Pleiotropic Effects of the *Arabidopsis* Cryptochrome 2 Allelic Variation Underlie Fruit Trait-Related QTL

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Abstract: **The previous molecular identification of a flowering time QTL segregating in the *Arabidopsis* L*er* × Cvi cross, demonstrated that natural allelic variation at the blue light photoreceptor *CRY2* gene affects flowering time (El-Assal et al., 2001). In addition, previous works on the same cross have mapped several QTL affecting other unrelated life history traits in the *CRY2* genomic region. In the present report, we have used a set of *Arabidopsis* L*er* transgenic plants carrying four different functional *CRY2* transgenes for phenotypic analyses, with the aim of exploring the extent of pleiotropy of *CRY2* allelic variation. It is concluded that previously identified QTL affecting fruit length, ovule number per fruit, and percentage of unfertilized ovules are caused by this same L*er*/Cvi *CRY2* allelic variation. In addition, dose effects of the *CRY2*-L*er* allele are detected for fruit length. A seed weight QTL at the map position of *CRY2* could not be confirmed and also no effect on seed dormancy was observed. Thus, it is shown that transgenic plants carrying different alleles can be a useful tool to attribute QTL for different complex traits to a specific locus, even when the relationship among the traits has not been previously suggested.**

Key words: **Cryptochrome 2 *(CRY2),* flowering, life history traits, ovule number, pleiotropy, quantitative trait locus (QTL), silique length.**

Introduction

There is increasing interest in finding the genes that underlie quantitative traits (Glazier et al., 2002; Darvisi and Pisanté-Shalom, 2002). Such traits are genetically complex because they are often controlled by multiple genes (multifactorial traits), with individual genes contributing to a limited extent to the trait variation and often showing epistatic interactions. In addition, environmental factors and low penetrance further complicate genetic analysis of these traits. Quantitative trait locus (QTL) analysis allows genetic dissection of complex traits into a number of individual loci, for which their approximate map positions, quantitative contribution to the trait variation, and mutual epistatic interactions can be determined. However, the cloning of individual QTL is still complicated and laborious, and few reports of QTL cloning in various organisms have been published (reviewed by Glazier et al., 2002; Paran and Zamir, 2003). Allelic variation in QTL is mainly based on naturally occurring genetic variation and this determines a major complication in QTL cloning as compared to cloning based on artificially induced genetic variation. Sequence analysis often does not provide sufficient evidence on the specific genes underlying the QTL that control the trait of interest, because there are a large number of nucleotide polymorphisms between individuals and many of them might have no effect. Plants are no exception to this, but provide an important advantage to QTL cloning because complementation by transformation of the candidate genes into a donor genetic background can be achieved relatively easily.

The cloning of individual QTL requires major efforts and loci with small effects and low penetrance still remain very difficult because of the difficulties to genotype and fine map such QTL. One solution is the selection of recombinants carrying recombination breakpoints at different positions within a defined region, followed by a very refined phenotypic characterization of this limited number of recombinants. Another alternative is to rely for some QTL on the pleiotropic effects caused by these loci. Pleiotropy can involve traits that are not obviously related, but the same genetic location of such apparently unrelated traits might suggest that a specific locus controls several traits. Ongoing QTL analyses provide such suggestions of pleiotropy when several traits are mapped in similar genomic regions in the same segregating population or, with less genetic accuracy, in different populations. Examples of pleiotropy in plants come from the extensive use of the Nipponbare × Kasalath rice recombinant inbred line (Ril) population (reviewed by Yano, 2001) and the Ril populations Columbia/Landsberg *erecta* (Col/L*er*) and Cape Verde Islands/Landsberg *erecta* (Cvi/L*er*) in *Arabidopsis* (reviewed in Koornneef et al., 2004). For instance, the co-location of QTL affecting circadian period length (Swarup et al., 1999), water use efficiency as determined by $\delta^{13}C$ discrimination (McKay et al., 2003) and flowering time (Alonso-Blanco et al., 1998) at the position of the *FLC* locus suggested unexpected pleiotropy, which could be confirmed by the use of loss of function mutants.

