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The involvement of the phytohormone ethylene in the adaptation of *Arabidopsis* rosettes to enhanced atmospheric carbon dioxide concentrations

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ABSTRACT

Atmospheric CO_2 levels are rising rapidly due to anthropogenic activities. Although plants require CO_2 to fuel photosynthesis, the relationship between CO_2 and plant growth is complex. In phytotrons elevated CO_2 was shown to stimulate growth of *Arabidopsis thaliana*. In nature, there is, however, a constant variation in temperature, the availability of sunlight, nutrients and water, in addition to biotic stresses. The aim of this study was to explore the impact of elevated CO_2 on rosette growth and transcriptome profiles in *Arabidopsis* plants grown under natural radiation and air temperature. Because of its regulatory function in cell elongation, photosynthesis, stomatal closure and leaf senescence, the importance of ethylene for the response to elevated CO_2 was investigated in wild-type plants (Col-0), and in plants with constitutive or reduced ethylene signaling (*ctr1-1* and *ein2-5*, respectively). Rosette area measurements indicated that increased atmospheric CO_2 did not increase vegetative growth in any of the genotypes. Moreover, both Col-0 and *ein2-5* plants grown at elevated CO_2 exhibited an increase in stress responsive gene expression compared to those grown at ambient CO_2. A downregulation of photosynthesis and an upregulation of metabolism were, apart from the overrepresentation of stress genes, the most important responses to elevated CO_2 in both wild-type and ethylene-insensitive plants. However, whereas in Col-0 starch biosynthesis and turnover were more strongly activated, lipid metabolism was enhanced in *ein2-5*. Through stomatal closure, sugar and lipid metabolism and leaf senescence, ethylene could be involved in the adaption of *Arabidopsis* rosettes to elevated CO_2.

1. Introduction

The concentration of carbon dioxide (CO_2) in the earth's atmosphere has been rising since the beginning of industrialization, currently reaching 408 ppm on average (https://www.esrl.noaa.gov/gmd/ccgg/trends/global.html). Global consumption of fossil fuels and deforestation are fundamental therein. Gradual increases in atmospheric CO_2 concentration have been reported for several periods of earth's history (*e.g.* rising to 3000 ppm during the Cretaceous period; Ehleringer et al., 1991). Nevertheless, the current anthropogenic emission is increasing at a much faster pace and is postulated to reach between 463 and 685 ppm by 2050 (www.ipcc.ch; Marchal et al., 2012). The main drivers for climate change include the Milankovitch cycles and solar activity, among others (Kutílek and Nielsen, 2010). Because CO_2 is a greenhouse gas, its increased levels cause global warming, which can also contribute to climate change. Together with global warming, an increase in the atmospheric levels of CO_2 has a direct impact on plants. They need CO_2 for photosynthesis and therefore productivity. Hence, increasing the atmospheric CO_2 concentration is expected to promote photosynthesis, though the latter is dependent on the photosynthetic machinery of a plant (*e.g.* C_3 *versus* C_4 plants; Robinson et al., 2012). In C_3 plants, elevated levels of CO_2 increase the carboxylation rate of RUBISCO while it inhibits the oxygenation of Ribulose-1,5-bisphosphate (RuBP), thus promoting photosynthesis (Drake et al., 1997). This is often partially counteracted by a reduction in stomatal conductance (Darwin, 1898), hence limiting transpiration and improving water-use efficiency, which adds to the direct promotion of light-use efficiency (LUE) by elevated atmospheric CO_2 (Drake et al., 1997). In contrast, C_4 plants have evolved both anatomical and biochemical adaptations that concentrate CO_2 at the place of carboxylation (Gowik and Westhoff, 2011). This allows for a more efficient assimilation of CO_2, even when stomata are closed, for instance in high temperature or drought conditions. At the current atmospheric CO_2 levels, carboxylation efficiency of RUBISCO in C_4 plants is close to

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saturation, thus increases in CO_2 are not expected to promote C4 photosynthesis – in contrast to C3 plants (Robinson et al., 2012).

CO_2 availability is not the only determinant of the rate of photosynthesis in C_3 plants; the access to nutrients dictates photosynthetic efficiency as well. Indeed, photosynthesis is a holistic physiological process, which integrates plant nutrition from atmosphere and rhizosphere (Kirschbaum, 2011). Also, water is indispensable for photosynthesis. The photosynthetic rate of plant leaves diminishes with decreasing water content (Lawlor and Cornic, 2002). Water stress also hinders photosynthesis through a decreased RuBP and ATP availability (Tezara et al., 1999). Water deprivation causes stomatal closure, which impedes the uptake of CO_2. The drought-induced reduction in stomatal conductance outweighs the reduction caused by elevated CO_2 (Morgan et al., 2004; Leakey et al., 2006). Hence, an increase in CO_2 availability should stimulate photosynthesis during growth under water deficiency (Xu et al., 2016). The response of photosynthesis to temperature depends on the kinetics of the RUBISCO enzyme and is strongly influenced by light intensity and CO_2 availability. With an increase in temperature, photorespiratory activity enhances due to both the stronger decrease in relative solubility of CO_2 as opposed to O_2 and the higher affinity of RUBISCO for O_2 at low CO_2 concentration. The optimal temperature for photosynthesis consequently increases with increasing CO_2. Nevertheless, photosynthesis is sensitive to inhibition by heat stress, causing RUBISCO inhibition; particularly the activation of RUBISCO by RUBISCO ACTIVASE is heat sensitive (Lin et al., 2012; Mathur et al., 2014).

Source activities (photosynthesis) control sink activities (growth, metabolism). However, when sink activities are inadequate and source and sink activities are in imbalance, photosynthesis is feedback-regulated by sinks because it has to be adjusted to the needs of the whole plant (Paul and Foyer, 2001; Ainsworth and Long, 2005; Thompson et al., 2017). A sugar-mediated downregulation of photosynthesis genes was postulated to facilitate this photosynthetic acclimation (Ainsworth et al., 2004). Because photosynthesis is such a highly integrated and regulated process, the relationship between increased atmospheric CO_2 and plant growth is complex and bears several feedforward and feedback mechanisms. The duration of exposure to high CO_2 concentrations is a key factor. A short-term increase in atmospheric CO_2 was often shown to stimulate photosynthesis and plant growth. Notwithstanding the initial promotion of photosynthesis, upon long-term-exposure to elevated CO_2 part of this stimulation is suppressed and the growth response to elevated CO_2 varies considerably across species and ecotypes within species (Long et al., 2004; Ainsworth and Long, 2005; Ainsworth and Rogers, 2007).

Ethylene plays a regulatory role in cell elongation (Feng et al., 2015). It interacts with sugar signaling pathways (Gazzarrini and McCourt, 2001) and is involved in the regulation of photosynthesis (Tholen et al., 2004, 2007 and, 2008). Ethylene-insensitive mutants grow larger rosettes and bolt and flower later than the wild-type (Bleecker et al., 1988; Van Der Straeten et al., 1993; Grbic and Bleecker, 1995; Hua et al., 1995). Constitutive ethylene response mutants are characterized by a severely dwarfed rosette and also by late flowering (Kieber et al., 1993). In addition, several reports associate ethylene with the regulation of stomatal conductance, though results are ambiguous. Ethylene alone promotes stomatal closure (Tanaka et al., 2005), while delaying it when applied in combination with abscisic acid (ABA), the prime signal for the control of stomatal movement (Desikan et al., 2006; Watkins et al., 2014). Ethylene could thus play a key role as regulator of the response of plants to elevated CO_2. Its biosynthesis has been shown to increase significantly in cucumber, rice and tomato plants under increased CO_2 (Dhawan et al., 1981; Bassi and Spencer, 1982; Mathooko et al., 1998; Seneweera et al., 2003; Wang et al., 2009). In *Medicago truncatula* elevated CO_2 down-regulated the ethylene signaling pathway (Guo et al., 2014). Because of an annotated genome and the availability of various ethylene-related mutants, *Arabidopsis thaliana* was used in this study.

Much of our knowledge on how plants will deal with future CO_2 concentrations relies on experiments in which plant performance was compared between current ambient CO_2 and experimentally increased CO_2 in phytotrons. A beneficial effect of various durations of elevated CO_2 exposure on *Arabidopsis* growth was observed (Summarized in Table S3). Plant performance strongly depends on environmental conditions. Hence, plants grown in a more natural environment may not experience the effects of increasing CO_2 the same way as plants growing in phytotrons. Therefore, the use of phytotrons to study effects of increased atmospheric CO_2 levels on plant growth is more and more being substituted by FACE (Free-Air Carbon dioxide Enrichment) experiments in the field (Ainsworth and Long, 2005). Interestingly, the beneficial effect of elevated CO_2 exposure on *Arabidopsis* growth in phytotron experiments (Table S3) remained absent in FACE experiments (Miyazaki et al., 2004; Li et al., 2006, 2008), emphasizing the importance of field experiments to provide insights in the impact of CO_2 on plant growth in nature and in agriculture. The number of studies on the consequences of elevated CO_2 on *Arabidopsis* growth in phytotrons, however, strongly exceeds those conducted in the field (Table S3). Therefore, we aimed to confirm the absence, under more natural conditions, of the growth-promoting effect of elevated CO_2 in *Arabidopsis*, as observed in phytotrons. Since no FACE system was at our disposal, field chambers with natural fluctuations in air temperature and light availability were used as a valuable alternative. Because of its regulatory role in vegetative growth and photosynthesis, involvement of ethylene in the adaptation of *Arabidopsis* to elevated CO_2 was expected. To provide novel insights rosette growth of Col-0, *ein2-5 (ethylene-insensitive 2)* and *ctr1-1 (constitutive triple response 1)* and transcriptome profiling of Col-0 and *ein2-5* were conducted.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana (L.) Heynh. Columbia 0 (Col-0) was purchased from the Nottingham Arabidopsis Stock Centre (NASC; University of Nottingham, Loughborough, United Kingdom) and *ctr1-1* and *ein2-5* originated from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University (Columbus, Ohio, USA). Both ethylene mutants are in Col-0 background.

2.2. Growth conditions

On 14/08/2014, seeds were sown directly on hydrated peat pellets (Jiffy Products International BV, Zwijndrecht, The Netherlands) and grown in a growth chamber at 22 °C and 100 µmol m^{-2} s^{-1} of photosynthetic active radiation (PAR) under a 16-h-light/8-h-dark cycle for 14 days. Subsequently, on 02/09/2014, the plants were transferred to the Drie Eiken Campus of the University of Antwerp (Belgium, 51°09´N, 04°24´E). To investigate the possible impact of an increased atmospheric CO_2 concentration on the growth of the *Arabidopsis* rosette, ventilated sunlit field chambers were used such that the day/night rhythm and air temperature are harmonious with the surrounding environment (De Boeck et al., 2007) (Fig. S1; Fig. 1 and Fig. S2). The aluminium-frame chambers covered a 150 cm x 150 cm usable interior ground area, with a height of 150 cm at the north side and 120 cm at the south side (Lemmens et al., 2006). They were covered with a colourless, UV-transparent polycarbonate plate (4 mm thick) on top, and polyethylene film (200 µm thick) on the sides, both with a PAR transmission of 86%. PAR was measured with a quantum sensor (type JYP 1000; manufactured by SDEC France, Reignac sur Indre, France) inside the chambers (data stored every half hour). The air temperature (Tair) inside the field chambers was measured continuously using a QFA66 sensor (Siemens, Berlin, Germany).

