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**Note**

# EMS mutagenesis and characterization of *Brassica rapa* mutants

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**Abstract** *Brassica rapa* is an important model crop in the genus *Brassica*, which includes various important vegetable crops such as Chinese cabbage and turnip, and is closely related to *Arabidopsis thaliana* (Arabidopsis). *B. rapa* var. *trilocularis* (yellow sarson), a rapid-cycling population of *B. rapa*, is commonly used for genetic research and is expected to bridge the gap between the model plant Arabidopsis and *Brassica* crops. In this study, 940 $M_2$ mutagenized lines of yellow sarson were produced using ethylmethanesulfonate (EMS) mutagenesis, and these $M_2$ plants were examined for alterations in visible phenotypes. In total, 293 independent $M_2$ lines (including 2188 $M_2$ plants) were investigated for phenotypic alterations, and 394 individual mutants were isolated. Subsequently, the observed mutant phenotypes were classified into 8 major categories and 18 subcategories. In addition, three mutants, namely, *early flowering 1* (*elf1*), *crane-like 1* (*crl1*), and *rosette 2* (*ros2*), were selected for further phenotypic and genetic analysis. *elf1* and *ros2* showed early and late flowering phenotypes, respectively. Based on their phenotypes, *crl1* and *ros2* are likely mutations in genes for auxin signaling and gibberellic acid biosynthesis, respectively.

**Key words:** *Brassica rapa*, ethylmethanesulfonate (EMS), flowering time, gibberellic acid (GA).

The Brassicacea family includes the model plant *Arabidopsis thaliana* (Arabidopsis) and the genus *Brassica*, comprising a group of six interrelated species of agronomic importance. Among the six *Brassica* crops, three monogenomic diploid species [*B. rapa* (AA, $n=10$), *B. nigra* (BB, $n=8$), and *B. oleracea* (CC, $n=9$)] have formed three amphidiploid species [*B. juncea* (AABB, $n=18$), *B. napus* (AACC, $n=19$), and *B. carinata* (BBCC, $n=17$)] through interspecific hybridization during the last 10,000 years (Rana et al. 2004), as described by U's triangle (Nagaharu 1935). These *Brassica* species are cultivated to produce vegetables, vegetable oils, condiments, and fodders. They contribute to ~10% of the world's vegetable crop production and 12% of edible oil supplies (Labana and Gupta 1993). In particular, *B. rapa* includes various important vegetable crops such as turnip, Chinese cabbage, and pak choi. Divergence of *Brassica* and Arabidopsis lineages from a common ancestor occurred between 14.5 and 20.4 million years ago (Mya; Yang et al. 1999). Subsequent whole genome triplication of *Brassica* (hexaploidization) is estimated to have occurred 13–17 Mya (Yang et al. 2006), indicating that triplicated homologous segments corresponding to counterpart segments of the Arabidopsis genome are present in the genomes of *Brassica* (Town et al. 2006). This triplication event resulted in massive genetic redundancy and functional diversification, and has likely allowed *Brassica* species to develop diverse morphological variants (Tang and Lyons 2012). After hexaploidization, three copies of the subgenome present within the same nucleus experience unequal gene losses (fractionation bias) that trigger expression differences among the least fractionated subgenome, the medium fractionated subgenome, and the most fractionated subgenome, which is called genome dominance (Cheng et al. 2011). This forms a mesohexaploid, which occurred between 5 and 9 Mya ago (Wang et al. 2011). Diploidization and divergence between *B. rapa*/*oleracea* lineages and *B. nigra* occurred ~8 Mya ago, while *B. rapa* and *B. oleracea* divergence occurred ~4 Mya ago (Yang et al. 2006).

Yellow sarson is a rapid-cycling population of *Brassica rapa*. It is a common model plant for *Brassica* genomic studies due its advantageous features. First, a wide array of *B. rapa* genetics and genomics information is available. For example, the draft genome sequence of the *B. rapa*

Abbreviations: Arabidopsis, *Arabidopsis thaliana*; CRL1, CRANE-LIKE 1; ELF1, EARLY FLOWERING 1; EMS, Ethyl methanesulfonate; GA, gibberellic acid; LL, continuous light; ROS2, ROSETTE 2.

