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Extensive phenotypic variation in early flowering mutants of Arabidopsis

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ABSTRACT

Flowering time, the major regulatory transition of plant sequential development, is modulated by multiple endogenous and environmental factors. By phenotypic profiling of 80 early flowering mutants of Arabidopsis, we examine how mutational reduction of floral repression is associated with changes in phenotypic plasticity and stability. Flowering time measurements in mutants reveal deviations from the linear relationship between the number of leaves and number of days to bolting described for natural accessions and late flowering mutants. The deviations correspond to "relative early bolting" and "relative late bolting" phenotypes. Only a minority of mutants presents no detectable phenotypic variation. Mutants are characterized by a broad release of morphological pleiotropy under short days, with leaf characters being most variable. They also exhibit changes in phenotypic plasticity across environments for florigenic related responses, including the reaction to light and dark, photoperiodic behavior, and sucrose sensitivity. Morphological pleiotropy and plasticity modifications are differentially distributed among mutants resulting in a large diversity of multiple phenotypic changes. The pleiotropic effects observed may indicate that floral repression defects are linked to global developmental perturbations. This first extensive characterization of phenotypic variation in early flowering mutants correlates with the reports that most factors recruited in floral repression at the molecular genetic level correspond to ubiquitous regulators. We discuss the importance of functional ubiquity for floral repression with respect to robustness and flexibility of network biological systems.

INTRODUCTION

Mutational analyses have proved very useful to identify gene functions (Bouché and Bouchez, 2001; Alonso et al., 2003). In turn, the realization that gene functions are involved in reticulate networks of interactions contributed to the emergence of systems biology which is based on exhaustive, simultaneous biological descriptions (Katagiri, 2003). Network systems reveal emergent properties that cannot be predicted from the properties of isolated constituents, but are specific of the interactive whole. In particular, the intricacy and flexibility of complex interactions indicates that gene functions are not only primary causal agents of specific processes but can also be recruited directly or indirectly in different processes of a system (Duboule and Wilkins, 1998; Greenspan, 2001). This functional versatility suggests that mutant phenotypes reflect not only specific functional effects but also distortions of wild type network systems. Canalization or robustness, the capacity of network biological systems to buffer a wide variety of perturbations (Waddington, 1942; Rutherford, 2000; Debat and David, 2001; Siegal and Bergman, 2002) can explain why numerous silent mutations are uncovered in insertion mutagenesis analyses (Bouché and Bouchez, 2001). But mutants are usually less canalized than wild types and most mutations can affect the expression of numerous unrelated genes and reveal multiple phenotypic variations (Waddington, 1942; Duboule and Wilkins, 1998; Rutherford, 2000; Wagner, 2000; Finnegan, 2001; Greenspan, 2001; Featherstone and Broadie, 2002; Bergman and Siegal, 2003). The degree of pleiotropy and distribution of phenotypic variation in mutants should thus help to characterize robustness and flexibility of network biological systems.

Both robustness and flexibility are crucial properties of network systems that allow developmental stability while enhancing coordinate, orderly dynamic variation necessary for growth and adjustment to the environment (Greenspan, 2001). Phenotypic plasticity, the mode of phenotypic variation that leads to a predictable and homogeneous subset of possible phenotypes for a given genotype in heterogeneous environments - also called its norm of reaction - is especially

striking in plants due to their extended and sequential mode of development (Via et al., 1995; Pigliucci, 1996; Sultan, 2000; Debat and David, 2001). Flowering time, a major modulator of plant sequential development (Kuittinen et al., 1997), is influenced by a large number of environmental cues, including light quality and intensity, photoperiod, temperature and nutrient availability (Garner and Allard, 1920; Bernier, 1988; Millar, 1999; Battey, 2000; Samach and Coupland, 2000). Physiological, biochemical, and genetic studies have shown that this plasticity relies on the adjustment between floral activation and repression processes and involves mobile florigenic and anti-florigenic signals (Lang et al., 1977; Bernier, 1988; Weller, 1997; Périlleux and Bernier, 2002). In Arabidopsis, the characterization of a large number of flowering time mutants and loci has led to models of the genetic regulation of flowering time (Koornneef et al., 1991; Mouradov et al., 2002; Périlleux and Bernier, 2002; Simpson and Dean, 2002). These models emphasize the interplay of multiple floral activation pathways whilst floral repression is still poorly integrated (Pouteau, 2001; Mouradov et al., 2002; Périlleux and Bernier, 2002; Simpson and Dean, 2002).

Mutant lines have been mostly used by developmental biologists for molecular genetic analyses, but the impact of mutations on phenotypic plasticity and pleiotropy has not been well studied (Bagnall, 1993; van Tienderen et al., 1996). Phenotypic plasticity has been mainly studied in the field of ecology and evolution and indirectly addressed in plant breeding in terms of genotype-environment interaction, by using natural accessions and recombinant inbred lines (RILs) (Karlsson et al., 1993; Zhang and Lechowicz, 1994; Clarke et al., 1995; Stratton, 1998; Maloof et al., 2001). However, heterochronic mutants exhibiting shifts in developmental timing can prove powerful tools to investigate developmental networks at the organism level (Wiltshire et al., 1994; Diggle, 1999). In this work, we used an extensive mutational analysis to investigate plant phenotypic stability and plasticity at a whole organism level based on phenotypic profiling of early flowering mutants in Arabidopsis. We report the diverse pleiotropic effects associated with reduced floral repression,

including variation in morphology and phenotypic plasticity, and discuss the possible role and specificity of floral repression in ontogenetic regulation of flowering time.

RESULTS

Altered rates of progression to flowering

In an initial screen for early flowering mutants under short days (SD), 81 mutants in Ws and Col-0 ecotypes were selected for further analysis (Pouteau et al., 2001; see Materials and Methods). Flowering time was measured by using two indicators: the bolting date and the number of nodes bearing leaves. A wide range of variation is observed in the mutant population under SD in controlled growth cabinets (Fig. 1). Whilst Ws produces about 34 rosette leaves and bolts after 7 weeks, flowering time varies between 5 to 28 rosette leaves and 3 to 7 weeks in *eav* T-DNA insertion mutants. The rate of progression to flowering (RPF) defined as the ratio between the number of nodes bearing leaves and the number of days to bolting is reduced in the 80 *eav* mutants compared to Ws and Col-0 wild types. No simple linear relation, i.e. relative RPF, can be found between wild types and the mutants (Fig. 1). Since temporal analyses in Arabidopsis indicated that the relative RPF is linear in different genotypes (Koornneef et al., 1991; Bagnall, 1993; Karlsson et al., 1993; Clarke et al., 1995; Kuittinen et al., 1997; Stratton, 1998), we used Ws and Col-0 RPF measurements to calculate a wild type relative RPF and compared the mutants to this wild type relative RPF. Whilst a large proportion of the mutants appear to fit in the wild type relative RPF, about one third of them can be classified as "relative early bolting" or "relative late bolting" genotypes (Fig. 1). This suggests that the two indicators of flowering time are not simply surrogates of each other but correspond to specific temporal components that can be uncoupled. In contrast to all *eav* mutants, *ebv1* only bolts early but produces the same number of leaves as the wild type and is characterized by an increased RPF. Preliminary analysis suggests that this is due to acceleration of

the rate of leaf initiation (data not shown), a phenotype also reported for the *amp1* mutant under long days (LD) (Chaudhury et al., 1993).

