Transgene expression and fitness of hybrids between GM oilseed rape and *Brassica rapa*

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Oilseed rape (*Brassica napus*) is sexually compatible with its wild and weedy relative *B. rapa*, and introgression of genes from *B. napus* has been found to occur over a few generations. We simulated the early stages of transgene escape by producing F_1 hybrids and the first backcross generation between two lines of transgenic *B. napus* and two populations of weedy *B. rapa*. Transgene expression and the fitness of the hybrids were examined under different environmental conditions. Expression of the transgenes was analyzed at the mRNA level by quantitative PCR and found to be stable in the hybrids, regardless of the genetic background and the environment, and equal to the level of transcription in the parental *B. napus* lines. Vigor of the hybrids was measured as the photosynthetic capability; pollen viability and seed set per silique. Photosynthetic capability of first generation hybrids was found to be at the same level, or higher, than that of the parental species, whereas the reproductive fitness was significantly lower. The first backcross generation had a significantly lower photosynthetic capability and reproductive fitness compared to the parental species. This is the first study that examines transgene expression at the mRNA level in transgenic hybrids of *B. napus* of different genetic background exposed to different environmental conditions. The data presented clarify important details of the overall risk assessment of growing transgenic oilseed rape.

Keywords: real time PCR / Fv/Fm / F_0 / heterosis / biosafety

INTRODUCTION

The intensified development and regulation of transgenic crops (James, 2004) calls for risk assessment studies to evaluate the consequences of intraspecific and interspecific transgene flow. Genetic engineering enables introduction into ecosystems of genes that confer fitness-related traits. If transgenes spread to wild populations, they have the potential to create or exacerbate weed problems by providing novel traits that allow these plants to compete better, produce more seeds, and become more abundant (Snow, 2002). Information about vigor of transgenic crop-wild hybrids in their natural environment is therefore needed to access the environmental and economic impact of the transgenic crops. It is also important that spontaneous hybrids and introgressed plants can be reliably identified, and that transgene expression in the environment is known. Therefore, we analyzed this in the *Brassica* crop-weed complex.

Oilseed rape (*Brassica napus*) is grown for oil production and as feed. In 2004, 19% of all *B. napus* grown worldwide was transgenic (James, 2004). One characteristic of oilseed rape is the large dispersal of seed and pollen. For example, a high percentage (5–10%) of the seeds are shed prior or during harvest (Gulden et al., 2003; Price et al., 1996). The small seeds are easily dispersed by wind and machinery, and feral populations of *B. napus* occur frequently along roadsides (Chèvre et al., 2004). Pollen of *B. napus* is dispersed by wind and insects, and has been found up to 3–4 km away from the pollen source (Rieger et al., 2002, Thompson et al., 1999). In addition, *B. napus* can hybridize with at least eight related species, *B. rapa* being the most likely hybridization partner. A high degree of spontaneous hybridization between oilseed rape and the weedy and wild relative *B. rapa* can occur under field conditions (Jørgensen and Andersen, 1994), and

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there is a well documented possibility of gene exchange between these species (Hansen et al., 2001; Hansen et al., 2003). We used two transgenic *B. napus* lines to produce the first hybrid generation (F_1) and the first backcross generation (BC_1) with two populations of *B. rapa*. The F_1 and BC_1 plants were grown in several environments, and the potential environmental effects were measured as 1) level of transgene expression, which is assumed to be without costs to plant fitness, 2) seed set as a measure of plant fitness and 3) photosynthetic capability as a physiological estimate of plant fitness.

Identification of transgenic plants at the protein level is quite common; however, transgene expression is not always stable as it can be influenced by environmental conditions such as temperature fluctuations (Down et al., 2001) and periods of heat stress (Broer, 1996; Meyer et al., 1992; Neumann et al., 1997). The environmental effect on the expression of the two transgenes, *barnase* and *barstar*, in *B. napus* × *B. rapa* hybrids was analyzed using quantitative PCR. The *barnase* gene confrers male sterility by preventing pollen production, and the *barstar* gene restores the fertility of *barnase* plants by inhibiting the *barnase*-encoded ribonuclease. The male sterile and fertility restorer lines represent a hybrid system enabling the production of vigorous hybrid seeds (DEFRA 1999; Mariani et al, 1992). Also, the reproductive fitness of the interspecific hybrids was measured. As a potential tool to quantify hybrid vigor, the photochemical capacity of Photosystem II (PSII) was analyzed. A lower photosynthetic rate, in terms of chlorophyll *a* fluorescence, has been observed to have a negative impact on fitness (Arntz et al., 1998; Arntz et al., 2000). Therefore, chlorophyll fluorescence can be considered a fitness-related parameter. Fluorescence is much simpler to measure than biomass; hence, if the photosynthetic capability reflects the plant vigor, the method would provide a fast alternative in fitness analyses.