Six field chambers were used in the experiment, all facing south. To ascertain the growth of *Arabidopsis* rosettes in response to increasing

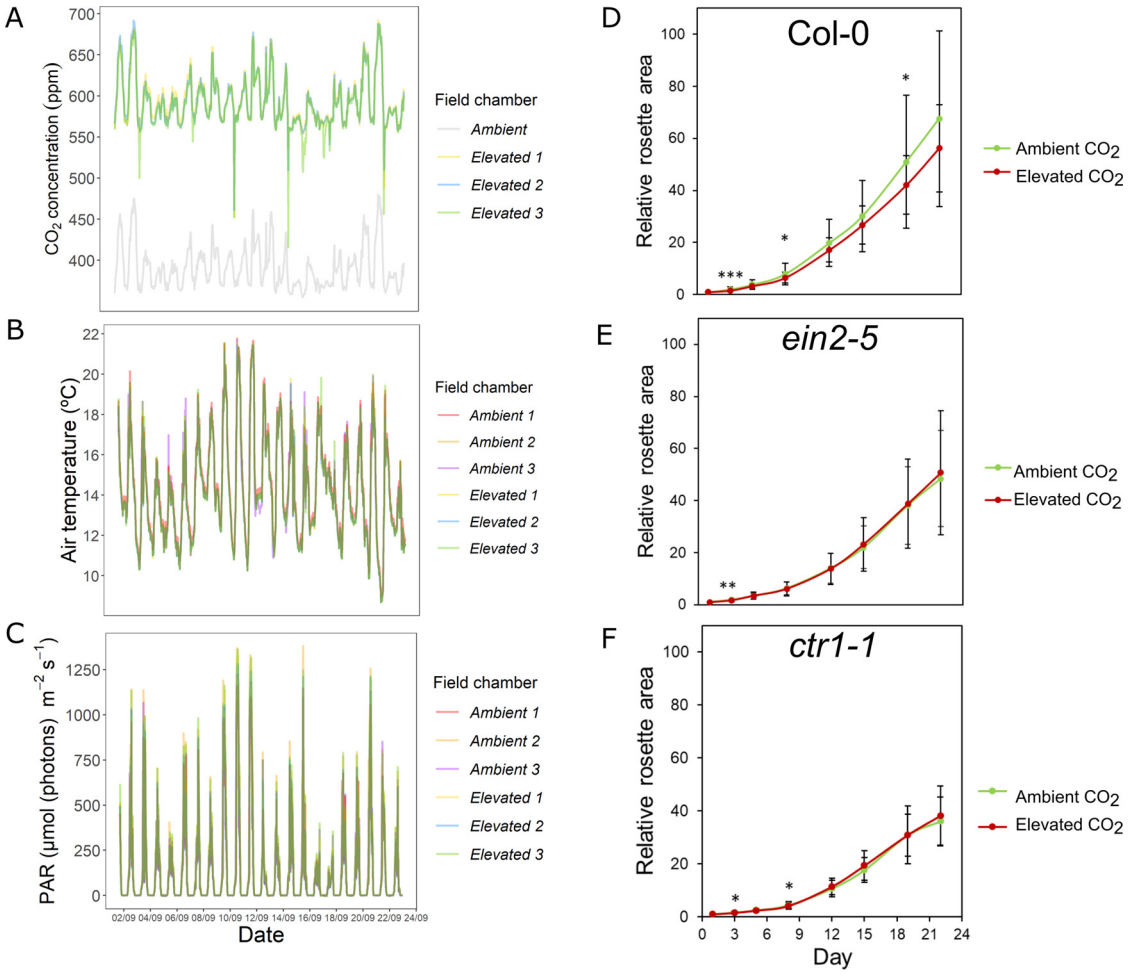

Fig. 1. (A–C) The fluctuations per field chamber (ambient and elevated) in (A) CO_2 concentration (ppm), (B) air temperature (°C) and (C) PAR from the sun (µmol (photons) m^{-2} s^{-1}). Three field chambers had ambient and three had elevated CO_2 concentrations (ambient + 200 ppm). The ambient atmospheric CO_2 concentration was measured in one chamber whereas the concentration of the elevated CO_2 was measured in each of the elevated CO_2 chambers. The temperature and PAR were measured in each of the six field chambers separately. (D–F) Relative rosette area of Arabidopsis plants under ambient and elevated CO_2 conditions of (D) Col-0, (E) *ein2-5* and (F) *ctr1-1*. The green line and the red line represent the relative rosette area in function of time for ambient and elevated CO_2 conditions, respectively. Dots represent mean relative rosette areas at a given day and error bars represent the respective SDs. The asterisks represent statistically significant differences in relative rosette area between plants grown under ambient and elevated CO_2 conditions for that day based on the Wilcoxon rank sum test. The number of asterisks represents the degree of significance of the difference. The sample size is at least 70 plants per genotype per treatment.

atmospheric CO_2 concentrations, a future rise of 200 ppm was selected as 'elevated' CO_2 treatment in three of the six chambers. The CO_2 concentration in each chamber was measured hourly and maintained close to the target concentration (24 h a day; for the elevated chambers) with an infrared analyser (WMA-4; PP Systems, Hitchin, UK). Over the total period of the experiment, the CO_2 concentration was on average 391 ± 26 ppm (SD) for ambient CO_2 chambers, and 593 ± 31 ppm (SD) for elevated CO_2 chambers. Each field chamber contained a wooden container with dimensions 1.2 × 1.2 x 0.25 m (LxBxH) delineated with a plastic sheet. The container was filled with potting soil (Substraat Lentse potgrond, Horticoop, The Netherlands), which consisted of 65% garden peat, 25% Baltic peat and 10% sand. The pellets with *Arabidopsis* rosettes, which had grown for two weeks in a growth chamber, were planted, on 02/09/2014, directly in the potting soil to avoid mechanical stress and wounding of the roots. The soil surface was watered regularly (at least two times per week) with a hose with a fine nozzle. Irrigation created a water table which was maintained throughout the experiment at 20 cm below the surface, both to preclude effects of water deficiency. Each field chamber contained 81 plants, 27 of each of the three genotypes (Col-0, *ctr1-1* and *ein2-5*), which were randomized in blocks of nine plants. Three different randomizations were employed for the six field chambers, with each genotype randomization plan being applied to one ambient CO_2 and one elevated CO_2 field chamber. The distance between the centres of the plants was around 14 cm. The genotypes were labelled with coloured labels (Fig. S1). Plants were exposed to ambient or elevated CO_2 for 22 days, which is linked to the start of bolting (bolting stem just appearing, not elongating). Samples for gene expression analysis (see 2.4 RNA extraction) were harvested on 22/09/2014. The experiment was repeated in September 2015, using the same experimental setup.

2.3. Image acquisition and analysis

An 18-Megapixel (5184 × 3456) digital single-lens reflex CCD camera (EOS 550D; Canon Inc., Tokyo, Japan) was used for plant imaging. The rosettes were photographed biweekly on fixed dates until the first plants presented evidence of bolting (approximately 22 days after start of treatment). The rosette area of the three *Arabidopsis* genotypes was quantified using the open source and in-house developed image analysis tool Rosette Tracker (De Vylder et al., 2012). This tool is not constrained by one specific monitoring system and was ideally suited for our imaging set-up. The minimal user input allowed rapid image processing. By dividing the absolute rosette area (mm^2) per plant, as measured using Rosette Tracker, by the rosette area at day one

the data was transformed to the relative rosette area. Using this transformation, the initial variation in rosette area at the beginning of the experiment was corrected for. Based on the relative rosette area in function of time (days) (Fig. 1D-F) the increase in relative rosette area was subdivided in a slow (Day 1–8) and a fast growth phase (Day 8–22). Relative rosette growth was calculated per growth phase by measuring the slope of the relative rosette area in function of the number of days (RRA/days).

2.4. RNA extraction

Only Col-0 and *ein2-5* plants were used for the transcriptome analysis. *Ctr1-1* plants are developmentally lagging behind Col-0 and *ein2-5* plants (Fig. 1); therefore, transcriptome differences could arise which are independent of the elevated CO_2 conditions. Therefore, complete rosettes were harvested when the first plants presented evidence of bolting, to avoid transcriptome changes due to the transition from vegetative to generative development rather than changes due to the elevated atmospheric CO_2 concentrations (Pouteau and Albertini, 2009). After removal of the roots, the rosettes were instantaneously frozen in liquid nitrogen. Per field chamber, half of the healthy rosettes of each genotype were pooled. The other half were pooled to serve as back-up. Hence, each sample contained complete rosettes originating from at least ten different plants. Out of the six samples per genotype (three ambient and three elevated), two ambient and two elevated CO_2 samples were selected for RNA sequencing. In addition, all six samples were used for real-time qPCR analyses. Plant tissues from each sample were homogenized using mortar, pestle and liquid nitrogen. RNA was extracted with the RNeasy Mini kit with on column DNAse treatment (Qiagen, Hilden, Germany). The extracted total RNA was quantified and qualified (260/280 and 260/230 ratios > 2), using an NP80 NanoPhotometer (Implen, Germany).

2.5. RNA sequencing and data analysis

The eight RNA samples were subjected to RNA sequencing using an Illumina™ platform following the protocol as described in Saini et al. (2017). Using the CLC Genomics Workbench v.6 with the *Arabidopsis thaliana* (Col-0 TAIR10) sequence database (www.arabidopsis.org) as a reference genome, the RNA sequencing data were analyzed. In short, using the default settings, the sequences were mapped against the reference genome after trimming. Based on the "reads per kilo base of exon model per million mapped reads" (RPKM) values (Mortazavi et al., 2008), the expression values were calculated. The RNA sequencing data was grouped accordingly and one to one group comparisons were conducted. Normalization of the expression values was done by scaling to the default setting of ten million reads. The Baggerly test (Baggerly et al., 2003) was used for moderated t-statistics pairwise contrasts, accounting for normal between-library variation. Genes with no counts in all replicates for at least one of the genotype/time combinations were discarded as not detectable above the background. Subsequently, the Baggerly's p-values were corrected for multiple testing for each of the one to one group comparisons based on the False Discovery Rate (FDR; Benjamini and Hochberg, 1995). The resulting FDR-corrected p-value was used as a cutoff for significantly differentially expressed genes. All significantly upregulated and downregulated genes were classified based on their gene ontology (GO) (http://www.geneontology.org/) using PANTHER (http://www.pantherdb.org/) (Mi et al., 2013). Therefore, the statistical overrepresentation test using default settings and an FDR < 0.05 as a cutoff were employed. As annotation dataset 'GO biological process complete' was preferred over 'PANTHER GO-Slim Biological Process'. A biological process term describes a series of events accomplished by one or more organized assemblies of molecular functions. Transcript changes in response to elevated CO_2 for known pathways were visualized using MapMan (Thimm et al., 2004). The identity of the concerned genes, their log2 fold change and fold change can be found in the supplementary data (SData1-4). A log2 fold change of < 0.25 was considered weak, between < 0.25 - 0.75 < modest and > 0.75 strong.

