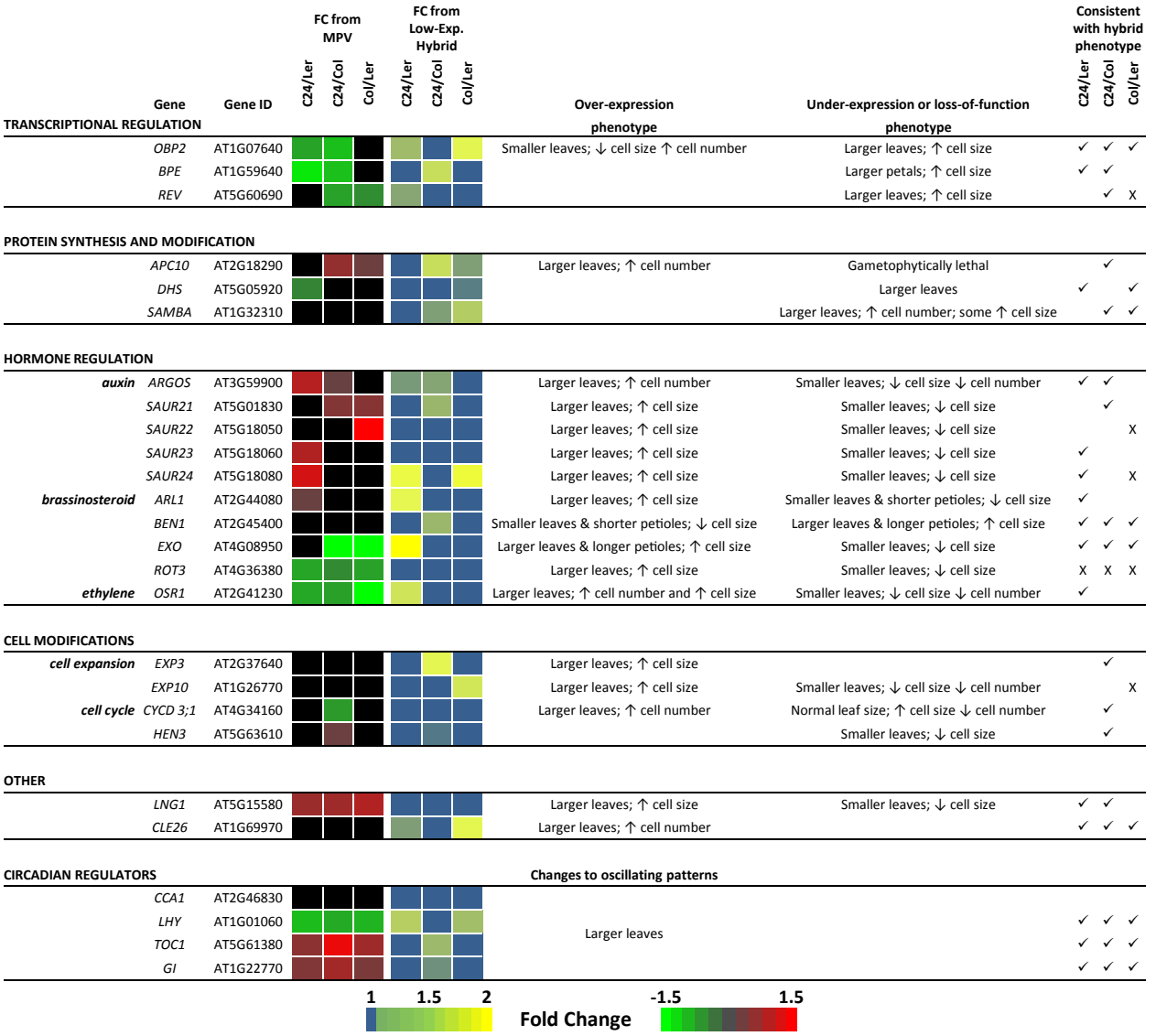

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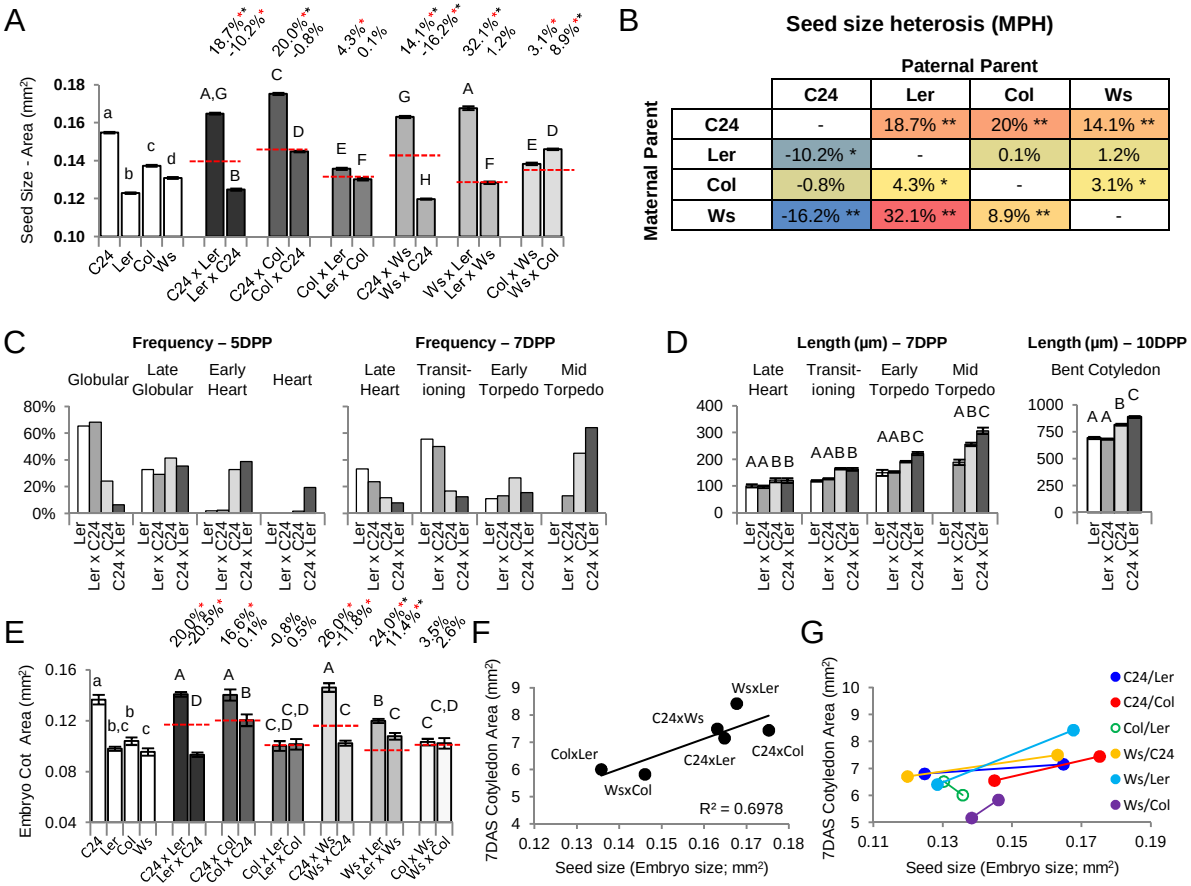

