

Ethylene is required in tobacco to successfully compete with proximate neighbours

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ABSTRACT

Plants sense neighbours even before these cause a decrease in photosynthetic light availability. Light reflected by proximate neighbours signals a plant to adjust growth and development, in order to avoid suppression by neighbour plants. These phenotypic changes are known as the shade-avoidance syndrome and include enhanced shoot elongation and more upright-positioned leaves. In the present study it was shown that these shade-avoidance traits in tobacco (*Nicotiana tabacum*) are also induced by low concentrations of ethylene. Furthermore, it was shown that transgenic plants, insensitive to ethylene, have a delayed appearance of shade-avoidance traits. The increase in both leaf angles and stem elongation in response to neighbours are delayed in ethylene-insensitive plants. These data show that ethylene is an important component in the regulation of neighbour-induced, shade-avoidance responses. Consequently, ethylene-insensitive plants lose competition with wild-type neighbours, demonstrating that sensing of ethylene is required for a plant to successfully compete for light.

Key-words: *Nicotiana tabacum*; competition; ethylene-insensitivity; leaf angle; neighbour signalling; phytochrome; red/far-red ratio; shade avoidance; stem elongation.

INTRODUCTION

The phenotype of a plant is strongly influenced by the presence of neighbouring plants. When growing at high plant densities with competition for light, the predominant strategy of individual plants is to avoid shading by neighbours. Plants achieve this through so-called shade-avoidance responses that are already induced well before the canopy closes (Ballaré, Scopel, & Sánchez 1990). These responses include enhanced shoot elongation, elevated leaf angles to the horizontal (hyponasty) and early flowering (Smith 2000). As these shade-avoidance responses increase the amount of light that can be captured, they result in increased plant fitness in crowded conditions (Schmitt 1997). In this paper we will demonstrate a component of

the regulation of such important phenotypical responses of plants that has been essentially unknown to date.

Proximate neighbours are sensed in the early phase of canopy development through the perception of reflected far-red (FR) light from the surrounding vegetation (Ballaré 1999; Smith 2000) by the phytochrome family of photoreceptors (Neff, Fankhauser, & Chory 2000; Smith 2000). The phytochromes are encoded by a small gene family with five members (Phy A–E) in *Arabidopsis* (Quail *et al.* 1995; Whitelam, Patel, & Devlin 1998). Phy B is thought to be the prime phytochrome involved in shade-avoidance responses (Smith & Whitelam 1997) and homologues have been described for several other plant species. In addition to the red/far-red (R/FR) ratio, other signals, such as blue light perceived by cryptochromes, may also provide information about the presence of neighbours in a closed canopy (Ballaré, Scopel, & Sánchez 1991; Ballaré 1999) and result in shade-avoidance responses.

In an entirely different situation, namely submergence, responses are induced that very much resemble shade-avoidance responses. Enhanced shoot elongation and hyponasty are induced in flooded semi-aquatic plant species by the gaseous plant hormone ethylene (Voeselek & Blom 1989; Kende, Van der Knaap, & Cho 1998). Ethylene is involved in many plant responses to biotic and abiotic stimuli (Abeles, Morgan, & Saltveit 1992) and is generally known as a growth inhibitor, although it can also stimulate elongation of certain plant organs (Voeselek & Blom 1989; Emery, Reid, & Chinnappa 1994; Kende *et al.* 1998). The ethylene-induced shoot elongation and hyponasty enable flooded plants to re-establish contact of leaf blades with the atmosphere when they are submerged by flood water. The remarkable similarity between shade-avoidance traits and submergence-induced growth suggests a role for ethylene in shade avoidance as well. This idea is strengthened by the suggestion that the constitutive over-production of ethylene may be responsible for the constitutive shade-avoidance phenotype of *Sorghum* plants that contain a null mutation in the gene encoding phytochrome B (*phyB-1*) (Finlayson, Lee, & Morgan 1998; Finlayson *et al.* 1999).