2.6. Real-time qPCR

cDNA was synthesized using a Bio-Rad iScript cDNA synthesis kit and according to the manufacturer's instructions. Subsequent real-time qPCR was carried out with the primers listed in Table S1. The reaction mix also contained qPCRBIO SyGreen Mix with Fluorescein (PCRBIO-SYSTEMS, London, UK). qPCR reactions were performed in an iCycler (Bio-Rad) with the following conditions: denaturation at 95 °C for 3 min and 40 cycles of denaturation at 95 °C for 15 s, annealing for 20 s at variable temperature (see Table S1), and extension at 72 °C for 30 s. Data analysis was performed using the qBASE software (Biogazelle, Zwijnaarde, Belgium). Expression values were based on three technical replicates and three biological replicates and were normalized to the expression of reference genes *elongation factor-1α* (*EF-1α*; AT1G18070), *ACTIN 2* (*ACT2*; AT3G18780) and *RGS1-HXK1 INTERACTING PROTEIN 1* (*RHIP1*; AT4G26410) (Czechowski et al., 2005).

2.7. Statistics

All statistics were conducted using R, an open source software for statistical computing and graphics (R Foundation for Statistical Computing). To ascertain normality of the data, the Shapiro-Wilk test (Shapiro and Wilk., 1965) was used. Homoscedasticity (homogeneity of variances) was assessed using Levene's test (Levene, 1960) and Bartlett's test (Snedecor and Cochran, 1989). When the assumptions of normality and homoscedasticity were compromised, a non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) instead of an independent two-group *t*-test was used to statistically test for significant difference in rosette area between plants of the same genotype grown under ambient and elevated CO_2. To compare rosette areas between the three genotypes, a non-parametric Kruskal-Wallis Dunn post hoc test (Dunn and Clark, 1969, 1971) with Benjamini-Hochberg adjustment (Benjamini and Hochberg, 1995) was used because of inflictions to the assumptions of normality and homoscedasticity. All plots/graphs were fitted using the GGPLOT2 package in R (R Foundation for Statistical Computing).

3. Results

3.1. Influence of elevated CO2 on rosette area

The growth of the plants was followed in time in CO_2 enrichment chambers in the field. The average minimum temperature (considering all six field chambers) over 22 days was 11.3 ± 1.1 °C. The average maximum temperature was 18.5 ± 1.8 °C (Fig. 1B). Throughout the experiment there was variation in cloudiness, which is reflected in the photosynthetically active radiation (Fig. 1C). The average maximum PAR was 790 µmol (photons) ± 302 m^{-2} s^{-1}. In the field chambers with elevated CO_2, the concentration clearly fluctuated harmoniously with the environment, at a 200 ppm higher CO_2 level (Fig. 1A).

The rosette growth of wild-type plants was not promoted by elevated CO_2 (Fig. 1D; Table 1). Although not significant, the relative rosette area was even smaller under elevated compared to ambient CO_2 after 22 days of growth. A significantly smaller relative rosette area was, however, observed at day 3 ($P < 0.001$), day 8 ($P < 0.05$) and day 19 ($P < 0.05$). Under elevated CO_2, however, in the fast growth phase, the relative rosette growth (Day 8–22) was significantly ($P < 0.05$) slower as opposed to under ambient CO_2 (Table 1). These results suggest that under natural conditions an increased concentration of 200 ppm CO_2 *in se* is insufficient to support enhanced *Arabidopsis* rosette expansion. When the experiment was repeated one year later during the same month, again no stimulation of rosette growth by CO_2

Table 1
Summary of the average relative rosette growth (Relative rosette area/days measured as slope of relative area in function of number of days) and SD for each of the three genotypes grown under ambient and elevated CO_2. A distinction was made between the relative rosette growth during the first 8 days (1–8) and subsequent days (8–22) based on the rate of growth, which were slow and fast, respectively.

Col-0			*ein2-5*			*ctr1-1*		
Ambient CO_2		Elevated CO_2	Ambient CO_2		Elevated CO_2	Ambient CO_2		Elevated CO_2	
Day 1-8	*Day 8-22*	*Day 1-8*	*Day 1-8*	*Day 8-22*	*Day 1-8*	*Day 1-8*	*Day 8-22*	*Day 1-8*	*Day 8-22*
1.00 ± 0.60	4.29 ± 2.17	0.81 ± 0.29	0.77 ± 0.36	3.54 ± 1.08	0.75 ± 0.39	3.09 ± 1.20	0.49 ± 0.18	0.46 ± 0.17	2.51 ± 0.81

was observed (Fig. S2D; Table S2). To gain insights in the importance of ethylene for rosette growth in response to elevated CO_2, the ethylene-insensitive mutant *ein2-5* and constitutive ethylene signaling mutant *ctr1-1* were investigated. Similar to wild-type plants, no deviation between the relative rosette area of *ein2-5* plants grown under ambient and elevated CO_2 emerged after 22 days of growth (Fig. 1E). Only at day 3 a significant difference was found ($P < 0.01$). The fast rosette growth (Day 8–22) was similar between *ein2-5* plants grown under ambient and elevated CO_2 (Table 1). Also in *ctr1-1*, growth under elevated CO_2 failed to promote the relative rosette area after 22 days (Fig. 1F). No significant differences were found in the fast growth (Day 8–22) between ambient and elevated CO_2 (Table 1). Furthermore, the relative rosette area of *ein2-5* plants after 22 days of growth in the field was significantly smaller (± 30%, $P < 0.001$) compared to wild-type plants under ambient CO_2 (Fig. 1D and E), which was also true for *ctr1-1 plants* (± 50%, $P < 0.001$) (Fig. 1D and E). However, when the experiment was repeated one year later no significant difference ($P > 0.05$) in relative rosette area was found between the three genotypes after 19 days of growth (Figure S2D-F). The difference in weather conditions could be at the basis thereof (Fig. 1D-F compared to Figure S2D-F). Together, our results indicate that an increase of 200 ppm in the atmospheric CO_2 concentration does not necessarily increase the relative rosette growth in *Arabidopsis* and that the growth response to elevated CO_2 is independent of ethylene sensitivity.

3.2. Expression of ethylene-related genes in Col-0 and ein2-5 in response to elevated CO_2

To gain further insights in the long-term impact of elevated atmospheric CO_2 on *Arabidopsis* growth, a transcriptome analysis was conducted on rosettes of Col-0 and *ein2-5*. This transcriptome profiling was done at the end of the vegetative growth phase (22 days in the field chambers) in order to avoid interference by the changes associated with floral transition. Clustering of the samples for all significantly differentially expressed genes (FDR adjusted p-value < 0.05) in a hierarchical tree clearly indicated grouping based on CO_2 treatment (Fig. S3). In Col-0, the elevated atmospheric CO_2 concentration caused a significant differential expression of 1240 genes (FDR adjusted p-value < 0.05), of which 636 were downregulated and 604 were upregulated compared to ambient CO_2 (Fig. 2A). In *ein2-5*, the elevated atmospheric CO_2 concentration caused a differential expression of 1040 genes (FDR adjusted p-value < 0.05), of which 519 were downregulated and 521 were upregulated compared to ambient CO_2 (Fig. 2A). In addition, real-time qPCR analyses were conducted on a subset of differentially expressed genes, in order to validate the results of the transcriptome profiling (Fig. S4).

First and foremost, the differentially expressed genes related to ethylene biosynthesis/metabolism and signaling were explored. Two important ethylene-related genes were differentially expressed in wild-type plants in response to elevated CO_2 (Table 2, *Col-0 eCO_2 vs. aCO_2*). *ACO1 (ACC OXIDASE 1)*, involved in ethylene biosynthesis (Fig. S4A), and *CTR1* were upregulated. Furthermore, also *RAP2.6* (*RELATED TO AP2*) and *RAP2.6 L*, belonging to the *AP2/ERF* family, were induced. *ERF6* (*ETHYLENE RESPONSE FACTOR 6*) was the only differentially expressed gene in ethylene-insensitive plants under elevated compared to ambient CO_2 (Table 2, *ein2-5 eCO_2 vs. aCO_2*). However, the expression of *ERF6* can be regulated independently from ethylene (Meng et al., 2013). No ethylene related genes were differentially expressed in *ein2-5* as opposed to Col-0 under ambient CO_2 (Table 2, *aCO_2 ein2-5 vs. Col-0*). Under elevated CO_2, however, various genes associated with ethylene biosynthesis and signaling were differentially expressed in ethylene-insensitive as opposed to wild-type plants (Table 2; *eCO_2 ein2-5 vs. Col-0*). *ACO1* (Fig. S4A) and *ACO2* was downregulated whereas *ACS8* (*ACC SYNTHASE 8*) was strongly upregulated. The ethylene receptors *ERS1* and *ERS2* (*ETHYLENE RESPONSE SENSOR*) were both strongly downregulated (Fig. S4B). The transcript abundance of *EBF2*

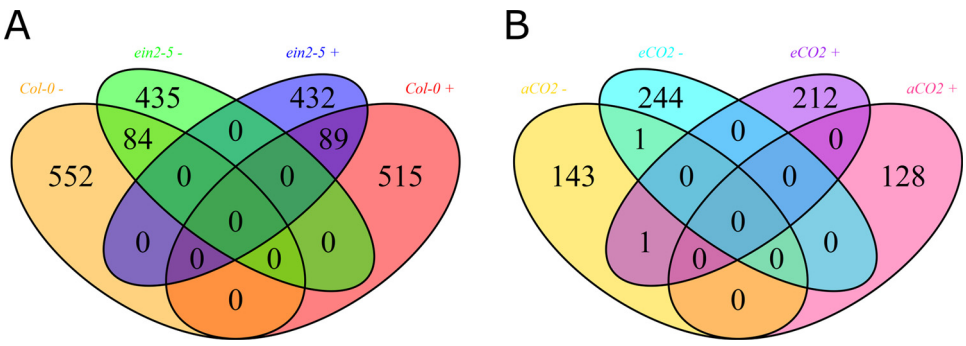

Fig. 2. Overview of the number of differentially expressed genes based on genotype and CO_2 treatment comparisons. (A) Venn diagram of the significantly upregulated genes (+) and downregulated genes (-) in Col-0 and *ein2-5* grown under elevated CO_2 as opposed to ambient CO_2. (B) Venn diagram of the significantly upregulated genes (+) and downregulated genes (-) under ambient (aCO_2) and elevated CO_2 (eCO_2) in *ein2-5* as opposed to Col-0.

(*EIN3-BINDING F-BOX 2*) was decreased whereas that of *EBF1* was increased. *EIN2* was strongly downregulated. Furthermore, *ERF1, ERF2, ERF011/CEJ1, RAP2.6 L* and *RAP2.3*, all characterized by an ethylene-inducible expression, were downregulated. Altogether, these results suggest that elevated CO_2 affect ethylene biosynthesis and signaling and that the consequences of ethylene insensitivity on the expression of ethylene-related genes is far more pronounced under elevated compared to ambient CO_2.

3.3. Common differentially expressed genes in Col-0 and ein2-5 in response to elevated CO_2

Wild-type and ethylene-insensitive plants shared 84 downregulated and 89 upregulated genes in response to elevated CO_2 (Fig. 2A). Interestingly, around half of the common downregulated genes encode for chloroplast localized proteins (SData 1), emphasizing the impact of elevated CO_2 on chloroplasts. Genes involved in the regulation of photosynthesis, chloroplast organization, chlorophyll metabolism and the response to light were significantly enriched (Table 3, *common ein2-5 and Col-0 eCO_2*; SData 1). Among the common upregulated genes between Col-0 and *ein2-5* on the other hand, there was an enrichment of genes related to abiotic stress

(Table 3, *common ein2-5 and Col-0 eCO_2*; SData 1). Altogether, these results suggest that independently of ethylene, elevated CO_2 suppresses the photosynthetic capacity and entails stress to *Arabidopsis* grown in the field, which corresponds to the absence of positive effect of elevated CO_2 on rosette growth in both genotypes (Fig. 1D and E).