Diversity in morphological pleiotropy

To recover the widest range of phenotypic effects associated with early flowering, the only phenotypic selection applied in the initial screen, apart from flowering time, was based on fertility and uniformity of transmission. Strikingly, the mutants exhibit a large range of morphological modifications, such as changes in plant architecture, modified leaf morphology, or altered pigmentation (Fig. 2). An example of a highly pleiotropic phenotype was described for the *lhp1-1* and *lhp1-2* mutants, including dramatic changes in plant architecture and leaf morphology, as well as dwarfism (Gaudin et al., 2001).

The range of morphological variation in the mutants under SD was investigated by a phenomenological survey at different developmental stages. We identified 28 variable morphological parameters ranging from vegetative features (for example elongation of petiole and hypocotyl) to floral and inflorescence features (Fig. 3). Only 4 mutants have no macroscopic morphological modification and the level of morphological pleiotropy per mutant (estimated as the percentage of modified morphological parameters) is 24.5 % on average. A large diversity is observed in the distribution of the variation among mutants (Fig. 3A). Each level of morphological variation (0 to 16 modifications per mutant) is equally represented in the population of mutants suggesting that the range of possible multiple morphological changes is not limited.

The 28 variable parameters are characterized by contrasting frequencies of variation in the mutant population. The variation level is highest for rosette pigmentation and rosette leaf shape (more than 60 % of the mutants) and lowest for floral features, silique shape, and elongation of secondary inflorescences (less than 10 % of the mutants) (Fig. 3B). Changes in rosette leaf shape

and pigmentation may be respectively associated with modifications in the leaf heteroblastic transition due to reduction in the number of leaves or temporal changes and perturbations either in photosynthesis and growth capacity or in light perception and signaling. The distribution of the level of morphological pleiotropy with flowering time also shows a wide range of variation among mutants (Fig. 2J). Most of the earliest mutants (less than 20 leaves) are highly pleiotropic whilst moderately early mutants (between 20 and 30 leaves) exhibit more variable levels of pleiotropy.

Genetic diversity

Gene tagging analysis indicates that morphological pleiotropy and early flowering are genetically linked in the T-DNA insertion mutants. The average frequency of tagging by a T-DNA expressing kanamycin resistance is 31 %. This suggests that each T1 parental line potentially bears 3 or 4 mutations. Yet, analyses of F2 and T2 populations for 52 T-DNA insertion mutants reveal no phenotypic segregation of the mutant features. This suggests that only one locus is affected in these mutants. The frequency of linkage is higher in T2 lines having two independent T-DNA insertion loci (55 %) than in T2 lines having one single T-DNA insertion locus (28 %). Multiple insertion may reflect a higher efficiency and lower rate of abortion of the T-DNA insertion process. Indeed, part of the non-tagged mutants are probably due to point mutations caused by aborted insertion. In total 16 mutants show linkage between early flowering and a T-DNA insertion and for 12 of them the genetic distance is less than 1%.

Despite potentially different underlying mechanisms, no significant difference is observed in the phenotypic behavior of semi-dominant (11) and recessive (49) T-DNA insertion mutants. Based on morphological similarities, alleles could only be detected for 5 complementation groups, each of them comprising no more than 2 or 3 alleles. Comparison between alleles reveals no significant difference in their phenotypic variation. Over 100 random crosses in a sample population of 24 mutants all resulted in complementation. This suggests that in the absence of morphological

similarities allelism is rare and that many mutants in the collection are probably unique members of their corresponding complementation group. The large phenotypic diversity in the collection is thus reflected by a low level of genetic redundancy. Because of this lack of redundancy, it is likely that the collection is not saturated and that the number of loci associated with floral repression is high.

Variation in phenotypic plasticity

To further characterize the phenotypic pleiotropy of early flowering mutants, their norms of reaction were assayed in response to environmental constraints having a florigenic influence. Two levels of the regulation of flowering time by photoperiod were investigated (Millar, 1999; Samach and Coupland, 2000). The input level corresponding to light/dark perception by photoreceptors and signaling was indirectly approached by measuring hypocotyl elongation, a classic example of developmental plasticity (Gendreau et al., 1997). The output level corresponding to the emission of rhythms by the circadian clock was analyzed through directly assessing flowering time in response to photoperiod (Samach and Coupland, 2000). Hormonal and metabolic signaling of flowering involves gibberellins (GAs) and sucrose (Bernier, 1988; Périlleux and Bernier, 2002). This was indirectly addressed by measuring reactions to paclobutrazol (an inhibitor of GA biosynthesis) and to a high concentration of sucrose, two conditions that have proved successful for the isolation of signaling mutants (Bethke and Russell, 1998; Gibson, 2000).

Comparison between the two genetic backgrounds shows that Ws is more sensitive to paclobutrazol than Col-0. Ws germination is fully inhibited at a concentration of 3×10^{-5} M whilst Col-0 still exhibits residual resistance at a concentration of 3×10^{-4} M (1.65 % +/- 1.23). Mutants in the two genetic backgrounds also show a differential response to paclobutrazol. No resistance is detected for 61 mutants in the Ws background whilst 3 out of 13 mutants in the Col-0 background exhibit a significant decrease in sensitivity (data not shown). The different flexibility of the response to paclobutrazol in the two ecotypes may indicate that mutational effects on phenotypic variation are

potentially bound to parental limitations. This may be tentatively explained by the fact that Ws is genetically constrained by a defect in phytochrome D and is an early flowering mutant itself (Auckerman et al., 1997). For the three other environmental factors analyzed, we observed similar levels of phenotypic plasticity and a similar range of mutant variation in both ecotypes and only 20 % of the mutant population have no or little changes in phenotypic plasticity.

