Plant–plant interactions mediate the plastic and genotypic response of *Plantago asiatica* to CO_2: an experiment with plant populations from naturally high CO_2 areas

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- **Background and Aims** The rising atmospheric CO_2 concentration ($[CO_2]$) is a ubiquitous selective force that may strongly impact species distribution and vegetation functioning. Plant–plant interactions could mediate the trajectory of vegetation responses to elevated $[CO_2]$, because some plants may benefit more from $[CO_2]$ elevation than others. The relative contribution of plastic (within the plant's lifetime) and genotypic (over several generations) responses to elevated $[CO_2]$ on plant performance was investigated and how these patterns are modified by plant–plant interactions was analysed.
- **Methods** *Plantago asiatica* seeds originating from natural CO_2 springs and from ambient $[CO_2]$ sites were grown in mono stands of each one of the two origins as well as mixtures of both origins. In total, 1944 plants were grown in $[CO_2]$-controlled walk-in climate rooms, under a $[CO_2]$ of 270, 450 and 750 ppm. A model was used for upscaling from leaf to whole-plant photosynthesis and for quantifying the influence of plastic and genotypic responses.
- **Key Results** It was shown that changes in canopy photosynthesis, specific leaf area (SLA) and stomatal conductance in response to changes in growth $[CO_2]$ were mainly determined by plastic and not by genotypic responses. We further found that plants originating from high $[CO_2]$ habitats performed better in terms of whole-plant photosynthesis, biomass and leaf area, than those from ambient $[CO_2]$ habitats at elevated $[CO_2]$ only when both genotypes competed. Similarly, plants from ambient $[CO_2]$ habitats performed better at low $[CO_2]$, also only when both genotypes competed. No difference in performance was found in mono stands.
- **Conclusion** The results indicate that natural selection under increasing $[CO_2]$ will be mainly driven by competitive interactions. This supports the notion that plant–plant interactions have an important influence on future vegetation functioning and species distribution. Furthermore, plant performance was mainly driven by plastic and not by genotypic responses to changes in atmospheric $[CO_2]$.

Key words: Canopy functioning, climate change, competition, elevated CO_2, evolutionary response, game theory, genotypic response, naturally high CO_2, Plantago asiatica L., plant–plant interactions, plastic response, selection pressure.

INTRODUCTION

Since the industrial revolution, there has been a rapid increase in atmospheric CO_2 concentration ($[CO_2]$), and it is predicted to increase further in the future. Understanding how plants respond to elevated $[CO_2]$ is important, due to their central role in ecological and production processes and because plants have a large influence on the local climate itself. The latter is, among others, because plants have a negative feedback on the atmospheric $[CO_2]$, as they take up $[CO_2]$ (Bonan, 2008). Hence, changes in $[CO_2]$ entail a global selective force due to their ubiquitous nature and importance for plant responses. Furthermore, plant–plant interactions may influence plant responses to elevated $[CO_2]$ as some plants may benefit more from rising $[CO_2]$ than others, and their traits therefore determine vegetation functioning. For example, C_3 species may gain a competitive advantage over C_4 species at elevated $[CO_2]$ as C_3 species benefit more from elevated $[CO_2]$ (Ziska, 2011), and this could lead to shifts in plant distribution patterns. So, it is important to know how plants respond to changes in the climate, and how this is potentially modified by plant–plant interactions.

When plants are placed in elevated $[CO_2]$, they respond immediately (instantaneous response hereafter, on the time scale of seconds to days), for example by increasing their assimilation rate (Ainsworth and Long, 2005). On a time scale of days to weeks of exposure to elevated $[CO_2]$, plants will respond plastically (response within a plant's lifetime), e.g. through reducing their leaf N content or changing their leaf area (Ainsworth and Long, 2005). However, it is still unclear what the magnitude of these responses is, and how this impacts on whole-stand characteristics. For instance, leaf area responses to

elevated [CO$_2$] and their effect on whole-stand leaf area index (LAI; leaf area per unit ground area) were found to differ between plant functional types. Increases in LAI were reported for young trees (Norby and Zak, 2011), while crops and grasses exhibited little change in LAI (Hirose *et al.*, 1997; Anten *et al.*, 2004; Ainsworth and Long, 2005). Furthermore, most of the experiments that investigated plant responses to elevated [CO$_2$] ran for a maximum of 10 years, while only a few studies investigated this on a longer time scale. One study investigated plastic responses within the life span of ferns, broadleaved trees and conifers in Florida that were probably associated with the last 150 years increase in [CO$_2$] (Lammertsma *et al.*, 2011), and found altered stomatal densities and reduced maximum pore size of the stomatal opening to reduce stomatal conductance (De Boer *et al.*, 2011).

Genotypic responses of plant populations to elevated [CO$_2$], which take place over several generations, can be studied with plants from natural CO$_2$ springs. In these natural elevated CO$_2$ areas, the atmospheric [CO$_2$] has probably been elevated for hundreds of years (Körner and Miglietta, 1994; Onoda *et al.*, 2007). The functioning of plants from these areas can be compared with those from ambient [CO$_2$] (Cook *et al.*, 1998; Vodnik *et al.*, 2002; Onoda *et al.*, 2007). Some studies (e.g. Fordham *et al.*, 1997; Nakamura *et al.*, 2011) show that plants originating from natural CO$_2$ springs have a higher relative growth rate compared with plants originating from ambient [CO$_2$] when grown at elevated [CO$_2$].

However, the few experimental studies investigating plant genotypic responses to elevated [CO$_2$] did not clearly investigate the potential mediating effect of plant–plant interactions. Plants often grow in dense communities where they share resources (e.g. light, water and utrients) with their neighbouring plants, and therefore there is a strong selective force on competition for those resources (Aerts, 1999). Furthermore, as noted above, it is likely that due to plant–plant interactions the species composition will change, because some plant species may benefit more than others from the rise of [CO$_2$] (Owensby *et al.*, 1999; Ziska, 2011). Those 'winning' plant species in turn will determine vegetation functioning. This indicates that plant–plant interactions may strongly mediate the trajectory of vegetation responses to elevated [CO$_2$]. For instance, a study that included competition through natural selection (i.e. game theoretical principles) accurately predicted the LAI, net photosynthesis and stomatal conductance of soybean stands grown at elevated [CO$_2$] obtained in free air carbon dioxide enrichment (FACE) experiments, and this prediction was much better than a model that did not consider these interactions (Van Loon *et al.*, 2014, 2015). However, the role of plant–plant interactions in mediating vegetation responses to elevated [CO$_2$] is still poorly understood (Bazzaz and McConnaughay, 1992; Song *et al.*, 2009). This is at least in part because an increase in atmospheric [CO$_2$] increases plant growth and thereby probably increases the competition for light and soil nutrients between plants.

