Forward and reverse genetics of rapid-cycling *Brassica oleracea*

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Abstract Seeds of rapid-cycling *Brassica oleracea* were mutagenized with the chemical mutagen, ethylmethane sulfonate. The reverse genetics technique, TILLING, was used on a sample population of 1,000 plants, to determine the mutation profile. The spectrum and frequency of mutations induced by ethylmethane sulfonate was similar to that seen in other diploid species such as *Arabidopsis thaliana*. These data indicate that the mutagenesis was effective and demonstrate that TILLING represents an efficient reverse genetic technique in *B. oleracea* that will become more valuable as increasing genomic sequence data become available for this species. The extensive duplication in the *B. oleracea* genome is believed to result in the genetic redundancy that has been important for the evolution of morphological diversity seen in today's *B. oleracea* crops (broccoli, Brussels sprouts, cauliflower, cabbage, kale and kohlrabi). However, our forward genetic screens identified 120 mutants in which some aspect of development was affected. Some of these lines have been characterized genetically and in the majority of these, the mutant trait segregates as a recessive allele affecting a single locus. One dominant mutation (*curly leaves*) and one semi-dominant mutation (*dwarf-like*) were also identified. Allelism tests of two groups of mutants (*glossy* and *dwarf*) revealed that for some loci, multiple independent alleles have been identified. These data indicate that, despite genetic redundancy, mutation of many individual loci in *B. oleracea* results in distinct phenotypes.

Introduction

Brassica oleracea is a species that demonstrates a great deal of morphological diversity. Common vegetables such as broccoli, Brussels sprouts, cabbage, cauliflower, kale and kohlrabi are all morphotypes of *B. oleracea*. It is

interesting to consider what characteristics of the ancestral *B. oleracea* genome provided the raw material for the morphological variety seen in today's *B. oleracea* crops.

While *B. oleracea* behaves genetically as a diploid, genome analysis has revealed that this species has undergone extensive gene duplication. Studies suggest that a genome triplication event occurred in a predecessor to modern *B. oleracea* (Lagercrantz and Lydiate 1996) and that this event was followed by many chromosomal rearrangements, endoreduplications and deletions (Kianian and Quiros 1992). Duplication events appear to be common in plant evolution as well-characterized species such as Arabidopsis, maize and soybean also all show evidence of duplication events (Blanc et al. 2003; Gaut and Doebley 1997; Initiative 2000; Shoemaker et al. 1996). Although, Arabidopsis and *B. oleracea* share a recent common ancestor many single-copy genes in Arabidopsis are present as multiple paralogous loci in *B. oleracea* (Lin et al. 2005; Lowman and Purugganan 1999; Schranz et al. 2002).

Gene duplication provides evolutionary opportunities for organisms. If a critical function is encoded by a single locus a mutation that changes the function of that gene is likely to have negative consequences for the organism. However, if genome duplication has created several loci that encode the same function, the selective pressure on those loci is reduced. One of the duplicate loci may retain the original function while permitting duplicates to undergo loss-of function without deleterious effects on the organism. For example *B. oleracea* has two orthologous genes for the Arabidopsis *APETALA1* (*AP1*) and, in all subspecies tested, one of these orthologous genes has undergone nonfunctionalization while the other is expressed and appears to be capable of encoding a functional protein (Lowman and Purugganan 1999). The reduction of selective pressure by duplication can also lead to neofunctionalization in which one or more duplicate genes mutate to assume new functions. Alternatively, subfunctionalization can occur in which duplicate loci retain different and complementary elements of the original function (Force et al. 2005). It is thought that redundancy in the *B. oleracea* genome and the subsequent reduction of selective pressure has created a situation favorable to the evolution of new morphotypes.

While gene duplication provides opportunities for organismal evolution the results of duplication can be problematic for geneticists. Many single-copy genes in *Arabidopsis* are represented by multiple loci in *B. oleracea* (Town et al. 2006). To determine the effectiveness of ethylmethanesulfonate (EMS) mutagenesis as a tool in *B. oleracea*, large-scale mutagenesis of a rapid-cycling *B. oleracea* was performed and the population tested using both forward and reverse genetics. Targeting induced local lesions in genomes (TILLING) is a reverse genetics technique that can be used to identify mutations in any target gene through heteroduplex analysis (Till et al. 2003). TILLING was used to determine that the effectiveness of EMS-mutagenesis in a sample of the *B. oleracea* population was similar to that found in EMS-mutagenized populations of other diploid species. Forward genetic screens were then used to identify mutants affecting different aspects of development. Many of the mutant phenotypes result from recessive alleles and segregate as single loci. These data suggest that despite extensive gene duplication there are many traits in *B. oleracea* that are determined by a single gene. The collection of mutants will be a valuable resource for plant research and genetics education.