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cultivar line 'Chiifu-401' has been published (Wang et al. 2011). In addition, the Brassica Database (BRAD; http://brassicadb.org/brad/index.php), which offers searching services and data mining tools, has been constructed and is used by researchers and breeders to efficiently apply *B. rapa* genome information to study *Brassica* plants (Cheng et al. 2011). Second, yellow sarson is diploid, self-compatible, and rapid life cycling (35–45 days), resulting in efficient genetics and breeding. Third, *B. rapa* species are closely related to the model plant Arabidopsis. The size of the *B. rapa* genome (529 Mbp) is only three to five times that of the Arabidopsis genome (125–130 Mbp; Johnston et al. 2005). The sequence of 283.8 Mb, covering more than 98% of the gene space, was assembled, and 259.6 Mb (91.13%) of the *B. rapa* genome assembly and 108.6 Mb (90.01%) of the Arabidopsis genome were included within collinear blocks (Wang et al. 2011). In addition, 41,174 protein coding genes were identified and 15,725 of 16,971 (93.0%) *B. rapa* gene families were shared with Arabidopsis (Wang et al. 2011). Therefore, the information obtained from Arabidopsis can be applied to *B. rapa* and a wide variety of *Brassica* relatives for research and breeding. Overall, yellow sarson is expected to play an important role as a new model crop.

In this study, EMS (ethyl methanesulfonate) mutagenesis was performed to construct mutant lines, and $M_2$ plants were inspected for visible altered phenotypes followed by collection of their phenotypic information. Subsequently, three mutants, namely *early flowering 1* (*elf1*), *crane-like 1* (*crl1*), and *rosette2* (*ros2*) were isolated, and the reproducibility of their phenotypes were verified in the $M_3$ generation (*elf1*, *ros2*) or reexamined in the $M_2$ generation (*crl1*). These results supported future prospects of *Brassica* research bridging the gap between the model plant Arabidopsis and *Brassica* crops.

To optimize EMS mutagenesis, seeds were treated with 0.8% EMS conditions (Figure 1). In total, 940 of 4000 mutagenized $M_1$ plants were fertile, and $M_2$ seeds were harvested from each single $M_1$ plant. Approximately 12 $M_2$ seeds produced from the same $M_1$ plant were sown as an $M_2$ line, and $M_3$ seeds were harvested from each $M_2$ line. In a controlled-environment room, 293 $M_2$ lines (3705 $M_2$ seeds) were sown. The germination rates of the $M_2$ lines varied from 8 to 100%.

The $M_2$ plants were screened for altered visible phenotypes at various developmental stages from seed germination to seed maturation (Figure 1). In total, 293 $M_2$ lines (2188 $M_2$ plants) were phenotyped and 29 $M_2$ plants showed phenotypic change in more than one phenotypic categories. As a result, 485 individual mutants were found in 394 mutant lines, and all phenotypes were classified into 8 major categories and 18 subcategories (Table 1, Saito et al. 2011). Figure 2 shows a representative mutant in some of the major categories. Our primary screening identified putative mutants with abnormal leaves (line B-6, Figure 2B), albino cotyledons (line C-23, Figure 2C), dwarf and curly leaves (line C-89, Figure 2D), and wax-less phenotypes (line C-42, Figure 2E) compared to wild-type (WT) plants (Figure 2A, 2E).

Of the 293 $M_2$ lines phenotyped, three mutants showing early (E1 and E2) or late (D1) flowering phenotypes were chosen for further analysis. To confirm whether the mutant phenotypes were inheritable, E1 and D1 plants were self-fertilized and phenotypes were reevaluated in the $M_3$ generation under different conditions. E2 plants were also reexamined in the $M_2$ generation because $M_3$ seeds were not obtained. In this experiment, 200 individual $M_2$ or $M_3$ plants were reevaluated for visible variations.

