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Abiotic stress and transgenics: Implications for reproductive success and crop-to-wild gene flow in *Brassica*s

Sari J. Himanen[a,b,*], Anne-Marja Nerg[b], Guy M. Poppy[c], C. Neal Stewart Jr.[d], Jarmo K. Holopainen[b]

[a]*MTT Agrifood Research Finland, Plant Production Research, Lönnrotinkatu 5, FIN-50100 Mikkeli, Finland*
[b]*Department of Environmental Science, University of Eastern Finland, Kuopio Campus, P.O. Box 1627, FIN-70211 Kuopio, Finland*
[c]*School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK*
[d]*Department of Plant Sciences, University of Tennessee, 2431 Joe Johnson Drive, Knoxville, TN 37996-4561, USA*

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Abstract

Various abiotic and biotic stressors affect crop and weed plant performance in agroecosystems. Ozone (O_3) tolerance in plants is partly regulated by the genotype and phenotypical properties, and it varies greatly in related species of wild and crop backgrounds. Thus, a continuous increase in atmospheric O_3 concentration could change population dynamics of sexually compatible crop and weed species, and consequently affect crop-to-wild gene flow in the future. One way to build resistance against a biotic stressor, in this case insect-mediated herbivory, in crop plants is transgene-mediated insecticidal toxin production. In this study we aimed to describe how the physiological and phenological responses in a crop *Brassica* and its weedy relatives functioned to affect their comparative O_3 tolerance. Furthermore, we studied how harbouring a transgene affects these responses in *B. napus* and *B. rapa* × transgenic *B. napus* BC_2F_2 backcross hybrid plants to reveal any within-plant trade-offs among toxin production, growth and O_3 tolerance. We found a higher number of O_3 symptoms but more effective compensatory assimilate allocation directed to reproduction for wild *B. rapa* compared to crop *B. napus* under elevated O_3. This result suggested that the invasion-orientated strategy of producing a high number of seeds when vegetative growth is limited might improve the performance of weedy species under elevated O_3. The probabilities for crop-to-wild transgene flow could be increased through higher seed production in hybrids under elevated O_3, but the germination of hybrid seeds in particular was hampered by O_3. The presence of transgenes did not perturb fecundity, within-plant biomass allocation or O_3 tolerance of *B. napus*.

Zusammenfassung

Verschiedene abiotische und biotische Faktoren beeinflussen die Performanz der Nutz- und Unkrautpflanzen in Agrarökosystemen. Die Ozon-(O3)-Toleranz der Pflanzen wird teilweise durch den Genotyp und durch phänotypische Eigenschaften reguliert und variiert in großem Maße bei verwandten Pflanzen mit einem wilden bzw. Nutzpflanzen-Hintergrund. Deshalb könnte eine kontinuierlich steigende O3-Konzentration in der Atmosphäre die Populationsdynamik von sexuell kompatiblen Nutz- und Unkrautarten verändern und in der Zukunft als Konsequenz den Genfluss von Nutz- zu Unkrautarten beeinflussen. Ein Weg um eine Resistenz gegenüber einem biotischen Stressor, in diesem Fall die Herbivorie durch Insekten, bei einer Nutzpflanze

*Corresponding author at: MTT Agrifood Research, Plant Production Research, Lönnrotinkatu 5, FIN-50100 Mikkeli, Finland. Tel.: +358 40 738 9873; fax: +358 15 226 578.
E-mail address: Sari.Himanen@mtt.fi (S.J. Himanen).

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aufzubauen, ist die transgen vermittelte Produktion von insektiziden Toxinen. In dieser Untersuchung war es unser Ziel, zu beschreiben, wie die physiologischen und phänologischen Reaktionen bei der Nutzpflanze Brassica und ihren verwandten Unkräutern funktionierten, um ihre O3-Toleranz im Vergleich zu beeinflussen. Darüber hinaus untersuchten wir, wie die Anwesenheit eines Transgens diese Reaktionen bei B. napus und transgenen B. rapa x B. napus BC2F2 Rückkreuzungshybriden beeinflusst, um irgendwelche "trade offs" zwischen der Toxinproduktion, dem Wachstum und der O3-Toleranz innerhalb der Pflanzen festzustellen. Im Vergleich zu genutztem B. napus fanden wir unter erhöhtem O3-Gehalt eine größere Anzahl von O3-Symptomen aber auch eine effektivere, kompensatorische Assimilate-Allokation in Richtung auf die Reproduktion bei wildem B. rapa. Dieses Ergebnis lässt vermuten, dass, wenn das vegetative Wachstum limitiert ist, die invasionsorientierte Strategie durch die Produktion einer großen Anzahl von Samen die Performanz der unkrautartigen Arten bei erhöhtem O3-Gehalt verbessern könnte. Die Wahrscheinlichkeit für den Genfluss von transgenen Nutz- zu Wildpflanzen könnte durch die höhere Samenproduktion bei Hybriden bei erhöhtem O3-Gehalt erhöht sein, wenn auch die Keimung der Hybridsamen bei erhöhtem O3-Gehalt behindert wurde. Die Anwesenheit von Transgenen störte bei B. napus weder die Fruchtbarkeit, noch die Biomassenallokation innerhalb der Pflanze oder die O3-Toleranz.