Our results clarify the impact of environment and genotype on fitness-related parameters, and we discuss the consequences of our results in respect to the risks of transgene flow from *B. napus* to populations of *B. rapa*.

RESULTS

Expression of the transgenes

The *barnase* and *barstar* genes were transcribed at the same level. Figure 1 shows the expression in *barstar* plants, and the *barnase* plants produced corresponding results (data not shown). No significant differences, in
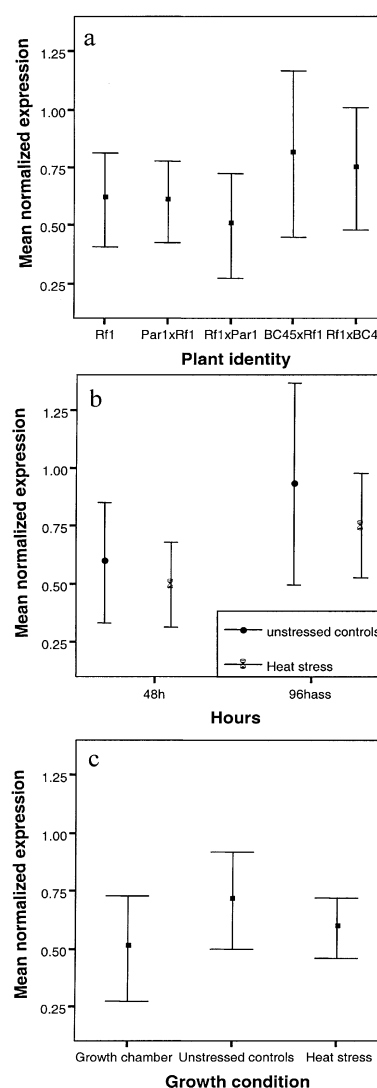
Figure 1. Transgene expression (*barstar*) in F_1 hybrids between *Brassica napus* and *B. rapa*. Error bar diagrams of mean normalized expression of the transgenes showing the 95% confidence intervals of: (A) plants of different identity where Rf1 is the transgenic parent, Par1 × Rf1 and Rf1 × Par1 are the reciprocal F_1 hybrids with the Par1 population of *B. rapa*, BC45 × Rf1 and Rf1 × BC45 are the reciprocal F_1 hybrids with the BC45 population of *B. rapa*. (B) heat-stressed plants and unstressed controls of all genotypes 48h after heat-stress and 96h after stress stop (96hass). (C) plants of all genotypes grown in growth chamber, as unstressed controls and under heat-stress, respectively.

mean normalized expression, were found between the generations (Tukey tests, $p > 0.702$), as the F_1 hybrids showed the same expression level as the transgenic parent (Fig. 1A). Hybrids from reciprocal crosses showed similar

expression (Fig. 1A). Also, the F_1 hybrids made with different *B. rapa* populations showed the same expression (Fig. 1A). Therefore, for the following analysis, the mean normalized expressions were pooled irrespective of the plant genotype.

The transgene expression during heat stress, as opposed to after heat stress, was found to be similar in all genotypes (t-test, $p = 0.072$) (Fig. 1B). There was no significant difference between the heat-stressed plants and the unstressed control plants (Fig. 1B). The expression in all three environments was compared, and showed that all genotypes had the same level of transgene expression irrespective of growth conditions (Tukey tests, $p > 0.764$) (Fig. 1C).

Impact of environmental conditions on plant vigor

Table 1 shows the mean values and standard deviations of pollen viability and photosynthetic capability for the various genotypes in the different environments. The BC_1 hybrids were only grown in the field; hence the environmental effects were not examined for this generation. For most of the analyzed genotypes, the percentage of viable pollen was lowest in the field (pooled F_1 mean: 38.4%) compared to plants grown under heat stress (pooled F_1 mean: 48.3%), unstressed controls (pooled F_1 mean: 46.8%), and in the growth chamber (pooled F_1 mean: 47.6%).

In most genotypes, the ratio of variable to maximum fluorescence (Fv/Fm) was significantly lowest in plants grown in the field, and significantly highest in the unstressed plants. Compared to the other environments, plants grown in the field showed large variances, as evident from the standard deviations.