Putative pleiotropic effects of one QTL can be confirmed in two different ways: i) by phenotypic analysis of the recombinants used to fine map one of the traits; ii) or by analysing transgenic

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plants transformed with specific alleles of a gene controlling one of the traits. Such phenotypic analyses can be used to test if pleiotropy is true, or if the phenotypic effects on the various traits are due to closely linked genes. In the *Arabidopsis* Cvi/L*er* RIL population a large number of traits has been identified by QTL mapping as affected by a locus on the top of chromosome 1. These include a locus called *EDI* (early, daylength-insensitive) which strongly affects flowering time and leaf number (Alonso-Blanco et al., 1998); a large number of life history traits such as seed size (measured as seed weight and seed length), or total fruit number; plant height, silique (the crucifer fruit) length, number of ovules per fruit, seed number per fruit, and percentage of unfertilised ovules (Alonso-Blanco et al., 1999); and several other seed-related traits, such as seed storability (Bentsink et al., 2000), seed dormancy (Alonso-Blanco et al., 2003), and phosphate content (Bentsink et al., 2003).

Using a positional cloning strategy, we identified the blue light photoreceptor cryptochrome 2 *(CRY2)* gene as being responsible for the *EDI* flowering time QTL (El-Assal et al., 2001). In addition, the allelic variation accounting for the flowering phenotypic differences was shown to be a single amino acid substitution of methionine (Met) to valine (Val) at position 367 of the *CRY2* protein. This amino acid substitution generated a gain of function allele, previously not identified by mutagenesis. Obvious and well known pleiotropic effects of the flowering time loci are the number of leaves and total number of fruits per plant. These effects of the *CRY2* QTL were shown first by analysing near isogenic lines (NIL) with small Cvi introgression fragments from the top of chromosome 1 into a L*er* genetic background. Conclusive proof of the flowering-related pleiotropic effects was obtained from analysis of transgenic L*er* plants carrying an extra *CRY2* gene from Cvi or from L*er,* and transgenic plants with an additional copy of the *CRY2* gene, where only the single amino acid substitution Val to Met at position 367 was introduced in *CRY2*-L*er* (El-Assal et al., 2001).

Thus far, the *CRY2* gene has been involved only in flowering time and photomorphogenesis (reviewed in Lin, 2002). In the present report, we have used a set of *Arabidopsis* L*er* transgenic plants carrying four different *CRY2* transgenes for further phenotypic analyses with the aim of determining if some other unrelated life history traits previously mapped on the top of chromosome 1 may be due to this allelic variation in the *CRY2* gene.

Materials and Methods

Plant materials

The genotypes used in this study have been previously described in El-Assal et al. (2001). Two non-transgenic lines were used: the laboratory reference accession Landsberg *erecta* (L*er*), and a near isogenic line named *EDI* NIL, carrying a 7 cM Cvi introgression spanning the *EDI* (*CRY2*-Cvi) locus from the top of chromosome 1, in an otherwise L*er* background. L*er* was the recipient genetic background of the functional transgenes and four different classes of transgenic plants were phenotypically characterized: i) L*er* plants carrying a 4.6 Kbp *CRY2* genomic fragment from L*er* (named T-*CRY2*-L*er*); ii) L*er* plants carrying the same 4.6 Kbp *CRY2* genomic fragment but from Cvi, (named T-*CRY2*-Cvi); iii) plants carrying a chimaeric transgene where 860 bp from the 4.6 *CRY2*-L*er* have been replaced with 860 bp from Cvi, the only sequence difference from L*er* being the nucleotide encoding the valine from L*er* at position 367 which is now encoding the methionine from Cvi (named T-*CRY2*-L*er* 367M); iv) plants carrying the reciprocal chimaeric transgene where 860 bp from the 4.6 *CRY2*-Cvi have been replaced with 860 bp from L*er,* the only sequence difference from Cvi being the nucleotide encoding the methionine from Cvi at position 367 which is now encoding the L*er* valine (named T-*CRY2*-Cvi 367V). For each class of transgenic line, plants of two independent homozygous lines carrying a single transgene at the T4 generation were used, and these were randomly selected from the lines previously characterized for flowering time (El-Assal et al., 2001).

Growth conditions and trait measurements

Plants were grown in an air-conditioned greenhouse in two different experiments, as described previously (Alonso-Blanco et al., 1998, 1999). In experiment 1, a total of 12 plants per genotype were grown in two rows of six pots (1 plant per pot) distributed in two blocks. Rows containing the various genotypes were randomised in each block and 4 plants were analysed (2 per block) for fruit and ovule traits. In experiment 2, four blocks with six plants per genotype were grown and four plants (1 per block) were analysed for fruit and ovule traits.