In order to test the contention that ethylene is involved in plant responses to neighbours and thus determines competitive ability, we investigated whether treatment of tobacco plants with ethylene could induce shade-avoidance responses. Furthermore, competition experiments were

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carried out with wild-type ethylene-sensing and transgenic ethylene-insensitive tobacco plants, in which we determined morphological changes as well as competitive success for the two genotypes.

MATERIALS AND METHODS

Plant growth

Seeds of ethylene-insensitive (Tetr) and wild-type (WT) tobacco (*Nicotiana tabacum* cv. Samsun NN) were germinated on moist white river sand covered with transparent polyethylene sheets to prevent dehydration (16 h light $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, 8 h dark; 20°C). Tetr is a transgenic ethylene-insensitive tobacco genotype obtained through introduction of an *Arabidopsis thaliana* ETR1 allele with the dominant activating mutation *etr1-1* (Knoester *et al.* 1998). After 9 d (two cotyledon stage), seedlings were selected for equal sizes both within and between genotypes and transplanted to pots (5 cm high \times 5.5 cm diameter) or competition plots as described below containing white river sand. Plants received full strength Hoagland's nutrient solution every day to maintain a high nutrient status in the substrate, reducing the likelihood of competition for nutrients during the experiments.

Ethylene experiment

WT and Tetr seedlings were grown in pots in a growth chamber (16 h light $220 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Philips HDS 600 W; Philips, Eindhoven, The Netherlands), 8 h dark; 70% relative humidity, temperature = 20°C). When the plants were 5 weeks old they were transferred to closed glass chambers (35 dm^3) that were flushed continuously (0.5 L min^{-1}) with various concentrations of ethylene (Praxair; Oevel, Belgium) in air, which were checked with a gas chromatograph. In such way the depletion and build-up of gases was prevented during the experiment. Measurements of leaf angles were taken after 24 h and of stem lengths after 7 d of treatment.

Far-red experiment

WT and Tetr seedlings were grown in pots in a growth chamber (16 h light $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, 8 h dark; 20°C). The FR treatment started when plants were 31 d old by addition of FR-emitting incandescent lamps (Paulmann Blacklight, Wernhout, The Netherlands) to the white light background of fluorescent lamps (Philips TLD 36 W/840), lowering the red (655–665 nm)/far-red (725–735 nm) (R/FR) ratio from 9 to 0.2 without affecting PAR levels. Stem length measurements of WT and Tetr in high and low R/FR were taken at the start of the experiment and after 4, 6 and 8 d of treatment.

Competition experiments

Competition experiments were carried out in a greenhouse with supplemental lighting (Philips PL 600 W) to maintain

a 16 h photoperiod with a minimum of $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR (temperatures at least 20°C , relative humidity approximately 70%). Plants were grown at a density of $1111 \text{ plants m}^{-2}$; earlier trials revealed that competition was intense, such that after 8 weeks of growth this density decreased individual shoot biomass to approximately 10% of the biomass of individually grown plants and to 20% of the biomass of plants grown at a density of 70 plants m^{-2} . Some variation in growth rates occurred between experiments due to seasonal variability, but this did not affect the relative differences between treatments.

To establish a time series of plant growth (see Fig. 2a & b), WT and Tetr were grown in their respective monocultures in square plots of 9×9 plants. The central nine plants of one plot of each monoculture were harvested and leaf angles and stem length were measured every week between 4 and 8 weeks after sowing. In the following experiments, WT and Tetr plants were grown in plots of their respective