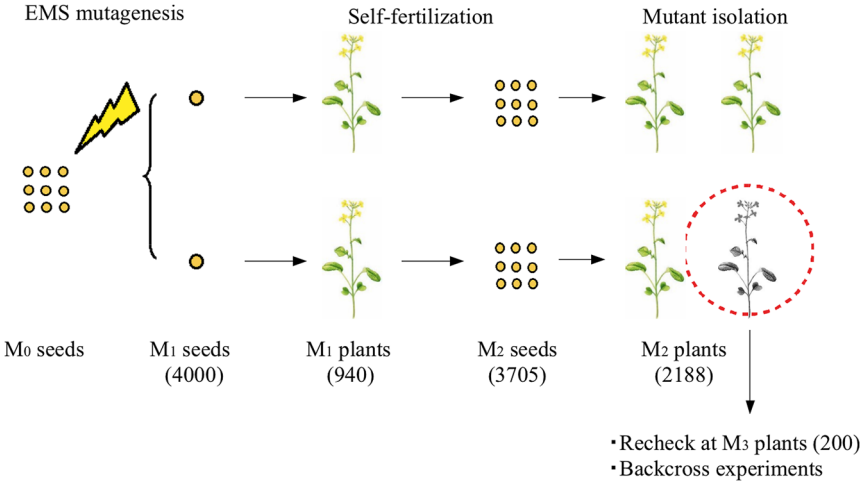


Figure 1. Strategy of ethyl methanesulfonate (EMS) mutagenesis of *Brassica rapa*. Wild-type (WT) *B. rapa* seeds ($M_0$) were mutagenized with EMS, and the resulting $M_1$ seeds were sown in a controlled-environment room to produce $M_2$ seeds from single $M_1$ plants. Twelve $M_2$ seeds were planted as an $M_2$ line, and whole $M_3$ seeds were obtained from the same $M_2$ line. During this process, each $M_2$ plant was inspected for altered visible phenotypes, and the self-fertilized seeds from each mutant were harvested. Numbers in parentheses show the number of seeds or plants.

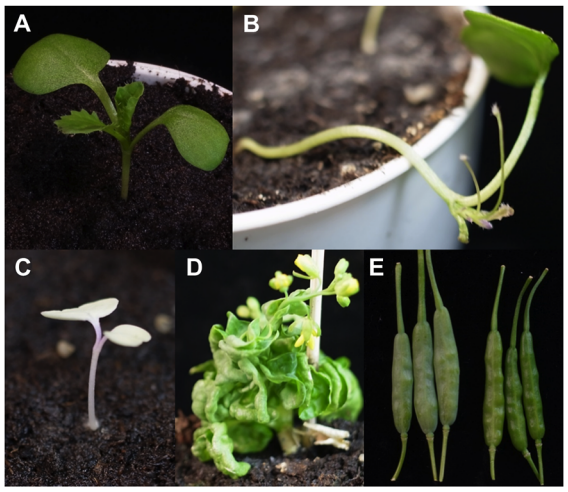


Figure 2. Mutant phenotypes obtained by ethyl methanesulfonate (EMS) mutagenesis. (A) Wild type (WT), (B) abnormal leaves (line B-6), (C) albino cotyledons (line C-23), (D) dwarf (line C-89), and (E) wax-less fruits (right; line C-42) and the WT (left).

Table 1. List of phenotypic categories and the number of mutants included in these classes.

| Major category | Subcate gory | No. of mutants (No. of mutant lines) |
|---|---|---|
| Plant size | Small plant | 38 (28) |
| Cotyledon morphorogy | One cotyledon | 65 (58) |
| | Three cotyledon | 12 (12) |
| | Four cotyledon | 1 (1) |
| Leaf morphology | Adaxially curled leaf | 4 (1) |
| | Other leaf morphology | 225 (197) |
| Cotyledon/leaf color | Yellow-green/Yellow-green | 15 (12) |
| | Yellow-green/green | 2 (2) |
| | gree/Yellow-green | 12 (7) |
| | Dull green/Dull green | 17 (12) |
| | White | 10 (9) |
| Flowering tinling | Early | 70 (41) |
| | Late | 1 (1) |
| Flower color | Pale yellow flower | 1 (1) |
| | Yellow-green sepal | 1 (1) |
| Flower morphology | Flower homeotic mutaion | 1 (1) |
| Other | Glossy leaves | 9 (9) |
| | Bolting suppression | 1 (1) |
| Total | | 485 (394) |

The number of phenotypic categories scored in $M_2$ plants is shown. Note that the total number of mutants exceeds the total number of mutant lines because a mutant sometimes presented more than one phenotype.

