

# Mitigation of establishment of *Brassica napus* transgenes in volunteers using a tandem construct containing a selectively unfit gene

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## Summary

Transgenic oilseed rape (*Brassica napus*) plants may remain as 'volunteer' weeds in following crops, complicating cultivation and contaminating crop yield. Volunteers can become feral as well as act as a genetic bridge for the transfer of transgenes to weedy relatives. Transgenic mitigation using genes that are positive or neutral to the crop, but deleterious to weeds, should prevent volunteer establishment, as previously intimated using a tobacco (*Nicotiana tabacum*) model. A transgenically mitigated (TM), dwarf, herbicide-resistant construct using a gibberellic acid-insensitive ( $\Delta gai$ ) gene in the *B. napus* crop was effective in offsetting the risks of transgene establishment in volunteer populations of *B. napus*. This may be useful in the absence of herbicide, e.g. when wheat is rotated with oilseed rape. The TM dwarf *B. napus* plants grown alone had a much higher yield than the non-transgenics, but were exceedingly unfit in competition with non-transgenic tall cohorts. The reproductive fitness of TM *B. napus* was 0% at 2.5-cm and 4% at 5-cm spacing between glasshouse-grown plants relative to non-transgenic *B. napus*. Under screen-house conditions, the reproductive fitness of TM *B. napus* relative to non-transgenic *B. napus* was less than 12%, and the harvest index of the TM plants was less than 40% of that of the non-transgenic competitors. The data clearly indicate that the  $\Delta gai$  gene greatly enhances the yield in a weed-free transgenic crop, but the dwarf plants can be eliminated when competing with non-transgenic cohorts (and presumably other species) when the selective herbicide is not used.

**Keywords:** *Brassica napus*, dwarfism, ecological competition, fitness, transgenic mitigation, volunteerism.

## Introduction

Transgene flow from cultivated crops to other crop varieties and to weedy relatives presents a potential agronomic and ecological risk from increased weediness and/or invasiveness of volunteer, feral or hybrid offspring, with increased potential of such progeny to compete with cultivated crops or natural populations (Snow and Palma, 1997; Dale *et al.*, 2002; Ellstrand, 2003; Messeguer, 2003; Stewart *et al.*, 2003; Gressel, 2005). Gene transfer between transgenic and non-transgenic *Brassica napus* varieties is well documented (Hall *et al.*, 2000, 2005; Rieger *et al.*, 2002; Beckie *et al.*, 2003). Oilseed rape cultivation traditionally results in significant volunteer populations during subsequent years because of

extensive seed shatter (Lutman, 1993; Price *et al.*, 1996); these may become feral, re-evolving some of the traits of weeds (Gressel, 2005). Transgenic volunteer offspring or feral strains, particularly herbicide-resistant populations, present additional management concerns (Hall *et al.*, 2000, 2005; Simard *et al.*, 2002).

Many 'containment' strategies to prevent transgene flow between crop varieties, and to related weeds or wild species, have been described in the literature (Daniell, 2002; Gressel, 2002; Jepson, 2002; Stewart *et al.*, 2003; Kuvshinov *et al.*, 2004; Maliga, 2004; Oliver *et al.*, 2004). There are also 'mitigation' strategies to preclude the impact of transgene flow amongst crop varieties (the subject of this paper) and between species (the subject of a companion paper;

Al-Ahmad and Gressel, 2005b) should containment fail (Gressel, 1999; Al-Ahmad *et al.*, 2004, 2005). The needs for containment and mitigation are most acute with rice and sunflowers, which have conspecific weeds, as well as with oilseed rape, sorghum and barley, which have closely related interbreeding weeds (Gressel, 2002; Ellstrand, 2003; Warwick and Stewart, 2005). Some transgenic traits e.g. *B. napus* with altered fatty acid contents (Walker *et al.*, 2004), probably have no selective advantage for outcrossing. Containment and mitigation are crucial for crops bearing pharmaceutical transgenes, where there must be no gene flow from the crop and its volunteers to edible varieties. Some gene flow (leakage) is inevitable with unidirectional containment mechanisms, i.e. either preventing gene outflow or gene influx, and leaked genes could then spread through the population of undesired species, unless the spread is mitigated.

A mechanism for mitigation has been proposed in which the primary transgene (e.g. herbicide resistance, pharmaceutical trait, etc.) is tandemly coupled with flanking genes that may be desirable or neutral to the crop, but unfit for the rare relative that introgresses the gene (Gressel, 1999). Such mitigator genes could include genes causing dwarfing, non-bolting, no secondary dormancy, no seed shattering, poor seed viability and inhibition of tillering or branching, all depending on the instance. If the transgene has a small fitness disadvantage, it will remain localized in a very small proportion of the population. Therefore, gene flow should be mitigated by lowering the fitness of recipients below the fitness of the wild-type, so that they will not spread (Gressel, 2002).

The validity of the transgenic mitigation concept was demonstrated in a tobacco (*Nicotiana tabacum*) model using dwarfing as the mitigator with herbicide resistance as the primary gene (Al-Ahmad *et al.*, 2004, 2005). Whereas transgenic tobacco plants with the tandem construct grew well when cultivated alone, they were unable to reach maturity when interspersed with the wild-type tobacco plants. Here, we report the use of the same transgenically mitigated (TM) tandem construct in the *B. napus* system. The transgene persistence in the TM *B. napus* volunteers was tested in crop-to-crop ecological competition experiments using TM vs. otherwise isogenic non-transgenic *B. napus* plants. There was no reason to expect the same level of mitigation as with the tobacco model, as *Brassica* has a totally different growth habit, with an extended period as a rosette followed by a rapidly bolting flower stalk.

Risk assessment of gene flow in oilseed rape has been widely studied using herbicide-resistant varieties, as this trait is easy to analyse in large populations (Hall *et al.*, 2000; Beckie

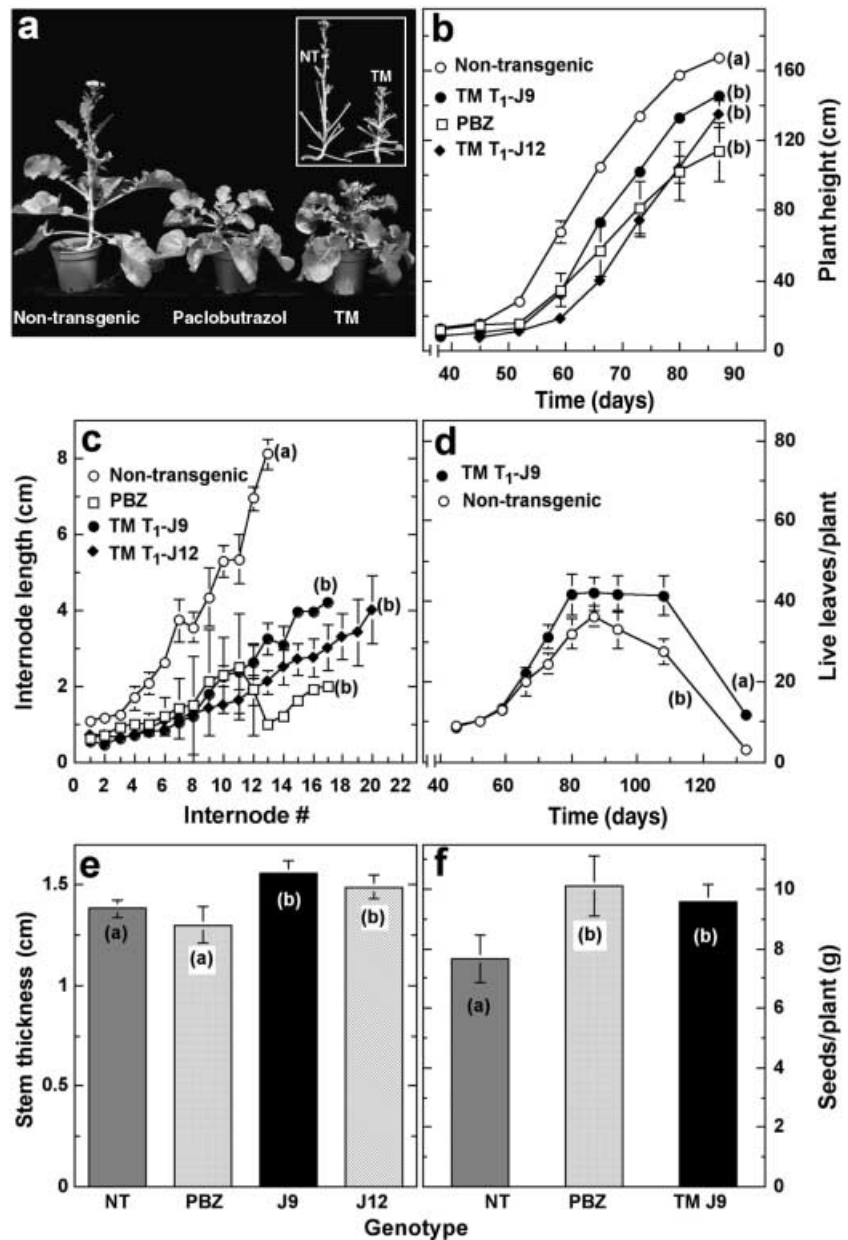
*et al.*, 2003). The use of mutant or transgenic herbicide-resistant *B. napus* has raised several management concerns, including the transfer of herbicide-resistant genes into weedy relatives (Rieger *et al.*, 1999; Snow *et al.*, 1999), the weediness of resistant volunteer *B. napus* (Squire *et al.*, 1997; Simard *et al.*, 2002) and the possibility that the transfer of herbicide resistance between varieties of *B. napus* may result in *B. napus* volunteers resistant to many herbicides. A small proportion (0.2%) of *B. napus* volunteer populations in western Canada had multiple resistance to glufosinate, glyphosate and imazethapyr within a few years of their use (Hall *et al.*, 2000). More recently, 10% of *B. napus* volunteers in Saskatchewan, Canada were doubly resistant to glyphosate and glufosinate (Beckie *et al.*, 2003). This illustrates the rapidity and distance to which resistance genes may transfer to future *B. napus* crops, as well as to related weeds.