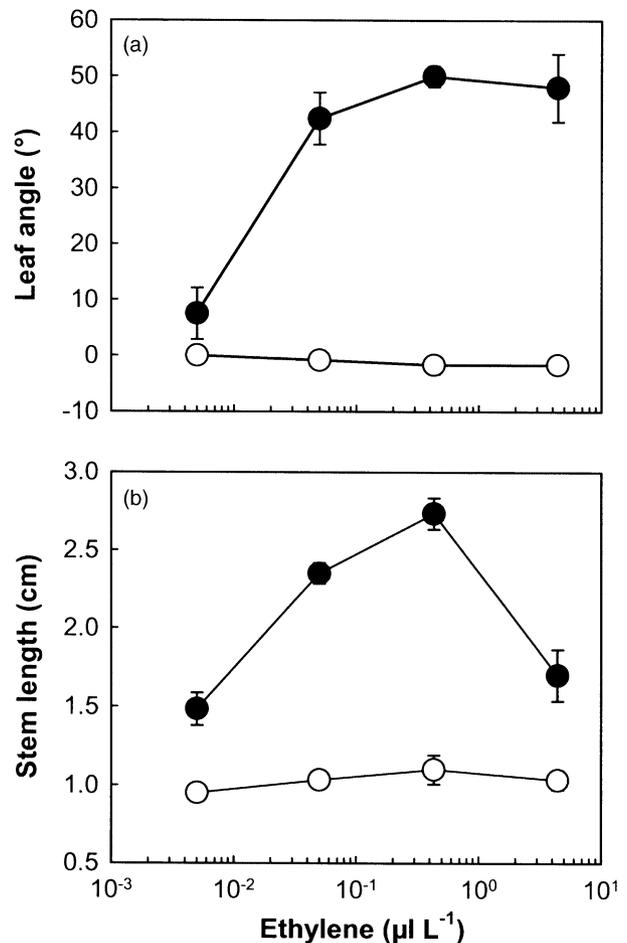


Figure 1. Inclination of the largest leaf (a), after 1 d of treatment and stem length (b), after 7 days of treatment of WT (black circles) and Tetr (open circles) tobacco at increasing exogenous ethylene concentrations. Experiments were started with 5-week-old plants ensuring that plants were beyond the rosette stage and thus stem formation had commenced. Data represent means of six plants \pm SE. Note that $0.005 \mu\text{L L}^{-1}$ ethylene is approximately the ambient atmospheric concentration.

monocultures and in 1 : 1 mixed plots following a checkerboard design. Plots of monocultures contained 9×9 plants, whereas mixed plots contained 10×10 plants. In all plots, the outer three rows of plants functioned to minimize possible edge effects. The central 9 (monocultures) or 16 (mixed cultures: 8 Tetr and 8 WT) plants of each plot were harvested and weighed 8 weeks after sowing (see Fig. 4a). In an additional experiment, plants were grown in pots for 5 weeks during the last three of which half of the Tetr plants received a 15 min day⁻¹ FR light treatment directly after the end of the photoperiod [end-of-day far-red (EODFR)], inducing a moderate shade-avoidance phenotype. Thereafter, WT and Tetr plants were transplanted to the mixed competition plots yielding two plot types: WT with EODFR-pretreated Tetr and WT with non-pretreated Tetr. Three replicates of each plot type were harvested and weighed after 14 d of competition, i.e. 8 weeks after sowing (see Fig. 4b).

RESULTS

Application of ethylene to the shoot of tobacco plants induced responses that show remarkable similarity with

classic shade-avoidance responses; increased stem elongation and leaf angles to the horizontal (Fig. 1). The highest ethylene concentration used appeared to be supra-optimal for stem elongation, but was still not inhibitory to it. Ethylene-insensitive (Tetr) plants appeared to be entirely unresponsive to ethylene, confirming their ethylene insensitivity.