Materials and methods

Mutagenesis

The rapid-cycling *B. oleracea* line, TO1000, was used for this project. TO1000 is derived from the rapid-cycling stock CrGC3-1 developed by the Crucifer Genetics Cooperative Madison, WI (Williams and Hill 1986), and has several attributes that make it well suited for both forward and reverse genetics. TO1000 has a shorter generation time (approximately 65 days) than commercially grown *B. oleracea* morphotypes, and is much smaller in size allowing thousands of plants to be grown and screened simultaneously. TO1000 is a doubled haploid that is expected to be homozygous at all loci and, thus, less genetically variable than most diploid *Brassica* species which are expected to be heterozygous at many loci.

Mutagenesis was performed on batches of seeds with a mass of 25 g (approximately 10,000 dry seeds). Seeds were surface sterilized for 10 min in 10% bleach solution, washed four times in sterile distilled H_2O and incubated in EMS for 14 h with gentle orbital shaking. EMS concentration was titrated to a level at which approximately 50% of the treated seeds did not germinate. For the batches of TO1000 seeds used in this project, 0.4% EMS was determined to be sufficient to achieve this level of seedling mortality. Treated seeds were transferred to a cheesecloth bag and rinsed for four hours in gently running water. Seeds incubated in distilled H_2O for 14 h and subjected to the same sterilization and washing steps as the EMS-treated seeds did not show any decrease in percent germination relative to untreated seeds (these received no sterilization, incubation or washing).

Planting and growth

Mutagenized seeds (the M_1 generation) were dried briefly then spread onto damp Whatman filter paper in plastic culture dishes and allowed to germinate for 2–3 days, in the

dark, at room temperature. Germinated seeds were transferred to Fafard Super-Fine Germination Mix in 2-in. tubular plastic cells and grown in a greenhouse at a constant temperature of 22°C with 16 h of light. Plants were top watered and fertilized once per week with Peters® fertilizer (The Scotts Company, Marysville, OH). When plants began to flower, paper pollination bags were placed over each plant to discourage outcrossing between M_1 individuals and to encourage self-pollination. The bagged plants were "rustled" daily to promote self-pollination within the bag.

Seed from 8,750 M1 plants were harvested and used to construct 875 M_2 seed pools each from 10 M1 plants. 200 M_2 seeds from each pool were planted in a 3.5 acre field plot in Arlington, Wisconsin, USA using a roll-planter. Putative mutants with visible phenotypes were transferred to a greenhouse (Biotron, University of Wisconsin, USA). Subsequent generations were also grown in a greenhouse under the same conditions listed above. (Agronomy Dept., University of Wisconsin, USA) and were bagged to promote self-pollination.

Seeds collected from 92 pools of 10 M1 plants each were sent to Agriculture and Agri-Food Canada, Saskatoon, SK, Canada in preparation for TILLING. Thirty-six individual M2 seeds were planted from each of the first 42 pools and 20 seeds each from the remaining 50 pools. Thus, a total of 2,512 M2 seeds were sown, of which approx 90% germinated to give a population of 2,263 individual lines from which leaf tissue was frozen for future analysis. DNA was extracted from all lines and a subset of 960 samples was sent to the University of British Columbia for identification of mutations using TILLING.

DNA extraction

Plant tissue was frozen in liquid nitrogen and freeze dried for a minimum of 24 h before milling 0.4 g of sample in a milling tube containing three ball bearings. Ground tissue was transferred to 50 ml centrifuge tubes and 15 ml of Kirby mix (Covey et al. 1981) was used as extraction buffer. DNA was then extracted using phenol/chloroform and precipitated using isopropanol. The pellet was treated with RNAase, re-extracted using phenol/chloroform as described above, dissolved in 0.25 or 0.5 ml 10 mM Tris, 1 mM EDTA pH 7.4, and quantified using fluorometric analysis.

Primer design PCR Amplification for TILLING

Primers were designed, using the web-based program CODDLE (proWeb Project 2003) and selecting "EMS in plants" as the Mutation Method. Primers were purchased from MWG Biotech, Inc. (High Point, NC, USA) and used at a final concentration of 0.2 mM as described by Colbert et al. (2001). Individual DNA samples were pooled fivefold for screening. PCR was performed according to Colbert et al. (2001) using 2.5 ng of genomic DNA for each amplification in 96-well or 384-well PCR. PCR cycles were as follows: 95°C for 2 min; eight cycles of [94°C for 20 s, 73°C for 30 s (decrementing 1°C per cycle), 72°C for 1 min]; 45 cycles of: [94°C for 20 s, 65°C for 30 s, and 72°C for 1 min]; 72°C for 5 min; 99°C for 10 min (denaturation and inactivation of taq enzyme); and 70 cycles of 20 s at 70°C (decrementing 0.3°C per cycle for random reannealing to allow hybridisation of mutant and wildtype molecules), hold at 4°C.