3.4. Differentially expressed genes in either Col-0 or ein2-5 in response to elevated CO_2

After omitting the common differentially expressed genes, specifically 552 and 435 were downregulated in Col-0 and *ein2-5,* respectively (Fig. 2A). Although several genes related to chloroplast organization were commonly downregulated in wild-type and ethylene-insensitive plants, genes involved in chloroplast relocation were also significantly

Table 2
An overview of the transcriptome data for the most important genes involved in ethylene biosynthesis, perception and signaling. The log2 fold change (fc) and fold change of the normalized expression values for the different combinations of genotype and CO_2 conditions and the corresponding FDR (False Discovery Rate)-adjusted p-value are represented. The p-values indicated in bold are smaller than the cut-off value of 0.05. Blue indicates a positive change, whereas red indicates a negative change.

		Col-0 eCO_2 vs. aCO_2		*ein2-5* eCO_2 vs. aCO_2			aCO_2 *ein2-5* vs. Col-0			eCO_2 ein2-5 vs. Col-0			
Accession ID	Gene	log2 fc	fc	p-value	log2 fc	fc	p-value	log2 fc	fc	p-value	log2 fc	fc	p-value
AT2G19590	*ACO1*	1.04	2.05	< 0.001	0.02	1.02	1.00	0.92	1.89	0.76	−0.09	−1.07	1.00
AT1G62380	*ACO2*	0.11	1.08	1.00	−0.59	−1.51	0.67	0.23	1.17	1.00	−0.47	−1.39	0.02
AT1G77330	*ACO5*	0.69	1.61	1.00	−0.05	−1.04	1.00	0.46	1.37	1.00	−0.29	−1.22	1.00
AT3G61510	*ACS1*	−1.85	−3.60	0.87	−0.38	−1.30	1.00	−1.59	−3.01	1.00	−0.12	−1.09	1.00
AT1G01480	*ACS2*	0.44	1.35	1.00	−0.88	−1.84	1.00	−0.58	−1.50	1.00	−1.90	−3.72	0.90
AT2G22810	*ACS4*	−1.48	−2.80	1.00	−0.18	−1.13	1.00	0.59	1.51	1.00	1.89	3.71	1.00
AT5G65800	*ACS5*	−0.36	−1.29	1.00	0.38	1.31	1.00	0.07	1.05	1.00	0.82	1.76	1.00
AT4G11280	*ACS6*	0.31	1.24	0.99	−0.13	−1.09	1.00	−0.01	−1.01	1.00	−0.45	−1.36	1.00
AT4G26200	*ACS7*	0.00	0.00	1.00	2.19	4.55	1.00	0.71	1.63	1.00	2.90	7.44	1.00
AT4G37770	*ACS8*	0.00	−1.00	1.00	1.09	2.13	0.14	0.27	1.21	1.00	1.37	2.58	0.01
AT3G49700	*ACS9*	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
AT4G08040	*ACS11*	−0.92	−1.90	1.00	−0.72	−1.65	1.00	0.92	1.89	1.00	1.12	2.17	1.00
AT3G50260	*CEJ1*	0.96	1.94	0.13	0.99	1.98	0.13	−0.66	−1.58	1.00	−0.63	−1.55	0.04
AT5G03730	*CTR1*	0.36	1.29	0.01	0.13	1.10	1.00	0.05	1.03	1.00	−0.18	−1.13	1.00
AT2G25490	*EBF1*	−0.52	−1.43	1.00	0.39	1.31	1.00	0.25	1.19	1.00	1.16	2.23	< 0.001
AT5G25350	*EBF2*	0.38	1.30	0.12	−1.23	−2.35	1.00	0.68	1.60	1.00	−0.94	−1.91	< 0.001
AT2G27050	*EIL1*	0.23	1.17	1.00	−0.28	−1.22	1.00	0.20	1.15	1.00	−0.31	−1.24	0.68
AT5G03280	*EIN2*	0.47	1.38	1.00	−0.42	−1.34	1.00	−0.22	−1.16	1.00	−1.10	−2.15	< 0.001
AT3G20770	*EIN3*	0.14	1.10	1.00	−0.04	−1.03	1.00	0.02	1.01	1.00	−0.16	−1.11	1.00
AT3G04580	*EIN4*	−0.23	−1.17	0.45	0.04	1.03	1.00	−0.17	−1.13	1.00	0.09	1.07	1.00
AT1G54490	*EIN5*	0.12	1.09	0.98	0.06	1.04	1.00	−0.10	−1.07	1.00	−0.16	−1.12	0.98
AT3G23240	*ERF1*	−0.84	−1.80	0.74	−1.73	−3.32	1.00	−2.41	−5.32	0.39	−3.30	−9.82	0.01
AT5G47220	*ERF2*	0.63	1.55	0.91	−0.34	−1.27	1.00	0.06	1.04	1.00	−0.91	−1.88	< 0.001
AT4G17490	*ERF6*	0.25	1.19	1.00	1.34	2.53	< 0.001	−1.21	−2.31	0.05	−0.12	−1.09	1.00
AT1G28370	*ERF11*	0.43	1.35	1.00	0.24	1.18	1.00	−0.27	−1.21	1.00	−0.46	−1.37	1.00
AT2G40940	*ERS1*	0.70	1.63	0.39	−0.15	−1.11	1.00	0.22	1.17	1.00	−0.63	−1.55	< 0.001
AT1G04310	*ERS2*	0.46	1.37	0.39	−2.47	−5.54	0.97	0.38	1.30	1.00	−2.54	−5.83	< 0.001
AT3G51770	*ETO1*	−0.11	−1.08	1.00	0.14	1.10	0.99	−0.21	−1.15	1.00	0.05	1.03	1.00
AT1G66340	*ETR1*	−0.04	−1.02	1.00	−0.12	−1.09	0.91	−0.15	−1.11	1.00	−0.23	−1.17	1.00
AT3G23150	*ETR2*	0.66	1.58	1.00	−0.60	−1.51	1.00	0.85	1.81	1.00	−0.40	−1.32	1.00

Table 3
Overview of the biological processes significantly overrepresented among the differentially expressed genes of different comparisons of genotype and CO_2 treatment, listed in the first column. "REF" indicates the number of genes in the Arabidopsis thaliana reference list that map to this particular annotation data category and "Expected" the number of genes you would expect in this list for a biological process, based on the reference list. "#DEGs" represents the number of differentially expressed genes that map to a particular biological process. "FDR" indicates the FDR-adjusted p-value. "FE" represents the Fold Enrichment of the genes observed for a biological process over the expected. Red values indicate negative values, which were used to indicate downregulated genes. Blue values indicate positive values, which were used to indicate upregulated genes. This table was created based on the PANTHER statistical overrepresentation test (Mi et al., 2013). Continued.

Comparison	Biological process	REF (27,502)	#DEGs	Expected	FDR	FE
Col-0 eCO2 vs aCO2	chloroplast relocation	14	5	0.28	5.77E-03	−18.06
Col-0 eCO2 vs aCO2	photosynthesis, dark reaction	17	6	0.34	1.21E-03	−17.84
Col-0 eCO2 vs aCO2	photosystem II repair	15	5	0.3	6.26E-03	−16.85
Col-0 eCO2 vs aCO2	response to low light intensity stimulus	18	5	0.36	1.04E-02	−14.04
Col-0 eCO2 vs aCO2	response to blue light	77	11	1.52	5.07E-04	−7.22
Col-0 eCO2 vs aCO2	photorespiration	52	7	1.03	1.96E-02	−6.81
Col-0 eCO2 vs aCO2	response to red light	66	8	1.31	1.36E-02	−6.13
Col-0 eCO2 vs aCO2	response to high light intensity	79	9	1.56	9.23E-03	−5.76
Col-0 eCO2 vs aCO2	response to virus	79	9	1.56	8.95E-03	−5.76
Col-0 eCO2 vs aCO2	cellular cation homeostasis	115	10	2.27	2.26E-02	−4.40
Col-0 eCO2 vs aCO2	cellular response to light stimulus	130	10	2.57	4.14E-02	−3.89
Col-0 eCO2 vs aCO2	response to heat	193	14	3.82	9.37E-03	−3.67
Col-0 eCO2 vs aCO2	phenylpropanoid metabolic process	155	11	3.07	4.20E-02	−3.59
Col-0 eCO2 vs aCO2	cofactor biosynthetic process	234	14	4.63	3.97E-02	−3.02
Col-0 eCO2 vs aCO2	response to metal ion	468	26	9.26	1.42E-03	−2.81
Col-0 eCO2 vs aCO2	response to water deprivation	329	17	6.51	4.47E-02	−2.61
Col-0 eCO2 vs aCO2	defense response to bacterium	331	17	6.55	4.62E-02	−2.60
Col-0 eCO2 vs aCO2	response to osmotic stress	618	26	12.22	4.29E-02	−2.13
Col-0 eCO2 vs aCO2	oxidation-reduction process	1331	49	26.33	9.06E-03	−1.86
Col-0 eCO2 vs aCO2	phosphate-containing compound metabolic process	1556	53	30.78	2.29E-02	−1.72
Col-0 eCO2 vs aCO2	response to hormone	1672	55	33.07	3.37E-02	−1.66
Col-0 eCO2 vs aCO2	starch catabolic process	17	5	0.32	3.83E-03	15.68
Col-0 eCO2 vs aCO2	energy reserve metabolic process	17	4	0.32	3.22E-02	12.54
Col-0 eCO2 vs aCO2	fatty acid catabolic process	33	7	0.62	8.40E-04	11.31
Col-0 eCO2 vs aCO2	protein transmembrane import into intracellular organelle	46	6	0.86	2.57E-02	6.95
Col-0 eCO2 vs aCO2	glutathione metabolic process	62	8	1.16	3.88E-03	6.88
Col-0 eCO2 vs aCO2	ribosome assembly	48	6	0.9	2.88E-02	6.66
Col-0 eCO2 vs aCO2	peptide biosynthetic process	567	51	10.64	7.05E-16	4.79
Col-0 eCO2 vs aCO2	protein targeting	142	11	2.66	9.50E-03	4.13
Col-0 eCO2 vs aCO2	microtubule-based process	121	9	2.27	4.03E-02	3.96
Col-0 eCO2 vs aCO2	response to cadmium ion	337	25	6.32	4.37E-06	3.95
Col-0 eCO2 vs aCO2	membrane organization	164	11	3.08	2.73E-02	3.57
Col-0 eCO2 vs aCO2	response to cold	375	24	7.04	7.66E-05	3.41
Col-0 eCO2 vs aCO2	response to cytokinin	236	15	4.43	5.72E-03	3.39
Col-0 eCO2 vs aCO2	response to water	338	21	6.34	4.56E-04	3.31
Col-0 eCO2 vs aCO2	response to jasmonic acid	211	13	3.96	2.01E-02	3.28
Col-0 eCO2 vs aCO2	response to salt stress	545	29	10.23	1.72E-04	2.84
Col-0 eCO2 vs aCO2	vesicle-mediated transport	369	17	6.92	4.96E-02	2.46
Col-0 eCO2 vs aCO2	response to abscisic acid	549	25	10.3	7.24E-03	2.43
ein2-5 eCO2 vs aCO2	photosynthetic electron transport chain	42	7	0.66	8.71E-03	−10.68
ein2-5 eCO2 vs aCO2	response to light stimulus	699	29	10.9	3.86E-03	−2.66
ein2-5 eCO2 vs aCO2	cytoskeleton organization	164	11	2.53	1.68E-02	4.34
ein2-5 eCO2 vs aCO2	response to water deprivation	329	20	5.08	4.56E-04	3.93
ein2-5 eCO2 vs aCO2	response to cold	375	20	5.8	1.84E-03	3.45
ein2-5 eCO2 vs aCO2	response to osmotic stress	618	26	9.55	3.47E-03	2.72
ein2-5 eCO2 vs aCO2	response to lipid	748	27	11.56	1.53E-02	2.34
ein2-5 eCO2 vs aCO2	carboxylic acid metabolic process	916	32	14.16	8.45E-03	2.26
ein2-5 eCO2 vs aCO2	cellular catabolic process	1126	37	17.4	7.91E-03	2.13
ein2-5 eCO2 vs aCO2	organic substance catabolic process	1208	39	18.67	8.36E-03	2.09
ein2-5 eCO2 vs aCO2	response to hormone	1672	49	25.84	7.56E-03	1.90
Comparison	Biological process	REF	#DEGs	Expected	FDR	FE
ein2-5 vs Col-0 aCO2	metabolic process	11388	86	59.21	4.62E-02	−1.45
ein2-5 vs Col-0 aCO2	cellular glucan metabolic process	196	8	0.9	2.54E-02	8.91
ein2-5 vs Col-0 eCO2	positive regulation of ethylene biosynthetic process	2	2	0.02	3.06E-02	−100.00
ein2-5 vs Col-0 eCO2	activation of MAPK activity	6	3	0.05	5.39E-03	−56.59
ein2-5 vs Col-0 eCO2	regulation of stomatal complex patterning	9	3	0.08	1.17E-02	−37.73
ein2-5 vs Col-0 eCO2	copper ion homeostasis	12	3	0.11	2.06E-02	−28.29
ein2-5 vs Col-0 eCO2	jasmonic acid and ethylene-dependent systemic resistance	15	3	0.13	3.12E-02	−22.64
ein2-5 vs Col-0 eCO2	porphyrin-containing compound catabolic process	16	3	0.14	3.27E-02	−21.22
ein2-5 vs Col-0 eCO2	negative regulation of ethylene-activated signaling pathway	17	3	0.15	3.67E-02	−19.97
ein2-5 vs Col-0 eCO2	floral organ abscission	26	4	0.23	1.14E-02	−17.41
ein2-5 vs Col-0 eCO2	cellular biogenic amine biosynthetic process	38	4	0.34	3.07E-02	−11.91
ein2-5 vs Col-0 eCO2	plant-type hypersensitive response	67	7	0.59	5.22E-04	−11.82
ein2-5 vs Col-0 eCO2	defense response to fungus, incompatible interaction	43	4	0.38	3.94E-02	−10.53
ein2-5 vs Col-0 eCO2	leaf senescence	90	8	0.8	3.49E-04	−10.06
ein2-5 vs Col-0 eCO2	toxin metabolic process	68	5	0.6	2.98E-02	−8.32
ein2-5 vs Col-0 eCO2	detoxification	71	5	0.63	3.24E-02	−7.97
ein2-5 vs Col-0 eCO2	ethylene-activated signaling pathway	174	11	1.54	1.27E-04	−7.15
ein2-5 vs Col-0 eCO2	defense response to bacterium	331	15	2.92	9.24E-05	−5.13