Keywords: *Brassica napus* ssp. *oleifera* (oilseed rape; canola); *Brassica rapa*; Ozone tolerance; Transgene introgression; Crop–weed population dynamics

Introduction

Diurnal background concentration of tropospheric ozone (O_3) already exceeds 40 ppb in many regions and trends towards increasing levels are predicted to continue for the coming decades (Sitch, Cox, Collins, & Huntingford 2007). This can make O_3 an important contributor on plant competitive dynamics. Oxidative stress by elevated O_3 is a severe environmental challenge for plants: phytotoxic symptoms arise and this affects photosynthetic processes, phenological development and yield (Booker et al. 2009). Most wild plant species are classified as susceptible to O_3 compared with crop species, which have been selected through breeding to be more robust under variable environments (Pleijel & Danielson 1997; Davison & Barnes 1998; Biswas et al. 2008). Genetically regulated O_3 tolerance (Biswas et al. 2008), but also differences in phenology (e.g. maturation age), life-histories (Pleijel & Danielson 1997) and phenotypes (Overmyer et al. 2008) affect O_3 responses of plants and could alter the competitive dynamics of co-species under elevated O_3.

The introduction of transgenic crops has initiated wide ecological research of potential gene escape into native species (Stewart, Halfhill, & Warwick 2003). The probabilities for transgene flow have been extensively studied on *Brassica*s (e.g. Warwick et al. 2003; Halfhill et al. 2004; Kelly, Bowler, Breden, Fenner, & Poppy 2005; Warwick, Légère, Simard, & James 2008), since wild *Brassica* species occur worldwide and exist commonly in agroecosystems. They are capable of hybridizing with cultivated *Brassica*s, which enables segregation of common genomic material among these plants (Wilkinson, Elliott, et al. 2003; Halfhill et al. 2004). Unwanted introgression of transgenes into wild species could have severe ecological consequences, although the pathway required for a transgene to be fully introgressed into a wild-plant genotype is a complicated one in which the success of the hybrids are crucial (Wilkinson, Sweet, & Poppy 2003). Environmental stresses, including elevated O_3, are candidates for affecting the performance and competitiveness of introgressed plant individuals and thus are critical in assessing the environmental risk of a transgene introgressing into the genome of a crop's wild relatives. Our study is the first to compare the performance of nontransgenic and transgenic crops, wild relatives and introgressed transgene-carrying back-cross hybrid plants under elevated O_3.

Bacillus thuringiensis (Bt) *Cry1Ac*-transgenic oilseed rape (*Brassica napus* ssp. *oleifera*), insecticidal against numerous key Lepidopteran *Brassica* pests, is a model plant used widely in ecological risk assessment studies for crop-to-wild gene flow (e.g. Halfhill, Richards, Mabon, & Stewart 2001; Warwick et al. 2003). Previously, we have shown that Bt toxin concentration in Bt-producing *B. napus* is not compromised by, but increased under high atmospheric O_3 (Himanen et al. 2009), and the plants exhibit similar responses to chronic and acute O_3 elevation during vegetative growth as their nontransgenic parent plants (Himanen et al. 2008). Here, our first aim was to test for a trade-off, as a result of intrinsic costs of constitutive Bt toxin production, among reproduction and O_3 tolerance in Bt *B. napus*. Secondly, we assessed whether elevated O_3 affects certain physiological characteristics (growth, allocation to reproduction, seed size) and therefore the performance of sexually compatible wild *B. rapa* and crop *B. napus* plants, representing different ecological life-history strategies (Moles and Westoby 2006), in a different way. Finally, we evaluated the O_3 responses and within-plant allocation patterns of introgressed Bt-transgene-carrying *B. rapa* × *B. napus* BC_2F_2 back-crossed hybrid plants to reveal whether elevated O_3 could affect the probability of transgene escape through altered performance or reproduction of hybrids. Our results could reveal important aspects for assessing competitive advantage of introgressed transgene-carrying hybrids, wild relatives and crop plants in future O_3-enriched atmospheres.

Materials and methods

Plants used in the experiments were: 1) non-transgenic *Brassica napus* ssp. *oleifera* (oilseed rape) cv. Westar (parent line), 2) its Bt-transgenic line GT1 F_4 (containing a synthetic Bt *Cry1Ac* gene and a green fluorescent protein (*gfp*) marker gene under CaMV 35S promoters, as described by Halfhill et al. 2001), 3) *Brassica rapa* wild accession 2974 (Milby, Québec, Canada; Halfhill et al. 2005) and 4) wild *B. rapa* (described above) × crop (Bt-transgenic GT1 *B. napus*) BC_2F_2 hybrid carrying the transgene and having *B. rapa* ploidy level and phenotype (hybridized as described in Halfhill, Millwood, Weissinger, Warwick, & Stewart 2003).