For the analysis of fruit traits, one fruit from the main inflorescence at position 6 to 12 from the bottom was removed just before maturity, the stage at which fruit and seed growth have stopped (Alonso-Blanco et al., 1999). The fruit length (FL) was measured with a caliper. Thereafter these fruits were dissected under a stereomicroscope to count the number of seeds and the number of unfertilized ovules, values that were used to determine the total number of ovules (ON) per fruit and the percentage of unfertilised ovules (Unf%).

In experiment 1, the seeds of the main inflorescence of the 12 plants per genotype were harvested once, when most of the siliques were mature (when fruits were brown due to loss of chlorophyll and were dry). The seeds were stored at room temperature and used for seed weight (SW) estimations and in germination experiments. The weight of 100 seeds was determined in three replicates from the same seed harvest of 12 plants per genotype, using a microbalance. In the second experiment seed weight determination was performed on 4 bulk samples (6 plants per harvest) of each block replicate. Seed germination tests were performed as described previously (Alonso-Blanco et al., 2003) by incubating seeds for 1 week in water, under white light, at 25 °C. Germination was assayed 1, 2, 3, 4, and 5 weeks after seed harvest, from plants grown in experiment 1 in long days. Three germination tests were performed per genotype at each time point using the seeds from a single seed harvest.

Statistical significances of the differences among genotypes ($p < 0.05$) were tested using the ANOVA test module of the SPSS v.11 statistical package and comparing treatments using the Tukey test. To test the effect of the *CRY2* alleles on the trait, the four transgenic lines carrying the L*er* variant at the 367 position were compared with the four transgenics carrying the Cvi variant at that position. Unf% data were first transformed into probits to improve normality of the data.

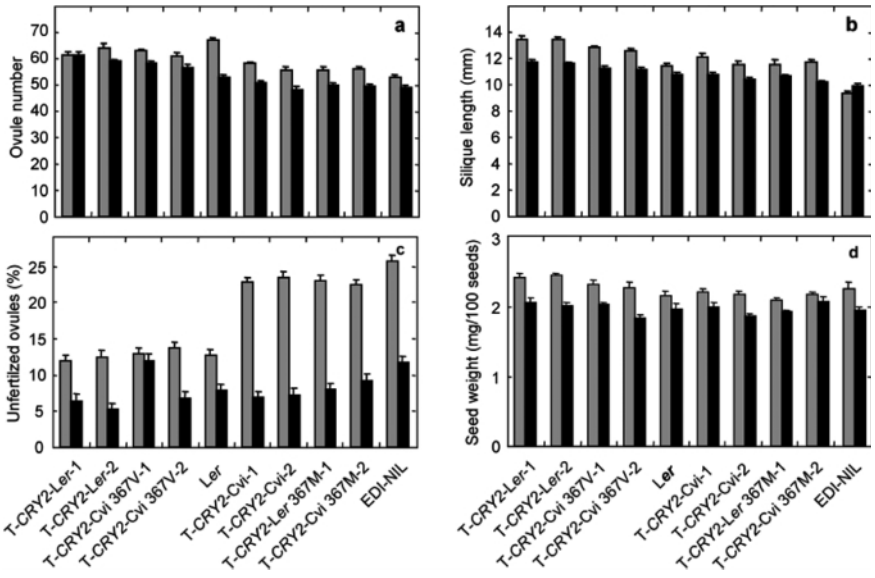

Fig. 1 Pleiotropic effects of *CRY2*/*EDI* QTL on: (**a**) ovule number; (**b**) silique length (mm); (**c**) unfertilised ovules (%); and (**d**) seed weight (mg per 100 seeds). Grey bars represent experiment 1 and black bars experiment 2. Mean ± standard error are shown (see Material and Methods for number of measurements per trait and per experiment).

Results and Discussion

A useful way to confirm and validate a QTL that was first identified in a segregating population is to observe the phenotypic difference predicted by the QTL analysis in a pair of near isogenic lines, differing only in the chromosomal region under study. The comparison of *Arabidopsis* genotypes L*er* and *EDI* NIL, differing only at the top of chromosome 1, confirms the previously mapped L*er*/Cvi QTL within that region (Alonso-Blanco et al., 1999) for SW, FL, ON, and Unf%. L*er* showed significantly larger FL and ON than the *ED*I NIL, while *EDI* NIL had significantly higher SW and Unf%, in agreement with the phenotypic additive effects predicted by the previously mapped QTL (Alonso-Blanco et al., 1999).