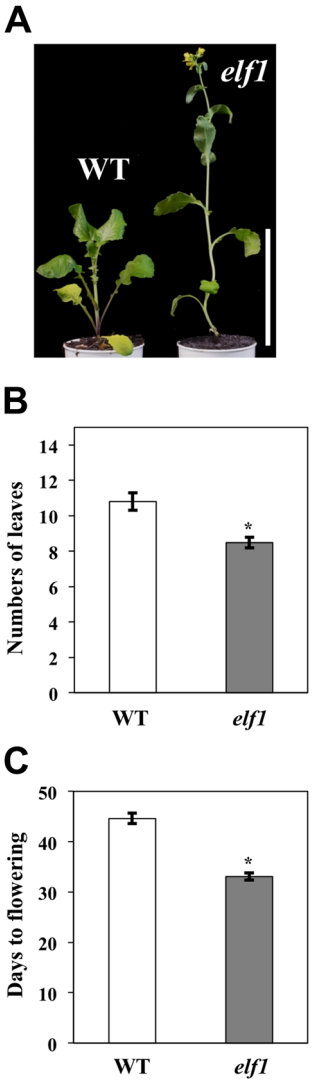


Figure 3. Flowering times of wild-type (WT) and *elf1* plants under continuous light (LL). (A) Appearance of WT and *elf1* at 31 days after sowing under LL. (B) The total number of leaves on the main stem when plants were flowered under LL. (C) The number of days to flowering under LL. Plants were grown on MS medium in growth chambers at 22°C for 9 days under long-days (LD; 16 h light/8 h dark) and then transplanted into soil in a controlled-environment room at 22°C under LL. Flowering time was scored by counting the total number of leaves on the main stem when plants flowered and the total number of days to flowering. Means±SE are shown. Twelve to 27 plants of each genotype were used for each trial. Scale bar represents 10 cm. Asterisk denotes statistical significance compared to the WT (Student's *t*-test, $p<0.05$).

All the E1 mutant plants showed early flowering phenotypes in the $M_3$ generation under continuous light (LL) conditions (Figure 3A), indicating that the mutant phenotype was heritable. Therefore, a mutation responsible for the early flowering phenotype of E1 was named *early flowering 1* (*elf1*). Flowering time was measured by scoring the number of leaves on the main stem when plants were flowered and the total number of days to flowering. Under LL, the total number of leaves was 8.5±0.13 for *elf1* and 10.3±0.28 for WT plants, respectively (Figure 3B). In addition, *elf1* (34.4±0.78 days) flowered ~8 days earlier than the WT (42.3±1.16 days) under LL (Figure 3C). These results suggested that the responsible gene for the mutation may be related to the flowering delay in WT plants under LL. It is still unclear whether the *elf1* behaves as a recessive or dominant mutation.

Because $M_3$ seeds of the E2 mutant plants were not produced from $M_2$ plants due to their low fertility, two $M_2$ seeds were reevaluated. In this experiment, all

plants germinated but the early flowering phenotype of the plants was minor. However, $M_2$ plants showed the same pleiotropic phenotypes as the E2 mutant plants observed in the previous trial, such as the suppression of lateral root formation (Figure 4A, 4D), upwardly curled leaves (Figure 4B), and dwarfism (Figure 4C). The total number of lateral roots (>0.3 mm) was 13.3±0.89 for the WT and 3.0±2.0 for *crl* (Figure 4D). In addition, all six $M_2$ plants (4 in the 1st evaluation and 2 in the reevaluation) showed mutant phenotypes, suggesting that this mutation was dominant. These results are similar to a previous study of the *crane* mutant in Arabidopsis (Uehara et al. 2008); thus, this mutant line was named *crane-like 1* (*crl1*). The low fertility phenotype of the *crl1* plants was reproduced in the reevaluation of two $M_2$ plants. A similar phenotype of low fertility was described in Arabidopsis *crane* mutant (Uehara et al. 2008).