The main aim of our study is therefore to determine the relative contribution of plastic vs. genotypic responses to elevated [CO$_2$] on plant performance, and to determine the extent to which these patterns are modified by plant–plant interactions. To investigate this, we use *Plantago asiatica* L., a perennial rosette herb. It was used in our experiment as it is known to adapt

to a wide range of environments by genetic differentiation. This makes it particularly suitable for testing adaptation to increased [CO$_2$] (Onoda *et al.*, 2009). The seeds originate from populations growing near natural CO$_2$ springs where elevated CO$_2$ levels have existed for hundreds of years (Onoda *et al.*, 2007), and from populations in nearby control areas with ambient [CO$_2$]. These are hereafter called spring and non-spring plants, respectively. Mono stands and mixed stands of either or both spring and non-spring plants, respectively, were created, to test whether differences in responses also lead to a higher performance. Plants were grown in walk-in growth chambers with low, intermediate and high [CO$_2$] (270, 450 and 750 ppm [CO$_2$], respectively; this is their growth [CO$_2$]) (Fig. 1). During the experiment, we measured leaf photosynthetic traits, plant dry weight, leaf area and total leaf N content. A model was used for scaling up from leaf to canopy photosynthetic traits to study the effect of the different treatments on canopy level and, secondly, to estimate the relative contribution of instantaneous responses, plastic responses within the lifetime of the plant and genotypic responses over several generations to elevated [CO$_2$] independently from competition.

We expect that plants respond plastically to elevated growth [CO$_2$] through partial closure of the stomata (Long *et al.*, 2004), increase of the rate of photosynthesis (Ainsworth and Long, 2005) and reduction of leaf N content per unit area (Ainsworth and Long, 2005). Furthermore, we expect that the genotypic response of plants to elevated growth [CO$_2$] will depend on the effect of plant–plant interactions. We hypothesize two possible outcomes. The first one assumes that regardless of plant–plant interactions (between or within populations), spring plants will do better at high [CO$_2$] and non-spring plants at low [CO$_2$]. The second possible outcome, with regard to the genotypic response of plants to elevated [CO$_2$], is that plants that would succeed under changing [CO$_2$] are not those that intrinsically perform best, but rather those that are the best competitors under those circumstances. This expectation is based on game theoretical principles, as, according to this, selection under competition tends to result in being a better competitor (i.e. selection for traits that maximize an individual plant's carbon gain) rather than having the highest performance (i.e. selection for traits that maximize carbon gain of the whole stand of plants) (Vermeulen *et al.*, 2013). So, this hypothesis predicts that the performance advantages of spring plants at high [CO$_2$] or non-spring plants at low [CO$_2$] will be apparent only in the mixed stands and not in the mono stands.

MATERIALS AND METHODS

Plant material and growth conditions

We used *Plantago asiatica*, a small (0·1–0·3 m) perennial rosette plant, predominantly self-pollinating species (Huh, 2013) with very limited pollen dispersal (0·1–0·4 m) (Onoda *et al.*, 2009). The seeds originate from three independent natural CO$_2$ springs in Japan: Asahi (38·2°N, 140·0°E, 540 m a.s.l., between 2123 and 2509 ppm in the elevated [CO$_2$] area), Kosaka (40·4°N, 140·8°E, 450 m a.s.l., between 503 and 7019 ppm in the elevated [CO$_2$] area) and Yuno-Kawa (40·7°N, 140·9°E, 560 m a.s.l., between 500 and 5339 ppm in the elevated [CO$_2$] area). In these CO$_2$ springs, most of the CO$_2$ is emitted from
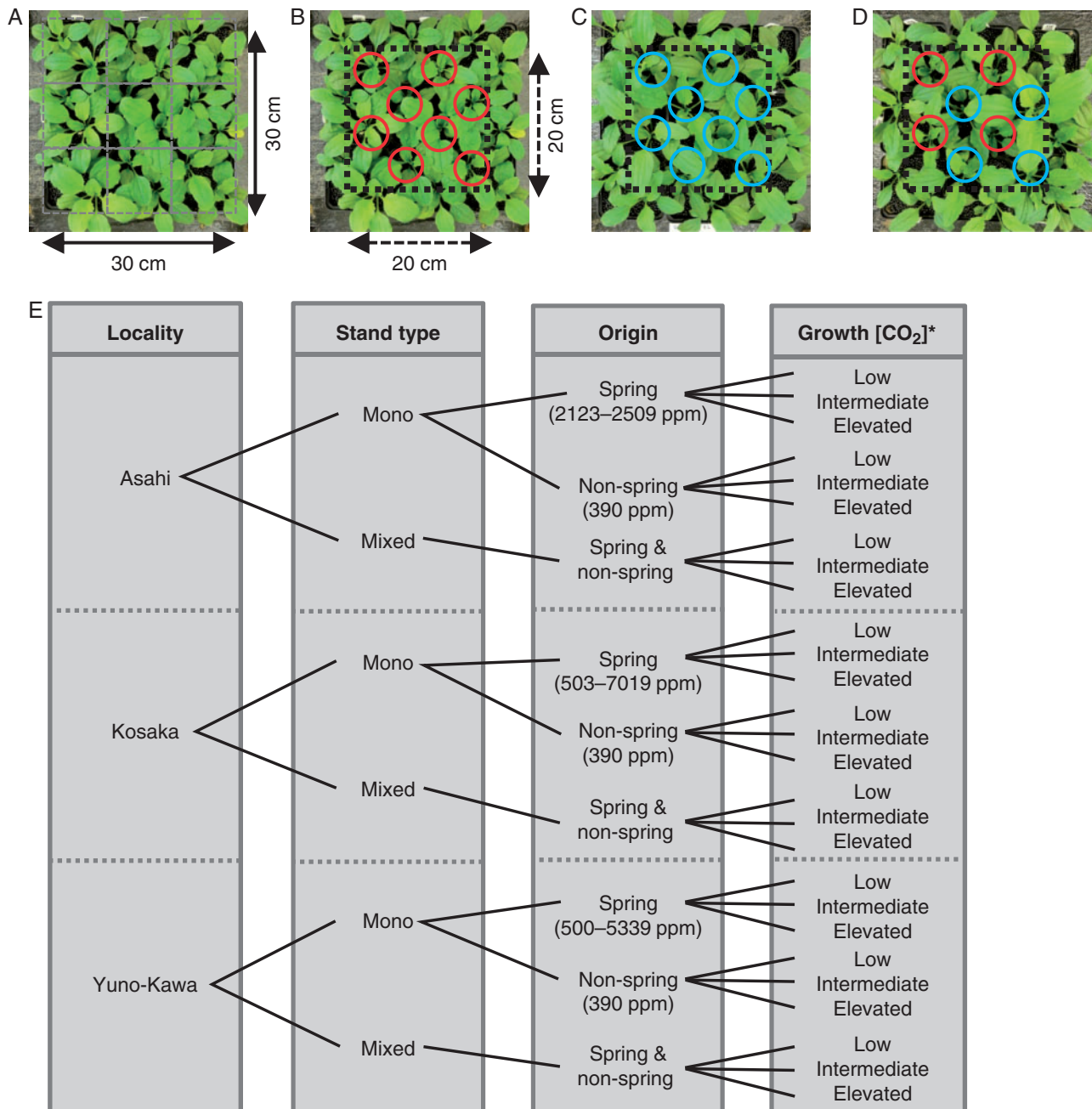
FIG. 1. Experimental set-up. Stands were established by putting three by three pots together; the total size of a stand is 30 × 30 cm (A). Stands could be a mono stand of either only spring plants (originating from a natural CO$_2$ spring spring) (B) or non-spring plants (originating from the control area) (C), or a mixed stand with both spring and non-spring plants in one stand (D). The plants within the area of the dashed square (20 × 20 cm) were used for measurements; these are the eight middle plants of a stand (B). Plants were grown under different CO$_2$ levels; this set-up was repeated for different localities. Photos (A–D) of the experiment were taken on 19 December 2013, 55 d after sowing. The different combinations of factors and their levels which were used in the experiment are shown in (E). *Low, intermediate and elevated growth [CO$_2$] are 270, 450 and 750 ppm, respectively.