Norms of reaction to photoperiod

Arabidopsis is a quantitative LD species that flowers later in SD than in LD. By comparing flowering time in LD and SD in the T-DNA insertion mutants and Ws, we identified four classes of response to photoperiod: class PhP1, flowering earlier in SD than in LD; class PhP2, photoperiod insensitive early flowering; class PhP3, early flowering under both SD and LD; and class PhP4, early flowering specifically under SD (Fig. 4). All EMS mutants fall into classes PhP3 and PhP4 (data not shown). A photoperiod sensitivity index (PSI) was determined as the ratio between the total number of leaves in SD and the total number of leaves in LD. Although a majority of mutants have a PSI lower than the wild type (between 0.8 and 2.2 compared to 2.8 for Ws), for about 20 % of them (mostly in class PhP3) a wild type PSI is observed.

Norms of reaction to light and dark

Hypocotyl elongation was determined in classical dark and LD light conditions and, for a number of mutants, in SD light condition. In Ws and Col-0 wild types, hypocotyl elongation is repressed in the light and is strongly activated in the dark (Gendreau et al., 1997). For Ws, no significant difference is observed in the two light conditions but the kinetics of hypocotyl elongation is slower in SD than in LD (data not shown). For about two thirds of the T-DNA insertion mutants, hypocotyl elongation is significantly different from the wild type in at least one of the conditions tested. However, only 7 mutants are affected in both light and dark conditions and 6 mutants exhibit changes only in SD but not in LD. Six classes of response to light and dark based on hypocotyl

elongation can be identified: class Hyp1, long in light; class Hyp2, long in light and short in dark; class Hyp3, short in light; class Hyp4, short in light and in dark; class Hyp5, short in dark; and class HypN, similar to the wild type (Fig. 5). Similar results were obtained in EMS mutants: among 10 mutants analyzed, only one is similar to the wild type and two are affected in both light and dark conditions (data not shown).

Norms of reaction to sucrose

For Ws and Col-0, the presence of 6 % sucrose delays germination and early development but subsequent development is little affected. The mutant behavior on 0 % sucrose is not significantly different from the wild type. We identified four classes of sensitivity to 6 % sucrose in the T-DNA insertion mutants (Fig. 5; see Materials and methods). Class Suc1 exhibits a high level of early growth arrest (up to 64 %) so that only a small proportion of seedlings develop into adult plants, a marked accumulation of anthocyanin and epinasty. For class Suc2, early growth arrest is limited and a majority of seedlings develop into adult plants characterized by a high level of shoot and leaf distortion and anthocyanin accumulation. Finally, class Suc3 shows only weak or variable germination or developmental defects and class SucN is similar to the wild type.

Multiple changes in phenotypic plasticity

The mutants are characterized by a large diversity in the distribution of the different norms of reaction (Fig. 5A, B). Multiple changes in response to photoperiod (P), sucrose hypersensitivity (S), and hypocotyl elongation (H) are more frequent than single changes (Fig. 5C). To estimate the distribution of phenotypic plasticity with flowering time, P, S, and H phenotypic indices were calculated and used to determine a global plasticity index Pi (0,66 on average in the mutant population). The indices appear differentially distributed in the different categories of mutants (Table I; Fig. 6). Despite a wide range of variation among the mutants, Pi tends to be lower with

earlier flowering time and some categories of mutants localize to different sectors of this distribution.

In most cases, S is associated with P and H together suggesting that it reflects global developmental perturbation (Fig. 5C). Accordingly, classes Suc1-3 comprising the "relative late bolting" sub-population are characterized by a low Pi. Because of their moderately early flowering time, they account for the main deviation from a putative linear relation between flowering time and plasticity in the mutant population (Fig. 6A). S is most often combined with class PhP3 and classes Hyp1 to Hyp4 (14 out of 17 mutants in both cases). The H index decreases from class Suc1 to SucN supporting the possibility of a positive correlation between H and S. In contrast, the P index is not significantly different in the different Suc classes suggesting that the correlation between P and S is indirect (Table I; data not shown).

P and H are combined in 50 % of the mutants (Fig. 5C). A positive correlation between P and H is suggested by the fact that the H index decreases from class PhP1 to PhP4 and that the P index is higher in classes Hyp1 to Hyp4 and intermediate in class Hyp5 compared to class HypN (Table I). The variation in P and H indices is reflected in the distribution of Pi variation with flowering time (Fig. 6B, C). Classes PhP1-2 and class PhP4 correspond to two separate clusters comprising respectively the earliest mutants with a low Pi and most of the least precocious mutants with highest Pi. In contrast, class PhP3 and most Hyp classes do not localize to a specific sector. However, most mutants in classes Hyp1 to Hyp4 have a low Pi whereas class Hyp5 exhibits an intermediate Pi and is clustered near class HypN characterized by the highest Pi.

According to a mechanistic understanding of photoperiodism, P and H alterations can reveal a reduced capacity to integrate environmental cues at the input level, including light photoreception and signaling, or at the oscillator or output levels due to clock dysfunction (Samach and Coupland,

2000). Indeed, defects in clock functions such as ELF3 have been identified among these mutants (Hicks et al., 2001; Carré, I, Gaudin, V, Pouteau, S, unpublished data). Specific light quality, such as red or blue light, may be needed for the detection of more subtle changes in mutants with a P phenotype but regular hypocotyl elongation in white light (classes Hyp5 and HypN). But other developmental defects are also involved. For example, reduction in hypocotyl elongation in *lhp1-1* and *lhp1-2* (*eav21* and *eav23*), two Hyp3 mutants, correlates with reduced cell size and dwarfism (Gaudin et al., 2001) and Hyp2 mutants that are also hypersensitive to sucrose may be defective in sugar metabolism or signaling. Finally, the Hyp5 phenotype also points to the possible contribution of photomorphogenesis in the dark or dark-signaling to the regulation of flowering time. In addition, Hyp5 like HypN is mostly independent from P and S phenotypes and is associated with moderate levels of morphological pleiotropy and a wild type relative RPF.

DISCUSSION

Based on the first extensive characterization of early flowering mutants in Arabidopsis, our work provides new perspectives on the regulation of floral repression in relation to phenotypic variation. Firstly, early flowering mutants exhibit modifications in the coordination of two temporal components of plant ontogeny: an exogenous component corresponding to environmental timing measured by the date of flowering, and an endogenous component related to organismic timing estimated by the number of leaves. The uncoupling of these two components results in "relative early or late bolting" phenotypes. In contrast, most previous analyses conducted with late flowering mutants or natural accessions and RILs indicated a strong correlation between the date of flowering and the number of leaves (Koornneef et al., 1991; Bagnall, 1993; Karlsson et al., 1993; Clarke et al., 1995; Kuittinen et al., 1997; Stratton, 1998). The difference in the behavior of early and late flowering mutants may be explained by the fact that the latter had been selected for a lack of pleiotropic effects (M. Koornneef, personal communication). A comparison of the early flowering

mutants with late flowering mutants not submitted to a phenotypic selective bias would thus be interesting. Because the "relative late bolting" phenotypes are most affected, it is possible that uncoupling of the two temporal dimensions in late flowering mutants would also be associated with profound disruptions of developmental stability. However, the high level of phenotypic variation in early flowering mutants is probably not coincidental of the type of selection applied but reflects endogenous constraints imposed by advanced ontogenetic phase change (see below). In addition, the uncoupling of endogenous and exogenous temporal components in "relative early bolting" phenotypes could reflect a slower rate of leaf initiation at early stages (Groot and Meicenheimer, 2000). Additional growth rate measurements would be needed to discriminate between different possible modes of variation in flowering time (Wiltshire et al., 1994; Diggle, 1999).