When the two genotypes were grown at the high plant density of 1111 plants m⁻² leaf area indices (total leaf area/corresponding substrate area) were identical for WT and Tetr plots at all time points (data not shown), indicating that competition intensity and light quality and quantity were comparable in WT and Tetr monocultures. However, WT plants showed the so-called hyponastic response (e.g. increased leaf angles to the horizontal) already at approximately 5 weeks after sowing (Fig. 2a & c), whereas Tetr's morphology was not yet affected (Fig. 2a & d) at that time. One week later, Tetr plants also began the hyponastic response and at the end of the experiment the genotypes had reached similar leaf angles. In addition to the reduced hyponastic response, stem elongation was also retarded in Tetr in comparison with WT (Fig. 2b). At the first harvest (30 d after sowing) WT and Tetr were still in the rosette

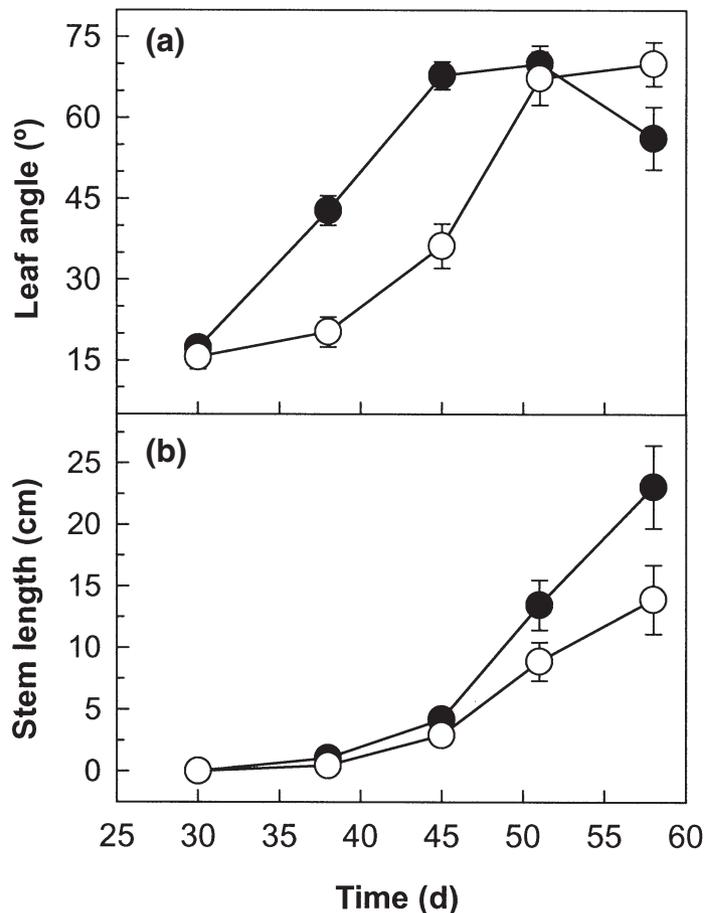


Figure 2. Time courses (in days after sowing) of (a), inclination of the largest leaf and (b), stem length of WT (black circles) and Tetr (open circles) tobacco in monocultures at 1111 plants m⁻². Data represent means of nine plants \pm SE. The photographs depict plants at the second harvest ($t = 38$ d) clearly showing the different leaf orientations of (c), WT and (d), Tetr.

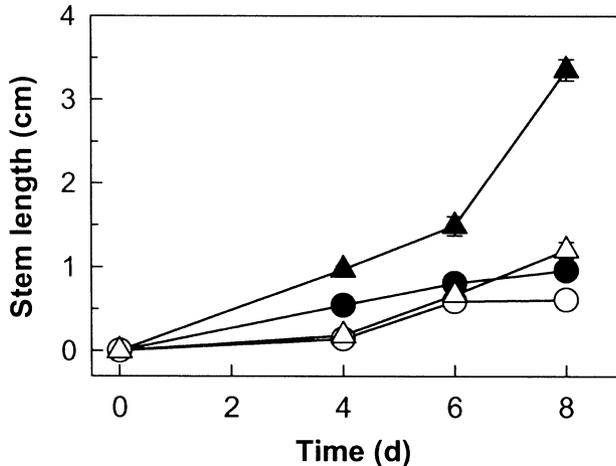


Figure 3. Stem elongation of WT (black symbols) and Tetr (open symbols) tobacco at high (circles) and low (triangles) red/far-red (R/FR) ratio. Light treatments were started ($t = 0$) with 31-day-old, pot-grown plants. Data represent means of six plants \pm SE.