$M_3$ seeds derived from D1 mutant plants were not harvested, but the other three $M_2$ plants that showed phenotypes similar to WT plants produced 154 $M_3$ seeds as bulk. Since self-fertilized $M_2$ plants heterozygous for mutations were expected to produce homozygous mutant progeny, these $M_3$ seeds were reexamined. As a result, 3 of the 38 $M_3$ plants derived from the same $M_2$ plant showed D1 phenotypes such as suppression of bolting (stem elongation) and flowering (Figure 5A, 5D). This mutant line was named *rosette2* (*ros2*) because these mutants showed similar phenotypes to the gibberellin$_1$ ($GA_1$)- and $GA_3$-deficient dwarf mutant *rosette* (*ros*) identified from *B. rapa* syn. *campestris* (Rood et al. 1989). Endogenous GAs are involved in the regulation of germination, reproductive development, and the control of leaf morphology in *Brassica*, although excess GA levels have only a minor effect on germination rate or floral development (Zanewich et al. 1990). To confirm whether *ros2* phenotypes were caused by endogenous GA deficiency, a 10 $\mu$l aliquot of 28.9 $\mu$M $GA_3$ solution that contained 0.1% ethanol was applied with pipette to the shoot apex of *ros2* seedlings at 42, 49, 56, and 63 day after sowing under long-days (LD) as reported (Rood et al. 1989). No bolting and reproductive development was observed after 42 days without $GA_3$ treatment, at which time WT plants were producing buds (Figure

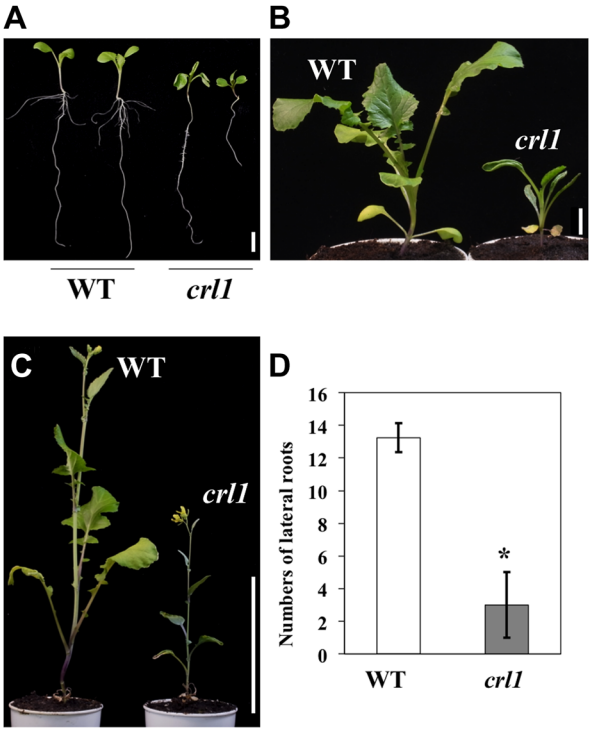


Figure 4. Phenotypes of wild-type (WT) and *crl1* plants. (A) Nine-day-old seedlings of the WT and *crl1*. Lateral root formation of *crl1* mutants was severely suppressed. (B) Leaves of the WT and *crl1* at 24 days. In *crl1* mutants, leaves were upwardly curled. (C) The 43-day-old WT and *crl1*. The *crl1* mutants were smaller than the WT. (D) Numbers of lateral roots (>0.3 mm) formed per plant of WT and *crl1* seedlings at 9 days. Plants were grown on MS medium in growth chambers at 22°C for 9 days under LD (16 h light/8 h dark) and then transplanted into soil in a controlled-environment room at 22°C under continuous light (LL). This experiment was performed twice with similar results. Means ±SE are shown. Scale bars represent 1 cm for (A and B) and 10 cm for (C). Asterisk denotes statistical significance compared with the WT (Student's *t*-test, $p<0.05$).