Secondly, early flowering mutants exhibit extensive phenotypic variation. This is characterized by a release of morphological pleiotropy under SD and a decrease in phenotypic plasticity across environments resulting in multiple variation profiles. Among the 28 variable morphological parameters identified, leaf characters are most often affected. The lower level of variation observed for floral features may be coincidental of the selection for fertility applied during the mutant screen. But this may also reflect their higher robustness against variation due to their more determinate character. Conversely, vegetative features may be more variable because of their higher flexibility as reflected by heteroblasty, i.e. the gradual change in leaf morphology exhibited by regular ontogenetic sequences (Diggle, 1999). Within a large diversity of alterations in response to light and dark, photoperiod and sucrose, hypersensitivity to sucrose coincides with more global developmental defects possibly due to osmotic, metabolic or signaling changes. Accordingly, many sugar response mutants previously described are also pleiotropic and show altered phytohormone response, metabolism or osmotolerance (Gibson, 2000). In contrast, most mutants with altered reactions to the dark, unlike those affected in the light, show only moderate developmental defects. This coincides with the finding that in natural populations hypocotyl elongation in the light but not

in the dark correlates with latitude and supports the notion that phenotypic plasticity profiles have different adaptive implications (Via et al., 1995; Maloof et al., 2001). The multiple variation profiles observed may indicate that the functional integrity of developmental network systems is compromised in early flowering mutants. This may prove instrumental to analyze how patterns of phenotypic covariation commonly observed for many morphological and life history traits (Armbruster and Schwaergerle, 1996) are conserved or disrupted by mutations imposing reduced floral repression.

Thirdly, the high level of pleiotropy in early flowering mutants emphasizes the importance of functional ubiquity for the interpretation of floral repression at the molecular genetic level. Indeed, floral repression seems to have recruited more ubiquitous factors than floral activation, including members of the MADS-box gene superfamily that act through complex multimeric protein interactions and epigenetic regulators (Goodrich et al., 1997; Michaels and Amasino, 1999; Hartmann et al., 2000; Finnegan, 2001; Gaudin et al., 2001; Gendall et al., 2001; Kinoshita et al., 2001; Pouteau, 2001; Scortecci et al., 2001; Yoshida et al., 2001; Mouradov et al., 2002; Noh and Amasino, 2003; Piñeiro et al., 2003; Ratcliffe et al., 2003; Sung et al., 2003). In addition to their intricate, interactive mode of action, most of these factors are also characterized by ubiquitous RNA expression. Among the floral repression-defective mutants analyzed in this work, 12 proved to be tagged by a T-DNA insertion. Genetic or molecular characterization of five mutants revealed defects in ubiquitous functions including chromatin remodeling and circadian clock regulation such as LHP1 and ELF3 (Gaudin et al., 2001; Pouteau et al., 2001; Carré, I, Gaudin, V and Pouteau S, unpublished data). In contrast, the seemingly more limited pleiotropy level in floral activation defective mutants coincides with the finding that many floral activators are involved in redundant pathways and correspond to more specific transcription factors (Mouradov et al., 2002; Simpson and Dean, 2002). These contrasting features suggest that floral activation and repression operate through different biological processes. Whilst redundancy, in addition to interaction between unrelated

genes, is often thought to be a common mode of canalization (Rutherford, 2000; Wagner, 2000; Featherstone and Broadie, 2002), functional ubiquity offers a global basis for different processes to interrelate with each other and undergo coordinated changes without compromising developmental network stability. Accordingly, loci associated with changes in floral activation and repression may contribute respectively to developmental canalization and flexibility.

By integrating a growing number and complexity of regulatory factors, the current genetic models have substantially contributed to advance our understanding of flowering time in Arabidopsis. More recently attempts have been made to include some repressors in regulatory networks, for example FLC and EFS (Michaels and Amasino, 1999; Sheldon et al., 1999; Soppe et al., 1999; Mouradov et al., 2002). Despite these efforts Arabidopsis models are still overall dominated by a floral activation rationale. One reason may be that analyses and interpretations are mostly conducted on a deterministic linear basis that is suited for simple chains of reaction but not for complex reticulate networks and ubiquitous processes. By showing differential relationships between flowering time and multiple variation profiles in early flowering mutants, our results point to the need to develop non-linear dynamic approaches to integrate floral repression and account for functional ubiquity (Pigliucci, 1996; Amzallag, 2001). Further multidimensional analyses will be needed to analyze the biological significance of the typology presented in this work and the possible reticulate correlations between multiple morphological and plastic features and flowering time.

MATERIALS AND METHODS

Plant Material

T-DNA insertion lines of Wassilewskija (Ws) ecotype were obtained from the Versailles collection, INRA, France (Bechtold et al., 1993). EMS mutagenized lines of Columbia (Col-0) ecotype were provided by Catherine Bellini, INRA, Versailles, France. A total of 7653 T-DNA insertion lines and

384 T2 EMS mutagenized lines were grown in SD and plants that flowered early were selected and selfed. The progenies were sown on soil in individual pots and grown in growth chamber under SD of 8 hrs light and 16 hrs dark under mixed white and incandescent light at 20 °C and 70 % RH. Progenies that showed uniform early flowering were selected, backcrossed, and outcrossed by standard genetic techniques (Pouteau et al., 2001). The 62 T-DNA insertion mutants and 19 EMS mutants obtained were named *eav1* to *eav80* for *early flowering from versailles* and *ebv1* for *early bolting from versailles*. The *eav21* and *eav23* mutants have been recently renamed respectively *lhp1-1* and *lhp1-2* since the two alleles proved to be altered in LHP1, a homologue of Heterochromatin Protein 1 (HP1), a member of the chromo domain protein family in *Drosophila* (Gaudin et al., 2001).