Plants were grown in four identical environment-controlled growth chambers (2.6 m^3, Bioklim 2600T, Kryo-Service Oy, Helsinki, Finland) under 16:8 h photoperiod, 20/16 °C thermoperiod and minimum 250 $\mu mol\, m^{-2}\, s^{-2}$ PAR irradiance. One hundred and four seeds from each genotype were individually sown into 0.66 l pots in 2:1:1 fertilized compost (Kekkilä, Finland, NPK: 100-30-200 $mg\, l^{-1}$): B2 *Sphagnum* peat (Kekkilä, Finland, NPK: 110–40–220 $mg\, l^{-1}$): sand (0.5–1.2 mm) mixture and placed in a randomised block design into each of the four chambers (26 plants of each genotype per chamber). Chronic O_3 fumigation (supplied as in Himanen et al. 2009) was started after emergence (7 days after sowing) with two chambers having close to 0 ppb O_3 (filtered air) and two having 100 ppb elevated O_3 treatment (8 h daily, 8.30 to 16.30 h). Elevated O_3 concentration of 100 ppb was used, as it caused severe but not detrimental phytotoxic challenge to the plants in earlier screening experiments (Himanen et al., unpublished). In these screenings, filtered air (0 ppb) and 50 ppb (high ambient concentration) were also compared and close to identical responses in the plants warranted the use of no O_3 elevation (filtered air) as the control treatment. To avoid any effects of chamber-specific growth conditions, the plants inside chambers and the treatments among chambers were rotated weekly (as in, e.g. Black, Stewart, Roberts, & Black 2007, except during flowering to prevent pollen contamination between treatments). The plants were transplanted into 1.4 l pots at 22 days after sowing. During flowering, flowers were hand-pollinated, using a fine brush, at two-day intervals within each O_3 treatment.

For biomass determination, the dry weight (DW) of leaves (dried at 60 °C) was measured individually for six plants per genotype and O_3 treatment (three plants per chamber) at 22 days and 33 days after sowing. Net photosynthesis was measured for the second growth leaf of six plants per genotype and O_3 treatment (three plants per chamber) 22, 28 and 35 days after sowing with a CI-510 Portable Photosynthesis System (Cid Inc., Vancouver, WA, USA) under saturating light intensity of 1800 $\mu mol\, m^{-2}\, s^{-2}$ PAR. The CO_2 input for the cuvette was taken from the growth chambers. O_3 damage (percentage of total leaf area damaged, assessed from photographs of the plants analyzed using Adobe Photoshop software) was determined for the same plants at days 28 and 35. The abscission of leaves for each genotype in each chamber was determined by collecting and determining DW of senesced leaves at two- to four-day intervals.

The plants were harvested when over 75% of all pods were dry (yellow in colour), but none had started to open, which occurred at 11–12 weeks after sowing. At harvest, stem height was measured and the number of branches was counted. The DW of stem, roots, pods and seeds in main rachis, and pods and seeds in branches for each plant were measured after drying. Harvest index was calculated as the ratio of seed DW to total plant DW. For presentation of the results (Table 4), harvest index and root:shoot ratio were multiplied by 100. The 1000-seed weight was calculated after weighing all or 100 seeds per plant, for main rachis and branch seeds separately. Germination of the seeds (collected from main rachis) was tested in 20 replicate Petri dishes (diameter 100 mm) lined with moisturised filter paper and placed in a growth chamber at 20/16 °C. Seeds from all eight treatments were randomly assigned to eight sectors in each dish, with five seeds per sector. Final germination percentage was determined after 14 days.

Before statistical analysis, all data were checked for normality and equality of residual error variances and then appropriately transformed (log or square root) if necessary to gain normality. Percentage values were arcsin-transformed prior to analysis. Results were analyzed using REML linear mixed models with plant genotype and O_3 treatment as fixed effects and chamber as a random factor. Post hoc tests based on estimated marginal means with Bonferroni correction were conducted both among O_3 treatments and genotypes, separately. Leaf senescence and seed germination results were tested with general linear models with genotype and O_3 as fixed effects. Correlation between root + stem DW and seed DW was tested using Pearson correlation tests separately for each genotype and O_3 treatment. All data were analyzed using SPSS for Windows 14.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Vegetative growth stage responses

At 22 days after sowing, transgene-harbouring BC_2F_2 hybrid and wild *B. rapa* plants had higher leaf DW than the *B. napus* genotypes (Tables 1 and 2). By day 33, O_3 reduced leaf DW in *B. rapa* ($P_{O_3} < 0.05$), whereas there was no difference in leaf DW between genotypes at this time-point. Photosynthesis rates were similar in all plant genotypes and at both O_3 levels at day 22 (Tables 1 and 2). By days 28 and 35, the photosynthesis rate was lower in hybrid and *B. rapa* plants than in *B. napus* genotype plants grown under elevated O_3.