Fv/Fm and the basal fluorescence (F_0), respectively, measured at different time-points during the stay in the conviron cabinets, were similar in hybrids and parental plants. Means based on data from all F_1 hybrid genotypes are shown in Figure 2. The heat-stressed plants (Fig. 2A), responded to the heat stress condition by a dramatic decrease in Fm/Fv. After heat stress was ended (96h after stress stop – hass), Fm/Fv rose to a pre-stress level (0h). F_0 also decreased during heat stress, and returned to a slightly higher level than the pre-stress level (0h) after stress stop (96hass) (Fig. 2C). The controls responded to the shift in environment by a continuous decrease in Fm/Fv that did not change significantly upon return to the growth chamber (96hass) (Fig. 2B). F_0 increased, and kept rising after return to the growth chamber (Fig. 2D).

Impact of genetic background on plant vigor

Table 1 shows the mean values and standard deviations of pollen viability, seed set, and photosynthetic capability, for the various genotypes of the different generations.

Impact of transgenes: impact could be studied for those crosses that segregated in transgenic and non-transgenic offspring. Among the male-fertile Non-Transgenic (NT-NMS1) plants and the transgenic male sterile sister plants (NMS1) of all generations, no differences were found in any of the analyzed fitness parameters. The same result was obtained from the transgenic and non-transgenic BC_1 hybrids from the Rf1 crosses.

Impact of generation: significant generation differences were found in all analyzed fitness parameters. Parental plants had on average 94% viable pollen, followed by the BC_1 hybrids with a mean of 53%, whereas the F_1 hybrids had a mean of less than 46% viable pollen. Parental plants also had significantly more seeds per silique than both hybrids generations, whereas the two hybrid generations produced a similar number of seeds per silique. In the field, the F_1 hybrids had the significantly highest photosynthetic capability. In the other environments, some F_1 genotypes showed higher fluorescence than the parents, whereas other genotypes showed fluorescence levels similar to or intermediary to the parents. The BC_1 generation had the significantly lowest chlorophyll fluorescence.

Impact of population and cytoplasm: no differences between the two populations of *B. rapa* were found in their pollen viability or in their number of seeds per silique. The chlorophyll fluorescence measurements showed that parental Par1 plants had a slightly higher photosynthetic capability than BC45 plants. In the hybrid offspring, fluorescence differed between the environments, but no clear trend was found in respect to populations.

In F_1 hybrids from reciprocal crosses, the pollen viability and the number of seeds per silique did not vary. The chlorophyll fluorescence varied depending on the growth condition, with no clear pattern of consistently higher fluorescence in any particular cytoplasmic background. In the BC_1 hybrid generation, no significant responses were found in any of the fitness parameters, irrespective of the populations used as parentals or the cytoplasmic background.

DISCUSSION

Transgene expression

Heat stressing temperatures of 35 °C – 37 °C have been found to inactivate or reduce transgene expression either

Table 1. Means ± standard deviations of the pollen viability (%), the number of seeds per silique and the ratio of variable to maximum fluorescence (Fv/Fm) in each parental, F_1 and BC_1 genotype grown at the respective environmental conditions. Numbers in brackets are the number of plants analyzed (the number of plants analyzed for the number of seeds per silique is similar to the number of plants measured for Fv/Fm in the field). ND – not determined. Genotypes are listed presenting the generation (P: parental; F_1: first hybrid generation; BC_1: first backcross hybrid generation). The hybrids are named with the female parent prior to the male parent. *B. rapa* populations: Par1 and BC45; *B. napus* lines: Rf1, NMS1 and NT-NMS1 (NT: non-transgenic).