Genetically engineered dwarfing is possible by suppressing genes for gibberellin (GA) production (Coles *et al.*, 1999), or the GA response itself (Peng *et al.*, 1997; Muangprom *et al.*, 2005). Dwarfing is disadvantageous for weeds that can no longer compete with the crop for light, but is desirable in many crops, including oilseed rape (Zhou and Xi, 1993), preventing lodging and improving the harvest index (grain to straw ratio) (Khush, 1999; Sakamoto and Matsuoka, 2004). GA biosynthesis-suppressing plant growth regulators (PGRs) reduce the height of oilseed rape and improve the yield (Scarlsbrick *et al.*, 1985; Baylis and Wright, 1990; Armstrong and Nicol, 1991; Zhou and Xi, 1993); this was confirmed with *B. napus* cv. Westar. This evidence suggests that the dwarfing mitigator transgene (gibberellic acid insensitive,  $\Delta gai$ ) in a tandem construct should produce dwarf plants, mitigating the risk of herbicide-resistant gene spread in field populations when it transfers to non-transgenic varieties, as reported below.

## Results and discussion

### GA biosynthesis-suppressing PGRs reduce the height and improve the harvest index in *B. napus*

Before embarking on the transformation of oilseed rape with dwarfing transgenes, it was imperative to ascertain that dwarfing would not reduce the yield. Thus, GA biosynthesis-suppressing PGRs were used to dwarf oilseed rape, as described briefly in Figure 1 and fully in the Supplementary material (Table S1 and Figure S1, available online). The findings with one PGR, paclobutrazol, support the proposal that the  $\Delta gai$  dwarfing gene may be useful for the generation of healthy, high-yielding oilseed rape by transgenic manipulation of the plant architecture, as demonstrated below.



**Figure 1** Compact nature and high productivity of the transgenically mitigated (*TM*) *Brassica napus* plants carrying a dwarfing gene in tandem with a herbicide-resistant gene. The plants were grown separately in 1-L pots in the glasshouse at wide spacing to avoid competition. The growth parameters were measured at intervals on independent non-transgenic plants and preselected hemizygous *TM* T<sub>1</sub> transgenic segregants (lines J9 and J12). A group of non-transgenic (*NT*) plants was sprayed with 0.2 mg a.i. (active ingredient) paclobutrazol (*PBZ*) as a chemically dwarfing control. The points are the mean ( $\pm$  SE) of  $n = 13$ – $15$ . No error bars are shown when the standard errors are smaller than the data points. Different letters within a panel indicate significantly different values at  $P \leq 0.05$  (least significant difference test).

### Expression of the *TM* construct in transgenic *B. napus* transformants

Sixteen independent, later verified, *TM* primary transformants and 16 escapes were regenerated using the *TM* construct in two *Agrobacterium tumefaciens* strains in the presence of 34  $\mu$ M kanamycin, which is just above the threshold for inhibiting the non-transgenics (Al-Ahmad, 2005). The integration of *ahas*<sup>R</sup> (acetohydroxy acid synthase; conferring resistance to imidazolinone herbicides) and  $\Delta$ *gai* as intact tandem genes within the genomes of the *TM* transformants was confirmed by the AHAS enzymatic assay (Figure S2a, available as Supplementary material) as a herbicide-resistant phenotype (Figure S2b, available as

Supplementary material), and by polymerase chain reaction (PCR) (Figure S3b, available as Supplementary material). Single gene copies of the *ahas*<sup>R</sup>- $\Delta$ *gai* tandem were inserted in *TM* T<sub>0</sub> lines J7, J8, J9 and J12, as shown by Southern hybridization of genomic DNA from *TM* *B. napus* T<sub>0</sub> transformants, using a probe that spans the tandem genes and the linker region between them (Figure S3c–f, available as Supplementary material).

This study presents findings with both hemizygous and homozygous *TM* transformants and their progeny. The hemizygote segregating offspring are analogous to field hybrids between *TM* transgenic and other non-transgenic varieties. Homozygous progeny are indicative of what might be expected of genes fixed by backcrossing.

### Vegetative phenotype of the TM *B. napus* transformants

The 16 primary TM *B. napus* transformants were all clearly dwarfed at early growth stages (stages 1–3; Figure S4, available as Supplementary material), but the dwarfing phenotype became less apparent after bolting (stages 4 and 5; Figure S2c, available as Supplementary material), as was also found with *B. napus* control plants treated with paclobutrazol (Figure 1a–c). The hemizygous TM T<sub>1</sub> plants of lines J9 and J12 had less apical dominance, were shorter ( $P = 0.01$ ) and thicker ( $P = 0.05$ ) stems, with shorter internodes ( $P = 0.01$ ) and more live leaves ( $P = 0.05$ ) than the non-transgenic plants (Figure 1). TM individuals of T<sub>2</sub> and T<sub>3</sub> (one homozygous to two hemizygous) segregating families and homozygous non-segregating families of lines J7, J9 and J12 were also dwarfed at early growth stages.

The homozygous TM T<sub>2</sub> plants from optimal line J9 formed leaves that were a darker green with shorter petioles ( $P = 0.01$ ; Figure S5a, available as Supplementary material), felt thicker and had a greater density ( $P \leq 0.01$ ) than the non-transgenics (Figure S5b, available as Supplementary material). However, most non-transgenic leaves had a greater area than the TM leaves ( $P \leq 0.01$ ; Al-Ahmad, 2005). The thicker TM leaves had > 25% more chlorophyll per unit area than the non-transgenics ( $P \leq 0.01$ ; Figure S5c, available as Supplementary material), but less carotenoids ( $P \leq 0.05$ ; Figure S5c,d, available as Supplementary material). The chlorophyll content was not significantly different between TM and non-transgenic plants when measured as pigment per unit fresh weight (Figure S5d, available as Supplementary material). Chlorophyll levels were also elevated in *Δgai* transformants of *Arabidopsis thaliana* (Peng *et al.*, 1999), *Chrysanthemum morifolium* (Petty *et al.*, 2003) and TM tobacco (Al-Ahmad *et al.*, 2004).