(*continued on next page*)

Table 3 (*continued*)

Comparison	Biological process	REF (27,502)	#DEGs	Expected	FDR	FE
ein2-5 vs Col-0 eCO2	response to nitrogen compound	297	12	2.62	2.22E-03	−4.57
ein2-5 vs Col-0 eCO2	response to cold	375	14	3.31	1.19E-03	−4.23
ein2-5 vs Col-0 eCO2	response to metal ion	468	17	4.14	2.35E-04	−4.11
ein2-5 vs Col-0 eCO2	response to oxidative stress	446	15	3.94	1.81E-03	−3.81
ein2-5 vs Col-0 eCO2	sulfur compound metabolic process	358	11	3.16	3.11E-02	−3.48
ein2-5 vs Col-0 eCO2	response to drug	529	15	4.67	9.43E-03	−3.21
ein2-5 vs Col-0 eCO2	cellular response to acid chemical	429	12	3.79	3.24E-02	−3.17
ein2-5 vs Col-0 eCO2	cellular response to oxygen-containing compound	578	16	5.11	7.41E-03	−3.13
ein2-5 vs Col-0 eCO2	response to abscisic acid	549	14	4.85	3.11E-02	−2.89
ein2-5 vs Col-0 eCO2	response to osmotic stress	618	15	5.46	3.11E-02	−2.75
ein2-5 vs Col-0 eCO2	organic acid metabolic process	1046	24	9.24	2.79E-03	−2.60
ein2-5 vs Col-0 eCO2	oxidation-reduction process	1331	29	11.76	1.70E-03	−2.47
ein2-5 vs Col-0 eCO2	cellular process	10702	113	82.11	1.18E-02	1.38
ein2-5 vs Col-0 eCO2	organic substance metabolic process	8574	94	65.78	2.59E-02	1.43
ein2-5 vs Col-0 eCO2	response to light stimulus	699	22	5.36	1.07E-04	4.10
ein2-5 vs Col-0 eCO2	response to water deprivation	329	12	2.52	1.04E-02	4.75
ein2-5 vs Col-0 eCO2	regulation of stomatal movement	74	7	0.57	3.20E-03	12.33
common ein2-5 and Col-0 eCO2	regulation of photosynthesis	47	5	0.14	0.00148	−35.25
common ein2-5 and Col-0 eCO2	chlorophyll metabolic process	59	4	0.18	3.31E-02	−22.46
common ein2-5 and Col-0 eCO2	chloroplast organization	188	7	0.57	0.00406	−12.34
common ein2-5 and Col-0 eCO2	response to extracellular stimulus	229	7	0.69	7.14E-03	−10.13
common ein2-5 and Col-0 eCO2	response to light stimulus	730	12	2.2	0.00324	−5.45
common ein2-5 and Col-0 eCO2	response to cadmium ion	339	9	1.08	2.53E-03	8.30
common ein2-5 and Col-0 eCO2	response to water deprivation	332	8	1.06	1.30E-02	7.53
common ein2-5 and Col-0 eCO2	response to cold	389	9	1.24	6.04E-03	7.23
common ein2-5 and Col-0 eCO2	response to salt stress	555	9	1.78	4.23E-02	5.07
common ein2-5 and Col-0 eCO2	organic substance catabolic process	1669	17	5.34	1.51E-02	3.18

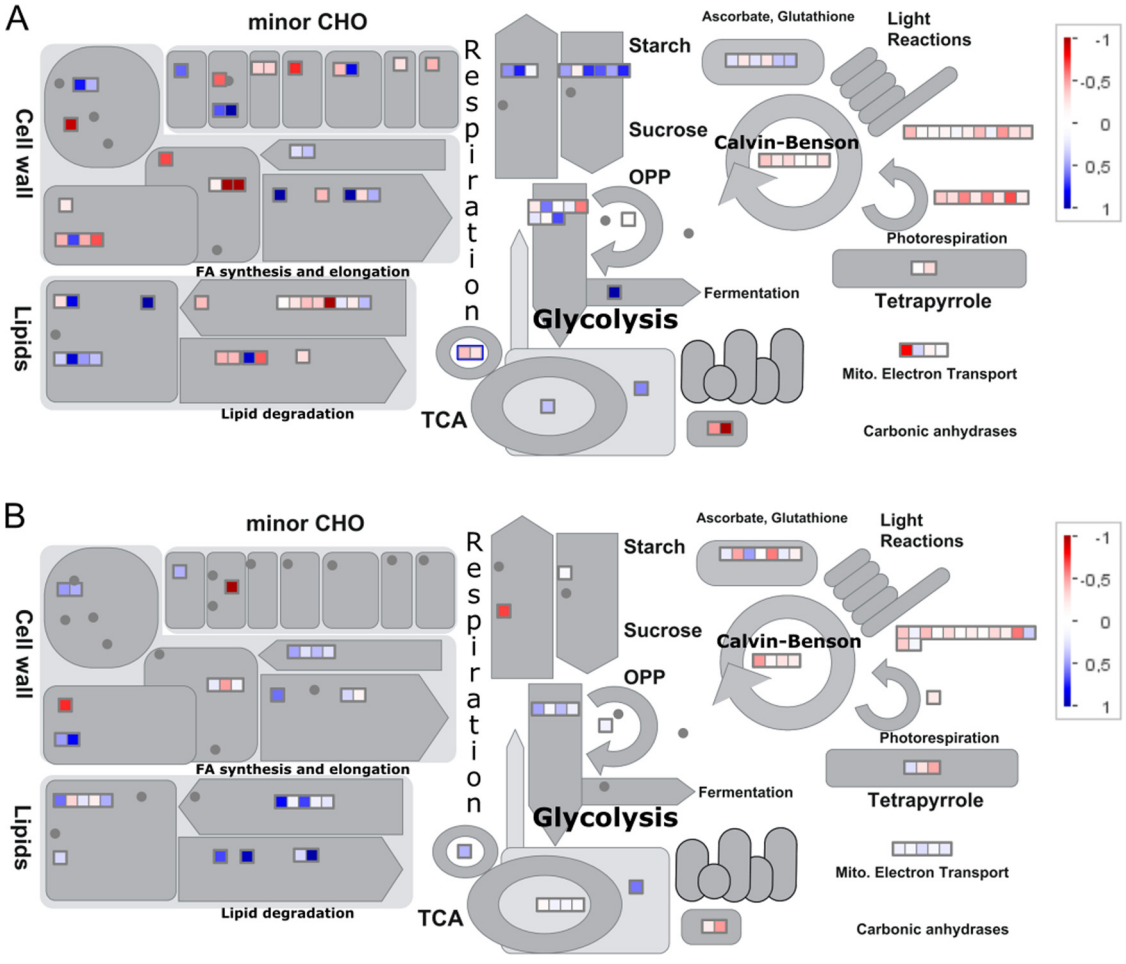

Fig. 3. Overview of transcript changes related to plant metabolism in response to elevated CO_2 in (A) Col-0 and (B) *ein2-5* as generated by Mapman. Blue and red blocks indicate significant (FDR adjusted $P < 0.05$) increases or decreases, respectively, of gene transcription, expressed as log2 fold change in elevated as opposed to ambient CO_2.

enriched among the downregulated genes in Col-0 (Table 3, *Col-0 eCO$_2$ vs aCO$_2$*; SData 2). Apart from the commonly downregulated photosynthesis genes, there were some that were only differentially expressed in either Col-0 or *ein2-5* (Fig. 3A and B; SData 2 and 3). Several photosynthetic electron transport chain genes were only downregulated in *ein2-5* (Table 3; SData 3), whereas several genes functioning in photosystem II repair and in the Calvin-Benson cycle (photosynthesis dark reaction) were only downregulated in Col-0 (Table 3; SData 2). Nevertheless, in *ein2-5* several genes encoding Calvin-Benson cycle enzymes were also downregulated (Fig. 3B; SData 3). Although these genes were different from those repressed in Col-0, our results suggest a reduced Calvin-Benson cycle activity in both Col-0 and *ein2-5* (Fig. 3A and B). Col-0 clearly differed from *ein2-5* in the regulation of photorespiration in response to elevated CO_2. Whereas in Col-0 eight genes functioning in photorespiration were downregulated, only one was supressed in *ein2-5* (Fig. 3A and B; SData 2 and 3).