stage and had similar sizes: shoot dry weight was 5.3 ± 0.7 mg for WT and 4.5 ± 0.3 mg for Tetr ($P = 0.257$; t -test) and leaf area was 1.78 ± 0.18 cm² for WT and 1.99 ± 0.17 cm² for Tetr ($P = 0.409$; t -test). When placed in a low R/FR environment, individually grown plants showed an elongation response similar to plants in the competition plots and again this response was reduced in the Tetr plants (Fig. 3).

To test the implications of Tetr's reduced shade-avoidance properties for its competitive ability, high-density mixtures of WT and Tetr were compared with high-density monocultures of the two genotypes. It appeared that the ethylene-insensitive plants did not suffer a stronger growth reduction than WT when both genotypes were grown in their respective monocultures (Fig. 4a). However, when Tetr had to compete with WT neighbours, the ethylene-insensitive plants were severely suppressed as shown by a five-fold lower shoot dry weight of Tetr plants compared to WT in the mixture (Fig. 4a) and the inability of Tetr to exploit the upper parts of the mixed canopy (Fig. 4c). Similar results were obtained with a different primary transformant line [Tetr20 (Knoester *et al.* 1998); data not shown] showing that Tetr's reduced competitiveness is not an artefact of the transformation process itself.

Finally, we checked whether this suppression of Tetr plants by WT neighbours was truly due to Tetr's delayed shade-avoidance properties. For this purpose, we conducted an experiment in which, before the onset of competition Tetr plants were pretreated with EODFR light for 3 weeks to induce a moderate shade-avoidance phenotype, thereby eliminating the delay in shade-induced phenotypic changes. The pretreatment resulted in an increase of the stem length of Tetr from 1.21 ± 0.09 to 1.90 ± 0.16 cm at the onset of competition, compared with WT plants with a stem length of 1.84 ± 0.18 cm (data are means \pm SE, $n = 15$). As a result, Tetr's competitive ability in a mixture with WT was

rescued completely; WT and EODFR-pretreated Tetr reached an equal biomass when competing with each other, whereas non-pretreated Tetr plants were strongly suppressed by WT neighbours (Fig. 4b).

DISCUSSION

Ethylene-insensitive Tetr plants lost competition for light with WT neighbours and our results show that this is related to their reduced shade-avoidance responses (i.e. a retarded vertical orientation of the leaves and reduced stem elongation). At this point it is important to note that this outcome is not due to an overall weak performance of the transgenic plants. Unlike, for instance, gibberellin mutants of *Arabidopsis* (e.g. *gal* and *gai*) that have a dwarf phenotype, Tetr plants do not have inherently reduced growth rates. Shoot biomass accumulation of Tetr is identical to WT when grown in high-density monocultures (Fig. 4a) and single-grown Tetr plants have relative growth rates identical to WT (unpublished results, D.J.H. Tholen, Utrecht University, The Netherlands). Although non-competing Tetr plants also seem to have a somewhat reduced stem elongation (Figs 1 & 3), competition experiments were started with young plants that were still in the rosette stage and, thus, had no stems yet. It is the retarded onset of elongation from this stage (Fig. 2b) that really mattered. The other important shade-avoidance trait, a more vertical orientation of the leaves, did not differ between single-grown plants of WT and Tetr (data not shown). In conclusion, it is unlikely that a factor other than the reduced shade-avoidance properties of the ethylene-insensitive plants was responsible for their reduced competitive ability. This is substantiated by the fact that we were able to fully rescue Tetr's competitive ability by eliminating the shade-avoidance delay (Fig. 4b).