To determine if the gene underlying these L*er*/Cvi QTL is also *CRY2,* which would then show pleiotropic effects on those traits, or any other gene located within the 7 cM introgressed region, we have phenotypically characterized four different classes of *CRY2* transgenic lines (see Materials and Methods). All the transgenic lines were in a L*er* genetic background, and therefore all transgenic plants carry two doses of the *CRY2*-L*er* endogenous alleles. In addition, the different transgenic lines carried two doses of another functional *CRY2* allele, namely: *CRY2*-L*er*, *Cry2*-Cvi, *CRY2*-L*er* 367M or *CRY2*-Cvi 367V alleles. Since the effect of L*er*/Cvi *CRY2* allelic variation on flowering time was determined by the single amino acid substitution at position 367 (El-Assal et al., 2001), we first tested if this same polymorphism accounted for the variation in other traits. For that, we compared two sets of transgenic lines, T-*CRY2*-L*er* and T-*CRY2*-Cvi-367V versus T-*CRY2*-Cvi and T-*CRY2*-L*er* 367M. Further comparisons of the phenotype of the transgenic lines with that of the non-transgenic lines, L*er* and *EDI* NIL, allowed us to determine dose effects of the *CRY2* alleles.

As shown in Fig. **1 a**, the L*er*/Cvi differences in ON are determined by the *CRY2* allelic variation. In both experiments, transgenic plants T-*CRY2*-L*er* or T-*CRY2*-Cvi 367V had significantly higher ovule numbers than transgenic plants carrying the *CRY2*-Cvi or *CRY2*-L*er* 367M transgenes. On average, the L*er* transgene increased the number of ovules per fruit in a L*er* genetic background from six (experiment 1) to nine (experiment 2) ovules, in agreement with the additive QTL effects previously described (Alonso-Blanco et al., 1999).

Comparisons of FL measurements also show that the transgenic plants T-*CRY2*-L*er* or T-*CRY2*-Cvi 367V were significantly different from the transgenic plants T-*CRY2*-Cvi or T-*CRY2*-L*er* 367M (Fig. **1 b**). In agreement with the additive effect observed in NIL comparisons and in the QTL mapping, the *CRY2*-L*er* transgenes increased the length of the fruit in experiments 1 and 2, on average by 1.4 mm and 0.9 mm, respectively. Therefore it is confirmed that the *CRY2* allelic variation affects FL and accounts for the FL QTL. However, fruits of the transgenic plants T-*CRY2*-L*er* or T-*CRY2*-Cvi 367V were significantly larger than the fruits of L*er* non-transgenic plants, and the fruits of transgenic plants T-*CRY2*-Cvi or T-*CRY2*- L*er*367M were significantly larger than those of *EDI* NIL. In contrast, fruits of the transgenic lines T-*CRY2*-Cvi or T-*CRY2*-L*er* 367M were not significantly different from fruits of the L*er* wild type. Since these transgenic plants carry two endogenous doses of the *CRY2*-L*er* allele, these differences indicate that L*er* allele is the allele increasing FL, and this is affected in a dose-dependent manner.

Related to FL, and partly explaining the shorter fruits of *EDI* NIL, this NIL also showed siliques with a mild club-like phenotype (Fig. **2**) reminiscent of the *clavata (clv)* mutant phenotype of *Arabidopsis* (Clark, 2001). However, none of the previously described *clv* genes maps to the top of chromosome 1. This phenotype is not observed in any of the transgenic plants analysed in this work (data not shown), nor in the Cvi accession itself, and therefore is not determined exclusively by allelic variation at the *CRY2* locus. It is therefore suggested that this phenotype results from an interaction of a Cvi allele located within the introgressed region on the top of chromosome 1 with a L*er* allele at another position. Since all the transgenic plants analysed in this work carry two doses of the L*er CRY2*

gene, we cannot conclusively discard the possibility that *CRY2* is not involved in this likely interaction. It is also possible that the presence of the *clavata*-like phenotype in *EDI* NIL further contributes to its reduced fruit length.