Specifically, 515 and 432 genes were upregulated in Col-0 and *ein2-5,* respectively (Fig. 2A). Starch catabolism and starch biosynthesis genes were significantly enriched in Col-0 but not in *ein2-5* (Table 3, *Col-0 eCO$_2$ vs aCO$_2$* and *ein2-5 eCO$_2$ vs aCO$_2$*). In *ein2-5 GLT1 (GLUCOSE TRANSPORTER 1)*, was the only, but weakly, upregulated gene related to starch metabolism (Fig. 3A and B; SData 2 and 3). *SUCROSE SYNTHASE 1 (SUS1)* was activated in both Col-0 and *ein2-5*. The starch biosynthesis gene *SUCROSE-6-PHOSPHATE PHOSPHOHYDROLASE 2 (SPP2)* was even strongly downregulated in *ein2-5* (Fig. S4C). Several glycolysis genes on the other hand were upregulated in both Col-0 and *ein2-5*. Genes involved in fatty acid catabolism were significantly enriched among the upregulated genes in Col-0 but not in *ein2-5*. Fatty-acid biosynthesis and elongation and lipid degradation genes were mainly downregulated in Col-0 but all upregulated in *ein2-5*. The same applied to genes involved in lipid turnover. Genes functioning in lipid metabolism (f.i. phospholipid and triacylglycerol synthesis) were predominantly upregulated in both wild-type and ethylene-insensitive plants, but more strongly in the former. Altogether these results suggest that in response to elevated CO_2 respiration and lipid metabolism changed, but that in the wild-type starch biosynthesis and turnover, whereas in ethylene-insensitive plants the lipid metabolism were more strongly activated.

In response to elevated CO_2, many genes were upregulated in Col-0 that were shown to be induced during leaf senescence (Guo et al., 2004;, 2012; Zhang et al., 2014) (Table 4). In *ein2-5*, however, only 3 of these senescence genes were weakly upregulated. Together these results suggest that leaf senescence is advanced under elevated CO_2 in wild-type plants, and that this response is mediated by ethylene.

Furthermore, both in Col-0 and *ein2-5*, a substantial part of the differentially expressed genes under elevated CO_2 were stress responsive (Fig. 4; SData 2 and 3). The most noticeable difference between both genotypes was in the expression of *GLUTATHIONE-S-TRANSFERASEs (GSTs)* and genes encoding enzymes that regulate the redox state of the cell. Genes involved in glutathione metabolism were significantly enriched in Col-0 but not in *ein2-5* (Table 3; Fig. S4D-E). Consistently, various genes functioning in the regulation of the cell redox-state were predominantly upregulated in Col-0 whereas in *ein2-5* more were downregulated. Furthermore, whilst in wild-type plants several *GSTs* were induced, only one was upregulated in ethylene-insensitive plants. Together these results suggest an impact of ethylene-insensitivity on redox homeostasis in response to elevated CO_2.

3.5. Differentially expressed genes in ein2-5 compared to Col-0 under ambient and elevated CO_2

Under ambient CO_2 273 genes were differentially expressed in ethylene-insensitive compared to wild-type plants, of which 145 were downregulated and 128 were upregulated (Fig. 2B). Metabolism genes were overrepresented among the downregulated and cellular glucan metabolism genes among the upregulated genes (Table 3). The consequences of ethylene insensitivity for transcript level changes thus seem limited under ambient CO_2 at the end of vegetative growth. Under elevated CO_2, however, 458 genes were differentially expressed, of which 245 were downregulated and 213 were upregulated in *ein2-5* compared to Col-0 (Fig. 2B). Only one upregulated gene was shared with ambient CO_2 (Fig. 2B). Ethylene-related genes were significantly overrepresented among the downregulated genes (Table 5; SData 4). Consistent with the CO_2-induced expression of many senescence genes in Col-0 but not in *ein2-5* (Table 4), genes involved in leaf senescence and chlorophyll degradation were downregulated in *ein2-5* compared Col-0 (Table 5; SData 4), indicating a delayed leaf senescence in the former. Furthermore, several genes encoding peroxidases, *GSTs* and proteins related to the cell redox-state were downregulated in *ein2-5* as opposed to Col-0 (Table 5; SData 4; Fig. S4D-E). Consistently, oxidative stress responsive genes were part of the overrepresented downregulated genes related to stress (Table 3; SData 4). Genes related to photosynthesis light reactions on the other hand were significantly enriched among the upregulated genes in *ein2-5* as opposed to Col-0 under elevated CO_2, but were only weakly upregulated. Genes involved in the response to water deprivation, such as *AT14A-LIKE1 (AFL1)*, and

Table 4
An overview of transcriptome data for genes involved in leaf senescence. The log2 fold change (fc) and fold change of the normalized expression values for the different combinations of genotype and CO_2 conditions and the corresponding FDR-adjusted p-value are represented. Blue indicates a positive change, whereas red indicates a negative change.

			Col-0 eCO$_2$ vs. aCO$_2$			*ein2-5* eCO$_2$ vs. aCO$_2$		
Accession ID	Gene	Gene description	**log2** fc	fc	p-value	**log2** fc	fc	p-value
AT1G73220	*OCT1*	*ORGANIC CATION/CARNITINE TRANSPORTER1*	3.02	8.11	< 0.01			
AT4G13250	*NYC1*	*NON-YELLOW COLORING 1*	0.82	1.77	< 0.001			
AT2G22300	*SR1*	*SIGNAL RESPONSIVE 1*	0.21	1.16	< 0.05			
AT1G20620	*SEN2*	*SENESCENCE 2*	1.01	2.02	< 0.001			
AT3G15730	*PLDALPHA1*	*PHOSPHOLIPASE D ALPHA1*	0.15	1.11	< 0.001	0.24	1.18	< 0.05
AT4G01610	*CATHB3*	*CATHEPSIN B3*	0.29	1.22	< 0.01			
AT2G29470	*GSTU3*	*GLUTATHIONE S-TRANSFERASE TAU 3*	2.30	4.91	< 0.01			
AT1G78380	*GSTU19*	*GLUTATHIONE S-TRANSFERASE TAU 19*	0.29	1.23	< 0.001			
AT1G80160	*GLYI7*	*GLYOXYLASE I 7*	1.58	2.98	< 0.05			
AT2G45570	*CYP76C2*	*CYTOCHROME P450 76C2*	1.98	3.94	< 0.05			
AT4G37990	*ELI3*	*ELICITOR-ACTIVATED GENE 3*	1.23	2.34	< 0.001			
AT1G52890	*NAC019*	*NAC DOMAIN CONTAINING PROTEIN 19*	1.11	2.17	< 0.001			
AT2G46680	*ATHB7*	*ARABIDOPSIS THALIANA HOMEOBOX 7*	0.51	1.43	< 0.05	0.29	1.22	< 0.01
AT3G26740	*CCL*	*CCR-like*	1.02	2.02	< 0.001	0.71	1.63	< 0.05
AT3G60140	*SRG2*	*SENESCENCE-RELATED GENE 2*	3.27	9.65	< 0.001			
AT5G13080	*WRKY75*	*WRKY DNA-BINDING PROTEIN 75*	0.91	1.88	< 0.001			

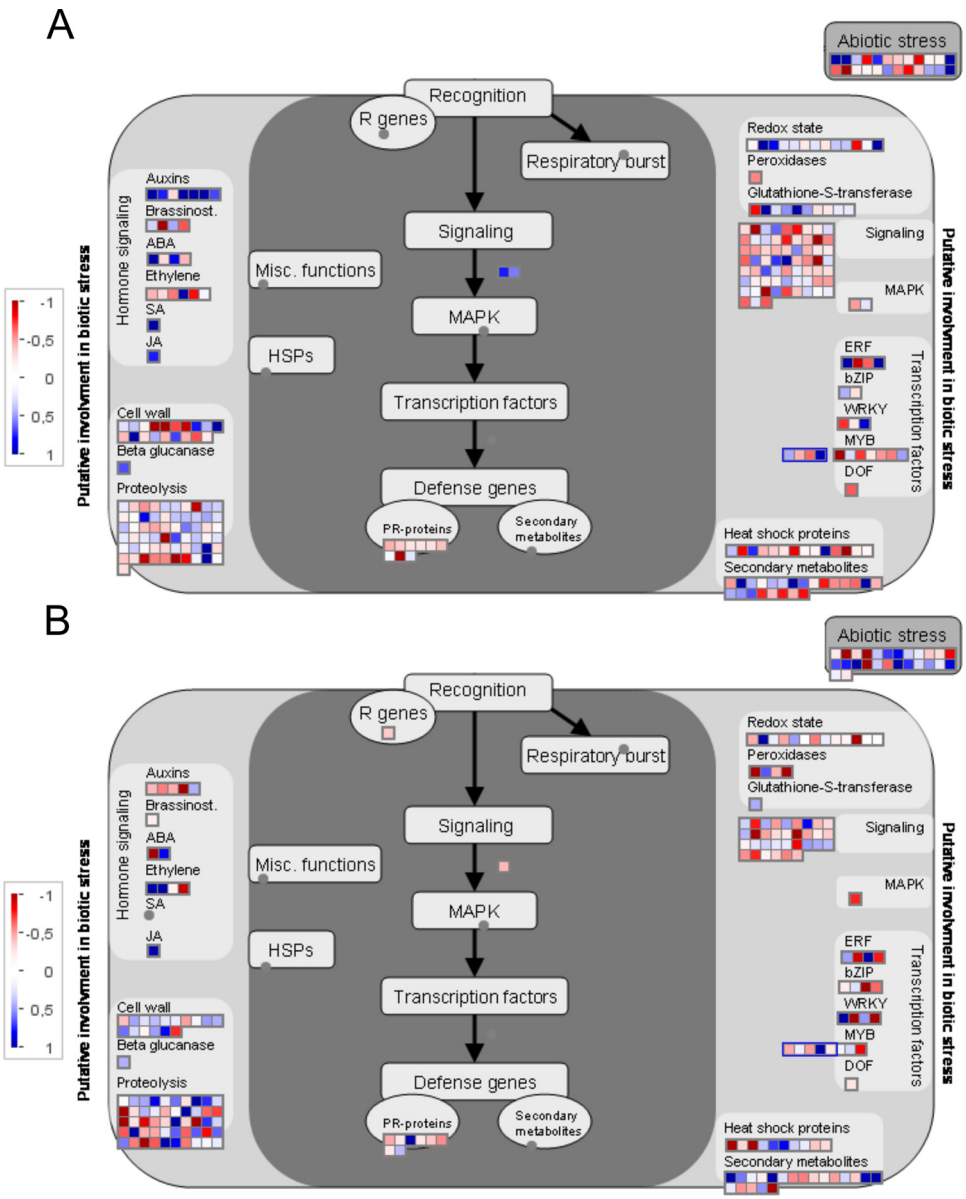

Fig. 4. Overview of transcript changes related to stress in response to elevated CO_2 in (A) Col-0 and (B) *ein2-5* as generated by Mapman. Blue and red blocks indicate significant (FDR adjusted $P < 0.05$) increases or decreases, respectively, of gene transcription, expressed as log2 fold change in elevated as opposed to ambient CO_2.

stomatal movement were also strongly enriched among the upregulated genes (Table, 3 *ein2-5 vs Col-0 eCO₂*; Fig. 5; Fig. S4F; SData 4). *SPP2*, involved in starch biosynthesis was strongly downregulated whereas *QUA-QUINE STARCH (QQS),* which plays a negative role in starch biosynthesis, was strongly upregulated (Fig. 5; Figure S4G; SData 4). These results suggesting a regulatory role for ethylene in photosynthesis, leaf senescence, stomatal movement and cell redox homeostasis in response to elevated CO_2. Also, the importance of ethylene for starch biosynthesis was indicated.