The homozygous TM T<sub>2</sub> plants from line J9 grown without competition accumulated double the shoot and root biomass by late season compared with the non-transgenics ( $P \leq 0.01$ ; Figure S6, available as Supplementary material). The dwarf TM plants formed 83% more leaves ( $P \leq 0.01$ ) with greater longevity (delayed senescence), and had 78% more branches ( $P \leq 0.01$ ; Figure S7d, available as Supplementary material). The higher level of chlorophyll may contribute to the prolonged photosynthetic activity in the TM plants. Thus, the mitigator *Δgai* gene had an advantageous effect on the dwarf TM crops grown without competition by prolonging and increasing productivity.

The homozygous dwarfed TM plants of line J9 failed to respond to exogenous GA<sub>3</sub> application, as expected, so that

the height, leaf number and branches formed per plant, and the time to flowering, were not significantly different from the non-treated TM plants (Figure S7, available as Supplementary material). However, GA<sub>3</sub>-treated non-transgenic plants bolted (Figure S7a, available as Supplementary material) and flowered (Figure S7c, available as Supplementary material) 20 days earlier than non-treated non-transgenic *B. napus* plants ( $P \leq 0.05$ ). *Arabidopsis* GA-insensitive mutants also failed to respond to exogenous GA treatment, and GA also accelerated flowering in GA-deficient mutants under non-inductive short-day conditions (Wilson *et al.*, 1992). GA<sub>3</sub> did not noticeably affect the TM plants, but the leaves of GA<sub>3</sub>-treated non-transgenic *B. napus* plants were yellowish and dehisced earlier than the green-bluish non-treated and solvent-treated controls (Figure S7b, available as Supplementary material).

### TM construct enhances seed yield of TM *B. napus* plants at wide spacing

Flower initiation was delayed by 7–10 days in the 2 : 1 mixed hemizygous and homozygous TM plants (progeny of hemizygous lines J7, J9 and J12) grown in pots without competition compared with the non-transgenics ( $P \leq 0.01$ ; Al-Ahmad, 2005). Flowering in *Arabidopsis* was similarly delayed in GA-deficient and GA-insensitive mutants under non-inductive short-day conditions (Wilson *et al.*, 1992).

The dwarfing *Δgai* gene driven by the *Arabidopsis thaliana* promoter did not decrease TM *B. napus* pollen viability (> 99%; Table S2, available as Supplementary material), despite causing kinetin-reversible male sterility in hemizygous TM T<sub>0</sub> tobacco and its homozygous progeny having the same TM 1 construct (Al-Ahmad and Gressel, 2005a). The reason for the species differences can only be speculated. Oilseed rape, unlike tobacco, is a rosette species, and may require a specific GA as a signal for bolting of the flower stalk, which may require a specific receptor that may not interact with *Δgai*.

There was no significant difference in the dry shoot biomass at harvest between the TM plants and the non-transgenics, with the exception of the strongly dwarfed TM line J16 ( $P \leq 0.05$ ), which was less reproductive than line J9 (Table S2, available as Supplementary material). The yield of the hemizygous TM T<sub>1</sub> *B. napus* line J9, grown separately in 1-L pots at wide spacing, was significantly higher than that of the non-transformed controls (Figure 1f). This was not the case with the other less productive hemizygous lines J12 and J16 ( $n = 10$  plants each; Table S2, available as Supplementary material). The highly productive TM line J9 was used in

glasshouse competition experiments, as it has the potential to be cultivated in future field experiments. Both TM and non-transgenic lines had equivalent > 98% normal and fully developed high-quality seeds (Table S2, available as Supplementary material).

### Co-segregation of the tandem traits in TM progeny

The selfed TM T<sub>2</sub> progeny of the hemizygous segregating families segregated according to the expected 3 : 1 Mendelian ratio of three linked imazapyr (herbicide) and kanamycin resistant as well as dwarf progeny to one linked sensitive and tall progeny (not significant from the  $\chi^2$  null hypothesis; Table S3, available as Supplementary material). These results were consistent with the Southern hybridization blots which showed that the TM construct was inserted into a single chromosome in these lines (Figure S3c–f, available as Supplementary material). All the non-transgenic segregant plants were tall and sensitive to imazapyr and kanamycin. The homozygous non-segregating families of lines J7, J9 and J12 were resistant to imazapyr and kanamycin (Table S3, available as Supplementary material), and were all dwarfed in the vegetative stages when grown in soil, indicating that the TM tandem traits remained linked.

The covalent *ahas*<sup>R</sup> and *Δgai* genes remained linked in T<sub>0</sub>, T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub> generations, as measured both genetically and by PCR amplification of different DNA segments within the TM construct T-DNA, including the 3' end of the *ahas*<sup>R</sup> gene across the linker region into the 5' end of the *Δgai* gene (Figure S3a,b, available as Supplementary material). The transgenic *B. napus* lines were at least 100-fold more resistant to imazapyr than the non-transgenic lines, when measured as growth or inhibition of AHAS enzyme activity (Figure S2a, available as Supplementary material). Seeds from hemizygous and homozygous TM lines produced normal leaves and roots when germinated on 42 μM imazapyr, whereas seeds of non-transformed plants could only grow normally on ≤ 0.42 μM imazapyr. The observed AHAS herbicide resistance as a result of the Ser<sub>653</sub> → Asn mutation was most clearly due to a single dominant nuclear gene, based on the segregation and the fact that there were no significant differences ( $P > 0.05$ ) in resistance to various herbicide doses between hemizygous and homozygous plants (Figure S2a, available as Supplementary material). This is not always the case: Vollenberg and Stoltenberg (2002) found semi-dominant inheritance of AHAS resistance in field-evolved imazethapyr-resistant populations of *Solanum ptycanthum* as a result of Ala → Thr mutation within domain C of the enzyme (Milliman *et al.*, 2003). The imidazolinone-resistant

(Trp<sub>552</sub> → Leu) mutant *Zea mays* (Bernasconi *et al.*, 1995) is also semi-dominant at certain imazapyr concentrations (Kanampiu *et al.*, 2002). Thus, the sensitive, hemizygous and homozygous TM *B. napus* progeny can be discriminated by Mendelian segregation by seed selection on imazapyr- or kanamycin-selective agar media, as described in 'Experimental procedures'.

### Transgenic mitigation prevents transgene establishment in volunteer offspring

The dwarf, herbicide-resistant, TM *B. napus*, used to simulate a transgenic volunteer crop, was planted in dense stands mixed with otherwise isogenic, tall, herbicide-sensitive, non-transgenic crop. Dense stands are expected from seed rain of shattering plants. These competitive interactions were assessed in internally replicated glasshouse and screen-house experiments.

### High productivity and low competitive fitness of TM *B. napus* plants cultivated at close spacing under glasshouse conditions

The productivity of TM and non-transgenic *B. napus* plants was determined by growing each genotype without competition, and the fitness was measured when they were cultivated together under competition at a spacing at which the roots and canopies overlapped. A single homozygous TM T<sub>2</sub> from line J9 that has an optimal agronomic phenotype was used.

### High productivity of TM *B. napus* cultivated without competition at close spacing

Homozygous, dwarf, imazapyr-resistant, TM T<sub>2</sub> line J9 plants formed more leaves than tall non-transgenic plants when grown alone at 2.5-cm spacing ( $P \leq 0.05$ ) and 10-cm spacing ( $P \leq 0.05$ ), measured after bolting (Figure S8, available as Supplementary material). TM homozygous plants also formed more leaves ( $P \leq 0.05$ ) and had higher fresh shoot biomass ( $P \leq 0.05$ ) than the mixed 2 : 1 hemizygous/homozygous, dwarf/semi-dwarf, imazapyr-resistant TM segregants when each genotype was grown alone without competition with the non-transgenics at 2.5- and 5-cm spacing (Figure S8, available as Supplementary material).