Comparison of the Unf% phenotype of the transgenic lines (Fig. **1 c**) shows that the *CRY2* allelic variation significantly affects fertility, since the *CRY2*-L*er* or *CRY2*-Cvi 367V transgenes increase the fertilization percentage as compared with *CRY2*-Cvi or *CRY2*-L*er* 367M. Larger environmental effects were detected on fertility than on previously described traits, as shown by the considerable differences in Unf% between experiment 1 and 2 (Fig. **1 c**). The Unf% was much smaller in experiment 2 than 1, and no significant differences among genotypes could be detected in this case, suggesting that the *CRY2* fertility effect is only expressed under conditions where fertilization is slightly hampered, as in experiment 1.

Comparisons of the SW of the transgenic lines did not confirm the SW difference detected in the non-transgenic lines L*er* and *EDI* NIL, where the Cvi introgression increased SW (Fig. **1 d**). Strong environmental effects where detected for SW, since differences were found between the experiments. In experiment 1, transgenic lines T-*CRY2*-L*er* or T-*CRY2*-Cvi 367V had significantly higher SW than transgenic lines T-*CRY2*-Cvi or T-*CRY2*-L*er* 367M, which is opposite to the difference in SW between the non-transgenic lines, L*er* and *EDI* NIL. In experiment 2, no significant SW difference between genotypes could be detected. Therefore, these data do not support the proposal that *CRY2* is the QTL responsible for the SW QTL, which was suggested to be a pleiotropic consequence of ON differences, such that more ovules per fruit would determine a trade-off with seed size (Alonso-Blanco et al., 1999). However, we cannot discard the possibility that *CRY2* allelic variation is not involved in the SW QTL because the relationship among the fruit and seed related traits is not linear and straightforward, partly due to the large environmental effects (detected for SW and Unf%) and to the *CRY2* dose effects (detected for FL). Alternatively, it is also possible that another locus is involved in the SW QTL that was previously mapped.

We also examined the germination behaviour of the allelic variation at *CRY2* because the 2-LOD support intervals of a seed germination/dormancy QTL span in the *CRY2* locus, although the maximum LOD score was about 5 cM below (Alonso-Blanco et al., 2003). As shown in Fig. **3**, the non-transgenic lines L*er* and *EDI* NIL did not differ in germination percentage at any of the five times after harvest where this was tested. Furthermore, the transgenic lines T-*CRY2*-L*er* or T-*CRY2*-Cvi 367V did not differ significantly from the other transgenic lines in any of the five assays. Therefore, we conclude that *CRY2* is not the gene underlying the germination QTL previously identified.

The present report shows that transgenic plants carrying different alleles can be useful tools to attribute QTL for different complex traits to a specific locus, even when the relationship among the traits has not been previously suggested. Accordingly, it is concluded that co-location of apparently unrelated traits may be an indication for true pleiotropy. However, it is shown that interpretation of phenotypes in these transgenic plans can be accomplished when functional allelic variants are compared in a no-null background, since dose effects might be involved. So far, natural variation at the *CRY2* gene has been demonstrated using similar approaches to those described here, in flowering transition (El-Assal et al., 2001) and photomorphogenic traits such as hypocotyls elongation and cotyledon opening (Botto et al., 2003). Here, we have shown that natural variation at the *CRY2* gene also affects traits like fruit length, ovule number per fruit, and fertility. However, how *CRY2* might affect the fruit-related developmental traits

Fig. 2 Inflorescence (**a**) and silique (**b**) phenotype of L*er* and the *EDI* NIL.

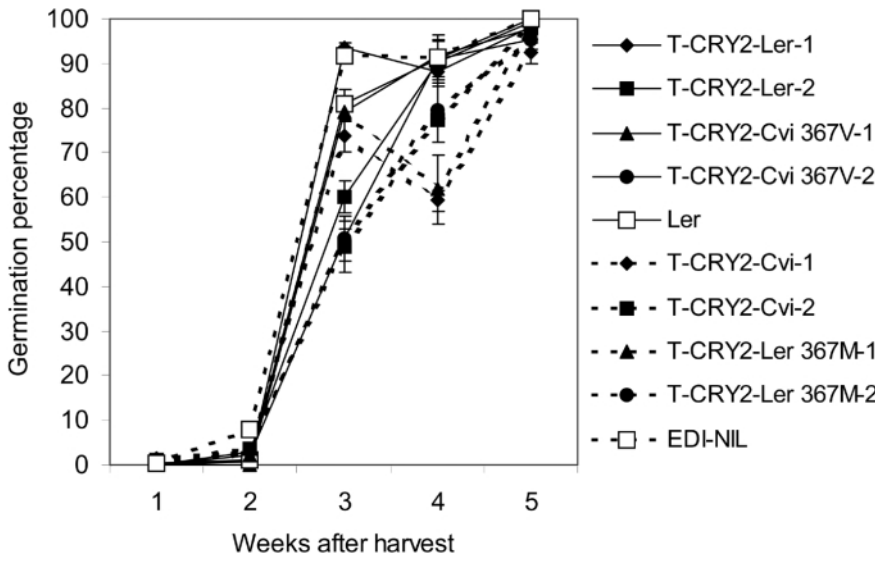

Fig. 3 Seed germination of genotypes differing in the *CRY2* region at various times after seed harvest.