Preparation of celery juice extract and TILLING

Crude celery juice extract (CJE) was prepared as described by (Till et al. (2004). Aliquots were stored at −70°C and were spun at approximately 2,000 G for 1 min before use to remove any tissue debris.

PCR products were digested with CJE as described by Till et al. (2003) and purified by passage through G50 Sephadex in 96-well Millipore Multiscreen® filtration plates (Millipore Corporation, Billerica, MA) before running on a 25 cm long LI COR acrylamide gel with a 0.4 mm wide, 96-well sharkstooth comb. Analysis of the gel images was done using GelBuddy (Zerr and Henikoff 2005) to define lanes and estimate sizes of cleavage products. In order to identify individual mutants in the fivefold pools used for screening, DNA from each individual in that pool was mixed 1:1 with wildtype DNA (from TO1000) and analyzed as above. Most identified mutations were sequenced in both directions using either the same forward or reverse primers as for PCR or an internal primer designed for sequencing. Sequence analysis was performed using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA) and the potential effect of the mutations was predicted using PARSESNP (Taylor and Greene 2003).

Scanning electron microscopy

SEM analysis of glossy mutants was performed at Cold Spring Harbor Laboratory using a S-3,500 N SEM system (Hitachi High Technologies America, Inc., Pleasanton, CA) as described by D. Jackson in (Jackson 2002).

Gibberellic acid treatment

Seeds were allowed to germinate on MS media with 0.6% phytagar. Two days after germination, seedlings were transferred to MS media containing 50 μM GA_3 (Sigma Corp.) Non-GA_3-treated controls were also transferred to fresh MS media. Hypocotyls were measured 6 days after germination.

Results

Population construction

The rapid-cycling *B. oleracea* line, TO1000, was used for this project. The homozygous background of this doubled haploid line increased the confidence that the mutations and novel phenotypes discovered in the mutagenized population were caused by new, induced mutations and not by genetic variability that existed in the parental line prior to mutagenesis. TO1000 is also self-compatible, which simplified the isolation of homozygous mutations because self-pollination in the first generation (M_1) can provide homozygous M_2 generation mutants that can be identified using forward genetic screens as well as through reverse genetics. Most mutations generated by chemical mutagenesis using EMS are recessive, loss-of-function mutations and, while TILLING can identify heterozygous mutations at the molecular level, the phenotypes that associated with these mutations may only be apparent in the homozygous state.

Mutagenesis of *B. oleracea* was achieved by treating TO1000 seeds with the mutagen, EMS at a concentration of 0.4% which resulted in approximately 50% germination. EMS-treated seeds were germinated and planted producing the M_1 generation that was self-pollinated. Following seed set, seeds from ten M_1 plants were harvested together resulting in a "pool" of M_2 seed. In this way, 875 M_2 pools were generated, representing the progeny of 8,750 individual M_1 lines that were screened for a number of visible phenotypes.

TILLING

TILLING was used to analyze the forward mutation frequency in *B. oleracea* using EMS. The target genes selected for screening were chosen based on their similarity to Arabidopsis genes that had been annotated as being involved in the response to abiotic stress. These genes were selected as targets in part because *B. oleracea* genomic or EST sequence was available publicly through the TIGR genome browser (http://atidb.org/cgi-perl/gbrowse/ati-browse). The list of DNA regions screened is given in Table 1 and the primers for each target gene are provided in Supplementary Table 1.

A population of 960 plants (10, 96-well PCR plates) from 40 pools of M_2 seeds was used for the TILLING experiment. We estimated that a sample size of at least 960 was necessary in order to assess the mutation rate and determine whether or not TILLING was feasible using this population. Twenty-seven mutations were identified in 15 different genes. Two of the mutations identified by TILLING were present in more than one individual so only 25 mutations were unique. The most likely explanation for this result is that these mutations were derived from siblings (progeny of the same M_1 parent.) and, therefore, were not genetically independent. We estimate (based on 2 out of 27 mutations) that approximately 93% of the 960 individuals (or 893 plants) screened are of unique genotypes. The mutation frequency in this mutagenised population can be calculated by dividing the total amount of DNA screened by the number of mutations detected, but as the last 100 bp

Table 1 Regions screened in 960 individual M_2 plants using TILLING, and number of mutations identified at each locus