spring water and not from the soil. We chose three springs instead of a single CO$_2$ spring, to have more replications and to test if plant responses to elevated [CO$_2$] occur independently of the environmental factors from their original habitats (Osada *et al.*, 2010). Furthermore, we chose these three springs, because these springs did not emit toxic gasses. The springs also did not have a significant effect on the air temperature; and the [CO$_2$] around these springs has been constantly high for

hundreds of years (Onoda *et al.*, 2007). Seeds from ambient [CO$_2$] were collected from plants in nearby areas (non-spring populations). In each location spring and non-spring populations were shown to be genetically distinct (Nakamura *et al.*, 2011). In the present study, offspring of each population (i.e. from each elevated and ambient CO$_2$ site) were used for experiments to avoid maternal effects (i.e. the effects of environment of parent generation). To obtain offspring of each population,

seeds obtained from field populations were separately sown in a common garden of Tohoku University (38·4°N, 140·6°E, Japan) in May 2010, and cultivated for 2 years. From July to September 2010, individuals germinated from seeds of the same population were mated. From October to December 2010 and 2011, offspring seeds were harvested.

Seeds originating from these three CO_2 springs (spring plants) and from their respective control areas (non-spring plants) were sown on 25 October 2013 on sandy soil. On 4 November 2013 the seedlings were transplanted to square (0·1 × 0·1 m) 0·15 L pots filled with sandy soil and 0·7 g of slow release fertilizer (N-P-K 16:9:12). Two plants were grown in each pot. Plants were grown in walk-in growth chambers (Reftech custom made, 20 °C, 70 % relative humidity, 10 h light at 400 µmol m⁻² s⁻¹) with three different CO_2 levels: 270 ppm (low [CO_2]), 450 ppm (intermediate [CO_2]) and 750 ppm (elevated [CO_2]). The low [CO_2] (270 ppm) chamber was about equal to the pre-industrial levels that have more or less persisted for several thousand years until about the 1800s. The intermediate level indicates the [CO_2] that can be expected within the next 1–2 decades, and the elevated [CO_2] is the level that is predicted for the end of this century. Plants were placed on an automatically watered irrigation mat.

Stands were established immediately after the seedlings were transplanted by putting three by three pots together for a total of nine pots and 18 plants (total stand size is 0·3 × 0·3 m; Fig. 1A), the eight middle plants of each stand were used for measurements (total area for the measurements in a stand is 0·2 × 0·2 m; Fig. 1B). Stands could be a mono stand of either only spring plants (Fig. 1B) or non-spring plants (Fig. 1C), or a mixed stand with both spring and non-spring plants with one of both in each pot (Fig. 1D). With this set-up, there is competition for both above- and below-ground resources. However, plants only interacted with a single other plant below-ground, but with 17 plants above-ground; therefore, competition was probably stronger above- than below-ground. In addition, there was ample availability of water and nutrients, while the light levels were relatively low (i.e. similar to a moderately overcast day), which also suggests that competition was predominantly above-ground. This set-up was repeated for all three localities. For each combination, there were four replicate stands (Fig. 1E). Thus, in total, 1944 plants were grown from which 864 plants were used for measurements, as stands of 18 plants were established from which eight plants were used for measurements for a total of 27 different combinations of factors which were replicated four times.

Gas exchange measurements

From 23 December 2013 (59 days after sowing) until 14 January 2014, photosynthetic CO_2 response curves were made using a portable open gas exchange system (LI-6400, LiCor) on attached fully expanded leaves. The system was equipped with an LED light source (LI-6400-02B, LiCor), which provided both red and blue light (the latter is important in controlling stomatal aperture). From every stand, one young fully expanded leaf was measured and, in the case of a mixed stand two leaves were measured (one of a spring plant and one of a non-spring plant). During the measurements, the leaf temperature was kept

at 25 °C, photon flux density was 1500 µmol m⁻² s⁻¹ and vapour pressure deficit was <2 kPa. For each leaf the photosynthesis was determined at a [CO_2] of (i.e. the order is the sequence of measuring): 500, 270, 150, 90, 50, 270, 500, 500, 700, 900, 1100 and 1500 ppm.

The CO_2 response curves were fitted with the biochemical model of Farquhar *et al.* (1980). Below an intercellular [CO_2] (C_i) of 300 ppm, the curve was fitted to the ribulose bisphosphate (RuBP) carboxylation-limited photosynthesis rate (P_{cl}):

$$P_{cl} = V_{cmax}[(C_i - \Gamma^*)/(C_i + K_m)] - R_d; K_m = K_c[1 + (O/K_o)]$$

where V_{cmax} is the maximum carboxylation rate, Γ^* is the [CO_2] compensation point in the absence of mitochondrial respiration, K_c and K_o are the Michaelis–Menten constants for carboxylation and oxygenation, respectively, and O is the oxygen concentration. For K_c, K_o and O, we assume a value of 404·9 µmol mol⁻¹, 278·4 mmol mol⁻¹ and 205 mmol mol⁻¹, respectively (Ishikawa *et al.*, 2007; for *P. asiatica*). R_d is the respiration rate in the light and was assumed to be 10 % of V_{cmax} (Von Caemmerer, 2000).

Above an intercellular [CO_2] of 300 ppm, the CO_2 response curve was fitted to the RuBP regeneration-limited photosynthesis rate (P_{jl}):

$$P_{jl} = J_{max}[(C_i - \Gamma^*)/(4C_i + 8 \Gamma^*)] - R_d$$

where J_{max} is the maximum electron transport rate.

From the outcomes of the CO_2 response curves, calculation of the gas exchange measurements taken under the growth CO_2 (i.e. 270, 450 and 750 ppm) could be carried out.