Percent damage to the total leaf area caused by O_3 was higher in the first (at 28 days) and second (at 28 and 35 days) growth leaves of hybrid and *B. rapa* plants than in

Table 1. Leaf dry weight and net photosynthetic rate (A) during vegetative growth (mean ± SEM) for non-transgenic and Bt-transgenic *B. napus*, *B. rapa* × transgenic *B. napus* BC_2F_2 hybrid and wild *B. rapa* plants grown under control conditions (filtered air) or elevated (100 ppb 8 h day^{-1}) O_3.

	Control				Elevated O_3			
	B. napus	Bt *B. napus*	BC_2F_2 hybrid	*B. rapa*	*B. napus*	Bt *B. napus*	BC_2F_2 hybrid	*B. rapa*
Leaf dry weight (g)								
22 days	0.18 ± 0.02a	0.12 ± 0.01a	0.36 ± 0.05b	0.42 ± 0.03b	0.17 ± 0.02a	0.15 ± 0.02a	0.33 ± 0.07ab	0.44 ± 0.05b
33 days	1.11 ± 0.21	0.91 ± 0.13	1.01 ± 0.18	1.17 ± 0.26	1.01 ± 0.14	0.87 ± 0.06	0.67 ± 0.12	0.61 ± 0.14
A (μmol m^{-2} s^{-1})								
22 days	22.5 ± 1.38	21.0 ± 1.40	21.2 ± 1.26	22.1 ± 0.96	23.1 ± 0.43	21.5 ± 0.68	23.0 ± 0.53	22.7 ± 0.63
28 days	22.5 ± 2.08a	22.3 ± 0.72ab	16.1 ± 1.65b	18.2 ± 0.62ab	21.1 ± 0.50a	22.2 ± 0.42a	7.80 ± 1.61b	9.95 ± 1.30b
35 days	17.0 ± 0.99	22.4 ± 2.39	17.8 ± 1.59	15.3 ± 1.79	19.2 ± 1.22a	21.1 ± 0.67a	6.67 ± 3.08b	10.3 ± 1.99b

Net photosynthesis was measured for 2nd growth leaves. Different letters indicate statistically significant ($P < 0.05$) difference between genotypes among control and elevated O_3 treatments.

Table 2. Mixed model results for main effects of genotype (non-transgenic *B. napus*, Bt-transgenic *B. napus*, *B. rapa* × transgenic *B. napus* BC_2F_2 hybrid and wild *B. rapa*) and O_3 treatment (control or 100 ppb elevated O_3) and their interaction on vegetative and reproductive growth stage parameters and within-plant allocation (as percentage of total plant DW) at harvest.

	Genotype $F_{3,151}$	O_3 $F_{1,151}$	Gt × O_3 $F_{3,151}$
Vegetative stage parameters:			
Leaf DW 22 days	**29.0*****	0.02	0.41
Leaf DW 33 days	0.74	**5.31***	1.12
A 22 days	0.87	1.73	0.21
A 28 days	**28.2*****	**22.8*****	**7.25*****
A 35 days	**12.5*****	**6.69***	**4.48*****
Reproductive stage parameters:			
Plant height	**42.2*****	0.07	**4.25****
Branch number	**13.1*****	0.41	1.35
Stem DW	**6.96*****	**27.2*****	**3.44***
Root DW	**16.4*****	**64.0*****	2.45
Root + stem DW	**9.18*****	**36.3*****	**3.61***
Total pod DW	**9.51*****	**15.3*****	1.34
Total plant DW	**12.5*****	**33.5*****	0.12
Main rachis seed DW	**83.6*****	**17.9*****	**4.09****
Branch seed DW	0.71	1.36	**3.06***
Total seed DW	**19.0*****	**9.13****	**3.80***
Seed germination	**114.1*****	**4.99***	1.98
1000-seed weight			
In main rachis	**120.5*****	0.22	1.17
In branches	**51.8*****	0.94	2.66
Within-plant allocation:			
Root: shoot ratio	**11.7*****	**44.3*****	1.32
Harvest index	**5.14****	1.99	**5.75****
Roots	**9.53*****	**39.9*****	**5.53*****
Stem	0.72	0.05	**4.16****
Pods in main rachis	**12.3*****	0.17	1.47
Seeds in main rachis	**35.8*****	0.01	2.07
Pods in branches	**26.0*****	0.14	0.75
Seeds in branches	**6.19*****	3.24	**3.41***

Statistical significance in bold ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$).

Table 3. Foliar O_3 damage (% of total leaf area, mean ± SEM) 28 and 35 days after sowing in non-transgenic and Bt-transgenic *B. napus*, *B. rapa* × transgenic *B. napus* BC_2F_2 hybrid and wild *B. rapa* plants grown under elevated (100 ppb 8 h day^{-1}) O_3. 1st growth leaf denotes the oldest leaf.