Amplicon	Description of *Arabidopsis thaliana* (*At*) homologue	*At.* homologue	Amplicon size (bp)	Total mutations	Effective mutations[a]
bo2	PgiC gene—glucose-6-phosphate isomerase	At5g42740	972	4	3^{hom}
bo4	COR314-TM2 similar to the cold acclimation protein WCOR413	At1g29390	1,100	2	0
bo5	Methyltransferase, putative	At3g18000	987	2	1
bo10	CEO1 Can protect yeast from oxidative damage	At1g32230	817	0	0
bo12	Early-responsive to dehydration protein-related/ERD protein	At1g62320	1,072	5	2
bo13	Stress-inducible protein, putative, similar to sti	At1g62740	805	2	0
bo17	ANNAT1 member of the annexin gene family	At1g35720	897	1	1
bo19	Dehydration-responsive protein-related, similar to ERD3 protein	At5g64030	1,501	0	0
bo20	SAL1 rescues sulfur assimilation mutants in yeast	At5g63980	1,078	0	0
bo21	LEA14 induced in response to wounding and light stress	At1g01470	928	0	0
bo25	Stress-responsive protein, similar to ethylene-inducible protein	At3g16050	866	2	1^{hom}
bo27	Dehydration-responsive protein-related, similar to ERD3 protein	At4g00750	918	3	2
bo29	Dehydration-responsive protein-related, similar to ERD4 protein	At4g02900	833	1	1
bo30	Dehydration-responsive protein-related, similar to ERD3 protein	At5g06050	1,453	2	1
bo31	NDPK2—maintains intracellular dNTP levels	At5g63310	1298	3	3^{1}
	Total		15,525	27	15

[a] Changes predicted to produce null or missense mutations in the target gene. Superscripts ^{hom} indicate a homozygous mutation that is predicted to be deleterious

from each end of the amplicon cannot be resolved on the TILLING gels the amplicon size has been reduced accordingly. In this small test population, we detected 25 mutations in 12,525 bp of DNA (sum of DNA in all amplicons −200 bp per amplicon) in 893 individuals. The mutation frequency in this population is, therefore, approximately 1 in 447 kb ((12,525 × 893)/25). The frequency and spectrum of mutations in this species was not substantially different from that observed in TILLING populations of other diploid species such as barley (1 mutation per 374 kb: Talamè et al. 2008), *Arabidopsis* (1 mutation per 300 kb: Greene et al. 2003) and rice (1 mutation per 300 kb: Till et al. 2007), but lower than that seem in polyploids such as wheat (1 mutation per 24 kb: Slade et al. 2005) or *Brassica napus* (1 mutation per 41.5 kb: Wang et al. 2008). This is as expected, since genetic redundancy in polyploids appears to allow higher mutational loads in these species. All of the mutations identified by TILLING in *B. oleracea* were G/C-to-A/T transitions, consistent with data from *Arabidopsis thaliana* and other plants where >99% of mutations have been shown to be this type of point mutations (Greene et al. 2003). Fifteen of the mutations were predicted to cause changes in the amino acid sequence of a protein, and the remaining 12 mutations were predicted to be silent either because they were in introns or because they affected the third bp of a codon triplet and do not change the amino acid sequence of the protein. Four of the missense mutations we identified were predicted by PARSESNP (Taylor and Greene 2003) to be deleterious mutations and three of these were homozygous in the M_2 plants we screened using TILLING.

Forward genetic screen for mutants

Seeds from the 875 M_2 pools were field grown (Arlington and Madison, Wisconsin, USA) over two consecutive summers. Approximately 200 seeds from each M_2 pool were planted and used for forward genetic screening during this period. These M_2 plants were screened for mutant phenotypes throughout the growth cycle. Putative mutants were transplanted to a greenhouse for further genetic analysis including confirmation of the mutant phenotype in the M_3. Mutants were considered to have arisen independently if they were isolated from different M_2 pools. Plants with similar mutant phenotypes, isolated from the same M_2 pool, could represent the same mutational event and so were not considered to be independent.

Mutations affecting many aspects of growth and development were identified (Fig. 1, Table 2). Detailed descriptions of phenotypes and genetic analysis, and additional pictures can be accessed on-line at: http://www.biochem.wisc.edu/brassicaclassroomgenetics/mutagenesis/index.html. Multiple independent isolates were discovered for almost all classes of mutant (i.e. very few phenotypes were represented by a single plant). Fertile mutants were allowed to self-pollinate permitting the mutant phenotype to be verified in the M_3 (and, in many cases, M_4 generation) confirming the genetic nature of the mutations (Table 2).