Canopy characteristics

Measurements of canopy structure, leaf area and biomass were done from 27 until 29 January 2014 (94–96 d after sowing). At this time, the stands had formed a closed canopy and there was clear mutual shading among neighbouring plants (see also pictures of the stands in Fig. 1A–C taken on 19 December 2013, >1 month before harvest). The height of each plant was measured from ground surface to the apex of the topmost leaf of all plants in the centre of the blocks (eight per stand, see Fig. 1A–D). Leaf vertical inclination angles were measured with a protractor and then categorized in three leaf angle classes (0–30°, 30–60° and 60–90°), which were used as input in the model (see below and also Goudriaan, 1988). The shoots (i.e. in this rosette plant consisting of only leaves) were then cut at ground level and leaf area was determined with a LI3100 leaf area meter (LICOR, Lincoln, NE, USA). Leaves were dried at 70 °C for 3 d, after which the dry weight per plant was determined. The leaf organic nitrogen content per plant was determined with a continuous flow analyser (SKALAR, The Netherlands) after Kjeldahl digestion.

Statistical analysis

Statistical analysis was performed using SPSS (version 21.0.0.1). A generalized linear model was applied to test the

TABLE 1. *Three types of responses to elevated [CO$_2$] that act on different time scales (a–c); these are modelled by subtracting different types of model runs (1–4). With the model, the canopy photosynthesis rate is calculated for a certain growth [CO$_2$] using the specific trait values (plant height, leaf angle, slope parameter of the stomatal sensitivity function, plant leaf N content and the leaf area index) to make the distinction between the three different responses to elevated [CO$_2$]. NSpr are non-spring plants originating from ambient [CO$_2$] areas and Spr are spring plants originating from naturally elevated [CO$_2$] areas*

Time scale of response				In the model	
	Type of response		Time exposed to elevated [CO$_2$]	Growth [CO$_2$]	Traits
		2	Seconds, transferred from low to elevated [CO$_2$]	Elevated	Low [CO$_2$]: NSpr
		1	None, whole lifetime in low [CO$_2$]	Low	Low [CO$_2$]: NSpr
(*a*) Immediate (seconds to days)	Instantaneous		2 – 1		
		3	Lifetime exposure to elevated [CO$_2$]	Elevated	Elevated [CO$_2$]: NSpr
		2	Seconds, transferred from low to elevated [CO$_2$]	Elevated	Low [CO$_2$]: NSpr
(*b*) Within lifetime of the plant (days to weeks)	Plastic		3 – 2		
		4	Several generations exposure to elevated [CO$_2$]	Elevated	Elevated [CO$_2$]: Spr
		3	Lifetime exposure to elevated [CO$_2$]	Elevated	Elevated [CO$_2$]: NSpr
(*c*) Several generations (evolutionary time scale)	Genotypic		4 – 3		

effects of growth [CO$_2$] (270, 450 and 750 ppm [CO$_2$]), origin (spring, non-spring) and stand type (mono stand, mixed stand), all as fixed factors. Location (Asahi, Kosaka and Yuno-Kawa) was treated as a random factor. A four-way analysis of vaiance (ANOVA) was used to test for significant differences. The average of a stand (average of eight spring or non-spring plants in a mono stand, the average of either the four spring or the non-spring plants in a mixed stand) was used for statistical analysis. For photosynthesis rate, stomatal conductance and leaf N, we measured one leaf in every mono stand and two leaves for every mixed stand (one of a spring plant and one of a non-spring plant).

Model analysis

Measured leaf photosynthetic traits were scaled up to canopy photosynthetic traits by using the canopy model of Van Loon *et al.* (2014). In short, this model calculates the light distribution within the canopy, following the approach of Spitters *et al.* (1986) that distinguishes between the distribution of a direct solar beam and diffuse radiation. The canopy photosynthesis model (Farquhar *et al.*, 1980) is a function of light (Spitters *et al.*, 1986), nitrogen (logarithmic relationship for leaf nitrogen vs. photosynthetic capacity), temperature (Johnson *et al.*, 1942; Farquhar *et al.*, 1980), [CO$_2$] and water (Tuzet *et al.*, 2003). Furthermore, this canopy model is based on steady-state assumptions of water transport (Sterck and Schieving, 2011) and of [CO$_2$] inflow and consumption. These steady-state assumptions were solved by using plant-specific parameters (Supplementary Data Table S1), and the measured climatic conditions of the growth chambers with their experimental constraints (total canopy leaf N content, water availability, incident light, temperature, atmospheric [CO$_2$]; Table S1). Details can be found in Van Loon *et al.* (2014).

The canopy photosynthesis model was used for two purposes. First, the model was used for scaling up from leaf to canopy photosynthetic traits to study the effect of the different treatments at the canopy level. This allowed us to assess effects of [CO$_2$], spring vs. non-spring and competition on whole-plant carbon gain. Secondly, the model was used to estimate the

relative contribution of three different types of responses to elevated [CO$_2$] on the canopy photosynthesis rate independently from competition. For this objective we therefore only conducted model calculations for the mono stands. The distinction between the three types of responses is based on the time scale: (*a*) instantaneous – immediate, time scale of seconds to days, which assesses what the effect of [CO$_2$] elevation would have been if plants would not have exhibited any trait changes; (*b*) plastic – within a plant's lifetime, days to weeks; and (*c*) genotypic – over several generations, evolutionary time scale (Table 1). We will further explain these responses and how they are modelled.

Instantaneous response. The instantaneous response is the difference in canopy photosynthesis rate between plants exposed to elevated [CO$_2$] for a short time because they are transferred from low to elevated [CO$_2$], and plants never exposed to elevated [CO$_2$] and that thus grew their whole life at low [CO$_2$]. In the model this is implemented by calculating the canopy photosynthetic rate of non-spring plants at elevated [CO$_2$], with the plants being given the same traits as measured from the non-spring plants growing in low [CO$_2$] (i.e. virtually transferring non-spring plants from low to elevated [CO$_2$]) (Table 1, model run 2) and from this subtracting the calculated canopy photosynthetic rate of non-spring plants grown in low [CO$_2$] (Table 1, model run 1).

Plastic response. The plastic response is the difference in canopy photosynthetic rate between plants that grew their whole lifetime at elevated [CO$_2$], and plants that have been exposed to elevated [CO$_2$] for a short time because they are transferred from low to elevated [CO$_2$]. In the model this is done by calculating the canopy photosynthetic rate of non-spring plants at elevated [CO$_2$] with their trait values as measured under those conditions (Table 1, model run 3) and subtracting the calculated canopy photosynthetic rate of the virtually transferred plants from low to elevated [CO$_2$] (Table 1, model run 2) from this.

Genotypic response. This response is the difference in canopy photosynthesis rate between plants that have existed for several generations under elevated [CO$_2$], and plants that grew their whole lifetime at elevated [CO$_2$]. In the model this is done by

taking the difference between the canopy photosynthetic rate of spring and non-spring plants grown at elevated [CO$_2$] with their trait values as measured under those conditions (Table 1, model run 4 and 3, respectively).