Genotype	Field			Growth chamber		Heat stress		Unstressed controls	
	Pollen viability	Seeds / silique	Fv/Fm	Pollen viability	Fv/Fm	Pollen viability	Fv/Fm	Pollen viability	Fv/Fm
P Par1	92.9 ± 2.4 (18)	16.6 ± 1.1	0.811 ± 0.011 (19)	84.4 ± 5.5 (14)	0.826 ± 0.004 (10)	88.0 ± 2.9 (10)	0.841 ± 0.001 (59)	97.6 ± 0.7 (11)	0.846 ± 0.000 (59)
P BC45	ND	15.1 ± 1.0	0.784 ± 0.013 (20)	95.9 ± 1.3 (17)	0.795 ± 0.006 (12)	86.4 ± 3.1 (10)	0.826 ± 0.001 (55)	91.5 ± 3.5 (11)	0.826 ± 0.001 (55)
P Rf1	92.0 ± 2.7 (20)	25.1 ± 1.2	0.784 ± 0.011 (21)	97.4 ± 1.8 (14)	0.792 ± 0.006 (11)	97.6 ± 1.0 (11)	0.822 ± 0.001 (59)	98.9 ± 0.9 (11)	0.825 ± 0.001 (60)
P NMS1	0 (7)	23.8 ± 1.2	0.803 ± 0.013 (7)	0 (8)	0.818 ± 0.002 (7)	0 (6)	0.817 ± 0.003 (25)	0 (5)	0.819 ± 0.004 (25)
P NT-NMS1	96.0 ± 2.3 (3)	25.2 ± 1.9	0.784 ± 0.018 (11)	98.9 ± 0.2 (15)	0.815 ± 0.003 (14)	96.7 ± 1.3 (7)	0.816 ± 0.003 (18)	97.8 ± 0.9 (8)	0.823 ± 0.003 (15)
F_1 Par1 × Rf1	33.4 ± 3.7 (18)	3.3 ± 0.4	0.811 ± 0.010 (17)	48.4 ± 2.5 (16)	0.800 ± 0.003 (16)	ND	0.834 ± 0.001 (58)	ND	0.834 ± 0.001 (59)
F_1 BC45 × Rf1	ND	4.6 ± 0.5	0.817 ± 0.009 (16)	51.3 ± 2.7 (17)	0.813 ± 0.002 (21)	59.0 ± 4.9 (12)	0.827 ± 0.003 (59)	55.3 ± 2.7 (12)	0.818 ± 0.003 (59)
F_1 Rf1 × Par1	34.0 ± 9.5 (3)	2.7 ± 0.4	0.818 ± 0.009 (18)	46.8 ± 3.1 (17)	0.822 ± 0.003 (16)	42.3 ± 4.7 (12)	0.826 ± 0.001 (44)	42.7 ± 2.9 (12)	0.831 ± 0.001 (45)
F_1 Rf1 × BC45	38.0 ± 3.2 (5)	3.5 ± 0.4	0.830 ± 0.006 (17)	48.9 ± 5.1 (17)	0.837 ± 0.001 (18)	54.6 ± 16.4 (7)	0.825 ± 0.002 (48)	52.3 ± 1.6 (6)	0.832 ± 0.001 (47)
F_1 NMS1 × Par1	0 (4)	3.6 ± 0.3	0.817 ± 0.008 (18)	ND	0.826 ± 0.002 (20)	0 (2)	0.827 ± 0.001 (60)	0 (2)	0.826 ± 0.001 (59)
F_1 NMS1 × BC45	0 (8)	4.1 ± 0.7	0.826 ± 0.008 (18)	0 (17)	0.831 ± 0.001 (19)	0 (5)	0.824 ± 0.002 (53)	0 (7)	0.829 ± 0.002 (53)
F_1 NT-NMS1 × Par1	48.0 ± 4.1 (5)	3.8 ± 0.5	0.828 ± 0.006 (17)	ND	0.827 ± 0.002 (16)	40.7 ± 5.3 (3)	0.825 ± 0.002 (54)	38.0 ± 9.2 (3)	0.823 ± 0.002 (53)
F_1 NT-NMS1 × BC45	ND	4.7 ± 0.6	0.822 ± 0.008 (18)	42.8 ± 4.9 (17)	0.832 ± 0.001 (17)	44.7 ± 12.7 (3)	0.825 ± 0.001 (54)	45.6 ± 4.4 (5)	0.826 ± 0.001 (54)
BC_1 Par1 × Rf1	65.1 ± 3.9 (34)	4.5 ± 0.7	0.763 ± 0.007 (31)	ND	ND	ND	ND	ND	ND
BC_1 BC45 × Rf1	ND	4.8 ± 0.9	0.746 ± 0.010 (30)	ND	ND	ND	ND	ND	ND
BC_1 Rf1 × Par1	ND	4.2 ± 0.6	0.760 ± 0.010 (25)	ND	ND	ND	ND	ND	ND
BC_1 Rf1 × BC45	ND	3.4 ± 0.7	0.746 ± 0.014 (17)	ND	ND	ND	ND	ND	ND
BC_1 NMS1 × Par1	27.2 ± 18.9 (5)	4.0 ± 0.7	0.785 ± 0.007 (12)	ND	ND	ND	ND	ND	ND
BC_1 NMS1 × BC45	ND	3.1 ± 0.7	0.776 ± 0.013 (13)	ND	ND	ND	ND	ND	ND
BC_1 NT-NMS1 × Par1	40.7 ± 23.1 (3)	6.2 ± 1.2	0.761 ± 0.013 (15)	ND	ND	ND	ND	ND	ND
BC_1 NT-NMS1 × BC45	ND	4.3 ± 0.9	0.723 ± 0.021 (15)	ND	ND	ND	ND	ND	ND
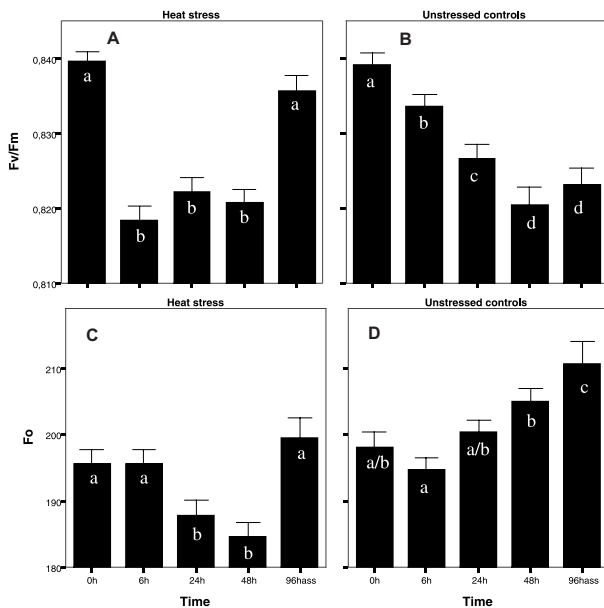
Figure 2. Photosynthetic capability in F_1 hybrids between *Brassica napus* and *B. rapa*. Bar diagram showing mean and 95% confidence intervals before stress (0h), during stress (6h, 24h and 48h) and 96h after stress stop (96hass) in interspecific *B. napus* $\times$ *B. rapa* F_1 hybrids grown in heat stress or as controls. (A) the ratio of variable to maximum chlorophyll fluorescence (Fv/Fm) in heat stressed plants; (B) the ratio of variable to maximum chlorophyll fluorescence in unstressed control plants; (C) the basal fluorescence level (F_0) in heat stressed plants; (D) the basal fluorescence level (F_0) in unstressed control plants. Statistical differences between the bars within the same subfigure are gives by the letters a, b, c and d.