Genetic analysis of mutants

Many M_2 plants with mutant phenotypes were back-crossed to the TO1000 parent in order to eliminate any additional

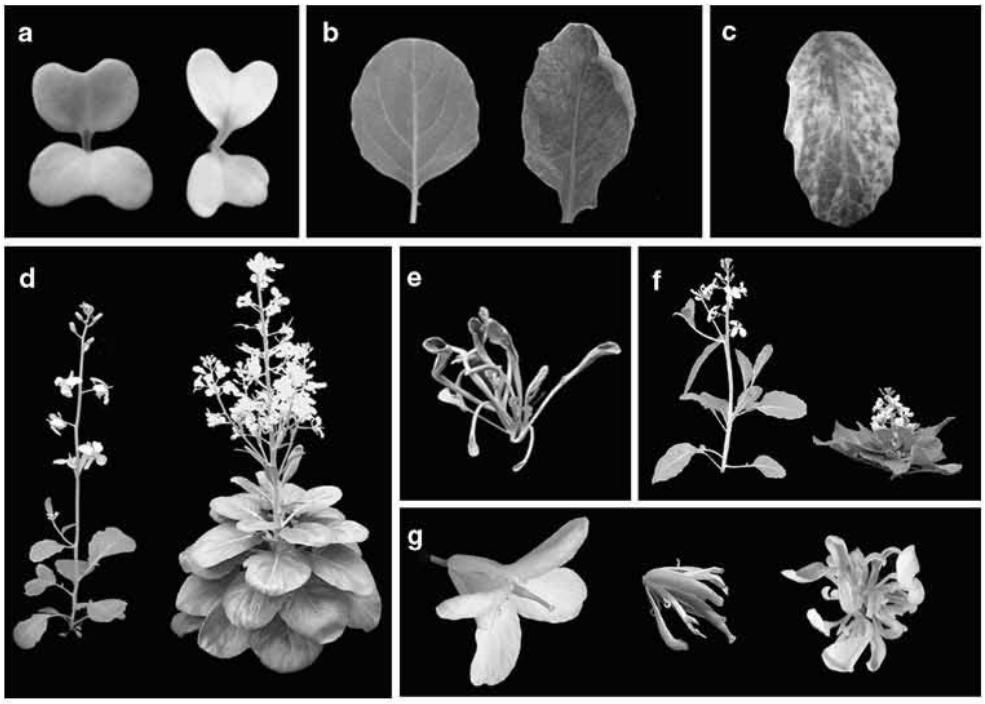

Fig. 1 Phenotypes of some *Brassica oleracea* mutants. **a** WT seedling (*left*) compared to *albino seedling1* (*right*). **b** WT leaf (*left*) shown beside *wrinkled-leaf 9* (*right*). **c** Leaf from *veriageted-albino1* (compare to WT leaf of similar age shown in B. **d** WT plant (*left*) compared to *late-flowering 8* (*right*). The *laf8* plant was planted several weeks before the WT plant shown. **e** *curly leaf1* (Compared to WT plant of similar age shown in panel d). **f** WT plant (*left*) compared to *dwarf4* (*right*). Both plants are the same age. **g** WT flower (*left*) compared to *apetala-like1* (*center*) and the flower of *altered leaf shape1* (*right*)

Table 2 Summary of 120 mutants that have been confirmed by growth of M_3 generation

Phenotype	Symbol	Number of independent lines[a]	Mode of inheritance	Special notes
Altered cotyledon shape and size	*act*	3	Recessive	Shape of true leaves also altered in some lines
Multiple cotyledons	*muc*	1	ND	3-4 cotyledons per plant
Albino seedlings, lethal	*alb*	2	Recessive	*alb1* and *alb2* are maintained in a heterozygous state
Albino-striped cotyledons	*sip*	1	Recessive	Venation on true leaves is albino
Curly leaves	*crl*	4	See notes	*crl1* allele is dominant, *crl2,3 and 4* are recessive
Variegated leaves	*vga*	2	ND	
Pale green leaves	*pal*	14	Recessive	Pale green when compared to parental lines
Yellow-green	*yel*	18	Recessive	Distinct yellow-green color, some show decreased vigor
Glossy, epicuticular wax is absent	*gly*	8[b] (4 loci)	Recessive	These 8 glossy lines represent 4 different genes (See Result)
Wrinkled leaves	*win*	11	Recessive	Cotyledon and petal shapes are also altered in some lines.
Bumpy leaf surface	*bum*	1	Recessive	
Altered leaf shape	*als*	4	Recessive	Misshapen leaves and flower petals
Thin serrated leaves	*tsl*	1	ND	
White leaf tips	*wlt*	2	Recessive	
Dwarf stature	*dwf*	18[b] (5 loci)	Recessive	These dwarf lines represent 5 different genes (See Results)
Dwarf-like	*dfl*	2	Semi-dominant	Unlike *dwf* group, stem elongation occurs in *dfl* but is inhibited compared to WT. *dfl* mutants also show altered leaf shape.
Long hypocotyl	*hy*	1	Recessive	
Albino stem	*abs*	2	Recessive	Petioles, leaves and floral buds are green
High anthocyanin	*han*	2	ND	Purple stems and leaf veins
Late flowering	*laf*	7	Recessive	Produce 12–20 leaves prior to flowering (Parental lines produce 6–8 leaves prior to flowering)
Yellow flowers	*yel*	9	Recessive	TO1000 parent has white petals
Apetala-like	*ap*	7	Recessive	No petals, reduced fertility and pleiotropic effects on leaf morphology