The TM plants cultivated alone at 2.5-, 5- and 10-cm spacing had an obvious 26–42-day delay of flower initiation and ripening (Figure 2b) compared with the non-transgenics grown at the same spacing. Despite the delay in flower initiation, the

homozygous TM plants were far more reproductive than the non-transgenic plants when both genotypes were grown separately at close spacing between the plants. The TM plants grown alone had approximately double the number of siliques ( $P \leq 0.05$ ), double the seed yield per plant ( $P \leq 0.05$ ) and double the yield per unit area ( $P \leq 0.05$ ; Figure 3c) at 2.5- and 10-cm spacing when compared with the tall non-transgenic plants (Table S4, available as Supplementary material). The TM plants had ~30% higher harvest index than the non-transgenic plants at these spacings ( $P \leq 0.05$ ; Table S4, available as Supplementary material). The mixed 2 : 1 hemizygous/homozygous TM segregants grown alone were less reproductive than the homozygous progeny (Figure 3; Table S4, available as Supplementary material), supporting the suggestion that the use of  $\Delta gai$  homozygous lines for agriculture would be advantageous, as this dwarfing gene more than doubled the crop yield under glasshouse conditions.

#### Low competitive fitness of TM *B. napus*

Strong competition between the plants at narrow spacing not only affects vegetative growth, but also has a large impact on bolting, flowering and the final outcome, as crowding reduces the photosynthetic ability throughout the plant life cycle. When the dwarf TM plants were cultivated alone, competing only with their TM siblings, they grew well and produced copious seed (see previous section). Conversely, the TM plants were poor competitors: (i) when in an equal mixture of seed from homozygous TM plants vs. non-transgenics from an otherwise isogenic background (Table 1; Figures 2 and 3c–e); and (ii) when the competing TM plants represented 75% of a segregating population (1 : 2 : 1 for homozygous : hemizygous : non-transgenic segregants; Table 1; Figure 3a,b). The competition experiments demonstrated that the semi-dwarf/dwarf TM plants were weak and

unfit when co-cultivated with the non-transgenic plants without using the herbicide.

The TM plants from both above groupings had reduced vegetative growth, measured as height ( $P \leq 0.01$ ), number of leaves ( $P \leq 0.01$ ) and shoot fresh biomass ( $P \leq 0.01$ ; Figure S8, available as Supplementary material). The TM plants developed more slowly than the non-transgenics (Figure 2). The relative vegetative fitness (based on the final dry shoot biomass) of the 2 : 1 hemizygous/homozygous TM segregants and the homozygous plants was about one-fifth of that of the non-transgenic plants (Table 1).

In a recent field study, genetically dwarf *B. napus* volunteers, grown at a wider spacing than used here, had a vegetative fitness of 17% at the flowering stage and only 7% at the silique stage relative to the tall plants (Fargue *et al.*, 2004). Thus, herbicide is required to curtail competition to obtain the yield advantage of a homozygous, herbicide-resistant, dwarf TM crop. Without the same herbicide, volunteers will disappear by competition in the rotational crop.

The survival of the 2 : 1 hemizygous/homozygous TM segregants grown in mixed cultures with 25% non-transgenics was more than 20% lower than that of the TM segregants grown alone at 2.5- and 5-cm spacing ( $P \leq 0.01$ ). At both spacings, ~28% of the TM segregants were dead after 116 days as a result of competition with the non-transgenic *B. napus* plants (Al-Ahmad, 2005).

The reproductive fitness of the TM plants was much lower than that of the non-transgenic competing plants. As a result of the high plasticity of oilseed rape, the non-transgenic plants mixed with unfit dwarf TM plants at 2.5- and 5-cm spacing were more reproductive than when grown alone. The short TM plants left more space, light and resources for the non-transgenics, elevating the yield of the non-transgenics. The surviving 2 : 1 hemizygous/homozygous TM segregants spaced at 2.5 cm completely failed to flower and, by the time the 25% of the population made up of tall non-transgenics

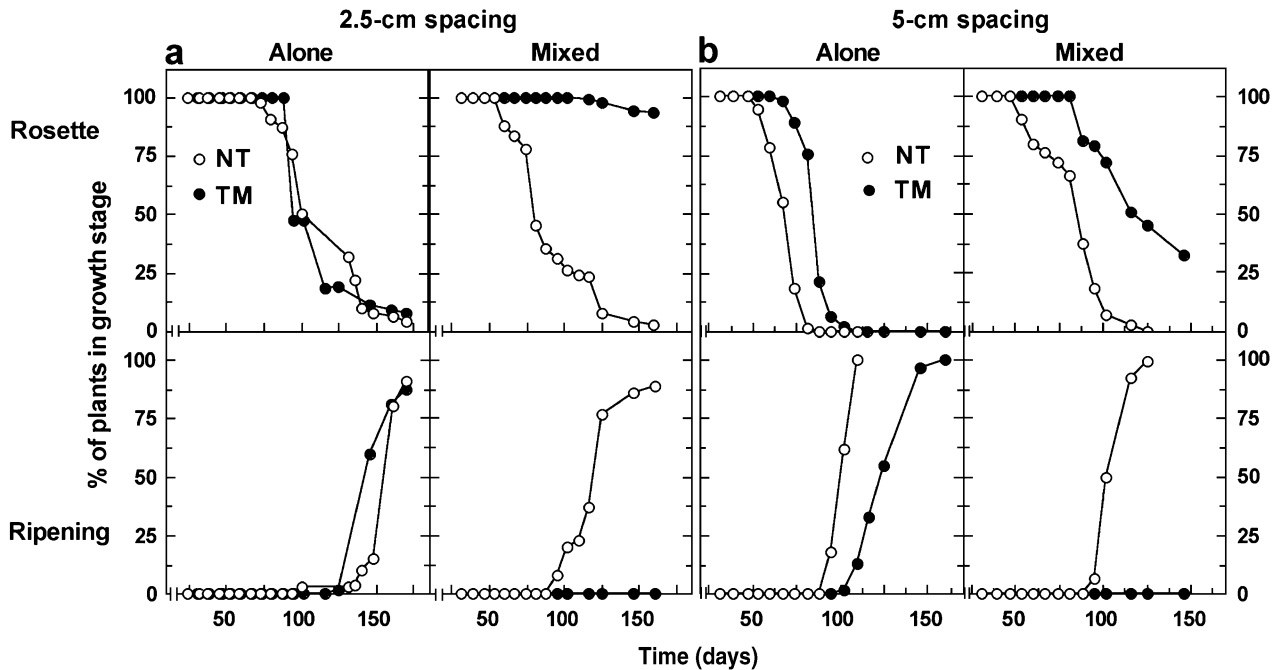
**Table 1** Very low relative fitness of transgenically mitigated (TM) *Brassica napus* families grown in competition with non-transgenic *B. napus*

Habitat	TM/NT ratio	Spacing (cm)	Shoot dry biomass	No. of seeds per plant	Weight of seeds per plant	Weight of seeds per unit area
Glasshouse*	Hemizygous TM segregants/NT	2.5	0.12	0	0	0
	Homozygous TM/NT	2.5	0.23	0	0	0
	Hemizygous TM segregants/NT	5	0.15	< 0.01	0.02	0.02
	Homozygous TM/NT	5	0.23	0.03	0.04	0.04
Screen-house†	Homozygous TM/NT	3	0.39	0.10	0.10	0.11

The data represent the TM transgenic/non-transgenic *B. napus* ratio. Data calculated from Tables S4 and S5 in the Supplementary material and Figures 3 and 5.

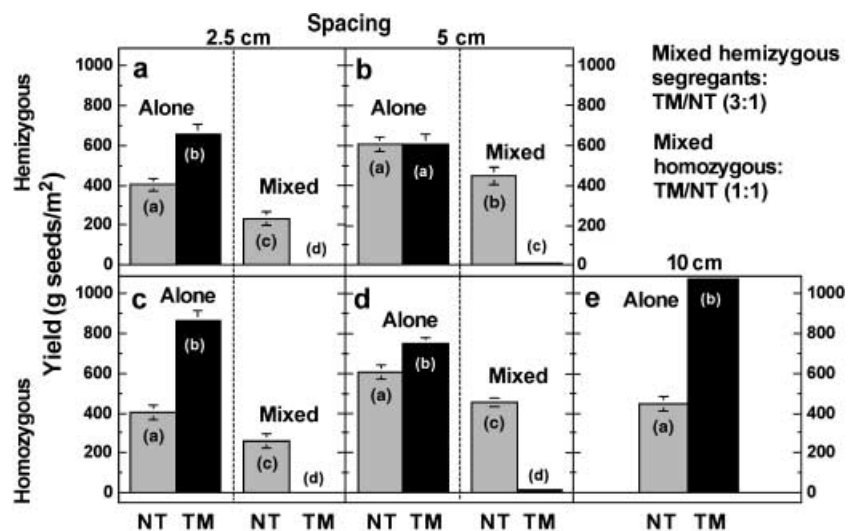
\*TM *B. napus* line J9 was used.