Growth leaf	*B. napus*	Bt *B. napus*	BC_2F_2 hybrid	*B. rapa*	$F_{3,39}$ genotype
28 days					
1	60.0 ± 3.3a	65.8 ± 2.5a	100.0 ± 0.0b	99.2 ± 0.8b	**100.6*****
2	10.0 ± 6.0a	12.5 ± 0.8a	95.0 ± 5.0b	94.2 ± 0.8b	**148.5*****
3	0	0	39.2 ± 9.2	35.8 ± 7.5	0.079
4	0	0	9.7 ± 5.3	11.3 ± 10.3	0.021
35 days					
1	53.8 ± 38.8	76.3 ± 13.8	100.0 ± 0.0	100.0 ± 0.0	1.166
2	7.5 ± 7.5a	20.0 ± 0.0a	91.3 ± 6.3b	96.3 ± 3.8b	**79.12*****
3	0	3.8 ± 3.8	68.8 ± 18.8	55.0 ± 12.5	6.746
4	0	0	37.5 ± 25.0	5.0 ± 5.0	1.625

Statistical significance in bold ($^{***}P < 0.001$). Different letters indicate statistically significant ($P < 0.05$) difference between genotypes.

B. napus genotypes (Table 3). Hybrid and *B. rapa* plants senesced earlier in the control treatment than the *B. napus* genotypes (Fig. 1A). Leaf senescence occurred earlier in all genotypes under elevated O_3 (by approximately one week) (Fig. 1B).

Reproductive growth stage responses

Plant height was lower in BC_2F_2 hybrid plants in the control treatment, and in *B. rapa* and hybrid plants under elevated O_3 than in the *B. napus* genotypes (Table 4). Height was reduced by elevated O_3 only in *B. rapa* plants ($P_{O_3} < 0.05$, Table 2, interaction gt × O_3). Hybrid plants had more branches than the other genotypes (Table 4).

In the control treatment, hybrid plants had lower stem DW than *B. rapa*, lower root DW than the *B. napus* genotypes, lower root + stem DW than non-Bt *B. napus* and *B. rapa* and lower total DW than non-Bt *B. napus* (Table 4). In the elevated O_3 treatment, stem, root, stem + root and total DWs were lower in *B. rapa* and hybrid plants than in *B. napus* genotypes. Elevated O_3 reduced root DW in all genotypes, but stem and root + stem were significantly reduced by O_3 only in *B. rapa* and hybrid plants ($P_{O_3} < 0.05$, Table 2, interaction gt × O_3).

Total pod and seed DW for *B. napus* genotypes were higher than for hybrid and *B. rapa* under control conditions, whereas seed DW under elevated O_3 differed only between non-Bt *B. napus* and hybrid plants (Table 4). Elevated O_3 reduced pod DW in all genotypes and seed DW in *B. napus* genotypes (Table 2, interaction gt × O_3).

Seed dry weights for the main rachis were lower in hybrid and *B. rapa* plants than in *B. napus* genotypes in the control treatment (Table 4). Non-Bt *B. napus* had also a higher seed DW in the main rachis than Bt *B. napus* under elevated O_3. Elevated O_3 reduced seed DW in the main rachis of *B. napus* genotypes ($P_{O_3} < 0.05$), whereas those of hybrid and *B. rapa* plants were unaffected (Table 2, interaction gt × O_3). On branches, O_3 reduced seed DW only for non-Bt *B. napus* plants ($P_{O_3} < 0.05$, Table 2, interaction gt × O_3).

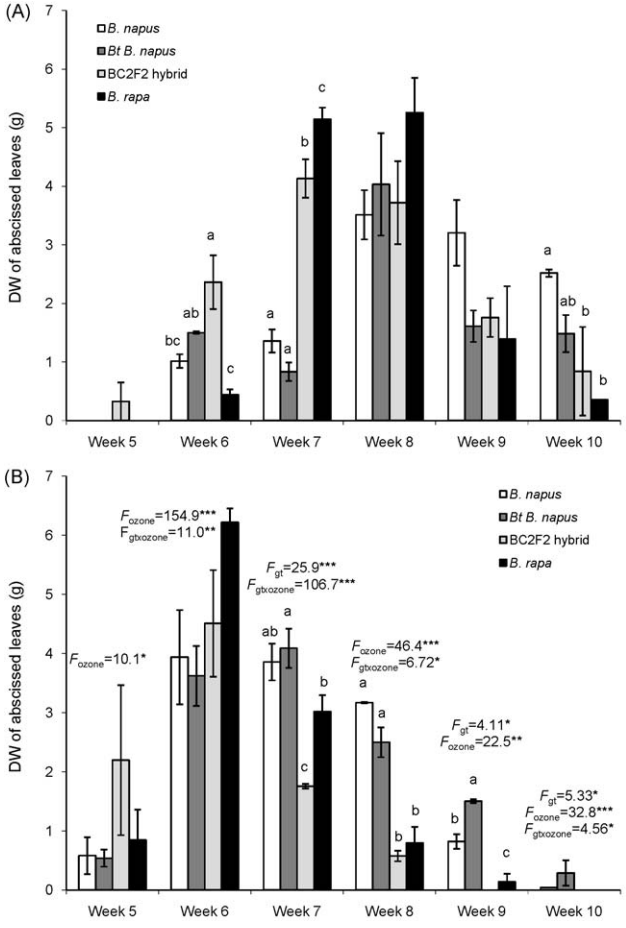

Fig. 1. Leaf senescence patterns (mean ± SEM) for non-transgenic and Bt-transgenic *B. napus*, BC_2F_2 *B. rapa* × transgenic *B. napus* hybrid and wild *B. rapa* plants grown under (A) control conditions (filtered air) and (B) elevated (100 ppb 8 h day^{-1}) O_3. Statistically significant main effects for genotype (gt) ($F_{3,7}$), O_3 ($F_{1,7}$) and their interactions ($F_{3,7}$) are shown ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Different letters above bars indicate statistically significant ($P < 0.05$) difference between genotypes among control and elevated O_3 treatments.