reversibly or irreversibly in transgenic petunia and tobacco (Broer, 1996; Meyer et al., 1992; Neumann et al., 1997). We investigated the stability of transgene expression during heat stress, in two lines of modified *B. napus* and in their F_1 hybrids, obtained with two populations of wild *B. rapa*. Our results show that the examined transgenes were very stably expressed, irrespective of genetic background, cytoplasmic origin and environmental conditions. Similar results have been found at the protein level of Bt toxin where stable expression in first generation, and backcross progenies (BC_1 to BC_4) of *B. napus* and populations of wild *B. rapa*, has been shown (Zhu et al., 2004). Our results on the mRNA level of the *barnase* and *barstar* transgenes confirm stability, also during heat stress. However, heat stress applied in our experiment was limited to 48h. Some transgenic lines of *Arabidopsis thaliana* showed silencing after heat treatment at 30 °C / 4 °C for 14 days (Meza et al., 2001). Hence, a longer period of stress might affect transgene expression. Stable expres-

sion of transgenes that are introgressed to wild relatives makes risk assessments and post-release monitoring more reliable and straightforward. Similar expression studies of introgressed transgenes in other species and environments are necessary to elucidate if the stable transgene expression that we found in the *B. napus* $\times$ *B. rapa* hybrids is a general phenomenon of lines that are selected for marketing.

Impact of the environmental conditions on plant vigor

Heat stress has been found to have severe detrimental effects on the percentage of viable pollen, the silique and seed production, and the dry matter accumulation in *B. napus* (Angadi et al., 2000; Morrison, 1993; Young et al., 2004). In our experiment, the majority of the parental plants and F_1 hybrids responded by producing less viable pollen when exposed to heat stress, but also under field conditions. This effect from field conditions on pollen viability is a new observation that confirms that plants are often more stressed when grown under natural conditions compared to growth chamber conditions.

The relationship between photosynthetic rate and fitness has been investigated in a wild-type *Amaranthus hybridus* and a mutant with a lower efficiency of electron transport through PSII (Arntz et al., 2000). The mutant had a lower rate of photosynthetic carbon assimilation that could translate into a 10–35% lower rate of photosynthesis. It was found that the mutant type had lower fecundity and a lowered seedling survivorship (Arntz et al., 2000), implying that a lower photosynthetic rate has a negative impact on fitness. We assume that the same relationship between a decline in Fv/Fm and a lower fitness was valid in our experiment; however, we did not investigate if this assumption was correct. We found decreases in Fv/Fm suggesting that the plants grown in the field were exposed to stress. Our results support previous findings in *Arabidopsis thaliana* where reductions in chlorophyll fluorescence were found when plants were grown in the field as opposed to in a growth chamber (Külheim et al., 2002).