4. Discussion

Plants are sensitive to the rising atmospheric CO_2 concentration. Because the current atmospheric CO_2 concentration is rate limiting for carbon fixation during photosynthesis, future increases could theoretically give rise to an amelioration of plant growth (Ludewig et al., 1998). However, the relationship between photosynthesis and plant growth under elevated CO_2 is complex (Long et al., 2004; Ainsworth and Long, 2005; Ainsworth and Rogers, 2007). In *Arabidopsis,* the impact of elevated CO_2 on vegetative growth varies depending on whether plants were grown in phytotrons or in the field (Table S3). In FACE studies with other species, elevated CO_2 promoted growth and productivity of above- and below-ground parts, though the effect was smaller than in experiments in a controlled environment (Long et al., 2006). This discrepancy might be due to the fact that fluctuations in the environment, inherent to field studies, can influence plant growth and physiological responses to elevated CO_2. On the other hand, in some FACE experiments - including those studying *Arabidopsis* (Miyazaki et al., 2004; Li et al., 2006, 2008) $-CO_2$ fumigation only occurs during the day (Miglietti et al., 2001), whereas phytotron studies generally analyze the effects of continuous CO_2 enrichment. In addition, differences in plant responses to continuous CO_2 fumigation *versus* fumigation during daytime only have been reported previously (Bunce, 2005, 2014). Therefore, the effect of continuously elevated CO_2 on rosette growth under more natural conditions was investigated. The hormonal regulation of the adaptation of plants to elevated CO_2 remains largely unexplored. Because ethylene is a prime factor in the regulation of vegetative development, its involvement in the adaptation to elevated CO_2 was

Table 5
Overview of interesting transcript changes in *ein2-5* compared to Col-0 under elevated CO_2 as generated by Mapman and PANTHER. The log2 fold change (fc) and fold change of the normalized expression values for the differentially expressed genes and the corresponding FDR-adjusted p-value are represented. Blue indicates a positive change, whereas red indicates a negative change.

Accession ID	Gene	Gene description	Annotation	log2 fc	fc	p-value
AT5G43450		*Encodes a protein whose sequence is similar to ACC oxidase*	Ethylene	−0.65	−1.57	< 0.001
AT4G37770	*ACS8*	*1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 8*	Ethylene	1.37	2.58	< 0.01
AT1G62380	*ACO2*	*ACC OXIDASE 2*	Ethylene	−0.47	−1.39	< 0.05
AT1G04310	*ERS2*	*ETHYLENE RESPONSE SENSOR 2*	Ethylene	−2.54	−5.83	< 0.01
AT2G40940	*ERS1*	*ETHYLENE RESPONSE SENSOR 1*	Ethylene	−0.63	−1.55	< 0.001
AT3G23240	*ERF1*	*ETHYLENE RESPONSE FACTOR 1*	Ethylene	−3.30	−9.82	< 0.05
AT3G50260	*CEJ1*	*COOPERATIVELY REGULATED BY ETHYLENE AND JASMONATE 1*	Ethylene	−0.63	−1.55	< 0.05
AT4G17500	*ERF-1*	*ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 1*	Ethylene	−1.24	−2.37	< 0.001
AT5G47220	*ERF2*	*ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 2*	Ethylene	−0.91	−1.88	< 0.001
AT2G25490	*EBF1*	*EIN3-BINDING F BOX PROTEIN 1*	Ethylene	1.16	2.23	< 0.001
AT5G25350	*EBF2*	*EIN3-BINDING F BOX PROTEIN 2*	Ethylene	−0.94	−1.92	< 0.001
AT5G03280	*EIN2*	*ETHYLENE INSENSITIVE 2*	Ethylene	−1.10	−2.14	< 0.001
AT3G30720	*QQS*	*QUA-QUINE STARCH*	Starch	2.12	4.36	< 0.001
AT3G52340	*SPP2*	*SUCROSE-6F-PHOSPHATE PHOSPHOHYDROLASE 2*	Starch	−0.67	−1.59	< 0.001
AT4G13250	*NYC1*	*NON-YELLOW COLORING 1*	Chlorophyll degradation	−0.40	−1.32	< 0.001
AT4G22920	*NYE1*	*NON-YELLOWING 1*	Chlorophyll degradation	−0.34	−1.27	< 0.001
AT5G44610	*MAP18*	*MICROTUBULE-ASSOCIATED PROTEIN 18*	Leaf senescence	−2.87	−7.33	< 0.05
AT3G15010	*UBA2C*	*UBP1-associated protein 2C*	Leaf senescence	−0.32	−1.25	< 0.05
AT5G03280	*EIN2*	*ETHYLENE INSENSITIVE 2*	Leaf senescence	−1.10	−2.15	< 0.001
AT1G69490	*NAC029*	*NAC DOMAIN CONTAINING PROTEIN 29*	Leaf senescence	−2.45	−5.48	< 0.001
AT4G02380	*SAG21*	*SENESCENCE-ASSOCIATED GENE 21*	Leaf senescence	−1.00	−2.00	< 0.01
AT5G08790	*NAC081*	*NAC DOMAIN CONTAINING PROTEIN 81*	Leaf senescence	−0.39	−1.31	< 0.05
AT1G73220	*OCT1*	*ORGANIC CATION/CARNITINE TRANSPORTER1*	Leaf senescence	−2.79	−6.91	< 0.05
AT1G54130	*RSH3*	*RELA/SPOT HOMOLOG 3*	Leaf senescence	−0.45	−1.36	< 0.001
AT1G44970		*Peroxidase superfamily protein*	Peroxidases	−3.13	−8.76	< 0.05
AT4G08770	*PRX37*	*PEROXIDASE 37*	Peroxidases	−2.62	−6.15	< 0.001
AT2G04700		*Ferredoxin thioredoxin reductase catalytic beta chain family protein*	Redox state	−0.09	−1.07	< 0.05
AT4G03520	*TRXM2*	*THIOREDOXIN M2*	Redox state	−0.13	−1.09	< 0.001
AT2G32920	*PDI9*	*PROTEIN DISULFIDE ISOMERASE 9*	Redox state	−0.45	−1.37	< 0.01
AT4G08390	*SAPX*	*STROMAL ASCORBATE PEROXIDASE*	Redox state	−0.25	−1.19	< 0.001
AT1G69920	*GSTU12*	*GLUTATHIONE S-TRANSFERASE TAU 12*	Glutathione-S-transferases	−4.93	−30.50	< 0.001
AT1G78380	*GSTU19*	*GLUTATHIONE S-TRANSFERASE TAU 19*	Glutathione-S-transferases	−0.16	−1.12	< 0.001
AT2G29460	*GSTU4*	*GLUTATHIONE S-TRANSFERASE TAU 4*	Glutathione-S-transferases	−1.07	−2.09	< 0.001
AT2G29470	*GSTU3*	*GLUTATHIONE S-TRANSFERASE TAU 3*	Glutathione-S-transferases	−5.13	−34.95	< 0.001
AT2G05070	*LHCB2.2*	*PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.2*	Water deprivation	0.07	1.05	< 0.05
AT1G52400	*BGLU18*	*BETA GLUCOSIDASE 18*	Water deprivation	0.52	1.43	< 0.001
AT1G56600	*GolS2*	*GALACTINOL SYNTHASE 2*	Water deprivation	0.53	1.44	< 0.01
AT5G40390	*SIP1*	*SEED IMBIBITION 1-LIKE*	Water deprivation	0.21	1.16	< 0.001
AT1G02205	*CER1*	*ECERIFERUM 1*	Water deprivation	0.15	1.11	< 0.05
AT1G78080	*RAP2.4*	*RELATED TO AP2 4*	Water deprivation	0.16	1.12	< 0.05
AT5G52300	*LTI65*	*LOW-TEMPERATURE-INDUCED 65*	Water deprivation	1.51	2.85	< 0.05
AT3G11410	*PP2CA*	*PROTEIN PHOSPHATASE 2CA*	Water deprivation	0.38	1.30	< 0.001
AT3G28270	*AFL1*	*AT14A-LIKE1*	Water deprivation	1.76	3.39	< 0.001
AT5G25610	*RD22*	*RESPONSIVE TO DESICCATION 22*	Water deprivation	0.35	1.28	< 0.01
AT3G18490	*ASPG1*	*ASPARTIC PROTEASE IN GUARD CELL 1*	Water deprivation	0.14	1.10	< 0.001
AT3G27690	*LHCB2.3*	*PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.3*	Water deprivation	0.37	1.30	< 0.001
AT2G05070	*LHCB2.2*	*PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.2*	Stomatal movement	0.07	1.05	< 0.05
AT4G03560	*TPC1*	*TWO-PORE CHANNEL 1*	Stomatal movement	0.15	1.11	< 0.01
AT1G51140	*FBH3*	*FLOWERING BHLH 3*	Stomatal movement	0.47	1.38	< 0.001
AT1G52400	*BGLU18*	*BETA GLUCOSIDASE 18*	Stomatal movement	0.52	1.43	< 0.001
AT1G69530	*EXPA1*	*EXPANSIN A1*	Stomatal movement	0.28	1.21	< 0.001
AT3G11410	*PP2CA*	*PROTEIN PHOSPHATASE 2CA*	Stomatal movement	0.38	1.30	< 0.001
AT3G45780	*PHOT1*	*PHOTOTROPIN 1*	Stomatal movement	0.27	1.20	< 0.001
AT2G05070	*LHCB2.2*	*PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.2*	Photosynthesis light reactions	0.07	1.05	< 0.05
AT2G34430	*LHB1B1*	*LIGHT-HARVESTING CHLOROPHYLL-PROTEIN COMPLEX II SUBUNIT B1*	Photosynthesis light reactions	0.26	1.20	< 0.001
AT2G40100	*LHCB4.3*	*LIGHT HARVESTING COMPLEX PHOTOSYSTEM II*	Photosynthesis light reactions	−0.26	−1.20	< 0.01
AT3G27690	*LHCB2.3*	*PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.3*	Photosynthesis light reactions	0.37	1.30	< 0.001
AT1G79040	*PSBR*	*PHOTOSYSTEM II SUBUNIT R*	Photosynthesis light reactions	−0.10	−1.07	< 0.01
AT3G50820	*PSBO2*	*PHOTOSYSTEM II SUBUNIT O-2*	Photosynthesis light reactions	0.12	1.09	< 0.01
AT1G03130	*PSAD-2*	*PHOTOSYSTEM I SUBUNIT D-2*	Photosynthesis light reactions	0.15	1.11	< 0.05
AT1G20340	*PETE2*	*PLASTOCYANIN 2*	Photosynthesis light reactions	−0.19	−1.14	< 0.001

investigated.