For many, additional genetic analysis has been performed

[a] Mutants with the identical phenotypes identified from different M_2 pools are assumed to represent different, independently-arising alleles

[b] Allelism tests were done on these mutants. Allelism data is not available for other mutant lines; therefore, the number of independent isolates provides no information about the number of loci responsible for the other traits described

background mutations that did not contribute to the mutant phenotype. In 42 of 44 mutant lines tested, the F_1 progeny of this back-cross resembled the TO1000 parent indicating that the mutant allele was recessive. The F_1 plants were allowed to self-pollinate and screening of F_2 plants revealed that, in most cases, the expected 3:1 ratio of wildtype to mutant phenotypes was observed, confirming that the mutation was recessive and indicating that the mutant phenotype was due to a mutation at a single locus (data not shown).

There were two cases in which the allele responsible for the mutant phenotype was not recessive. The F_1 plants resulting from a cross between TO1000 and the line *curly-leaf1* (*crl1*) (Fig. 1e), produced a 1:1 ratio of curly to parental leaves indicating that *crl1* is dominant and that the *crl1* parent used in this test was heterozygous. This was confirmed by analysis of the F_2 population in which a 1:3 ratio of wildtype to curly-leaf plants was observed. Another mutation that did not segregate as a recessive allele was *dwarf-like1* (*dwl1*). The *dwl1* plants have limited internode elongation, similar to dwarf mutants, however, the mutation is pleiotropic also affecting leaf shape and phyllotaxy (not shown). The F_1 plants from a cross between TO1000 and *dwl1* were of intermediate height suggesting that the mutant allele was semi-dominant or incompletely dominant to the wildtype allele.

The TO1000 parent has white flowers. Nine independent mutants were identified with yellow flowers (*yel1* to *yel9*) all of which are recessive. This is consistent with observations from other *B. oleracea* morphotypes that white flowers are dominant over yellow and that flower color is determined by a single locus (Spini and Kerr 2000).

Seven independent mutants with floral defects resembling the Arabidopsis *apetala1* mutant were identified

(Fig. 1g, Table 2). The *B. oleracea apetala* (*ap*) mutants showed reduced fertility and pleiotropic effects on leaf morphology. These phenotypes are not associated with the Arabidopsis *apetala1* mutation. All *ap* mutants were determined to be recessive and to segregate as a single locus. The fact that *ap* mutants were detected is consistent with the observations that following duplication of the *B. oleracea APETALA1* ortholog, one copy underwent non-functionalization (Lowman and Purugganan 1999) A phenotype is expected in our forward genetics screen only if a single locus in *B. oleracea* encodes the *AP1* function since, then, loss-of-function at this locus would result in the apetala-like phenotype observed in our forward genetics screen.

Characterization of mutants

Allelism tests were completed for two classes of mutants. The goal of these tests was to determine whether mutants with similar phenotypes had mutations affecting the same locus or different loci.

Eight *glossy* (*gly*) mutants were identified with shiny, bright-green leaves in sharp contrast to the matte leaves of the TO1000 parent. Leaves of TO1000 are covered with a delicate mesh of epicuticular wax (Fig. 2a). The *gly* mutants appear to be deficient in the production of this wax or in its deposition above the leaf cuticle (Fig. 2a). A similar class of mutants has been identified in Arabidopsis and is found to include a large number of wax biosynthesis genes (Kunst and Samuels 2003). Allelism tests revealed that *glossy* mutants identified in our study fell into four allelism groups. This indicates that there are at least four genes in *B. oleracea* that, when mutated, produce the glossy phenotype. Several alleles of *gly2* were identified. The *gly2-1* allele appears to be a partial loss-of-function capable of producing limited epicuticular wax while *gly2-2* and *gly2-3* show greater reduction in wax deposition (Fig. 2).