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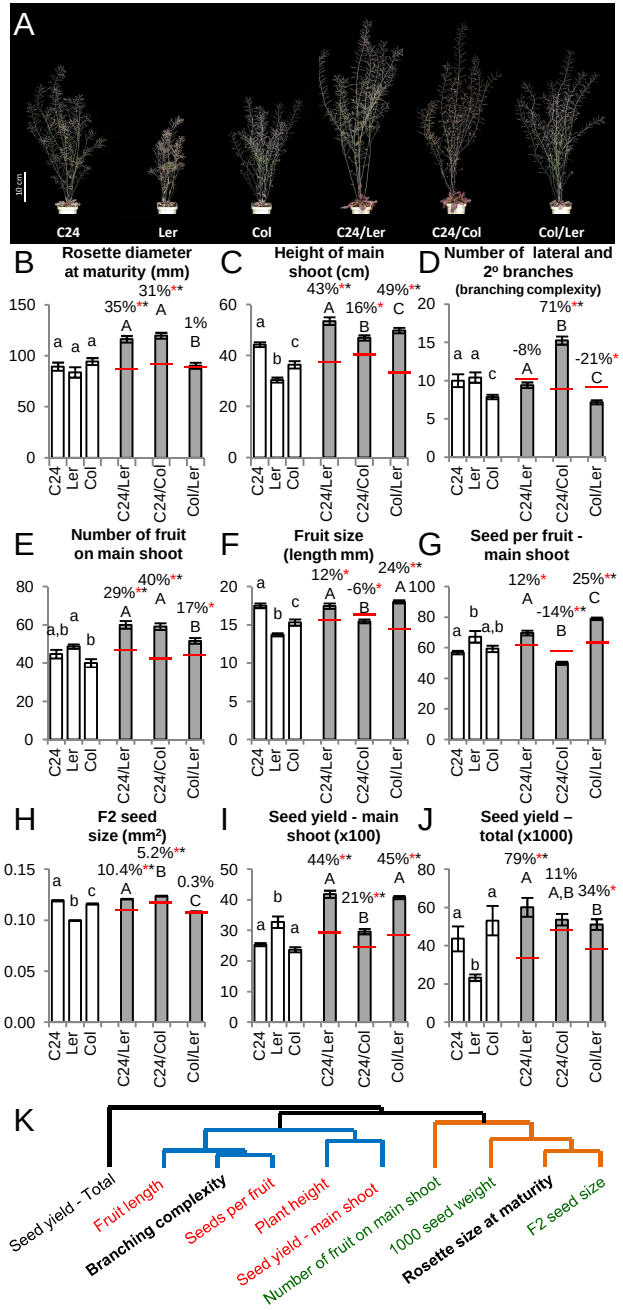

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