Growth conditions for flowering time assays

Mutant seeds were sown on soil (Stender A240, Bluemendenwerk Stender GmbH, Germany) and grown in Sanyo Gallenkamp SGC660 growth cabinets at 20 +/- 0.2 °C and 70 % +/- 2 % RH. The soil was kept moist by application of nutrient solution three times a week. The light was provided by mixed fluorescent and incandescent tubes and the Photon Flux Density (PFD) measured at soil level was 230 +/- 20 µE/m2/s and 2 +/- 0.2 µE/m2/s, respectively. SD corresponded to 8 hrs light and 16 hrs dark and LD conditions consisted in 16 hrs light and 8 hrs dark. Developmental uniformity was obtained by selecting the 10 most uniform plants on average about 12 days after sowing, bringing the plant density to one plant per pot, and rotating the trays three times a week. Bolting time was measured as the number of days from sowing to the first elongation of the floral stem at 0.1 cm height. The number of true leaves produced by the apical meristem was recorded on bolted plants. No major variation was observed in 2 to 4 independent repeats (12 repeats in SD and 9 in LD for Ws).

Evaluation of the morphological pleiotropy under SD

Repeated characterizations of mutant phenotypes under SD in successive generations obtained from the initial screen and in genetic analyses were further investigated in an exhaustive survey. Four individuals per genotype were selected for developmental uniformity and grown in parallel in the same growth room in SD. Aerial morphological features (vegetative, floral, inflorescence and plant architecture) were examined after 4 and 10 weeks. Apart for floral features that were observed under a dissection microscope, all parameters were macroscopically recorded. Size or quantity parameters were semi-quantified by recording the corresponding ranges of variation for each genotype compared to the wild type. The other parameters such as fertility or leaf surface wrinkling were visually assessed on a qualitative basis.

Growth conditions in vitro

Mutant seeds were sterilized and sown on Petri dishes containing a standard nutrient medium as described by Santoni et al. (1994). Uniform germination was obtained by allowing the seeds to imbibe at 4°C for 48 hrs and exposing the plates to white light at 200 µE/m2/s for 4 hrs before transfer to a growth chamber. Standard growth conditions corresponded to LD of 16 hrs fluorescent light at a PFD of 200 µE/m2/s and 20°C and 8 hrs dark at 15 °C. For dark growth conditions, the plates were placed in opaque bags under LD conditions. SD conditions consisted in a 8 hrs light period at 20°C and a 16 hrs night at 15°C.

Phenotypic plasticity assays *in vitro*

For hypocotyl length measurements, seedlings were grown on standard nutrient medium without sucrose during 10 days for LD and dark conditions according to Gendreau et al. (1997) and during 15 days for SD conditions (Lefebvre, D, Pouteau, S, unpublished data). Seedling spreading, camera recording, and image analysis with Optimas 6.1 software (Bioscan, Imasys, Suresnes, France) was as described by Gendreau et al. (1997). An average of 40 uniform seedlings was analyzed per

genotype and per repeat. No major variation was observed in 2 to 4 independent repeats (17 repeats in LD, 8 in SD, and 12 in the dark for the wild type).

For sucrose sensitivity measurements, seedlings were grown on standard nutrient medium without sucrose or with 6 % w/v sucrose. Germination and early development were recorded at different times during a period of 3 to 17 days of growth. Early development was divided into 3 stages: root emergence (stage 1); shoot emergence (stage 2); and cotyledon expansion and greening (stage 3). Later developmental stages, recorded as stage 3, were examined for morphological features in the initial population and in a sub-population transferred to fresh medium after 10-15 days of growth. The sensitivity of the mutants to 6 % sucrose was determined by measuring the following parameters: inhibition of germination, early growth arrest (at stage 1 or stage 2), developmental delay (later transition from stage 1 to stage 2 and stage 3), morphological features including shoot or leaf distortion, anthocyanin accumulation, epinasty, and root phenotype. An average of 80-100 seeds was analyzed per genotype and per repeat. A consistent behavior was observed in 2 to 4 independent repeats performed for 34 mutants comprising all mutants exhibiting sensitivity to 6 % sucrose (10 repeats for the wild type).

For paclobutrazol resistance assays, an average of 80-100 seeds was sown per genotype and per repeat on a 0.7 % agar medium containing paclobutrazol (Sopra s.a., Zeneca Agrochemicals, France) and the percentage of germination was scored after 7 to 10 days in LD. Mutant seeds in Ws and Col-0 backgrounds were assayed at a concentration of 10^{-5} to 3×10^{-5} M and 3×10^{-4} M paclobutrazol, respectively. Ws and Col-0 responses were assayed in 5 and 4 independent repeats, respectively.

Evaluation of phenotypic and plasticity indices

The PSI photoperiod sensitivity index was determined as the ratio between the total number of leaves in SD and the total number of leaves in LD.

To estimate the deviations of the mutants from the corresponding wild type plasticity response to photoperiod, sucrose, and light/dark, P, S, and H phenotypic indices were calculated. Each index

was based on three criteria analyzed: P1) early in LD, P2) reduced PSI, P3) photoperiod insensitive (PSI //1); S1) morphological modifications and/or developmental delay, S2) moderate inhibition of development (10-30 % early growth arrest), S3) strong inhibition of development (50-65 % early growth arrest); H1) modification in LD, H2) modification in SD, H3) modification in the dark. Each criterion was given a value of 0 or 1 depending on the absence or presence of a significant difference with the wild type based on t test scores.

The P, S, and H phenotypic indices were used to calculate a Pi plasticity index: $Pi = [9 - (P + S + H)]/9$.

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FIGURE CAPTIONS AND LEGENDS

Figure 1. Variation in the rate of progression to flowering in SD. Relation between the number of rosette leaves and the number of days to bolting for *eav1* to *eav61* T-DNA insertion mutants (blue diamonds), *eav62* to *eav80* EMS mutants (yellow diamonds), *ebv1* (green square), and the Ws (red circles) and Col-0 ecotypes (solid circles). Independent repeats are presented (2 on average for *eav1* to *eav61* and 12 for Ws). Standard deviations are shown for Ws and Col-0 only for the sake of clarity. Thin line: linear regression for the population of T-DNA insertion mutants. Dotted red line: linear regression calculated with Ws and Col-0 (out of scale: average 66.2 rosette leaves and 61.3 days to bolting) scores. Area between black dotted lines: range of variation of wild type relative RPF.

Figure 2. Morphological pleiotropy of early flowering mutants in SD. A, Ws, rosette and base of the floral stem showing the first cauline leaf. B, *eav2*. C, *eav6*. D, *eav43*. E, *eav1*. F, *eav15*. G, *eav49*. H, *eav40*. I, *ebv1*. J, Variation of morphological pleiotropy (proportion of modified parameters in 28 variable morphological parameters) with flowering time in T-DNA insertion mutants (diamonds) and Ws (solid circles). Standard deviations are shown for 12 Ws independent repeats. Thin line: linear regression.