Eighteen mutants with dwarf stature were recovered from the M_2 population. These plants were characterized by reduced internode elongation and slightly delayed flowering compared to WT (Fig. 1f). Allelism tests revealed that the mutants fell into five complementation groups. Eight alleles of *dwf1*, four alleles of *dwf2*, three alleles of *dwf3*, two alleles of *dwf4* and one allele of *dwf5* were identified. Studies in Arabidopsis reveal that some dwarf mutants are deficient in the biosynthesis of gibberellic acid (GA) and that these mutants can by rescued by exogenous application of GA (Olszewski et al. 2002). Other Arabidopsis dwarf mutants do not respond to application of GA. These GA-insensitive mutants are deficient in some aspect of the GA response pathway. Response of the mutants to fungal gibberellin (GA_3) was examined in seedlings to determine whether the *B. oleracea* dwarf lines were GA-deficient. Seeds were germinated on media with and without GA_3 and hypocotyl lengths were compared (Fig. 3). The *dwf1* seedlings were not responsive to GA_3 suggesting that *dwf1* is deficient in the GA response pathway. Seedlings of *dwf2*, *dwf3*, *dwf4* and *dwf5* were GA_3-responsive (data for *dwf5* not shown). Adult plants of *dwf2*, *dwf3*, *dwf4* and *dwf5* were also responsive to exogenously-applied GA_3 (data not shown). Therefore, these dwarf mutants are considered to be deficient in GA biosynthesis.

Discussion

The rapid-cycling, doubled haploid *B. oleracea* line, TO1000, was mutagenised using EMS and characterized using both forward and reverse genetics. TILLING was used to screen a subset of 893 independent M_2 individuals

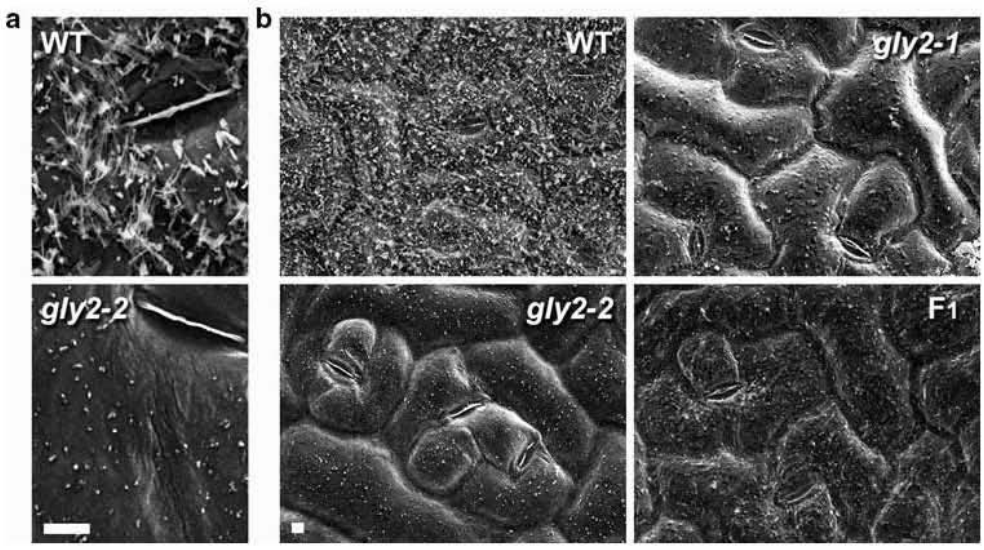

Fig. 2 Phenotypic analysis and allelism test of *glossy* mutants. **a** Epicuticular wax on adaxial surface of wildtype (WT) and *gly2-2* leaves. **b** Wildtype (WT) is evenly covered with epicuticular wax. *gly2-1* and *gly2-2* both lack epicuticular wax to varying degrees. Both *gly2-1* and *gly2-2* are recessive (data not shown). The wildtype phenotype is not restored in the F_1 of *gly1-2* × *gly2-2* (F_1) indicating that *gly2-1* and *gly2-2* are allelic. *White bars* represent 10 μm

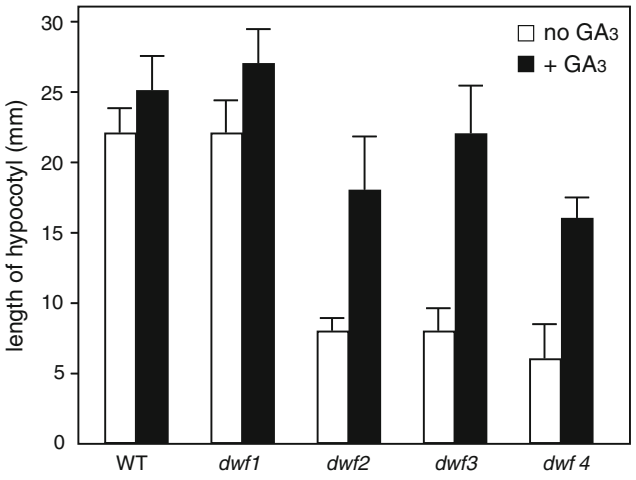

Fig. 3 Effect of GA_3 on *dwf* mutant seedlings. Seedlings were grown on media without (*white bars*) or with (*black bars*) 50 μM GA_3. Hypocotyls were measured 6 days after germination. *Error bars* represent the standard deviation of the mean