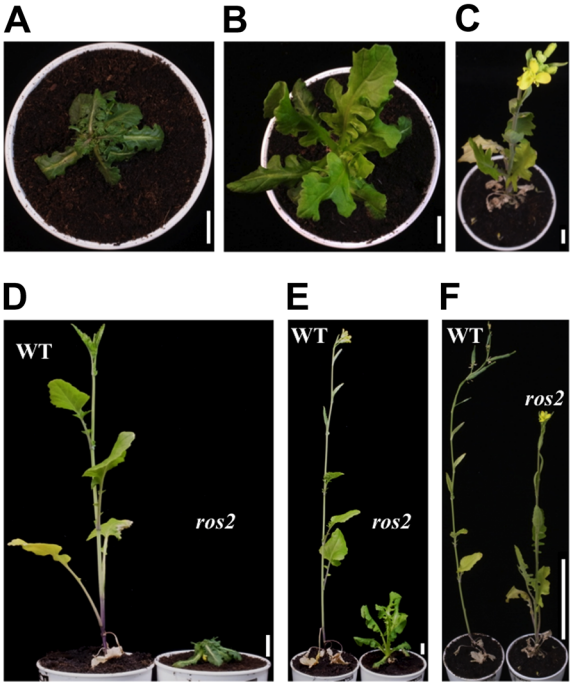


Figure 5. Phenotypes of wild-type (WT) and *ros2* plants. (A–C) The aerial regions of *ros2* at 42 days without application of $GA_3$ (A), 49 days with an application (42 days after sowing) of $GA_3$ (B), and 66 days with four applications (42, 49, 56, and 63 days after sowing) of $GA_3$ (C). (D–F) Comparison of the WT and *ros2* at 42 days without application of $GA_3$ (D), 49 days with an application of $GA_3$ (E), and 66 days with four applications of $GA_3$ (F). WT and *ros2* plants were grown on MS medium in growth chambers at 22°C for 9 days and 42 days, respectively, under LD (16 h light/8 h dark) and then transplanted into soil in a controlled-environment room at 22°C under LL. Scale bars represent 1 cm for (A–E) and 10 cm for (F).

5A, 5D). However, $GA_3$ induced upper curling and size increases in *ros2* leaves after 1 week (Figure 5B, 5E), and additional application of $GA_3$ induced bolting and floral development at 66 days (Figure 5C, 5F). These results suggested that *ros2* is sensitive to GA and that mutant phenotypes are caused by decreased endogenous GA concentrations.

Genetic analysis of $M_2$ progeny suggested that the *crl1* mutation was dominant. In addition, the *crl1* plants showed visible phenotypes similar to the *crane* mutant (Figure 4). In Arabidopsis, 9 dominant mutants of *Aux*/*IAA* genes were reported, but only the *crane* mutant showed unique phenotypes including up-curled leaves and lateral root suppression (Uehara et al. 2008). The *crane* mutant contained two dominant mutant alleles, *crane-1* and *crane-2*, which are gain-of-function mutations in domain II of IAA18 (Uehara et al. 2008). *IAA18* is a member of the 29 *Aux*/*IAA* genes and acts as a transcriptional repressor of the signaling pathway of the plant hormone auxin. In Arabidopsis, domain II of IAA18 is involved in the instability of IAA18 protein, and gain-of-function mutations in this gene prevent proteins from being degraded by the $SCF^{TIR1/AFBs}$ ubiquitin–ligase complex composed of auxin receptors, TRANSPORT INHIBITOR RESPONSE1 (TIR1) and its homologous proteins AUXIN-SIGNALING F-BOX PROTEINS (AFB1/2/3/4/5; Dharmasiri et al. 2005a; Dharmasiri et al. 2005b; Greenham et al. 2011; Kepinski and Leyser 2005). Hence, the missense mutations in domain II block the interaction between Aux/IAA and TIR1/AFBs, resulting in constitutive suppression of the auxin response.

According to BRAD, two copies of *IAA18* homologs in *B. rapa* are located on chromosomes A6 (Bra018938) and A8 (Bra014303), respectively. Moreover, a blastp search revealed that Arabidopsis IAA18 (At1g51950) shares high amino acid identity with Bra018938 protein (*E* value$=9e^{-98}$, identity 75%) and Bra014303 protein (*E* value$=e^{-104}$, identity 75%). These data suggest that gain-of-function mutations in either or both of the *crane* homologous genes conserved in *B. rapa* may trigger constitutive suppression of auxin response in *crl1* plants, such as *crane*.