Our results reveal ethylene as an important new element in the transduction pathways leading to the development of shade-avoidance traits. Increased stem elongation and leaf angles, were delayed but not entirely absent in ethylene-insensitive plants (Figs 2 & 3). Our data thus show that ethylene is not an indispensable component of shade-avoidance responses but rather acts as a positive modulator that accelerates these responses (Fig. 2). This is comparable to the function of ethylene as a positive modulator of leaf senescence in *Arabidopsis* (Grbic & Bleeker 1995).

The fact that individually grown Tetr plants showed a reduced stem elongation response to a low R/FR ratio (Fig. 3) suggests a role for ethylene in phytochrome mediated shoot elongation. Here we show for the first time directly that ethylene is involved in shade-avoidance responses to neighbours (Fig. 2) and to low R/FR ratios (Fig. 3), consistent with the correlative data of Finlayson *et al.* (1998, 1999). There are numerous possible sites for ethylene to interact in the regulation of shade-avoidance processes. Research to date has shown that photomorphogenic responses are complex webs of cross-talking signalling pathways of various photoreceptors (Ballaré 1999; Briggs & Olney 2001; Chory & Wu 2001) that include the action of a number of plant hormones, such as gibberellins,

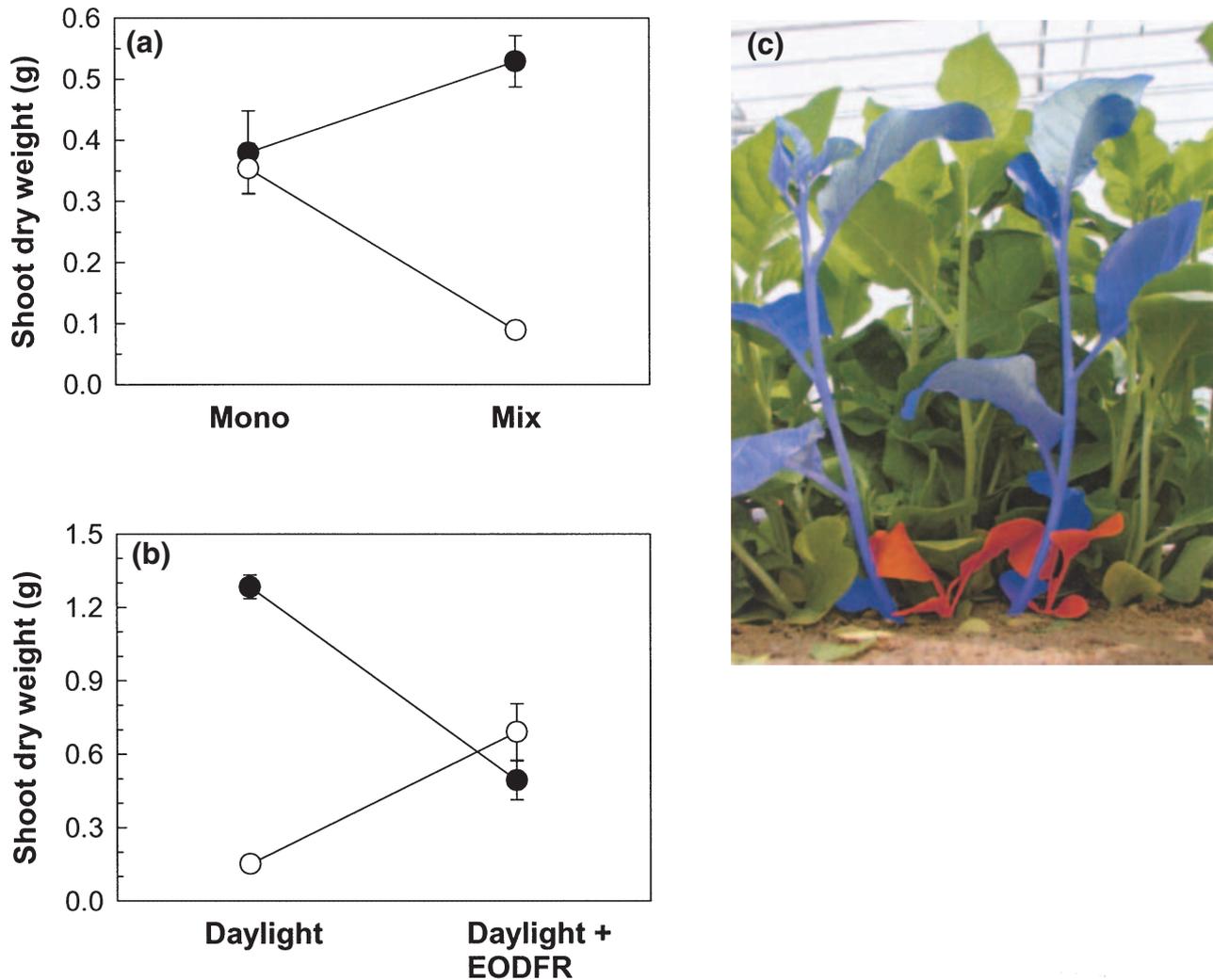


Figure 4. Performance of WT (black circles or purple plants) and Tetr (open circles or orange plants) tobacco at 1111 plants m^{-2} . (a), Plants were placed in monoculture or 1 : 1 mixture approximately 1 week after germination and grown in these competition plots for an additional 7 weeks. Data represent means of five plot means \pm SE. (b), Plants were placed in a 1 : 1 mixture of WT and Tetr or WT and EODFR-pretreated Tetr approximately 6 weeks after germination (and thus 3 weeks after the start of the pretreatments) and grown in these competition plots for an additional 2 weeks. Data represent means of three plot means \pm SE. (c), Depicts WT (purple) and Tetr plants (orange) in the non-pretreated mixture at the time of harvest. These target plants were digitally false-coloured to clearly discriminate WT and Tetr. These plants, which were in the centre of the mixed competition plot, were representative of the entire canopy.