remains to be elucidated. This might be related to the function of photoreceptors with cell elongation processes, although we cannot discard that these phenotypic effects are an indirect consequence associated with flowering differences determined by *CRY2* allelic variation.

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References

Alonso-Blanco, C., Bentsink, L., Blankestijn-De Vries, H., Hanhart, C. J., and Koornneef, M. (2003) Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana.* Genetics 164, 711 – 729.

Alonso-Blanco, C., Blankestijn-De Vries, H., Hanhart, C. J., and Koornneef, M. (1999) Natural allelic variation at seed size loci in relation to other life history traits of *Arabidopsis thaliana.* Proc. Natl. Acad. Sci. USA 96, 4710 – 4717.

Alonso-Blanco, C., El-Assal, S. E.-D., Coupland, G., and Koornneef, M. (1998) Analysis of natural allelic variation at flowering time loci in the Landsberg *erecta* and Cape Verde Island ecotypes of *Arabidopsis thaliana*. Genetics 149, 749 – 764.

Bentsink, L., Alonso-Blanco, C., Vreugdenhil, D., Tesnier, K. J. Y., Groot, S. P. C., and Koornneef, M. (2000) Genetic analysis of seed soluble oligosaccharides in relation to seed storability of *Arabidopsis thaliana.* Plant Physiol. 124, 1594 – 1603.

Bentsink, L., Yuan, K., Koornneef, M., and Vreugdenhil, D. (2003) The genetics of phytate and phosphate accumulation in seeds and leaves of *Arabidopsis thaliana,* using natural variation. Theor. Appl. Genet. 106, 1234 – 1243.

Botto, J. F., Alonso-Blanco, C., Garzaron, I., Sanchez, R. A., and Casal, J. J. (2003) The Cape Verde Islands allele of cryptochrome 2 enhances cotyledon unfolding in the absence of blue light in *Arabidopsis.* Plant Physiol. 133, 1547 – 1556.

Clark, S. E. (2001) Cell signalling at the shoot meristem. Nat. Rev. Mol. Cell Biol. 2, 276 – 284.

Darvisi, A. and Pisanté-Shalom, A. (2002) Complexities in the genetic dissection of quantitative traits loci. Trends in Genet. 18, 489 – 491.

El-Assal, S. E.-D., Alonso-Blanco, C., Peeters, A. J. M., Raz, V., and Koornneef, M. (2001) The cloning of a flowering time QTL reveals a novel allele of *CRY2.* Nature Genetics 29, 435 – 440.

Glazier, A. M., Nadeau, J. H., and Aitman, T. J. (2002) Finding genes that underlie complex traits. Science 298, 2345 – 2349.

Koornneef, M., Alonso-Blanco, C., and Vreugdenhil, D. (2004) Naturally occurring genetic variation in *Arabidopsis thaliana.* Ann. Rev. Plant Biol. 55, 141 – 172.

Lin, C. (2002) Blue light receptors and signal transduction. Plant Cell 14, s207-s225.

McKay, J. K., Richards, J. H., and Mitchell-Olds, T. (2003) Genetics of drought adaptation in *Arabidopsis thaliana:* I. Pleiotropy contributes to genetic correlations among ecological traits. Mol. Ecol. 12, 1137 – 1151.

Paran, I. and Zamir, D. (2003) Quantitative traits in plants: beyond the QTL. Trends in Genetics 19, 303 – 306.

Swarup, K., Alonso-Blanco, C., Lynn, J. R., Michaels, S., Amasino, R. M., Koornneef, M., and Millar, A. J. (1999) Natural allelic variation identifies new genes in the *Arabidopsis* circadian system. Plant J. 20, 1 – 11.

Yano, M. (2001) Genetic and molecular dissection of naturally occurring variation. Curr. Opion. Plant Biol. 4, 130 – 135.

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