The traits that are changed for the model simulations to make the distinctions between the three different responses to elevated [CO$_2$] are plant height (Supplementary Data Fig. S1A), leaf angle determining the extinction coefficient (Supplementary Data Fig. S1B–D), the slope parameter of the stomatal sensitivity function (a_ψ), plant leaf N content and the LAI [the leaf area of a plant divided by the ground area (in this case per plant half of the ground area of a pot)]. We defined LAI of one individual to distinguish between the LAI of spring and non-spring plants in mixed stands. Changes in a_ψ per locality, origin and stand type were estimated based on measurements of the stomatal conductance at growth [CO$_2$]. We calculated the average stomatal conductance per growth CO$_2$ level and compared this value with the stomatal conductance for a specific locality, origin and stand type. If this value was 10 % higher than the average, then a_ψ was also increased by 10 % for this specific locality, origin and stand type. The a_ψ values can be found in Supplementary Data Table S2. Other parameter input values were kept constant for all simulations (Table S1, Supplementary Figs S2 and S3). For each treatment in the model, we separately used the parameter values as measured for that treatment for each replicate plant from each of the three localities (i.e. Asahi, Kosaka and Yuno-Kawa). So, the model outcome of one treatment is the average of 12 model runs.

RESULTS

Results of growth chamber experiment

There was no plant mortality observed. Further, no significant difference was found among the three localities (Asahi, Kosaka and Yuno-Kawa) for all measured traits, except for stomatal conductance (see further below).

Lumped together for locality, origin and stand type, leaf area significantly increased with increase in growth [CO$_2$], this increase being stronger from low to intermediate than from intermediate to elevated [CO$_2$] (13·6 and 3·3 %, respectively) (Fig. 2A). Notably, there was a significant three-way interaction of growth [CO$_2$] × origin × stand type. At low [CO$_2$], spring plants had larger leaf areas than non-spring plants in the mono stand, but this was not the case at intermediate and elevated [CO$_2$]. Conversely, in the mixed stands, non-spring plants had larger leaf areas than spring plants under low [CO$_2$], while for intermediate and elevated growth [CO$_2$] spring plants had larger leaf areas (Fig. 2A). In other words, the better performance of non-spring plants at low [CO$_2$] and of spring plants at intermediate and elevated [CO$_2$] was only apparent when there was competition between the different populations of origin.

A similar pattern was observed for whole-plant dry weight (Fig. 2B). Dry weight increased significantly with increasing growth [CO$_2$] when data of the different localities, origins and stand types were lumped together; a stronger increase was observed if [CO$_2$] increased from low to intermediate compared with intermediate to elevated (43·0 and 10·1 %, respectively) (Fig. 2B). There was a significant three-way interaction of

growth [CO$_2$] × origin × stand type. The dry weight of spring and non-spring plants in mono stands did not differ significantly. On the other hand, in mixed stands, non-spring plants had a significantly greater dry weight under low [CO$_2$], while this held for spring plants at intermediate and elevated growth [CO$_2$] (Fig. 2B).

Lumped together for locality, origin and stand type, there was a significant decrease in specific leaf area (SLA; leaf area divided by leaf dry weight) with increasing growth [CO$_2$], with a larger difference between low and intermediate [CO$_2$] than for intermediate and elevated levels (–19·6 and –6·7 %, respectively) (Fig. 2C). A significant growth [CO$_2$] × origin interactive effect was observed. Although not significant, spring plants tended to have higher SLA compared with non-spring plants at low growth [CO$_2$], while non-spring plants had a significantly higher SLA compared with spring plants at intermediate [CO$_2$]. At elevated [CO$_2$] there was no clear trend (Fig. 2C).

None of the effects (locality, growth [CO$_2$], origin and stand type) was significant for total leaf N content (i.e. total amount of N in the canopy of a plant), but the general pattern resembled that of leaf area and dry mass; that is, the spring and non-spring plants in mono stands did not have a significantly different total N, but in mixture spring plants had a significantly greater total N compared with the non-spring plants under intermediate and elevated growth [CO$_2$] (Fig. 2D). Total leaf N of plants was strongly and positively correlated to leaf area (Supplementary Data Fig. S4).

The measured leaf photosynthesis rate at growth [CO$_2$] (A_{growth_co2}) significantly increased with increasing growth [CO$_2$] (lumped together for locality, origin and stand type) (Fig. 3A). There was a significant origin × stand type interactive effect. The non-spring plants tended to have a higher A_{growth_co2} in mono stands, while in a mixed stand they tended to have a lower A_{growth_co2} compared with spring plants, a trend that appeared consistent across CO$_2$ levels (Fig. 3A).

Stomatal conductance significantly decreased with increasing [CO$_2$] (lumped together for locality, origin and stand type) (Fig. 3B–D). There was a significant interaction of growth [CO$_2$] × origin. Spring plants had a higher stomatal conductance at low growth [CO$_2$] compared with non-spring plants for Kosaka and Yuno-Kawa, whereas this was the case for non-spring plants at intermediate [CO$_2$] for Kosaka (Fig. 3C, D).

Results of the canopy model

A canopy model was used for two different purposes: first, to scale up from leaf photosynthetic traits to plant and canopy photosynthetic traits, to study the effects of the different treatments at the canopy level; and, secondly, to investigate the contribution of phenotypic trait responses to elevated [CO$_2$] and genotypic trait differences between spring and non-spring plants regarding canopy photosynthesis (Table 1).