Table 4. Variables measured at harvest (mean ± SEM) for non-transgenic and Bt-transgenic *B. napus*, *B. rapa* × transgenic *B. napus* BC_2F_2 hybrid and wild *B. rapa* plants grown under control conditions (filtered air) or elevated (100 ppb 8 h day^{-1}) O_3.

	Control				Elevated O_3			
	B. napus	Bt *B. napus*	BC_2F_2 hybrid	*B. rapa*	*B. napus*	Bt *B. napus*	BC_2F_2 hybrid	*B. rapa*
Plant height (cm)	118.5 ± 2.0a	119.1 ± 2.6a	97.6 ± 3.3b	111.1 ± 3.4a	122.8 ± 3.0a	126.6 ± 2.0a	97.7 ± 2.4b	101.0 ± 1.8b
Branch number	3.1 ± 0.2a	3.2 ± 0.2ab	4.3 ± 0.1c	3.8 ± 0.2bc	3.5 ± 0.2ab	3.4 ± 0.2a	4.0 ± 0.2b	3.8 ± 0.1ab
Stem DW (g)	4.61 ± 0.25ab	4.39 ± 0.22ab	3.85 ± 0.18b	4.73 ± 0.33a	4.17 ± 0.21a	4.02 ± 0.15a	3.10 ± 0.17b	3.14 ± 0.14b
Root DW (g)	0.83 ± 0.06a	0.84 ± 0.04a	0.61 ± 0.04b	0.78 ± 0.07ab	0.65 ± 0.05a	0.65 ± 0.03a	0.33 ± 0.03b	0.38 ± 0.03b
Root + stem DW (g)	5.44 ± 0.29a	5.23 ± 0.26ab	4.46 ± 0.22b	5.51 ± 0.36a	4.82 ± 0.25a	4.67 ± 0.18a	3.43 ± 0.19b	3.52 ± 0.17b
Total pod DW (g)	6.77 ± 0.37a	6.52 ± 0.39a	5.36 ± 0.26b	5.05 ± 0.29b	5.52 ± 0.24	5.47 ± 0.29	4.57 ± 0.36	4.90 ± 0.21
Total plant DW (g)	12.3 ± 0.66a	11.8 ± 0.63ab	9.81 ± 0.32b	10.6 ± 0.40ab	10.3 ± 0.46a	10.1 ± 0.45a	8.00 ± 0.36b	8.42 ± 0.29b
Seed DW (g)								
In main rachis	1.93 ± 0.09a	1.85 ± 0.08a	0.74 ± 0.07b	0.95 ± 0.11b	1.64 ± 0.09a	1.31 ± 0.05b	0.69 ± 0.08c	0.88 ± 0.05c
In branches	1.39 ± 0.15	1.20 ± 0.10	1.33 ± 0.15	1.04 ± 0.11	0.92 ± 0.07	1.12 ± 0.11	1.26 ± 0.15	1.27 ± 0.11
Total seed DW (g)	3.32 ± 0.16a	2.99 ± 0.17a	2.07 ± 0.15b	1.99 ± 0.18b	2.56 ± 0.13a	2.43 ± 0.14ab	1.95 ± 0.19b	2.15 ± 0.11ab
Germination (%)	84.7 ± 3.6a	87.5 ± 4.0a	40.0 ± 7.9b	10.6 ± 4.6c	81.2 ± 6.3a	85.6 ± 4.2a	16.3 ± 5.8b	7.1 ± 3.4b
1000-seed DW (g)								
In main rachis	4.21 ± 0.11a	4.13 ± 0.13a	2.25 ± 0.22b	2.25 ± 0.13b	3.98 ± 0.16a	4.33 ± 0.12a	2.33 ± 0.17b	2.01 ± 0.14b
In branches	4.04 ± 0.14a	3.69 ± 0.23a	2.39 ± 0.11b	2.42 ± 0.14b	3.62 ± 0.03a	4.20 ± 0.28a	2.09 ± 0.27b	2.12 ± 0.13b
Root: shoot ratio	18.0 ± 8.0ab	19.1 ± 5.0a	15.9 ± 9.0b	16.9 ± 1.4ab	15.4 ± 8.0a	16.2 ± 5.0a	10.7 ± 6.0b	12.0 ± 8.0b
Harvest index	27.4 ± 5.0a	25.4 ± 4.0a	21.1 ± 1.3b	19.3 ± 1.8b	25.1 ± 10.0	23.8 ± 5.0	23.6 ± 1.9	25.5 ± 0.9

Different letters indicate statistically significant ($P < 0.05$) difference between genotypes among control and elevated O_3 treatments.