The *ros2* mutant showed a typical severe dwarf phenotype, as is commonly observed in GA-deficient or GA-insensitive mutants (Figure 5A, 5D). In addition, exogenously applied $GA_3$ restored plant height and leaf size of *ros2*, indicating that this phenotype was caused by endogenous GA deficiency derived from the suppression of GA biosynthesis (Figure 5C, 5F). Over 100 GAs have been identified from plants (MacMillan 2002), but only a small number function as biologically active GAs in higher plants, such as $GA_1$, $GA_3$, and $GA_4$. Bioactive GAs regulate plant growth and development, including bolting (stem elongation), leaf expansion, and flower induction throughout the life cycle. At least 14 genes encoding six GA biosynthetic enzymes (CPS, KS, KO, KAO, GA20ox, and GA3ox) catalyzing the formation of bioactive GAs have been isolated in Arabidopsis (Supplemental Figure 1; Yamaguchi 2008). In addition, two cytochrome P450 genes, *CYP714B1* and *CYP714B2*, which encode GA 13-oxidase (Supplemental Figure 1), have recently been identified in rice (Magome et al. 2013). However, the isolation and phenotypic analysis of *ga13ox* mutants has yet to be reported in Arabidopsis. A common phenotype caused by mutation in the GA biosynthetic genes is dwarfism, which can be rescued by applying exogenous GA. Historically, dwarf genes have been utilized in plant breeding as represented by the success of the "Green Revolution" in rice and wheat (Hedden 2003; Khush 2001). Furthermore, $GA_1$- and $GA_3$-deficient dwarf mutant *rosette* (*ros*; Rood et al. 1989) and the semidominant GA-insensitive dwarf mutant caused by a gain-of-function mutation in the C-terminal GAI, RGA, SCARECROW (GRAS) domain of a REPRESSOR OF GA1-3 (RGA) homolog, Brrga1-d (Muangprom et al. 2005), have been reported in *B. rapa*, and are expected to reduce lodging problems of oilseed *Brassica* cultivars without affecting the harvest.

To identify candidate genes, the amino acid sequences of GA biosynthetic enzyme proteins from Arabidopsis were used to select *B. rapa* homologous genes. Nineteen candidate genes were identified, and ~90% of Arabidopsis proteins share more than 80% amino acid identity with their *B. rapa* homologous proteins (Supplemental Table 2). Furthermore, CPS, KS, and KO were each encoded by a single gene in Arabidopsis (Yamaguchi 2008) and only *AtKS* and *AtKO*/*CYP701A3* contained a single homolog of *B. rapa* (Bra035120 and Bra009868, respectively; Supplemental Table 2). These data, together with the observation that the *ros2* mutation was recessive, suggested that these two genes are candidates for *ros2*. However, the possibility exists that several mutations induced at the same time or even a single mutation in one redundant gene is sufficient to cause the *ros2* phenotype. To identify the responsible gene, backcross experiments and comprehensive analysis of all candidates are required.

The *elf1* and *ros2* exhibited early and late flowering phenotype, respectively (Figure 3 and 5). Flowering time is a quantitative trait that regulates an appropriate timing of reproduction and affects the yield potential. In vegetative crops such as Chinese cabbage (*Brassica rapa* ssp. *pekinensis*), early bolting and flowering can cause severe losses in yield and quality. By contrast, in rapeseed (*Brassica napus*) cultivated for seed production, late flowering can shorten the spring cultivation period in the northern hemisphere where plants are exposed to cold temperatures from late fall to early spring. Therefore, flowering time adaptation for specific local environments is one of the major breeding goals. Further investigations

are necessary for the identification of mutations responsible for the altered flowering phenotypes to address this issue.

In conclusion, we have developed the EMS mutant collection in the diploid Brassica species, *B. rapa*. This collection is suitable for forward genetics studies on *B. rapa*. Based on the current study, we are planning to perform a large-scale mutagenesis of *B. rapa* with EMS. These mutant collections would be attractive tools for molecular breeding of the Brassica species.