†TM *B. napus* line J7 was used.



**Figure 2** Suppression of growth of homozygous, dwarf, herbicide-resistant, transgenically mitigated (TM) *Brassica napus* plants (filled symbols) when competing with non-transgenic (NT) *B. napus* plants (open symbols). The plants were grown either alone or co-cultivated (mixed) at (a) 2.5-cm and (b) 5-cm spacing in large containers in the glasshouse without using the selective herbicide.

**Figure 3** Transgenically mitigated (TM) *Brassica napus* plants (black data bars) have high productivity when grown alone under self-competition, but are severely unfit when competing with the non-transgenic (NT) *B. napus* plants (grey data bars). The plants were grown at 2.5 and 5 cm from themselves or each other in the glasshouse without the herbicide. A spacing of 10 cm allowed the demonstration of oilseed rape plasticity, a situation that occurs when non-transgenic plants compete out unfit TM plants grown at 5-cm spacing. No error bars are shown when the standard errors (SEs) are too small to be seen. Different letters within a panel indicate significantly different values at  $P = 0.05$  (least significant difference test).



finished flowering and formed siliques and copious seed, 95% and 70% of the TM plants at 2.5- and 5-cm spacing, respectively, remained stunted at the rosette stage (stage 2) (Figure 2). Of the homozygous TM plants that survived, 93% and 45% of plants were still at the rosette stage at 2.5- and 5-cm spacing, respectively, when the non-transgenics had completed their life cycle (Figure 2). None of the hemizygous or homozygous TM plants competing with non-transgenic plants at 2.5-cm spacing produced any viable seed

( $P < 0.05$ ; Figure 3a,c). Their reproductive fitness relative to the non-transgenic competitors was zero at 2.5-cm spacing (Table 1). At 5-cm spacing, some TM plants growing at the edge of the containers developed some flowers, forming a few siliques and seeds after the harvested non-transgenic plants had been removed from the containers (Figure 3b,d). The reproductive fitness of the TM plants relative to the non-transgenics was never more than 4% (Table 1). Similarly, the reproductive fitness of the genetically dwarfed volunteer

*B. napus* relative to the tall crop competitors was about 47% at wider outdoor spacing (60–100 crop plants/m<sup>2</sup> and 1–5 volunteers/m<sup>2</sup>) (Fargue et al., 2004).

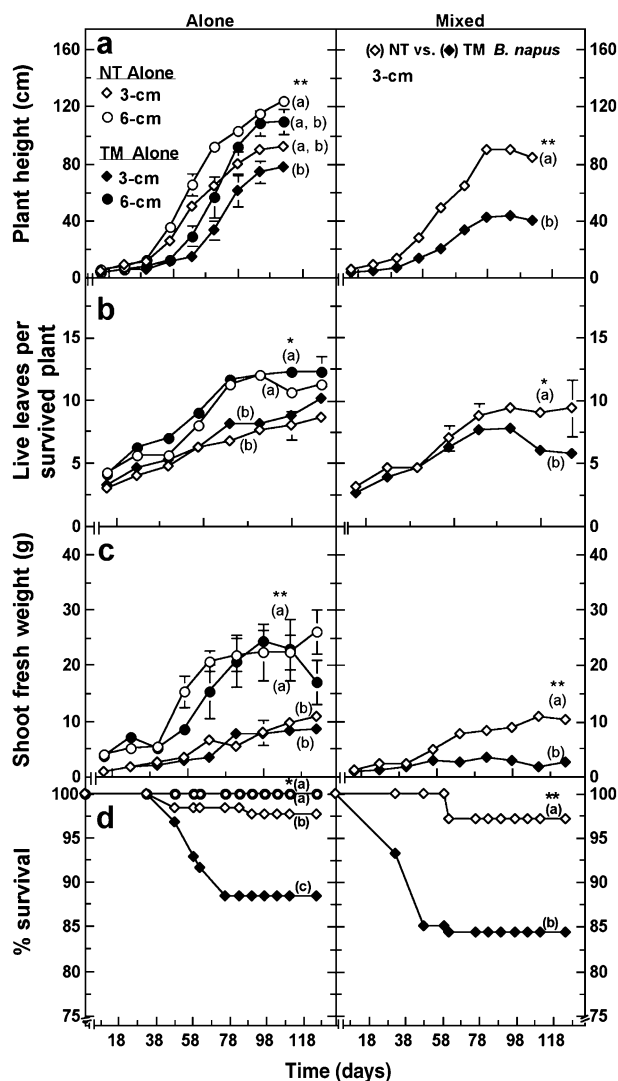
In a field situation, any volunteer transgenics that survived would probably be killed during harvesting of the following crop, before the TM plants could set seed. Even if the TM plants were to set seed, further generations of their progeny would still be non-competitive with wild-type cohorts, other weeds or crops in subsequent generations, except when the selective herbicide was used. In summary, the homozygous TM *B. napus* had a much higher productivity than the non-transgenics when grown alone under typical field spacing, but were weakly productive and hardly reproductive when grown in competition with tall plants at close spacing, and would represent little threat in any situation in which herbicides were rotated.

#### High productivity and low fitness of TM *B. napus* cultivated at close spacing in biocontainment screen-house conditions

The previous oilseed rape competition experiments were conducted under a controlled glasshouse environment, where temperature and humidity fluctuations during the growing season were limited. To further evaluate the efficacy of the transgenic mitigation concept in environments that mimic a real field habitat, biocontainment screen-house competition experiments were conducted between TM and non-transgenic *B. napus* genotypes. A single homozygous TM T<sub>2</sub> from line J7 that has an optimal agronomic phenotype was used. The plants were all cultivated at 3-cm spacing between the individuals. Monocultures of non-transgenic and TM *B. napus* were additionally cultivated at 6-cm spacing to assess their productivity under self-competition and to mimic a situation in which non-transgenic crop or weed plants cultivated at 3-cm spacing had competed out unfit TM plants, so that the newly available spacing between individual plants was doubled to about 6 cm (Figure 4).

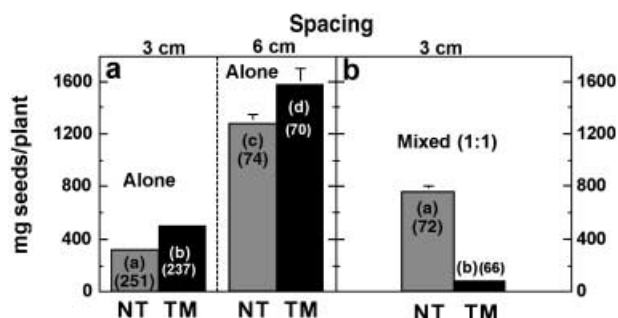
#### Higher productivity of TM *B. napus* cultivated without competition in the biocontainment screen-house

The dwarf, imazapyr-resistant, TM T<sub>2</sub> line J7 homozygous plants grown alone at 3- and 6-cm spacing accumulated 49% and 15% more dry shoot biomass at harvest, respectively, than the non-transgenic plants grown alone at similar spacing ( $P \leq 0.05$ ; Table S5, available as Supplementary material). Both TM and non-transgenic genotypes had > 99% pollen viability. The homozygous TM plants grown



**Figure 4** Reduced growth and poor survival of unfit homozygous transgenically mitigated (TM) *Brassica napus* competing with non-transgenic *B. napus* plants: (a) the height was measured from the base of the plant to the apex; (b) the number of live leaves formed per plant; (c) the above-ground fresh shoot biomass; and (d) the survival of plants measured during the cultivation period. Non-transgenic (open symbols) and TM dwarf/herbicide-resistant plants (filled symbols) were planted alone or co-cultivated at 3 and 6 cm from each other in the biocontainment screen-house without using herbicide. NT, non-transgenic. The points are the mean ( $\pm$  SE) of  $n = 4-10$ . No error bars are shown when the standard errors are smaller than the data points. Different letters within a panel indicate significantly different values at  $*P \leq 0.05$  or  $**P \leq 0.01$  (least significant difference test).

alone at 3- and 6-cm spacing formed 70% and 48% more seeds per plant ( $P \leq 0.05$ ; Table S5, available as Supplementary material), and 57% and 23% more seed mass per plant ( $P \leq 0.01$ ; Figure 5), respectively, than the non-transgenic plants grown without competition at similar spacing. The high plasticity of oilseed rape could account for the higher yield of plants grown at 6-cm than at 3-cm spacing (Figure 5).