Figure 3. Variability of morphological pleiotropy in early flowering mutants under SD. A, Distribution of morphological changes in *eav1* to *eav61* T-DNA insertion mutants. Empty and filled boxes indicate respectively the absence and presence of macroscopically detectable morphological changes. B, Frequency of changes in 28 morphological parameters. Elongation of hypocotyl (1) and petiole (2). Rosette size (3), raising (4), and pigmentation (5). Rosette leaf shape (6), serration (7), surface (8), and trichomes (9). Cauline leaf size (10), shape (11), serration (12), and surface (13). Flower size (14) and number (15). Perianth organ shape (16) and reproductive organ shape (17).

Silique size (18), shape (19), and fertility (20). Floral stem elongation (21), length of first internode (22), length of other internodes (23), thickness (24), and appendage arrangement (25). Co-florescence elongation (26), number of secondary inflorescence (27), and secondary inflorescence elongation (28).

Figure 4. Response to photoperiod. Flowering time under SD (shaded bars) and LD (open bars) in *eav* T-DNA insertion mutants (numbered from 1 to 61) grouped in 4 different classes based on t test scores: PhP1 (later in LD than in SD), PhP2 (insensitive to photoperiod), PhP3 (early in SD and in LD), PhP4 (early specifically in SD). The Ws control is shown on both rows (average of 12 and 9 repeats in SD and LD, respectively). Standard deviations are indicated.

Figure 5. Norms of reaction to light and dark, photoperiod, and sucrose. A-B, Hypocotyl elongation in LD (solid bars), in SD (red bars) and in the dark (blue bars) in *eav* T-DNA insertion mutants (numbered from 1 to 61) and in Ws (average of 17 repeats in LD, 8 in SD, and 12 in the dark). Error bars correspond to standard deviations. A, Classes of mutants differing from Ws in the light (Hyp1, Hyp3) or in the dark (Hyp5), or both (Hyp2, Hyp4) based on t test scores. B, Mutants similar to Ws in the light and in the dark (HypN). Corresponding reactions to photoperiod (classes PhP1 to 4) or to 6% sucrose (classes Suc1 to N) are indicated. C, Combined changes in photoperiod response (P), sucrose sensitivity (S) and hypocotyl elongation (H) or absence of changes (+).

Figure 6. Variation of phenotypic plasticity with flowering time in *eav1* to *eav61* T-DNA insertion mutants (diamonds) and Ws (solid circles), showing the tendency of the different norms of reaction. A, Response to 6 % sucrose, classes Suc1 to 3 (brown diamonds), class SucN (open diamonds). B, Response to photoperiod, classes PhP1 to 2 (green diamonds), class PhP3 (red diamonds), class PhP4 (open diamonds). C, Hypocotyl elongation in response to light and dark, class Hyp1 (blue diamonds), class Hyp2 (indigo diamonds), classes Hyp3 to 4 (red crosses), class Hyp5 (yellow

diamonds), class HypN (open diamonds). Plasticity index (Pi; see Materials and Methods). Thin line: linear regression. Standard deviations are shown for 12 Ws independent repeats.

Table I. P, S, and H phenotypic indices in the plasticity classes

Class	Number of mutants	P index	S index	H index
PhP1-2	6	3.00 +/- 0.00	0.00 +/- 0.00	1.83 +/- 1.13
PhP3	30	1.77 +/- 0.43	0.90 +/- 1.13	1.28 +/- 0.82
PhP4	25	0.84 +/- 0.37	0.16 +/- 0.47	0.48 +/- 0.70
Suc1-3	17	1.59 +/- 0.62	1.82 +/- 0.81	1.56 +/- 0.58
SucN	44	1.48 +/- 0.82	0.00 +/- 0.00	0.80 +/- 0.94
Hyp1-4	31	1.77 +/- 0.76	0.90 +/- 1.14	1.71 +/- 0.68
Hyp5	9	1.44 +/- 0.53	0.33 +/- 0.50	0.94 +/- 0.17
HypN	21	1.14 +/- 1.14	0.00 +/- 0.00	0.00 +/- 0.00