auxins and brassinosteroids (Chory & Li 1997; Morelli & Ruberti 2000; Neff *et al.* 2000). Ethylene can interact with any of these hormones (Abeles *et al.* 1992) as well as with the phytochrome system itself (Vangronsveld, Clijsters, & Van Poucke 1988; Finlayson *et al.* 1999). In conjunction with this modulating effect, ethylene may accumulate in a dense canopy as has been described once in the literature for canopies of cotton (Heilman, Meredith, & Gonzalez 1971). As such, ethylene may serve as a previously unknown environmental signal parallel to the well-established light signals (e.g. R/FR ratio, blue light). Since ethylene production rates are generally low and diffusion rates to the atmosphere will be high, high levels of ethylene accumulation are not to be expected. Still, elongation processes may be very sensitive to ethylene (Fiorani *et al.* 2002)

and low levels of ethylene are already sufficient to stimulate shade-avoidance responses (Fig. 1).

As a consequence of their disturbed shade-avoidance responses, the ethylene-insensitive plants suffered a severely reduced competitive ability when competing with WT neighbours (Fig. 4). In their crowded monoculture, however, Tetr plants did surprisingly well. This was probably due to the fact that all neighbouring plants suffered the same disruption contrary to the mixture where WT plants were able to profit from Tetr's delayed response. When these differences in shade-avoidance capacities were eliminated in the mixtures by the FR pretreatment of Tetr, WT and Tetr plants reached equal dry weights again (Fig. 4b). Such an increase of gain in dry weight upon restoration of shade-avoidance responses is in accordance with data from

others, where the degree to which plants can display shade-avoidance responses is closely linked to the ability to compete for light (Schmitt, McCormac, & Smith 1995; Dudley & Schmitt 1996).

We conclude that ethylene can induce shade-avoidance traits in tobacco, whereas the lack of ethylene perception in transgenic, ethylene-insensitive plants leads to an attenuated shade-avoidance reaction. As a consequence, these Tetr plants lose competition for light with WT neighbours. Our results thus demonstrate that ethylene sensing is an important, yet previously unappreciated requirement for successful competition for light.

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