**Figure 5** Homozygous transgenically mitigated (TM) *Brassica napus* plants have high productivity when grown alone under self-competition, but are severely unfit when in competition with the non-transgenic *B. napus* plants. The plants were grown at 3- and 6-cm spacing between each other in the screen-house without the herbicide. The bars are the mean ( $\pm$  SE) of  $n$  indicated in parentheses. No error bars are shown when the standard errors are too small to be seen. Different letters within a panel indicate significantly different values at  $P = 0.01$  (least significant difference test).

#### Low competitive fitness of TM transgenic *B. napus* cultivated in the biocontainment screen-house

The dwarf TM transgenic *B. napus* could hardly compete with the non-transgenic *B. napus* plants in the screen-house, when co-cultivated without using the herbicide, as observed in the glasshouse experiments. There was a strong growth suppression of the weak TM transgenic plants, which were shorter ( $P \leq 0.01$ ) and formed fewer leaves ( $P \leq 0.01$ ) with less shoot biomass ( $P \leq 0.01$ ) than the non-transgenic competitors (Figure 4).

The survival of the TM plants was 13% less than that of the non-transgenics after 2 months of growing together at 3-cm spacing ( $P \leq 0.05$ ; Figure 4d). The biomass produced by the TM plants was 40% of that of the non-transgenics (Table S5, available as Supplementary material). Despite the viable pollen, the female reproductive fitness of the TM plants was much lower than that of the competing non-transgenic plants (Table S5, available as Supplementary material). The seed set of the TM *B. napus* plants under competition was 11% the number ( $P \leq 0.05$ ), 10% the weight per plant ( $P \leq 0.05$ ; Figure 5) and 11% the weight per unit area of the competing non-transgenic plants. Thus, the TM reproductive fitness was no more than 11% of the non-transgenic *B. napus* (Figure 5b; Table 1). The harvest index of the TM plants was less than 40% of that of the non-transgenic competitors ( $P \leq 0.05$ ; Table S5, available as Supplementary material).

In conclusion, despite the slight yield drag, probably imposed by the herbicide resistance gene, the mitigating dwarfing gene enhanced the yield when the TM *B. napus* plants were cultivated without competition. The dwarfing gene fulfilled its mitigating function, as the TM progeny

were highly unfit when competing with tall non-transgenic *B. napus* plants. TM individuals may produce a few offspring under competition, but these are not a threat to crop production, as the linked unfitness would be continuously manifested in future generations, keeping the herbicide transgene at a low frequency, and lowering the potential risk of transgene flow and subsequent establishment in related species.

The effective use of TM technology would be favoured in areas in which crops and herbicides are rotated, so that the TM dwarf/herbicide-resistant volunteer weeds could not persist for many seasons. The volunteers possess a very low Haldanian fitness (Haldane, 1960) in the absence of the selective herbicide. Oilseed rape is typically followed by a 2-year rotation with wheat, so that TM volunteers should be competed away. Most volunteers and hybrid progeny would be removed by auxinic herbicides [2,4-dichlorophenoxyacetic acid (2,4-D), dicamba] used in wheat fields. The remaining TM volunteers would be far less fit to compete with taller wheat plants.

#### Experimental procedures

The methods of application of GA biosynthesis-suppressing PGRs to validate the concept that dwarfing should be usable with *B. napus* are described in the Supplementary material available online.

#### Plasmid construction

The pPZP212-*ahas*<sup>R</sup>- $\Delta$ *gai*-1 tandem construct (TM 1) includes the *Arabidopsis thaliana ahas*<sup>R</sup> mutant gene (Ser<sub>653</sub>  $\rightarrow$  Asn) (Sathasivan *et al.*, 1990) for herbicide resistance, as the primary gene of choice, in tandem with the dwarfing  $\Delta$ *gai* mutant gene [ $\Delta$ <sub>82-132</sub> open reading frame (ORF); Peng *et al.*, 1997] as a mitigator. Both genes were tightly linked to each other in the same orientation with a 15-base pair linker (Figure S3a,c, available as Supplementary material). The assembly and verification of the TM 1 construct are described in Al-Ahmad *et al.* (2004). The native pPZP212 binary vector (Hajdukiewicz *et al.*, 1994) also carried the *aadA* gene conferring bacterial resistance to spectinomycin (outside the T-DNA), and the *kan* gene encoding neomycin phosphotransferase II (NPTII) conferring plant resistance to kanamycin (within the T-DNA).

#### Transformation of the TM 1 construct into *B. napus*

*A. tumefaciens* strains EHA101 and EHA105 containing the TM 1 construct were used to transform excised cotyledonary

petioles of *B. napus* L. cv. Westar seedlings, based on the transformation protocol of Moloney *et al.* (1989). All verified transformants are designated as 'TM'. Non-transgenic *B. napus* cv. Westar and their progeny, used for this study, were regenerated from tissue culture, using the same protocol as employed to generate the TM transformants, but without using *Agrobacterium*.

### Molecular and enzymatic analyses

The putative TM transformants were analysed by PCR for the presence of intact tandemly linked *ahas*<sup>R</sup> and  $\Delta$ *gai* genomic inserts, as described in Al-Ahmad *et al.* (2004).

Southern hybridization analysis (Sambrook *et al.*, 1989) was used to test the stability and to ascertain the number of copies of the integrated *ahas*<sup>R</sup> and  $\Delta$ *gai* tandem genes in the primary TM *B. napus* transformants. Leaf genomic DNA was isolated by the Puregene<sup>®</sup> kit for genomic DNA purification (Gentra Systems, Minneapolis, MN, USA), digested overnight with *Xba*I, and then fractionated on agarose gels and transferred onto membranes according to Sambrook *et al.* (1989). The ~ 1-kb probe used in the hybridization was amplified by PCR with primers designed to the *ahas*<sup>R</sup>-linker- $\Delta$ *gai* tandem region (Figure S3c, available as Supplementary material), and labelled with  $\alpha$ -<sup>32</sup>P-dCTP by the random primer method (Feinberg and Vogelstein, 1984).

The rapid leaf disc AHAS enzyme assay test, pioneered by Shaner (2002) and described in Al-Ahmad *et al.* (2004), was used to further confirm the integration and expression of the TM construct in the TM plants with a discriminatory dose of 5  $\mu$ M imazapyr.

### Inheritance of the TM construct

#### *Seed germination in the presence of kanamycin or imazapyr*

A discriminatory dose of 260  $\mu$ M kanamycin or 0.5  $\mu$ M imazapyr, inhibiting the growth of non-transformed plants, was determined in Nitsch (1969) agar medium. Surface sterilized seeds of TM and non-transgenic *B. napus* were planted on medium supplemented with either selector. Sensitive seedlings stopped growing within 10 days and formed only short roots and cotyledons, whereas resistant seedlings formed long roots and true leaves and continued normal growth. The segregation ratio of TM to non-transgenic seedlings of each line was then calculated. Resistant/dwarfed seedlings were grown to maturity in soil and the plants were analysed for resistant AHAS by enzymatic assay and for the presence of the *ahas*<sup>R</sup> and  $\Delta$ *gai* inserts by PCR.