The P, S, and H indices are estimates of the deviations from the corresponding wild type plasticity response to photoperiod, sucrose, and light/dark (see Figures 4 and 5 and Materials and Methods). By definition, wild type P, S, and H indices are 0. Mean values and standard deviations are indicated for each class.
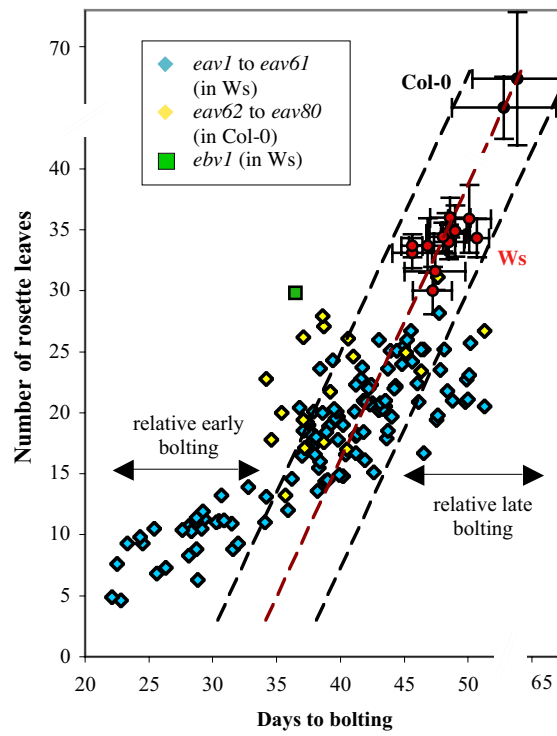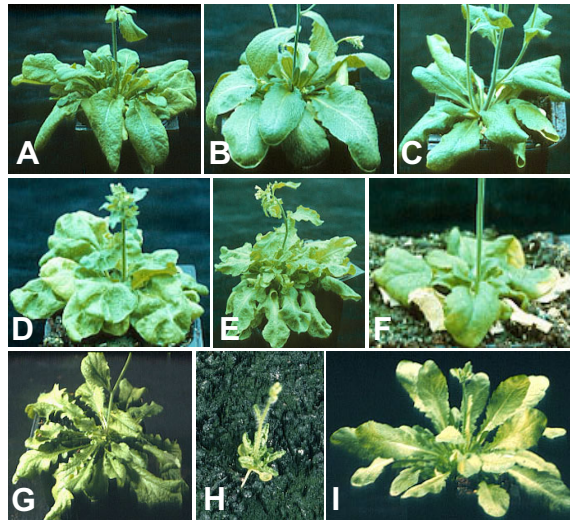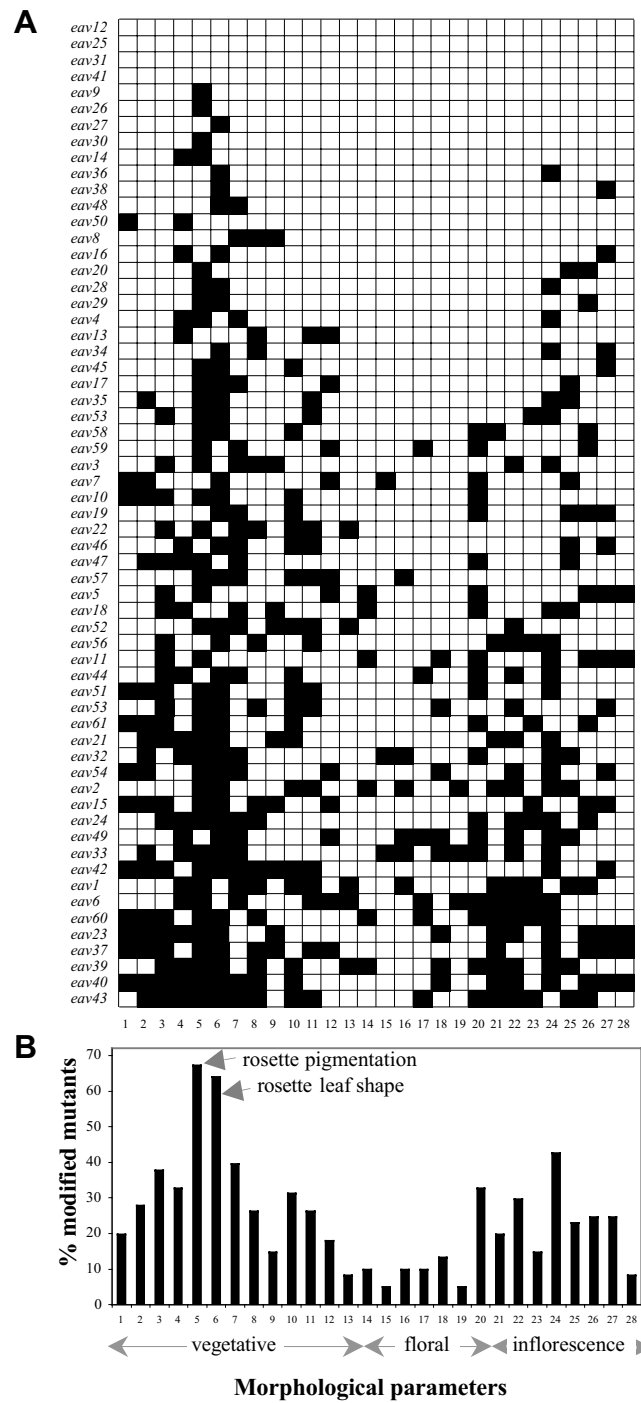
Figure 1

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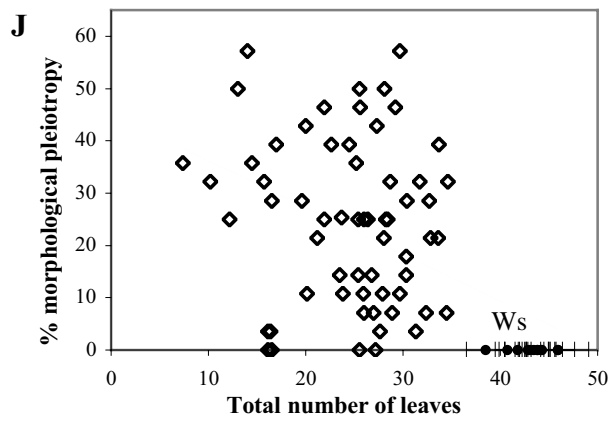
Figure 2