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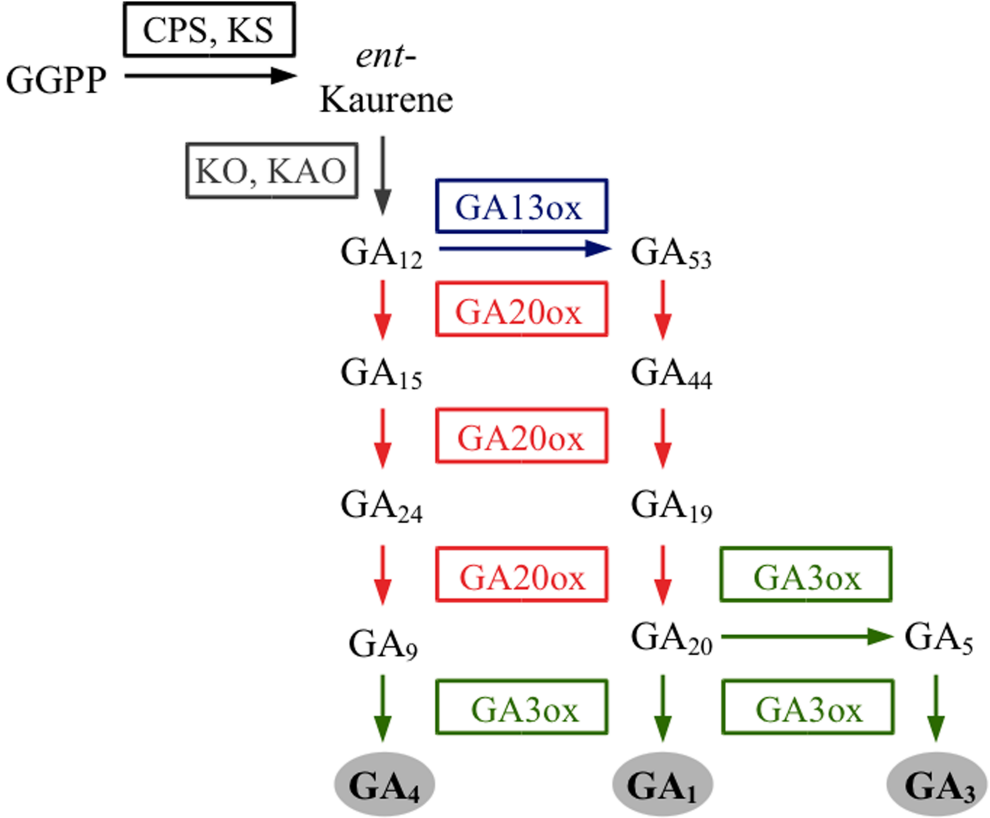
**Supplemental Table and Figure**

**Supplemental Figure legend**

**Supplemental Figure 1.** The gibberellin (GA) biosynthesis pathways in plants. Three bioactive GAs found in various plant species are shown (highlighted grey). GGPP, *trans*-geranylgeranyl diphosphate; CPS, *ent*-copalyl diphosphate synthase; KS, *ent*-kaurene synthase; KO, *ent*-kaurene oxidase; KAO, *ent*-kaurenoic acid oxidase; 3ox, GA 3-oxidase; 13ox, GA 13-oxidase; 20ox, GA20-oxidase.

**Supplemental Table 1.** List of GA biosynthetic genes and their homologs in *Brassica rapa*.

| Gene name | Accesion number | *B. rapa* gene ID | Identity |
|---|---|---|---|
| *AtCPS* [*GA1*] | At4g02780 | Bra036239 | 664/809 (82%) |
| | | Bra000864 | 591/729 (81%) |
| *AtKS* [*GA2*] | At1g79460 | Bra035120 | 651/785 (82%) |
| *AtKO/ CYP701A3* [*GA3*] | At5g25900 | Bra009868 | 430/478 (89%) |
| *AtKAO1/ CYP88A3* | At1g05160 | Bra015394 | 449/490 (91%) |
| *AtKAO2/ CYP88A4* | At2g32440 | Bra005596 | 401/489 (82%) |
| *AtGA20ox1* [*GA5*] | At4g25420 | Bra013890 | 321/378 (84%) |
| | | Bra019165 | 300/346 (86%) |
| *AtGA20ox2* | At5g51810 | Bra028277 | 328/376 (87%) |
| | | Bra022565 | 332/378 (87%) |
| *AtGA20ox3* | At5g07200 | Bra009285 | 328/380 (86%) |
| | | Bra028706 | 322/380 (84%) |
| *AtGA20ox4* | At1g60980 | Bra027106 | 291/376 (77%) |
| *AtGA20ox5* | At1g44090 | Bra014019 | 305/356 (85%) |
| *AtGA3ox1* | At1g15550 | Bra026122 | 319/359 (88%) |
| | | Bra026757 | 312/358 (87%) |
| *AtGA3ox2* | At1g80340 | Bra008480 | 311/350 (88%) |
| *AtGA3ox3* | At4g21690 | Bra020909 | 302/351 (86%) |
| *AtGA3ox4* | At1g80330 | Bra008480 | 187/335 (55%) |




**Mizuno et al. Supp Figure 1**