from this collection. Twenty-five unique mutations in 15 different genes were identified using this reverse genetics technology. This mutation frequency and the type of mutations observed in *B. oleracea* is similar to that seen in other diploid plants after treatment with EMS (reviewed in Haughn and Gilchrist 2006). Forward genetic screens identified 120 mutations affecting many different genes from a population of approximately 175,000 M_2 individuals (generated from 8,750 mutagenized M_1 lines). Complementation tests among 26 mutants from two distinct phenotypic classes identified 9 separate loci.

The fact that both forward and reverse genetic characterization of multiple loci was done on the same mutagenized population provides a unique opportunity to compare the mutation frequency per amplicon with the frequency of deleterious mutations per gene. TILLING identified an average of 1.8 mutations per amplicon (~800 bp) in this small test population. However based on PARSESNP, the predicted frequency of deleterious mutations in the population was only 0.15 per amplicon. Since an average gene size (2,000 bp; The Arabidopsis Genome Initiative 2000) is approximately 2.5 times that of the TILLING amplicon, and the population used for forward genetics screens was 9.8 times the size of the one used for TILLING (8750/893), the expected number of deleterious mutations per gene in the entire population would be $0.15 \times 2.5 \times 9.8 = 3.6$. This predicted frequency is only slightly higher than the frequency of loss-of-function alleles (2.9 per gene) observed for those genes characterized (glossy and dwarf = 26 alleles for 9 loci) and probably reflects the fact that multiple independent mutants within a pool were not counted in the forward genetics screen since they could not be distinguished from siblings, or from the fact that not all of the predicted deleterious alleles will result in an obvious phenotype. It has been previously shown that many single-copy genes in Arabidopsis are present as multiple, paralogous loci in *B. oleracea*, reflecting one or more genome duplication events in the latter since the two species evolved from their common ancestor (Lin et al. 2005; Lowman and Purugganan 1999; Schranz et al. 2002; Town et al. 2006). Forward genetics has revealed, however, that many traits in *B. oleracea* can be determined by a single locus so that mutations at these loci result in a visible phenotype.

The rapid-cycling characteristic of the line of *B. oleracea* used for mutagenesis makes the mutants identified in this study potentially valuable tools for education in plant genetics. The rapid-cycling *Brassica rapa* known as Wisconsin Fast Plants has been used extensively in education (Himelblau et al. 2004) but the lack of robust, easily-identifiable mutant phenotypes displaying Mendelian inheritance limits the usefulness of this background in genetics education (although FastPlants *are* effective models for teaching quantitative traits). The *B. oleracea* mutants described here could become a novel resource for educators wishing to create inquiry-based laboratories in which students examine Mendelian inheritance and genetic complementation in *Brassica* crop species.

We have identified several morphological and developmental mutants whose phenotypes are similar to those seen in Arabidopsis. In this study, we report two cases where there were multiple, independently-identified alleles of a gene. Although it is unlikely that the genome was saturated for mutations (Poisson distribution predicts that, for saturation to be achieved, five alleles should be found for every single locus trait), the mutagenesis was sufficient to produce multiple "hits" in most loci that resulted in a visible mutant phenotype. Mapping and molecular analysis of these mutations in the future will help those who seek to identify the functions of *B. oleracea* genes using a comparative genomics approach. In such an approach, a single well-characterized gene from Arabidopsis may very likely identify several paralogous loci in *B. oleracea*, and mutant phenotypes will help to determine which of the paralogs encodes the function in question and which have undergone neofunctionalization or nonfunctionalization. For genes with redundant or overlapping functions, it is difficult to identify them using forward genetics since the probability of a mutation occurring in more than one of the genes in the same plant is extremely low. In situations like this, TILLING provides an ideal reverse genetics tool for isolating mutations in all of the members of a gene family since it enables the identification of putative loss-of-function mutations at each locus regardless of phenotype. Double or multiple mutants could then be constructed using standard genetic crosses to create a plant with combined mutations in all members of the gene family in order to determine the function of these genes collectively.

We have shown the feasibility of TILLING in *B. oleracea*. This reverse genetic technique, together with the wealth of information in Arabidopsis concerning gene function, could be used to identify genetics mutations in key *B. oleracea* to explore the evolution of gene function or to generate lines of *B. oleracea* with novel, commercially significant phenotypes. These data can also be used to predict the function of paralogous genes in related *Brassica* species, such as the economically important oilseed *B. napus* (canola).

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