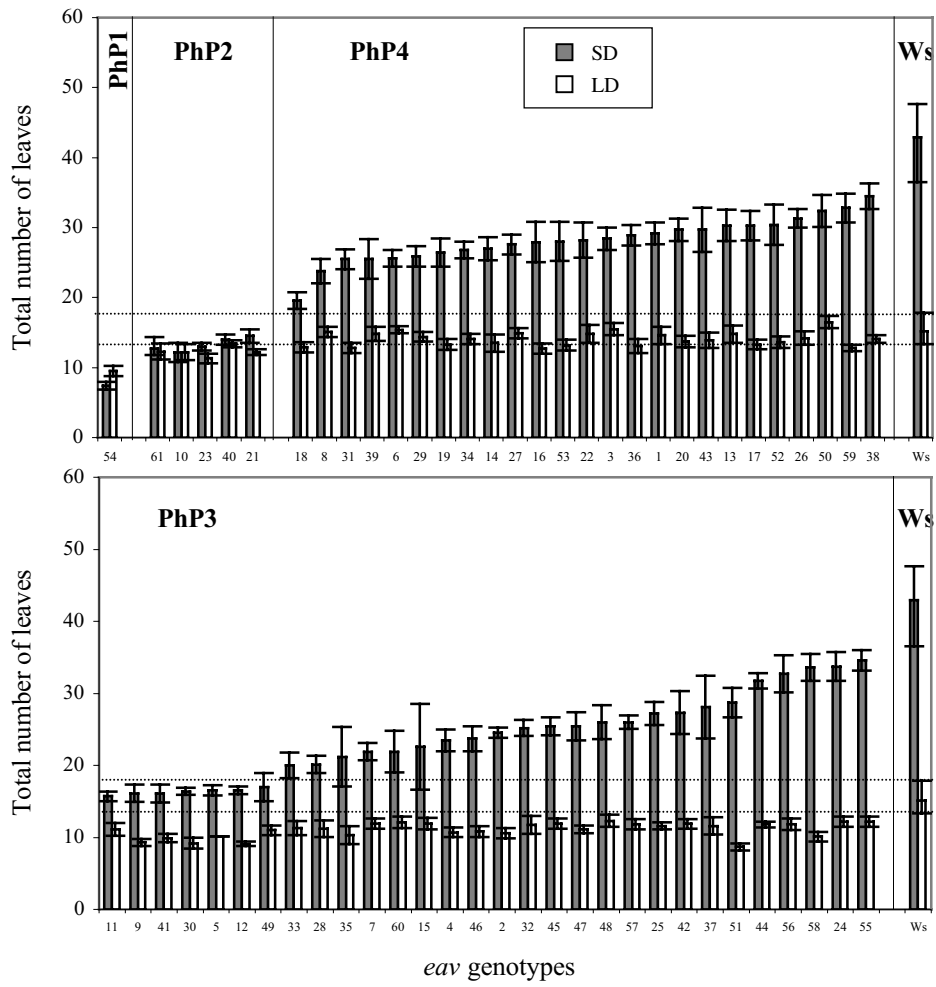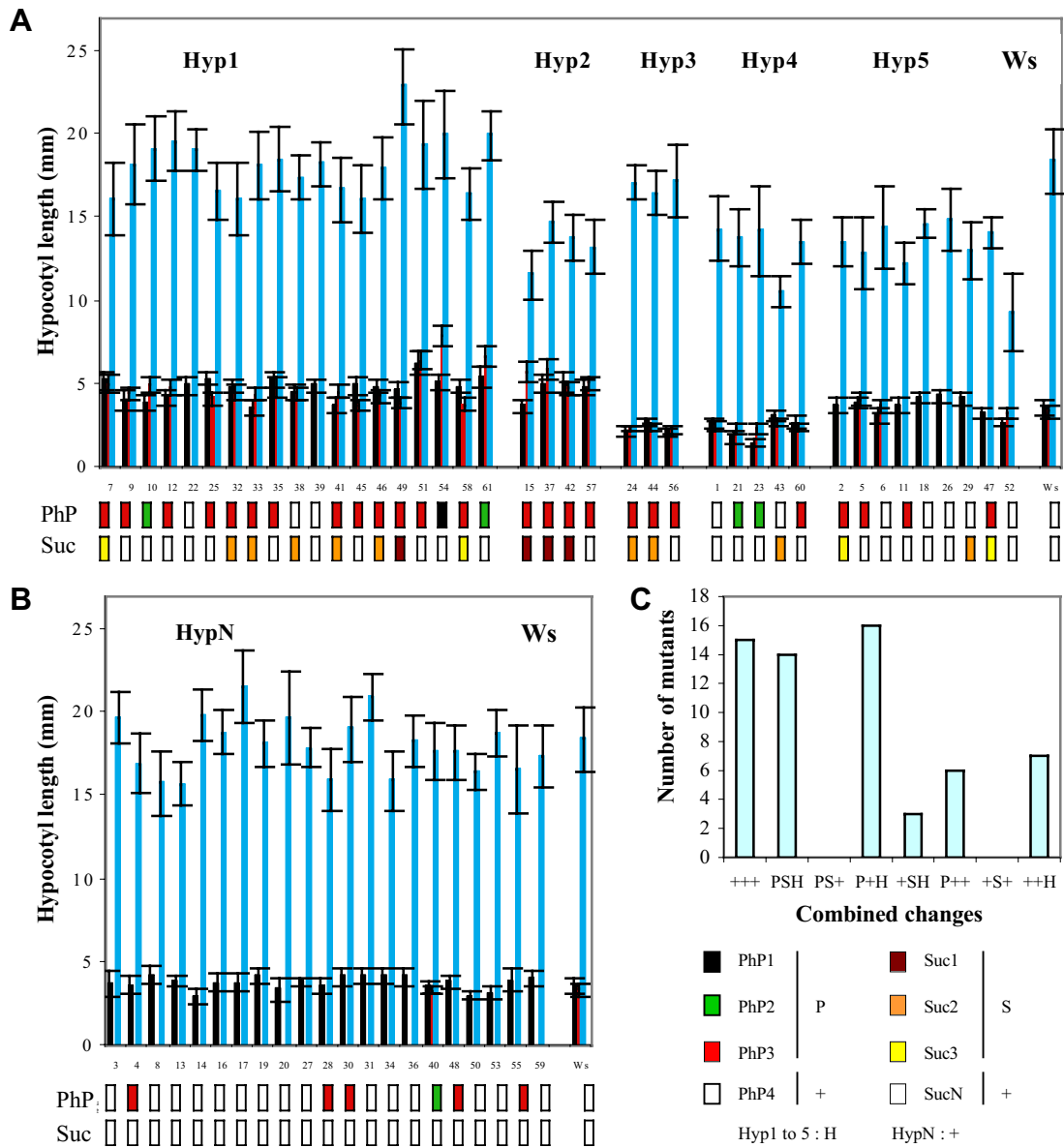Pouteau et al.

Figure 3

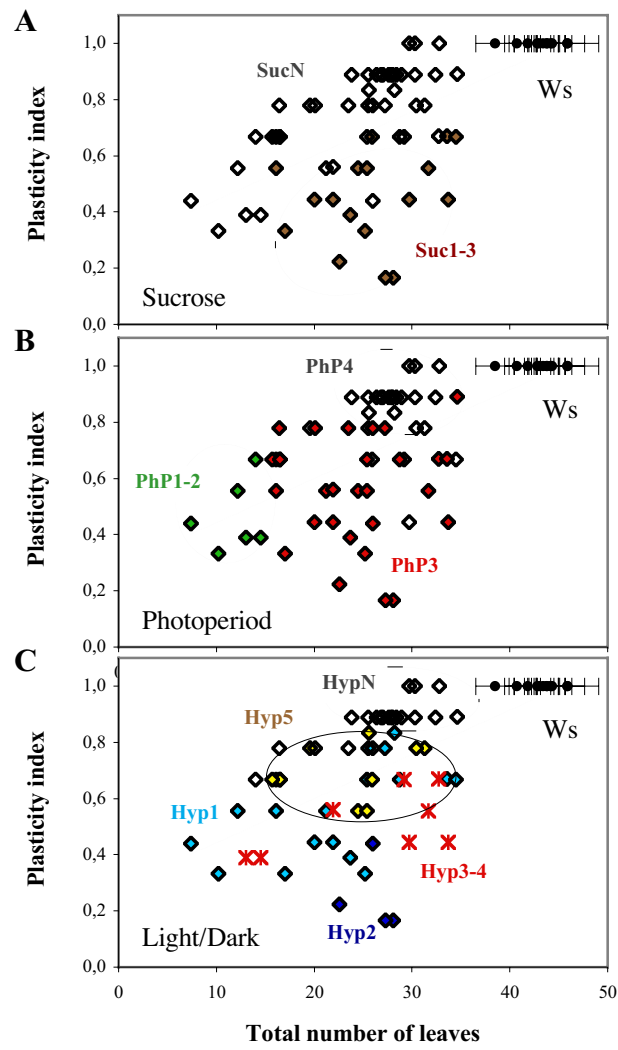Pouteau et al.

Figure 4

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Figure 5

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Figure 6

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