We compared Fv/Fm and F_0 during and after heat-stress. Unexpectedly, Fv/Fm fell continuously in the control plants. This could be a response to the different light conditions in the growth cabinet as opposed to the growth chamber, even though the irradiance did not reach levels that ought to be stressful. F_0 increased in our control plants – a response that is normally characteristic to environmental stress (Krause and Weis, 1984). Hence, the symptoms of a stress-response were present in the supposedly unstressed control plants. This complicates

Table 2. Estimate of fitness parameters of F_1 and BC_1 hybrids from crosses between *B. napus* and *B. rapa* relative to *B. rapa*. Estimates are based on present and previous studies.

Parameter	*B. rapa*	F_1 hybrids	BC_1 hybrids	References
Survival	1	0.925	0.953	Hauser et al., 1998a, b
Fv/Fm	1	1.029	0.950	Present study
Pollen viability	1	0.409	0.473	Present study
Total number of seeds per plant	1	0.783	0.202	Hauser et al., 1998a*, b*; Present study**
Average	1	0.786	0.644	

* Number of pods per plant.
** Number of seeds per pod.

analysis of the response in the heat stressed plants where a dramatic fall in Fv/Fm was observed. In contrast to previous findings (Havaux, 1993; Krause and Weis, 1984; Yamane et al., 1997), F_0 decreased in our heat-stressed plants. Such a decrease has been observed in *Vicia faba* and *Spinacia oleracia* (Misra et al., 2001), when high temperature was applied together with high irradiance. Since our control plants responded with a rise in F_0, it seems reasonable to believe that the heat-stressed plants experienced both a temperature and an unintended light stress. Using chlorophyll fluorescence in stressed plants as an expression of vigor may not provide straightforward results, as the method is very sensitive to any change in the environment. Also, since plants readily acclimate to alterations in the environment, the results may reflect acclimatization responses that are not representative of the general physiological fitness. In the field, rapid acclimatization processes are known to efficiently adjust the thermostability of PSII in response to various environmental stimuli such as water stress and light (Havaux, 1993). A low Fv/Fm may be an advantage in some situations if it serves to protect the plant on a long-term perspective. Therefore, other parameters such as biomass measurements should be included in examinations of plant vigor despite the intensive labor such measurements require.

Impact of genetic background on plant vigor

Transgenic plants were just as fit as their non-transgenic siblings in pollen viability, seeds produced per silique and photosynthetic capability. This supports findings from other fitness-related parameters when comparing transgenic and non-transgenic hybrids of *B. napus* and *B. rapa*. Previously, no significant differences in survival or the number of seeds per plants has been found (Snow et al., 1999). Together these findings indicate that costs associated with these transgenes are negligible.

No clear effect of cytoplasmic origin or parental population could be observed from our experiments.

Effects from different generations on pollen viability and seed production showed that the parental generation was most fit followed by the BC_1 generation and then the F_1 generation. This is in agreement with previous findings in hybrid generations between *B. napus* and *B. rapa* (Hauser et al., 1998b; Snow et al., 1999). This also agrees with the observation that in both plants and animals, hybrids with genotypes more similar to one or the other parental have a higher fitness compared to genetically intermediate hybrids, such as F_1 hybrids (Arnold and Hodges, 1995). However, our findings on the reproductive fitness are in contrast to our chlorophyll fluorescence measurement, where some F_1 hybrids had the most efficient photosynthetic capability, followed by the parental generation and then the BC_1 hybrids. Heterosis, a fitness boost due to hybridization can result in extreme characteristics in first generation hybrids (Arnold, 1997; Ellstrand, 2003; Rieseberg, 1997), and may be the reason for the observed increases in photosynthetic capability of the F_1 hybrids. Hence, the different fitness-related parameters used in our experiments provided opposite directed results as to the vigor of the plant generations.