Figure 4A shows the outcomes for the first modelling exercise. For the total canopy net photosynthesis rate, a similar pattern was found to that for leaf area and whole-plant above-ground dry weight. Canopy net photosynthesis rate increased significantly with increasing growth [CO$_2$] when data were lumped together for locality, origin and stand type (Fig. 4A). A significant three-way interaction of growth [CO$_2$] × origin ×
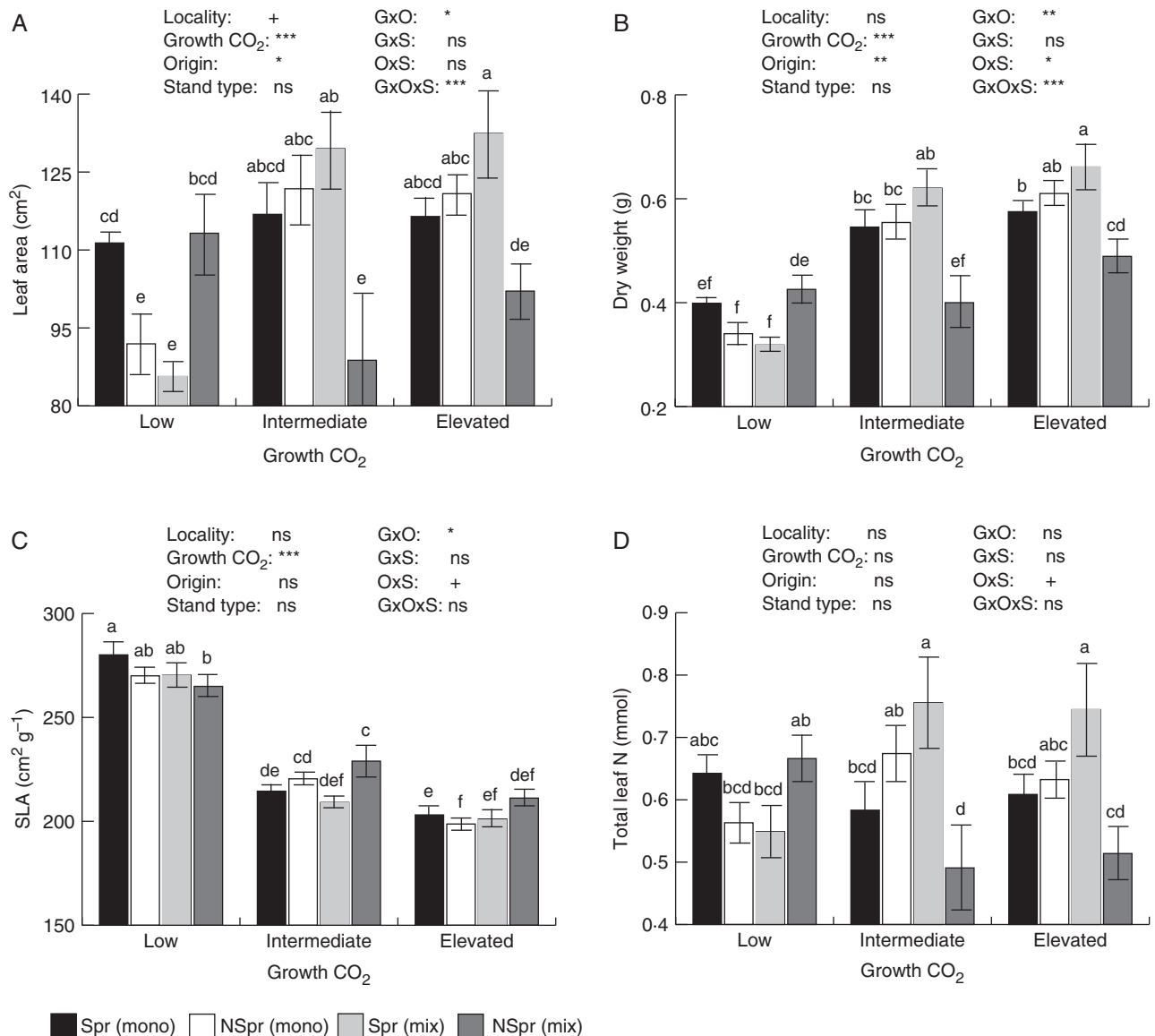
FIG. 2. Leaf area (A), above-ground dry weight (B), specific leaf area (SLA) (C) and total canopy leaf N content (D) of plants grown under low (270 ppm), intermediate (450 ppm) or high (750 ppm) [CO$_2$] and originating from either spring areas (Spr) or non-spring areas (NSpr), and these are grown either in a mono stand (mono) or in a mixed stand (mix) (*n* = 12). Values are mean ± s.e. Different letters indicate significant differences between the treatments (*P* < 0·05). Results of the generalized linear model are shown: ***P* < 0·001; **P* < 0·01; *P* < 0·05; +*P* < 0·1.

stand type was found. There was no significant difference in canopy net photosynthesis rate of spring and non-spring plants in mono stands, while in mixed stands non-spring plants had a significantly higher canopy photosynthetic rate under low [CO$_2$], and this held for spring plants at intermediate and elevated growth [CO$_2$] (Fig. 4A).

Figure 4B shows the outcomes for the second modelling exercise. Canopy photosynthesis rate was simulated to increase by 28 % by an instantaneous response, i.e. when plants that were grown in low [CO$_2$] were virtually transferred to elevated [CO$_2$] (Fig. 4B). The simulated plastic response resulted in an additional 15 % increase in photosynthesis at elevated [CO$_2$] compared with the instantaneous response (Fig. 4B). Conversely, the simulation showed that non-spring plants had a

similar canopy photosynthesis rate to spring plants at elevated [CO$_2$], indicating that in mono stands the genotypic differences contributed little to whole-stand photosynthesis (Fig. 4B). So, both the direct effect of elevated [CO$_2$] and the plastic response to this condition contributed to increased whole-plant photosynthesis, but effects of the genotypic response were negligible.

DISCUSSION

In our study we showed that changes in whole-stand photosynthetic rate, SLA and stomatal conductance of *Plantago asiatica* in response to changes in growth [CO$_2$] were mainly driven by instantaneous responses and plastic responses within the lifetime
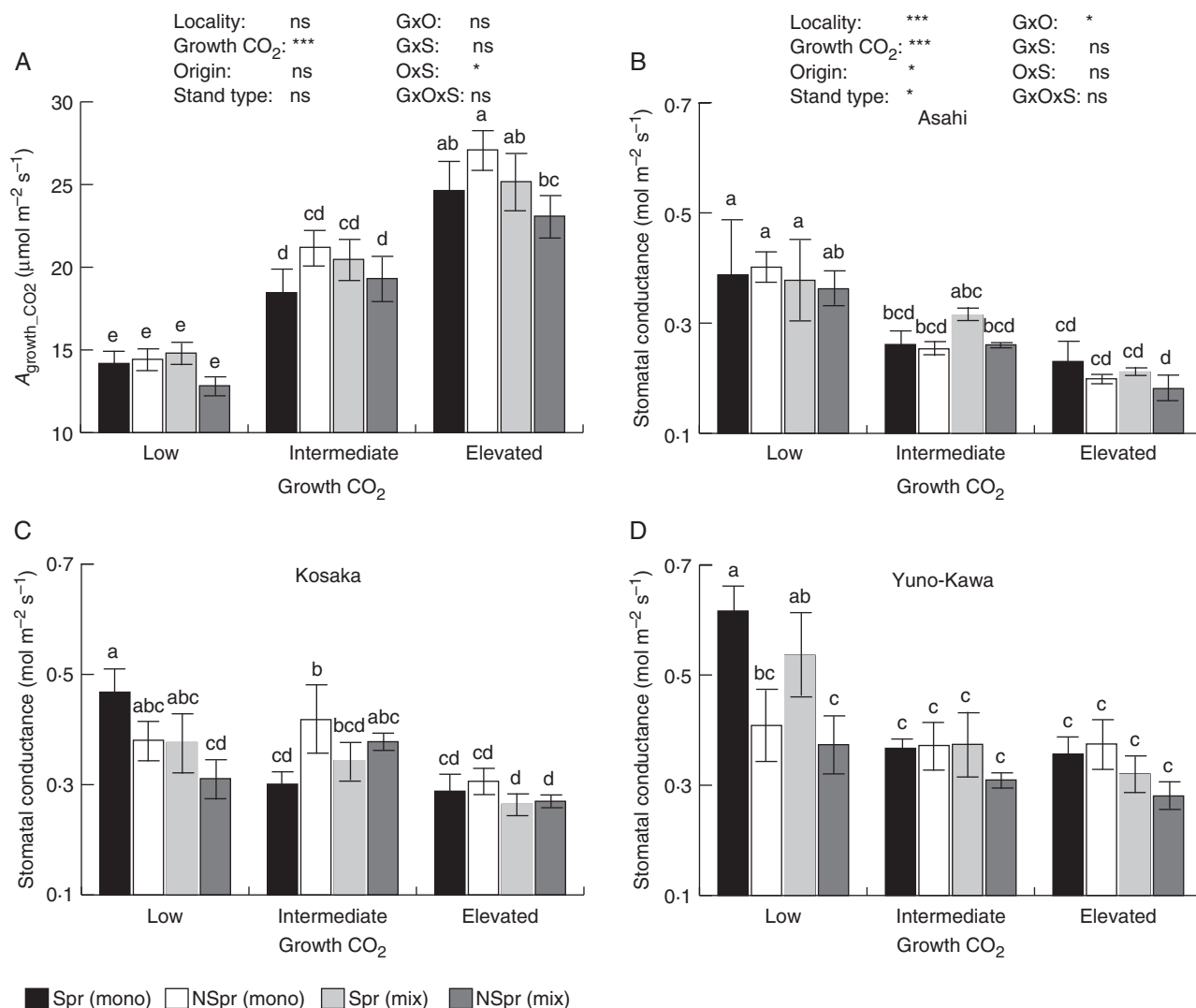
Fig. 3. Photosynthesis at growth [CO$_2$] (A_{growth_co2}) ($n = 12$) (A) and stomatal conductance for the areas Asahi (B), Kosaka (C) and Yuno-Kawa (D) ($n = 4$) of plants grown under low (270 ppm), intermediate (450 ppm) or high (750 ppm) [CO$_2$] and originating from either spring areas (Spr) or non-spring areas (NSpr), and these are grown either in a mono stand (mono) or in a mixed stand (mix). Values are mean ± s.e. Different letters indicate significant differences between the treatments ($P < 0.05$). Results of the generalized linear model are shown: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; +$P < 0.1$.