#### *Zygoty determination*

Individual TM T<sub>1</sub> plants were grown in soil under glasshouse conditions and were self-pollinated to distinguish between the segregating hemizygous families and the non-segregating homozygous families. Seeds were separately collected from each plant at full maturity. The T<sub>0</sub> primary plants are expected to be hemizygous for the transgenes, and thus T<sub>1</sub> plants with a single gene copy should have a Mendelian ratio of 1 : 2 : 1 or 3 : 1, depending on the type of dominance. The T<sub>2</sub> families should have 25% non-segregating homozygous resistant families. The zygoty was determined by germinating at least 20 seeds from each selfed T<sub>1</sub> parent, and the T<sub>2</sub> progeny were screened for imazapyr or kanamycin resistance on selective agar medium, as described above. The T<sub>1</sub> plants were classified as homozygous (non-segregating families) if all their T<sub>2</sub> progeny were imazapyr and/or kanamycin resistant, and hemizygous (segregating families) if approximately 25% imazapyr- and/or kanamycin-sensitive seedlings were found. Kanamycin-resistant families were all imazapyr resistant, as expected.

#### **Characterization and productivity of the TM *B. napus* transformants grown at wide spacing without competition**

TM and non-transgenic *B. napus* plants were each grown separately in 13-cm diameter, 1-L pots filled with soil, with considerable spacing between pots such that the plants were not competing with each other. The plants were grown under glasshouse conditions, and were enzymatically assayed for AHAS resistance, as above. A number of non-transgenic plants were sprayed with 0.2 mg a.i. (active ingredient) paclobutrazol per plant for comparison as chemically dwarfed controls.

Phenotypic characteristics were measured weekly. The main stem thickness was measured 5 cm above the soil at maturity, and the internode length was measured from the first basal leaf towards the first apical branch carrying flowers and siliques. Five randomly chosen plants were harvested at each growth stage (Figure S4, available as Supplementary material) to obtain fresh weights.

The season-long seed production was quantified to compare the performance of the indeterminate TM and non-transgenic *B. napus* plants. When the plants stopped flowering, the number of siliques (containing at least one fully developed seed) on each plant was counted. Ten mature, undehisced siliques were randomly selected from two to three branches per plant for seed counts. The average number of fully developed seeds per silique and the number

of seeds per plant (number of siliques times the average number of seeds per silique) were then calculated. The harvested seeds were sorted and counted as fully developed, prematurely germinated within siliques (oviparous) or aborted (small and shrunken). The mature plants were cut at the soil surface, dried and the shoot biomass was measured for each plant. The weight of cleaned, fully developed seeds per plant was then measured. Finally, the harvest index (the ratio of seed mass per plant/total plant shoot biomass) was calculated.

### Chlorophyll measurement

Chlorophyll was extracted in 80% acetone (Porra *et al.*, 1989) from ten 8-mm-diameter leaf discs from four leaves per plant. Chlorophyll *a* and *b* were determined from the absorbance at 470, 646.6 and 663.6 nm using the equations in Porra *et al.* (1989), and total carotenoids using the equations in Hill *et al.* (1985).

### Pollen viability test

Young anthers were collected from three or more flowers per plant, at least three times, from a minimum of three plants per genotype. Pollen was stained with cotton blue (Phillips, 1981), and the proportion of viable, deeply stained grains having normal morphology vs. non-viable, abnormal, shrunken and non-stained grains was measured.

### Exogenous GA application

Homozygous TM T<sub>2</sub> line J9 and non-transgenic *B. napus* plants were sprayed once a week with 0.15 mM GA<sub>3</sub> (Sigma, St Louis, MO) in 0.1% (v/v) Tween-20, beginning 26 days after planting in soil (early stage 2 = 2–3 true leaves per plant). Control plants were sprayed with an aqueous solution containing Tween-20 and 0.1% (v/v) methanol (the solvent used to dissolve GA<sub>3</sub>).

### Competitive fitness of TM vs. non-transgenic *B. napus* plants and the productivity of each alone with narrow spacing under glasshouse conditions

The competitive interactions between TM and non-transgenic *B. napus* plants were assessed in replicated glasshouse experiments. In the first experiment, seeds obtained from the selfed homozygous TM T<sub>1</sub> line J9 were used to grow the TM T<sub>2</sub> plants in mixed cultures with the non-transgenic plants at a 1 : 1 ratio. All the T<sub>2</sub> progeny were TM, as confirmed by selecting a few seeds on 0.5 μM imazapyr,

as described above. In the second experiment, we used the T<sub>2</sub> progeny of the selfed hemizygous TM line J9, which segregated at a 1 : 2 : 1 ratio of TM homozygous : TM hemizygous : non-transgenic. Both experiments were conducted in 55 × 41 × 22-cm plastic containers filled with a mixture of peat, crushed tuff rock and loam (1 : 1 : 1). Each container was divided to give four microplots. The TM vs. non-transgenic *B. napus* seeds were hand-sown in soil at 2.5- and 5-cm spacing (about 360 and 90 seeds/container, respectively), without using the selective herbicide. Monoculture controls of non-transgenic alone and TM alone were also grown at the same spacing, and at 10-cm spacing (about 30 seeds/container). Plants were grown under ambient glasshouse light (988 ± 53 μE/m<sup>2</sup>/s) at 25.5 ± 1.9 °C. The light intensities are the averages (± SE) of noontime measurements made on 10 occasions during the growing season. The young plants were assayed for resistant AHAS enzyme activity by the rapid leaf disc test. The non-transgenic segregants within the hemizygous monoculture controls were replaced by TM segregants grown as spares.

The performance and productivity of surviving TM transgenic vs. non-transgenic *B. napus* plants grown separately were quantified by measuring various growth parameters in a randomly chosen microplot of each genotype in each container and at each time interval. A destructive measurement procedure was followed in these competition experiments. Thus, each week, 5–18 plants were cut at the soil surface and the vegetative productivity was determined by measuring the plant height, number of live leaves, shoot fresh weight and the developmental growth stage. Plants grown at the periphery of the containers (less than 5 cm from the container edges) were excluded from the measurements. The number of surviving plants of each genotype was recorded at maturity. The lifetime seed production of each plant was quantified as described above for the productivity of *B. napus* without self-competition. A 25 × 25-cm cultivated area was chosen in the middle of each container to exclude edge effects.

The competitive vegetative fitness (based on shoot dry biomass) and the competitive reproductive fitness (based on the number of seeds formed per plant, weight of seeds per plant and weight of total seed output/genotype/unit area) of the TM vs. non-transgenic plants were calculated at time intervals as a TM/non-transgenic ratio. The seed size was determined by measuring the weight of six random samples of 100 seeds each. The below-ground competition was not measured in our experiments as a result of the extremely intertwined root systems at all spacing, but it was assumed that below-ground competition would manifest itself in above-ground growth parameters, and vice versa.

### Competition of TM vs. non-transgenic *B. napus* and self-competition of each alone under biocontainment screen-house conditions

The competition between homozygous TM T<sub>2</sub> line J7 and non-transgenic *B. napus* plants was independently evaluated in an internally replicated experiment in the screen-house mandated by biosafety authorities. Seeds were hand-planted in each microplot in winter without using the selective herbicide. Microplots of a single container were seeded either wholly as one genotype at 3- or 6-cm spacing (for genotype monoculture productivity) or with mixed seeds (1 : 1 ratio) at 3-cm spacing for mixed-culture competitive fitness. Germination and growth were followed at all stages, and rain was supplemented by irrigation when needed, until plant maturity. A destructive measurement procedure and the determination of growth parameters were as described above for the glasshouse experiments. The male fertility was determined as the percentage pollen viability. The productivity (when grown alone) and fitness (when in competition) of each genotype were determined per plant and per unit area. Entire above-ground portions of the ready-to-harvest mature plants were harvested, bagged, dried and then separated into vegetative and reproductive (seed) components and weighed. The harvest index and relative TM/non-transgenic vegetative and reproductive fitness were calculated as described above for the glasshouse competition trials.

### Statistical analyses

Tandem trait segregation data were statistically analysed by the chi-squared ( $\chi^2$ ) test. The plant growth parameters, productivity and fitness data were analysed using the JMP® program (version 4.0.1; SAS Institute 2000, Cary, NC) by one-way analysis of variance (ANOVA) and by comparing the least significant differences (LSD). Probability levels were considered to be statistically significant at  $P \leq 0.05$  and highly significant at  $P \leq 0.01$ . Differences were not considered to be statistically significant at  $P > 0.05$ .