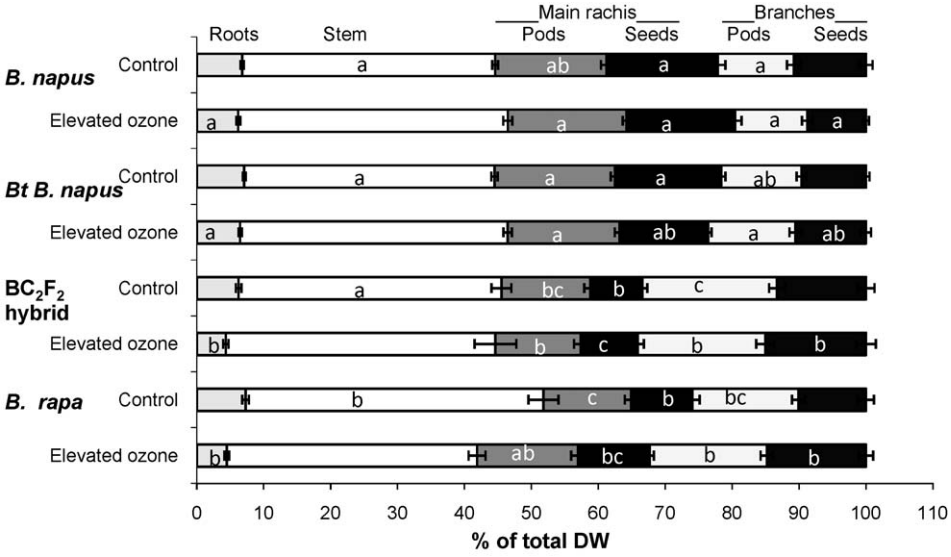

Fig. 2. Within-plant distribution of DW at harvest (mean % of total plant DW), for non-transgenic and Bt-transgenic *B. napus*, BC_2F_2 *B. rapa* × transgenic *B. napus* hybrid and wild *B. rapa* plants grown under control conditions (filtered air) or under elevated (100 ppb 8 h day^{-1}) O_3. Different letters in bars indicate statistically significant ($P < 0.05$) difference between genotypes among control and elevated O_3 treatments.

B. napus genotypes had a higher germination rate than hybrid and *B. rapa*, irrespective of O_3 treatment (Table 4). Hybrid plants had also higher germination than *B. rapa* in the control treatment. Elevated O_3 reduced the germination percentage for hybrid plant seeds only ($P_{O_3} < 0.05$). The 1000-seed weights were higher in the *B. napus* genotypes than in *B. rapa* and hybrid plants in main rachis and branch seeds, and the weights were not affected by elevated O_3 (Table 2).

Within-plant allocation responses

In the control treatment, hybrid plants had the lowest root:shoot (R:S) ratio, and Bt *B. napus* the highest, this difference being the only statistically significant one (Table 4). The R:S ratio was reduced by elevated O_3 in all genotypes (Table 2) and *B. napus* genotypes had higher R:S ratios than hybrid and *B. rapa* plants under elevated O_3. Harvest index (HI) was higher in *B. napus* genotypes than in hybrid and *B. rapa* plants in the control treatment. Under elevated O_3, HI was equal in all genotypes. Elevated O_3 increased HI solely for *B. rapa* ($P_{O_3} < 0.05$, Table 2, interaction gt × O_3).

The allocation of DW to roots was higher under elevated O_3 for *B. napus* genotypes than for BC_2F_2 hybrid and *B. rapa* (Fig. 2). Elevated O_3 reduced percentage DW in roots in all other genotypes except non-transgenic *B. napus* (Table 2, interaction gt × O_3). More biomass was allocated to stem DW in *B. rapa* than other genotypes in the control treatment. Elevated O_3 increased percentage allocation to stem for the *B. napus* genotypes, but reduced it for *B. rapa* (interaction gt × O_3). The allocation to DW of pods was higher towards branches in hybrid and *B. rapa* than *B. napus* genotypes under elevated O_3. In the control treatment, *B. napus* genotypes had a higher percentage allocation to seeds in main rachis than hybrid and *B. rapa*, whereas hybrid plants had the lowest allocation to main rachis seeds under elevated O_3. Percent biomass of seeds in branches was lower for non-Bt *B. napus* than for hybrid and *B. rapa* plants under elevated O_3. Elevated O_3 increased percentage allocation to branch seeds only in *B. rapa* ($P_{O_3} < 0.05$, interaction gt × O_3).

Root + stem DW correlated positively with total seed DW for non-Bt and Bt *B. napus* genotypes in the control treatment ($r = 0.879$, $P < 0.001$ and $r = 0.897$, $P < 0.001$, respectively), and for Bt plants the correlation was significant also under elevated O_3 ($r = 0.777$, $P < 0.001$). In contrast, seed DW was negatively correlated to stem + root DW for *B. rapa* plants in the control treatment ($r = -0.556$, $P = 0.011$) and a marginally statistically significant negative correlation was found for hybrid plants grown under elevated O_3 ($r = -0.432$, $P = 0.057$).