of a plant and not by genotypic responses, which take place over several generations. We further found that plants native to high [CO$_2$] habitats (spring plants) performed better in terms of whole-stand photosynthesis, biomass and leaf area, than those from ambient [CO$_2$] habitats (non-spring plants) at elevated [CO$_2$] only when plants of the two [CO$_2$] habitats competed. Similarly, plants from ambient [CO$_2$] habitats performed better at pre-industrial [CO$_2$] only when competing with plants from elevated [CO$_2$] habitats. Our results build upon the work of Manea and Leishman (2011) who illustrated the large role of plant competition in determining community-level outcomes under elevated [CO$_2$] by showing that the relative success of invasion by exotic species was increased under elevated [CO$_2$]. Together, our findings suggest that selection under changing [CO$_2$] is strongly mediated by competitive interactions between plants, as more competitive plants benefit more from elevated [CO$_2$], and this may have important consequences for species distribution and vegetation functioning.

Although we found an increase in whole-plant photosynthetic rate to elevated [CO$_2$] by both instantaneous and plastic responses, this increase was small compared with the [CO$_2$] increase. Yet, FACE studies found similar responses to elevated [CO$_2$] (Ainsworth and Long, 2005). An important cause could be the concave form of the photosynthesis–CO$_2$ response curve, as photosynthesis tends to saturate at high [CO$_2$] (Leakey and Lau, 2012). In addition, light levels in growth chambers were around 400 µmol m^{-2} s^{-1} which is comparable with the light levels on a moderately overcast day at our site, but which tends to be below the level that would saturate photosynthesis. So, the RuBP regeneration rate was probably predominantly limiting photosynthesis, resulting in a relatively moderate increase in photosynthesis by [CO$_2$] elevation. Additionally, this low photosynthetic response to [CO$_2$] elevation is also generally thought to be at least partly the result of photosynthetic downregulation (e.g. Von Caemmerer *et al.*, 2001). An extensive review on elevated [CO$_2$] studies showed that on average an
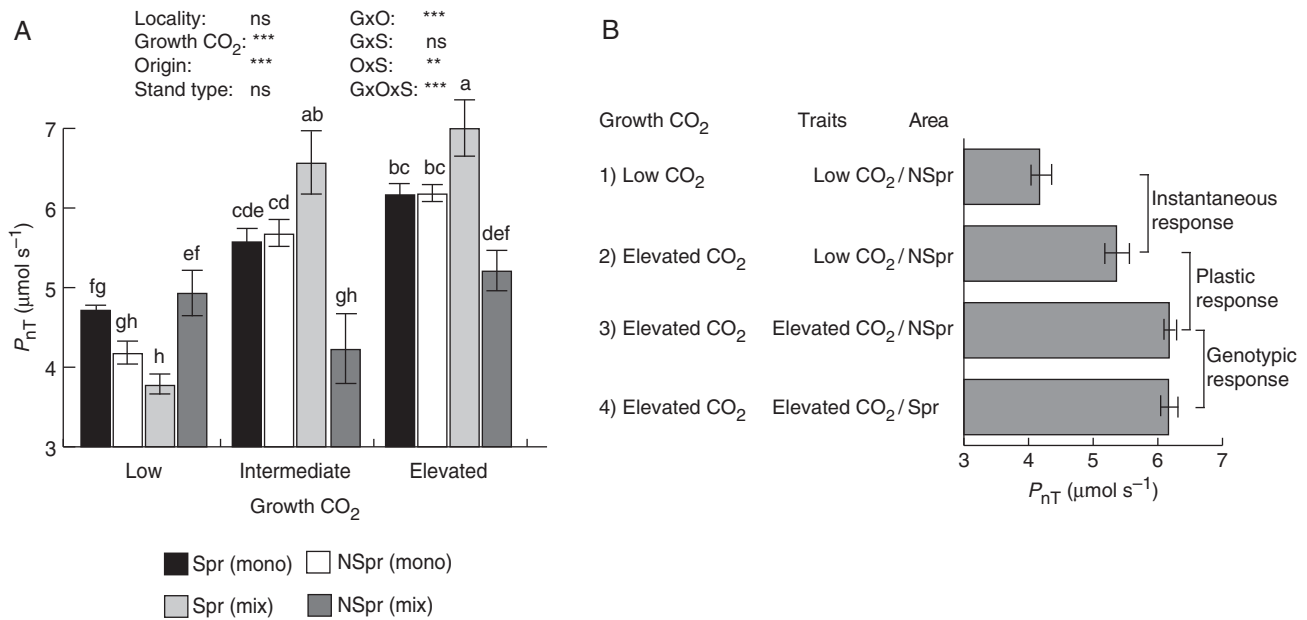
FIG. 4. (A) Modelled canopy photosynthesis rate (P_{nT}) of plants grown under low (270 ppm), intermediate (450 ppm) or high (750 ppm) [CO_2] and originating from either spring areas (Spr) or non-spring areas (NSpr), and these are grown either in a mono stand (mono) or in a mixed stand (mix) ($n = 12$). Values are mean ± s.e. Different letters indicate significant differences between the treatments ($P < 0.05$). Results of the generalized linear model are shown: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; +$P < 0.1$. (B) Modelled canopy photosynthesis rate of (1) non-spring plants grown at low [CO_2], (2) non-spring plants grown at elevated [CO_2] which have the traits of those grown at low [CO_2] (non-spring plants transferred from low to elevated [CO_2]), (3) non-spring plants grown at elevated [CO_2] and (4) spring plants grown at elevated [CO_2]. Difference 1 and 2, instantaneous response; difference 2 and 3, plastic response; difference 3 and 4, genotypic response (see also Table 1). Values are mean ± s.e.

increase in [CO_2] only leads to small increases in growth due to photosynthetic downregulation, but that this response is also highly species dependent (Poorter 1993).