An attempt to estimate the compounded fitness of the F_1 and BC_1 hybrids compared to *B. rapa* – based on published data and the present data – is shown in Table 2. The data implies, that since F_1 hybrids produce more silique per plant than BC_1 hybrids (F_1 hybrids: 993 silique per plant; BC_1 hybrids: 225 silique per plant (Hauser et al., 1998a, b)), the total number of seed set per F_1 hybrid plant by far exceeds the total number of seed set per BC_1 hybrid, as both F_1 and BC_1 produce the same number of seeds per silique (Tab. 1). When averaging the values included in Table 2, both generations of hybrids have a lower vigor than *B. rapa*, and F_1 hybrids have a higher vigor than BC_1 hybrids. Other fitness parameters than the ones presented in the table, for example seed viability, could influence the compounded fitness values. Different environmental conditions might also affect the estimated values; hence, it is difficult to give precise estimates.

Conclusion

Investigations of two transgenes showed that transgene expression was stable, and being transgenic or not had no impact on fitness. Therefore, weediness or invasiveness is not likely to increase under non-selective conditions. Photosynthetic capability in F_1 hybrids of *B. napus* × *B. rapa* was sometimes higher than their parents, however, their reproductive fitness was lower. The higher photosynthetic capability suggests that F_1 hybrids may have enhanced vegetative vigor compared to their parents, and may present a larger potential for transgene spread than formerly expected. The compounded fitness estimate suggests a high seed set per F_1 plant, and spontaneous introgression of genes from *B. napus* × *B. rapa* hybrids to wild species has been reported (Hansen et al., 2001; Hansen et al., 2003). BC_1 hybrids form the next step of transgene introgression into wild species, but our results suggest that the vegetative vigor of the BC_1 plants is limited. In conclusion, we can recommend that compounded fitness estimates be used in risk assessments of GM plants, as different fitness factors often give different trends. Therefore, the combination of more factors in a compounded estimate has its benefits, and here the photosynthetic capability could be a valuable parameter indicating the vegetative fitness. Our results also showed that the fitness of both parents and hybrids are generally lower in the field, compared to a growth chamber. This is worth considering if risk assessment studies are made under non-natural conditions.

MATERIALS AND METHODS

Plant materials

Two transgenic *B. napus* ssp. *oleifera* (DC.) lines of the variety "Drakkar" were used (provided by Plant Genetic Systems, Belgium). The "Nuclear Male Sterility" line (NMS1) expresses the ribonuclease barnase, as a hemizygous trait, so that half the offspring are Non-Transgenic NMS1 (NT-NMS1) siblings that were used as controls. The homozygous *B. napus* "Restorer of fertility" line (Rf1) expresses barstar, which restores the fertility in hybrids between NMS1 and Rf1 by specific inhibition of the barnase (Mariani et al. 1990). A promoter that limits their expression to the tapetum cells of the pollen sac during anther development directs both transgenes. Both transgenic lines contain the *bar* transgene encoding a phosphinothricin acetyl transferase making the plants resistant to phosphinothricin-based herbicides. The *bar* gene was inserted in the same construct as the *barnase* and *barstar* genes. The two transgenic *B. napus* lines were used to produce F_1 and BC_1 hybrids with two Danish populations of wild *B. rapa* ssp. *campestris* (L.); Par1 (Snow et al., 1999) and BC45 (Landbo and Jørgensen, 1997). For the F_1 generation, pair-wise crosses were made by hand pollination using 12 NMS1 plants and six *B. rapa* plants of each of the two populations. To analyze the impact of cytoplasmic origin, reciprocal crosses were made with the Rf1 line using 12 Rf1 and six *B. rapa* plants of each of the two wild populations. For the production of the BC_1 generation, 12 *B. rapa* plants were pair-wise pollinated by six F_1 hybrids from each of the reciprocal crosses between Rf1 and Par1 or BC45, respectively. Likewise, pair-wise pollinations of 12 F_1 male-sterile hybrids with six Par1 plants, and six BC45 plants, were performed. A Basta dot-test was performed on all hybrid plants to validate expression of the transgene encoded herbicide resistance trait. For the cross with the hemizygous NMS1 line, the Basta dot-test was also used to identify the transgenic male-sterile hybrids from their non-transgenic siblings.

Growth conditions

Table 1 gives the four different environments, the genotypes, and the number of plants grown in the different environments. Seedlings were grown in a growth chamber (16h light / 18 °C, 8h dark / 16 °C), with both natural and artificial light (~ 400 µmol), until initiation of flowering. Plants were kept in the growth chamber until seeds were collected. For heat stress treatment, F_1 and parental plants were moved from the growth chamber to a conviron growth cabinet (16h light / 37 °C, 8h dark / 20 °C) for 48h, and after heat-treatment they were moved back to the growth chamber. Since the light and air circulation was somewhat different in the conviron cabinets compared to the growth chamber, control plants were moved to a conviron cabinet without heat treatment (48h; 16h light / 18 °C, 8h dark / 16 °C) during the same period as heat stress was performed. In the conviron cabinets there was only artificial light. Light intensities were; top-leaves: 600–700 µmol; mid-leaves: 430–450 µmol; basal-leaves: 350 µmol. For the field experiment, seedlings were transplanted from the growth chamber to the field at the two-leaf stage, and Basta dot-tests performed. Plants were placed in the field in early April and watered when needed. The plants were left to random pollination.