Discussion

Ozone tolerance of wild and cultivated *Brassica*s: implications for crop–weed population dynamics

The high percentage of O_3 lesions and reduced photosynthesis rates in wild *B. rapa* and transgene-carrying back-crossed hybrid plants suggested high O_3 sensitivity, which is typical for native species (Davison & Barnes 1998). *B. rapa* plants had earlier senescence under elevated O_3 than crop *B. napus* plants, and O_3-induced differences in phenology might reduce overlap of their flowering periods and, hence, cross-fertilization. Important from the aspect of reproduction was also the increased harvest index and higher percentage of total DW allocated to branch seeds, resem-

bling indeterminate growth, in *B. rapa* under elevated O_3. Interestingly, seed DW correlated positively with root + stem DW in the *B. napus* genotypes indicating better reproduction with higher vegetative biomass, whereas these were inversely related in *B. rapa*. Wild *B. rapa* seems to have a stronger assimilate sink in seeds that leads to a more pronounced within-plant trade-off and allocation to seed production than that in *B. napus*. This might benefit *B. rapa* population development under abiotic stresses such as elevated O_3. The transgenic back-crossed hybrid plants lacked such a high allocation as the parent *B. rapa* to reproduction, which might be attributable to interference with the *B. napus* genome (Halfhill et al. 2003).

Previously, Black et al. (2007) also found compensation for O_3 damage during the vegetative stage in *B. campestris* (syn. *B. rapa*); as a result, O_3-exposed plants produced a mature seed yield comparable to control plants. Similarly, Sutherland, Justinova, and Poppy (2006) reported a higher compensation for herbivore defoliation in wild *B. rapa* (increase in biomass) than in *B. rapa* × *B. napus* F_1 hybrid or *B. napus* plants. In nature, it is beneficial for a wild weedy species such as *B. rapa* to rely on a fast growth cycle, high seed number and relatively small seed size in a bet-hedging strategy to maximize possibilities of finding suitable habitats to invade and establish a seedbank in the soil (Moles & Westoby 2006). In contrast, human-bred *B. napus* produces a lower number of high-quality seeds (Zia et al. 2008), which can restrict their compensatory potential. Based on our results, the crop *B. napus* showed less foliar O_3 damage and therefore would have a higher chance of survival after high O_3 episodes, yet wild *B. rapa* had higher compensatory allocation to reproduction, which can enhance its persistence under O_3. This emphasizes the need for follow-up field studies on the ecological fitness and competitive dynamics (Monaghan, Metcalfe, & Torres 2009) of *Brassica* species from weedy origins and agricultural backgrounds in a natural O_3-enriched agroecosystem.

We also found reduced germination percentage for the back-crossed hybrid seeds under elevated O_3, which could be important for competitive abilities and invasiveness. Small seeds of weeds have been earlier suggested to be more vulnerable to environmental stresses (Harbur & Owen 2004) and hybrid plants typically have lower seed size than their parent types (Wei & Darmency 2008). The 1000-seed weight for the *B. napus* cultivar used here was approximately double that of *B. rapa* and hybrid seeds. Field tests to ascertain whether O_3 affects hybrid seed dormancy or actual viability should be performed.

Bt transgene interaction and abiotic stress effects on transgene flow

There were no observed trade-offs between transgenicity and reproductive ability in *B. napus*, as was reported earlier (Mason, Braun, Warwick, Zhu, & Stewart 2003). Lower main rachis seed DW in Bt plants compared to the non-Bt genotype under elevated O_3 did, however, suggest that there was either a temporal phenological or allocational difference, which was then balanced for in total seed DW. The growth and reproductive responses to elevated O_3 were comparable in Bt-transgenic and non-transgenic *B. napus* genotypes.

The fecundity of the Bt-transgenic *B. rapa* back-crossed hybrids was equal to *B. rapa* and lower than those of the *B. napus* genotypes in control conditions. The similarity of O_3 responses in wild *B. rapa* and the transgene-harbouring back-crosses revealed that the impact of phenotype properties exceeded the potential effects conferred by this particular transgene on O_3 responses. However, from an ecological perspective it could be meaningful that the reproductive outcome of the back-crossed hybrids was less affected by elevated O_3 than that of the *B. napus* parent plants. Thus, it is important to continue assessments for potential changes in transgene flow dynamics by elevated O_3. Assessment of whether differential O_3 sensitivity will render competitive advantage that might affect crop–weed population dynamics or the probability of unwanted exchange of genetic material between crop and wild plants would benefit from measuring comparative pollen release and synchronization of flowering in O_3-enriched field environments. Another important aspect to consider is the role of low germination rate of the back-crossed hybrid seeds (Wei & Darmency 2008) under O_3 stress that we observed here, which could serve to restrict transgene flow.

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