A decrease in SLA with elevated [CO_2] was found, and this might have caused the induction of photosynthetic downregulation. The decreased SLA at elevated [CO_2] can be the result of the accumulation of non-structural carbohydrates at elevated [CO_2], and this might have caused induction of photosynthetic down-regulation (Roumet *et al.*, 1999).

A decrease in stomatal conductance with elevated [CO_2] was found; this mostly resulted from instantaneous closure of the stomatal apparatus and to a lesser extent to decreased stomatal density across the populations from the different [CO_2] habitats (data not shown). No clear differences between spring and non-spring plants were observed, suggesting that there are no genotypic differences in stomatal response. This finding in contradiction to the results of Haworth *et al.* (2011) in the grass species *Agrostis canina*. Here spring plants had a higher stomatal conductance compared with non-spring plants when grown at ambient [CO_2], and spring plants reduced their stomatal conductance much less compared with non-spring plants when grown at elevated [CO_2], which increased the difference in stomatal conductance between spring plants and non-spring plants. The lack of genotypic differences in the stomatal responses to elevated [CO_2] in our study could be associated with the costs of stomatal control (Haworth *et al.*, 2011). There is possibly a trade-off between physiological and morphological control of stomatal conductance to changes in [CO_2] (De Boer *et al.*, 2012; Haworth *et al.*, 2013). Species with active control of the stomatal apparatus generally show little reduction in stomatal density, whereas species with little or no active control of the

stomatal apparatus are more likely to reduce their stomatal density (Haworth *et al.*, 2013). Furthermore, many species show little or no response in stomatal density to [CO_2] elevation (Haworth *et al.*, 2013).

Although the spring plants originating from three different localities in Japan (Asahi, Kosaka and Yuno-Kawa) with rather different natural CO_2 levels (500–7019 ppm), there was no location effect found for most of the measured traits. This might be due to the non-linear photosynthetic response of plants to CO_2 and the concentration at which CO_2 is saturating (Leakey and Lau, 2012), i.e. above a certain [CO_2], photosynthesis only responds very little and thus variation in [CO_2] probably no longer exerts a strong influence on plant performance.

Our results confirm our second hypothesis regarding genotypic responses, i.e. that spring plants performed better under intermediate and elevated [CO_2] than non-spring plants only when the two competed and not when each one was grown alone. The reverse also held; non-spring plants performed better under low [CO_2] only when they competed with spring plants and not when grown alone. So, our findings clearly suggest that adaptation to changing [CO_2] can be strongly mediated by plant–plant interactions at least for our species. The study of Bazzaz *et al.* (1995) also supports this, as plants with the highest growth response to elevated [CO_2] in the absence of competition did not have the highest growth response in competitive stands. Game theory could help to explain our findings, as it proposes that in vegetation stands where plants interact, natural selection would not favour plants with the highest performance (e.g. growth or reproduction) *per se*, but rather those that exhibit competitive advantages over neighbours (Anten and During, 2011). For example, game theoretical models predicted

that selection might favour plants with more leaf area or that grow taller than would be needed for maximized production (Anten, 2002; Falster and Westoby, 2003). In addition, a game theoretical model, which assumed that the best competitors will prevail under elevated [CO$_2$], gave better predictions of measured LAI and photosynthesis and net ecosystem productivity in elevated [CO$_2$] experiments than models that assume that plants with the inherent highest photosynthesis will prevail (Van Loon *et al.*, 2014, 2015). Together with the findings reported here, this also suggests that competition strongly mediates plant adaptations to elevated [CO$_2$] and this may have important consequences for future species distribution and vegetation functioning.

The higher performance of the competing spring plants at elevated [CO$_2$] and competing non-spring plants at low [CO$_2$] is the result of a higher dry weight and leaf area, but not of higher leaf photosynthetic rates per unit leaf area. Differences in dry mass allocation might explain the differences in dry weight and leaf area while having a similar photosynthetic rate per unit leaf area for the spring and non-spring plants in a mixture. Several studies (e.g. Nakamura *et al.*, 2011; Polle *et al.*, 2001) show that spring plants allocate more biomass to leaves than to roots, resulting in higher growth rates (Nakamura *et al.*, 2011). In addition, it has been shown that a larger leaf area is an important trait in plants' competitive ability under elevated [CO$_2$] (Ziska *et al.*, 2004, 2005), as competition for light becomes more intense (Hikosaka *et al.*, 2003). This could explain why spring plants had a competitive advantage when competition would be primarily for light. However, this would not explain why in our study non-spring plants were competitively superior under pre-industrial [CO$_2$]. In addition, as spring and non-spring plants shared pots in the mixed stands, below-ground interactions could also have contributed to the observed competitive differences. Although it is most likely that with our set-up competition was predominantly above-ground, below-ground competition might still play a role. The fact that leaf area and biomass were correlated with whole-plant nitrogen suggests that differences in nitrogen uptake and thus competition for that resource may have partly driven our results. However, as we did not measure roots, we cannot confirm these conclusions. So, more research is needed to identify the traits that may explain the competitive interactions observed in this study.

Due to its ubiquitous nature and importance for plant growth, changes in [CO$_2$] entail a global selective force. Although the selection pressure is high, changes in plant performance were mainly determined by plastic responses, with genotypic differences contributing very little. However, our results also suggest that genotypic selection under changing [CO$_2$] is determined more strongly by differences in competitive ability than by differences in performance *per se*. This is because we found that *P. asiatica* plants originating from naturally high [CO$_2$] areas and from ambient [CO$_2$] areas showed similar responses to variations in [CO$_2$] when grown separately, but when grown together they appeared to differ in their competitive ability.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: canopy height and leaf angle. Figure S2: maximum carboxylation capacity (V_{cmax}) vs. leaf N content per unit leaf area. Figure S3: maximum electron transport rate (J_{max}) vs. leaf N content per unit leaf area. Figure S4: total leaf area of a plant vs. the N content of its leaves. Table S1: model parameters with input value and unit.

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