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## Supplementary material

The authors have supplied additional data which are available online. These include a supplementary results section [GA biosynthesis-suppressing plant growth regulators (PGRs) reduce the height and improve the harvest index of *B. napus*] and a supplementary experimental procedures section (PGR validation that dwarfing should be usable with *B. napus*).

**Figure S1** Paclobutrazol reduced the height and improved the harvest index in non-transgenic *Brassica napus*. The plants were sprayed at the two true-leaf stage (3 weeks) and then twice more at biweekly intervals. The harvest index of a plant equals the ratio of seed output to total plant dry biomass. No error bars are shown when the standard errors are smaller than the data points. Different letters within a panel indicate significantly different values at  $P \leq 0.01$  (least significant difference test).

**Figure S2** Transgenically mitigated (TM) *Brassica napus* transformants are herbicide resistant (a, b) and dwarf (c, d). (a) AHAS (acetohydroxy acid synthase) enzyme activity was assayed spectrophotometrically in leaf discs as described in 'Experimental procedures', and is presented as the percentage activity of controls tested in the absence of imazapyr. The individuals assayed were non-transgenic (○), preselected TM segregating transgenics (as 1 TM homozygous to 2 TM hemizygous) of lines T<sub>1</sub> J7 (□), T<sub>1</sub> J9 (■) and T<sub>1</sub> J12 (●), and preselected TM homozygous plants of line T<sub>2</sub> J9 (△). The TM line represents the mean of the four independent transgenic events. The enzyme activities of the non-transgenic and TM controls were 0.058 and 0.077  $\mu\text{mol acetolactate}/\text{cm}^2$  leaf discs/h, respectively. One square centimetre of untreated leaf discs contains  $88.5 \pm 2.5$   $\mu\text{g}$  soluble proteins. (b) Appearance of representative susceptible non-transgenic vs. resistant homozygous T<sub>2</sub> line J9 seedlings at 12 days, picked from agar medium containing 0.5  $\mu\text{M}$  imazapyr. (c, d) Dwarfism induced by  $\Delta\text{gai}$  in TM transgenic *B. napus*. Non-transgenic control, 0.2 mg a.i.

paclobutrazol-treated (as a chemically dwarfed) control and a TM T<sub>1</sub> line J9 plant are shown before (c) and after (d) bolting. The inset in (c) shows the more compact internode length of the TM plants after removing the leaves.

**Figure S3** Detection of the transformed transgenically mitigated (TM) genes in primary transgenic *Brassica napus* by polymerase chain reaction (PCR) amplification (a and b) and Southern hybridization (c–f). (a) Circular map of the plasmid pZP212-*ahas*<sup>R</sup>- $\Delta\text{gai}$ -1 (TM 1 construct) used for transformation. Arrows indicate gene orientation; A, B, C and D on the map denote the sites chosen for PCR amplification used to ascertain transformation with intact TM T-DNA (modified from Al-Ahmad et al., 2004). (b) Electrophoresis of 15  $\mu\text{L}$  of PCR product through a 1% (w/v) agarose gel at 50 V for 45 min; M, molecular marker (1-kb DNA ladder); NT, non-transgenic; (–), control without template DNA; P, TM 1 plasmid control; numbers below lanes, e.g. 7, 9 and 12, of (b) denote representative independent TM *Brassica* lines; A–D denote the sites chosen for PCR amplification as shown in (a). (c–f) Southern hybridization confirmed the transformation of intact TM tandem genes into the primary transgenic *B. napus*. Total genomic DNA was isolated from the putative T<sub>0</sub> *B. napus* transformants, digested with *Xba*I, fractionated on a 0.8% agarose gel, transferred to nitrocellulose filter and hybridized to an ~1-kb probe designated by the bar shown in (c) over the 3' terminal region of *ahas*<sup>R</sup>, the linker and the 5' region of  $\Delta\text{gai}$ . (d) The probe was amplified by PCR. (e) A hybridized signal of the TM 1 plasmid shown in (a) used as template. (f) Single gene copies of the tandem insert in transgenic *B. napus* lines J7, J8, J9 and J12. No signals in non-transgenic control (NT) and in the non-transgenic escapees of lines J10 and J11. The 5712-bp labelled fragment equals the TM *Xba*I-*ahas*<sup>R</sup> fragment shown in (c), and the > 7610-bp fragment includes the  $\Delta\text{gai}$  insert and part of *B. napus* genomic DNA.

**Figure S4** Vegetative and reproductive developmental growth stages of *Brassica* (copied by permission from Harper and Berkenkamp, 1975). They used a decimal system to describe the intermediate growth stages as follows: pre-emergence (stage 0); seedling (stage 1); rosette (2.1 first, 2.2 second, etc. true leaves expanded; this system has a limitation as the number of leaves cannot exceed nine, regardless of the type of plant); bud (3.1 flower cluster visible at centre; 3.2 above level rosette; 3.3 lower buds yellowing); flower (4.1 first flower open; 4.2 many flowers open; 4.3 lower siliques start to fill; 4.4 complete flowering); ripening (5.1 lower siliques with full seeds; 5.2 lower siliques with green seeds; 5.3 lower siliques with green–brown seeds; 5.4 lower siliques with brown seeds; 5.5 plant senescent).

**Figure S5** Homozygous transgenically mitigated (TM) *Brassica napus* plants had shorter and thicker leaves with more chlorophyll content per unit area. No error bars are shown when the standard errors are too small to be seen. Different letters within a panel indicate significantly different values at  $P \leq 0.05$  (least significant difference test).

**Figure S6** Enhancement of root and shoot growth of transgenically mitigated (TM) *Brassica napus* plants after bolting. The shoot and root productivity of the homozygous TM T<sub>2</sub> line J9 vs. non-transgenic *B. napus* at different growth stages of development (Figure S4) is shown ( $n = 5$  plants per measurement). No error bars are shown when the standard errors are smaller than the data points. Different letters within a panel indicate significantly different values at  $P \leq 0.05$  (least significant difference test).

**Figure S7** Dwarf transgenically mitigated (TM) *Brassica napus* fail to respond to exogenous GA<sub>3</sub> (gibberellin) application, whereas GA treatment hastens bolting (a) and flowering (c) of the non-transgenics. Non-transgenic and homozygous TM T<sub>2</sub> line J9 plants were sprayed once a week with an aqueous solution of 0.15  $\mu\text{M}$  GA<sub>3</sub> during the rosette stage of growth ( $n = 10$ ). The growth stage of each of 10 plants was recorded as a decimal considering intermediates (e.g. growth stage of 2.4 = rosette with four leaves; Figure S4), and the average number of each measurement datum is given as a point. The plant branches were measured once at maturity. No error bars are shown when the

standard errors are smaller than the data points; significant points at  $P \leq 0.05$  (\*) are shown for areas delineated within a panel; ns, not significant. Similar letters within the lower panel indicate a non-significant difference ( $P > 0.05$ ) between the values (least significant difference test).

**Figure S8** Vegetative growth suppression of transgenically mitigated (TM) *Brassica napus* (simulating unfit volunteer weeds) when in competition with non-transgenic *B. napus*. Non-transgenic (open symbols) and TM dwarf/herbicide-resistant plants (filled symbols) were planted alone or co-cultivated at 2.5, 5 and 10 cm from each other in the glasshouse without using herbicide. The points are the mean ( $\pm$  SE) of  $n = 4$ –17. No error bars are shown when the standard errors are smaller than the data points.

**Table S1** Chemically induced dwarfism in non-transgenic *Brassica napus* treated with paclobutrazol

**Table S2** Vegetative and reproductive productivity of transgenically mitigated (TM) *Brassica napus* grown separately in 1-L pots in the glasshouse at wide spacing between the plants without competition

**Table S3** Co-segregation of imazapyr and kanamycin resistance in selfed transgenically mitigated (TM) *Brassica napus* transformants

**Table S4** Reproductive productivity and fitness of *Brassica napus* under glasshouse conditions

**Table S5** Productivity and fitness of homozygous transgenically mitigated (TM) *Brassica napus* grown under screen-house conditions