Transgene expression

An equal volume of the smallest flower buds was collected from each plant 48h after initiation of heat-stress, and also

from corresponding control plants and plants in growth chamber conditions. Thereafter all plants were returned to the growth chamber. Flower buds were collected again from all plants 96h after the heat-stress period had ended. The flower buds were kept at –80 °C until RNA purification. The buds were homogenized in a Mixer Mill (MM300, Retsch) after freezing in liquid nitrogen, and mRNA was isolated from crude lysate using Dynabeads® mRNA Purification Kit. RT-PCR was made using the Superscript™ II RNase H⁻ Reverse Transcriptase protocol (Invitrogen). Expression of the transgenes was analyzed on a RotorGene 2000 Real-Time cycler (Corbett Research), initial denaturing 98 °C, 180 s – 40 cycles: 98 °C, 20 s; 58 °C, 30 s; 72 °C, 30 s; acquiring at 88 °C, 20 s – hold: 50 °C, 30 s – melt: 50 °C–99 °C rising by 1 °C each step, hold for 15 s on first step, then 5 s on each step afterwards. Sequence-specific primers were designed for actin (forward: 5'-TGACATTAAAGAGAAGCTTG-3'; reverse: 5'-CACTTCATGATGGAGTTGT-3'), which was used as an internal standard, and for *barstar* and *barnase* amplification (sequence information is confidential). The mean normalized expression (Muller et al., 2002), as calculated by averaging three concomitant threshold cycle values of the target gene and of the reference gene, respectively, was found using the Q-gene tool (Muller et al., 2002). The actin used as internal standard was similar to the *A. thaliana* actin 2/7 according to a BLAST search. Expression of the actin standard was stable during heat treatment (*t*-test, $p = 0.305$).

Pollen viability and seed set

Pollen from two flowers per plant was collected from parental plants, F_1 hybrids and BC_1 hybrids (see Tab. 1 for distribution between genotypes). The pollen grains were stained with cotton blue. The percentage of viable pollen grains was determined by finding the ratio of viable to non-viable pollen grains from 200 pollen grains per plant.

The seed set was only measured in plants grown in the field, where pollinators were present. Siliques were collected at maturity from parental plants, F_1 hybrids and BC_1 hybrids (see Tab. 1 for distribution between genotypes). The number of seeds per silique was determined by collecting 20 siliques from each plant.

Chlorophyll fluorescence

The chlorophyll *a* fluorescence was measured using a Plant Efficiency Analyzer (Handy PEA, Hansatech Instruments Ltd., UK), applying one light flash for one second. The leaves were dark-adapted for 20 minutes before measuring. Three leaves of each plant were measured, one at the base, one halfway up the stem, and one at the top, respectively. These measurements were pooled when analyzing the different genotypes. In the field experiment, measurement on only one leaf (halfway up) per plant was made. Chlorophyll fluorescence was measured just before opening of the first flower buds when all plants were still in the growth chamber (0h). Measurements were then taken 6h, 24h and 48h after moving the plants to the heat-stress or control conviron cabinets. After returning the plants to the growth chamber, measurements were taken 96h after stress stop (96hass). At this time point, the plants that had remained in the growth chamber were also measured, ensuring similarity in age in all plants. Plants in the field were measured in the early flowering period, corresponding to the developmental stage of the plants at the 96hass time-point measurements. The chlorophyll fluorescence data are presented as the ratio of variable to maximum fluorescence (Fv/Fm) or as the basal fluorescence level, F_0 (for further information on these parameters, see Strasser et al, 2000).

Data analysis

All statistical analyses were made using the statistical software package SPSS for Windows (version 11.5) (SPSS Inc., Chicago, US). In all tests performed, the significance level used was $\alpha = 0.05$. When data followed a normal distribution, comparisons were made using a *t*-test or a Univariate Analysis of Variance, including Post Hoc Tamhane or Tukey B test. Otherwise the non-parametric Mann-Whitney U test or the Kruskal-Wallis H test was used (SPSS base system and on-line guides).

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