4.1. Rosette growth

Phytotron studies indicated that higher levels of CO_2 allow *Arabidopsis* plants to grow larger rosettes (Table S3). In the sunlit field chambers with natural fluctuations of irradiation and air temperature no increase in relative rosette area of wild-type plants in response to elevated CO_2 was found (Fig. 1D; Table 1; Fig. S2D; Table S2). These findings are consistent with other field trials using the model organism (Miyazaki et al., 2004; Li et al., 2006, 2008) (Table S3). Also in ethylene-insensitive plants and plants with a constitutive ethylene activity, elevated CO_2 did not promote rosette growth (Fig. 1E and F and Fig. S2E and F). Various explanations as to the cause of the absence of increased growth in the field under elevated CO_2 can be proposed. The enhanced expression of stress responsive genes upon elevated CO_2

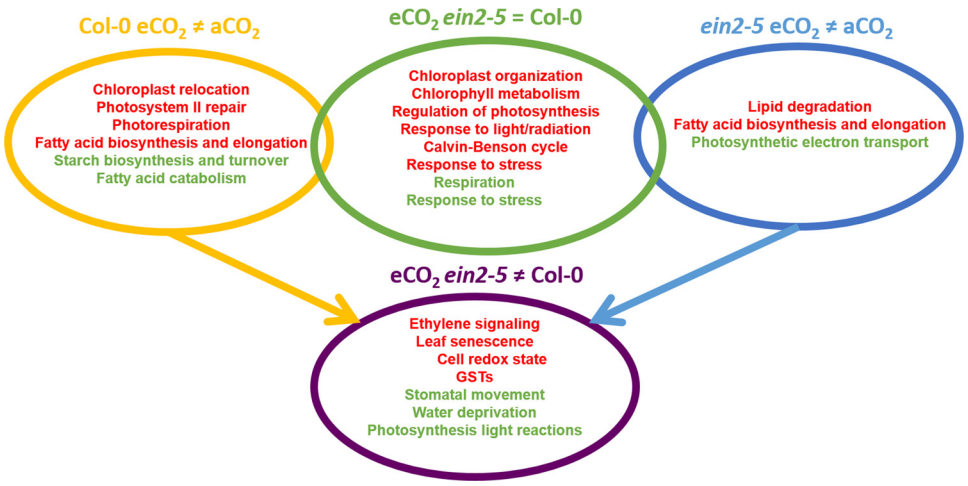

Fig. 5. Overview of the biological processes associated with the significantly differentially expressed genes in response to elevated CO_2. The upregulated biological processes are represented in green, whereas the downregulated processes are represented in red. The yellow circle indicates changes in Col-0 under elevated compared to ambient CO_2. The blue circle indicates changes in *ein2-5* under elevated compared to ambient CO_2. The green circle indicates common changes in Col-0 and *ein2-5* under elevated compared to ambient CO_2. The purple circle indicates changes in *ein2-5* compared to Col-0 under elevated CO_2.

levels, such as *GSTs* and those related to redox status (Fig. 4) is consistent with comparable studies (Miyazaki et al., 2004; Li et al., 2006, 2008) and could have impeded the stimulation of growth by higher concentrations of CO_2. The observed photosynthetic acclimation in response to long-term exposure to elevated CO_2 could also have limited the growth potential at later stages of vegetative development (Fig. 3). Furthermore, nutrient constraints could have restricted the growth promoting effect of elevated CO_2. A high carbon allocation to the roots, to increase carbon-sink capacity and nutrient uptake, can limit the growth of aerial tissues under elevated CO_2 (Crookshanks et al., 1998). Roots, however, were not the subject of this study. The discrepancy between phytotron and field studies emphasizes the importance of the latter to provide further insights in the response of plants to future increases in atmospheric CO_2. It is important to consider the timing of plant transfer, duration of the CO_2 treatment, and CO_2 concentration, when comparing phytotron and field studies. In this study, plants were grown for 14 days before transfer to the field chambers after which they were treated with 590 ppm CO_2 for approximately three weeks. It is conceivable that the duration of treatment was insufficient to provoke any observable growth response. In phytotron studies, plant transfer occurred between 0–5 weeks (Table S3), while treatment lasted between 7 days and 6 weeks. Since in most phytotron studies a stimulation of growth was observed, we believe that the duration of treatment was sufficient for the plants to respond to the CO_2 treatment. Noteworthy, the concentrations used for elevated CO_2 treatments were generally between 100 and 200 ppm higher in phytotron studies (Table S3), which could explain the presence of an observable growth effect in the latter.

4.2. A role for ethylene in the response to elevated CO_2

A function for ethylene in the adaptation of plants to elevated CO_2 is suggested by the upregulation of *ACO1* in wild-type plants (Table 2). Because ethylene was shown to regulate both transcript and protein levels of *CTR1* (Shakeel et al., 2015), its upregulation in Col-0 in response to elevated CO_2 corroborates an increase in ethylene biosynthesis (Table 2). In support, earlier studies showed an elevated ethylene production under increased atmospheric CO_2 in various plant species (Dhawan et al., 1981; Bassi and Spencer, 1982; Mathooko et al., 1998; Seneweera et al., 2003; Wang et al., 2009). Moreover, the reduced transcript abundance of various ethylene-related and -inducible genes in *ein2-5* as compared to Col-0 in response to elevated CO_2, and the absence thereof under ambient CO_2 (Table 2 and 3), support a function for ethylene in the adaptation to increased atmospheric CO_2. Our results suggest the involvement of ethylene in photosynthesis, stomatal closure, starch and lipid metabolism, leaf senescence and plant stress. Noteworthy, high concentrations of CO_2 (*e.g.* > 1% or 10,000 ppm CO_2) can inhibit ethylene biosynthesis, a feature often exploited to delay the ethylene-mediated onset of ripening of stored fruit (Thiamann, 1972). However, the concentration used in our study, which is approximately 17-fold lower, does not evoke such a response, as evidenced by the upregulation of *ACO1* (Table 2).

4.2.1. Photosynthesis and stomatal closure

Efficient plant primary growth strongly depends on photosynthesis. Genes related to photosynthesis, chlorophyll metabolism and chloroplast movement were downregulated in both wild-type and ethylene-insensitive plants, indicating photosynthetic acclimatisation due to the long-term exposure to elevated CO_2 (Table 3; Fig. 3; Fig. 5). Several photosynthesis related genes were weakly upregulated in *ein2-5* compared to Col-0 under elevated CO_2 (Table 5). Ethylene-insensitive plants were shown to have a reduced photosynthetic capacity compared to wild-type plants (Tholen et al., 2004, 2007,2008). The latter could contribute to the observed smaller to similar relative rosette area of ethylene-insensitive plants compared to wild-type plants in field chambers (Fig. 1D-F; Fig. S2D-F), in contrast with phytotron studies (Bleecker et al., 1988; Van Der Straeten et al., 1993; Grbic and Bleecker, 1995; Hua et al., 1995).

Several genes involved in stomatal movement were upregulated in *ein2-5* as opposed to Col-0 in response to elevated CO_2 (Table 3; Fig. 5). Stomatal closure has been shown to occur during growth under CO_2 enrichment (Betts et al., 2007; Xu et al., 2016). Several studies suggest a role for ethylene in the regulation of stomatal movement. Ethylene was shown to induce stomatal closure (Desikan et al., 2006), whereas it inhibited the ABA-induced closure of stomata in *Arabidopsis* (Tanaka et al., 2005). Ethylene-insensitive *Arabidopsis* plants had a substantial lower stomatal conductance than wild-type plants (Tholen et al., 2007). Because glucose is able to trigger stomatal closure (Li et al., 2018) and because ethylene was shown to play a role in sugar signaling (Zhou et al., 1998; Gazzarrini and McCourt, 2001), the impact of ethylene-insensitivity on stomatal movement (Table 3; Fig. 5) could be linked to its impact on starch biosynthesis and turnover (Fig. 3B and Fig. 5). Taken together, a role for ethylene in the regulation of stomatal closure in response to elevated CO_2 is suggested.

4.2.2. Plant respiration: starch and lipid metabolism

During photosynthesis, photosynthates (sugars) are being produced, which can be stored as starch for later use. The increased expression of sucrose synthase and genes involved in starch synthesis and turnover in wild-type plants (Fig. 3A; Fig. 5), suggests an accumulation of non-structural carbohydrates (NSCs) because of the elevated CO_2 (Ainsworth et al., 2004). An increase in starch biosynthesis and turnover in response to elevated CO_2 was also observed in previous studies (Cheng et al., 1998; Teng et al., 2006; Ekman et al., 2007; Li et al.,

2008; Jauregui et al., 2018). Starch metabolism is an important factor in *Arabidopsis* growth through its regulatory role in soluble sugar availability (Caspar et al., 1991; Gibson et al., 2011; Ragel et al., 2013). Furthermore, the starch turnover was shown to dictate the growth response to elevated CO_2 (Sun et al., 2002; Baslam et al., 2017; Jauregui et al., 2018). Jauregui et al., 2018 showed that in contrast with starchless mutants, growth was not enhanced by elevated CO_2 in starch excess mutants. Because NSCs function as signaling molecules, they are able to affect gene transcription. The negative feedback of NSC accumulation on photosynthesis (Ainsworth and Bush, 2011; Thompson et al., 2017) provides an explanation for the downregulation of photosynthesis-related genes under elevated CO_2 (Table 3; Fig. 3A). The more starch accumulates the more severe photosynthetic acclimation (Long et al., 2004; Jauregui et al., 2018). Genes involved in starch synthesis and turnover were, however, not upregulated in ethylene-insensitive plants, suggesting the importance of ethylene therein. Ethylene signaling was shown to be involved in the transcriptional regulation of starch biosynthesis in rice (Wuriyanghan et al., 2009; Fu and Xue, 2010; Zhu et al., 2011). In starch accumulating *Arabidopsis sweetie* plants, the expression of *ERF1* and *ERF2*, here downregulated in *ein2-5* in response to elevated CO_2 (Table 2), showed an increased expression (Veyres et al., 2008). Nevertheless, photosynthesis was also downregulated in ethylene-insensitive plants in response to elevated CO_2 (Fig. 3B), suggesting an alternative carbohydrate storage molecule apart from starch and/or an alternative underlying cause for the observed photosynthetic acclimation in *ein2-5*.

Both in Col-0 and *ein2-5*, several genes encoding enzymes involved in glycolysis and the citric acid cycle were upregulated in response to elevated CO_2 (Fig. 3A and B; Fig. 5). An increase in plant respiration upon growth under elevated CO_2 is consistent with previous studies (Cheng et al., 1998; Li et al., 2008; Leakey et al., 2009) and enfeeble a regulatory role for ethylene. The increase in respiration could be a mechanism to buffer the increased availability of respiratory substrates (photosynthates) (Gonzelez-Meller et al. (2004)) and/or to the export of carbohydrates to sink tissues, a process which requires a substantial amount of energy (Leakey et al., 2009). Elevated CO_2 increased transcript abundance of genes involved in fatty acid catabolism in wild-type plants, whereas those involved in fatty acid biosynthesis and elongation and lipid degradation were generally downregulated (Fig. 3A; Table 3; Fig. 5). Ekman et al. (2007) found a significant decrease in the fatty acid concentration of the leaves in response to high CO_2. In ethylene-insensitive plants on the other hand, genes involved in fatty acid biosynthesis and elongation, and lipid degradation were promoted (Fig. 3B; Fig. 5). Together with the pronounced lipid changes by ethylene during leaf senescence in *Arabidopsis* (Jia and Li, 2015), our results suggest a role for ethylene in the regulation of lipid metabolism under elevated CO_2.

4.2.3. Leaf senescence

The transcript abundance of many senescence genes was increased under elevated compared to ambient CO_2 in Col-0 but not in *ein2-5* (Table 4). These results suggest an acceleration of leaf senescence, mediated by ethylene, in response to elevated CO_2 (Fig. 5). However, the rosette leaves exhibited no visible signs of senescence (Fig. S1), suggesting the very onset. Chlorophyll degradation only occurs when leaf senescence is in an advanced stage (Diaz et al., 2005). The accelerated leaf senescence could therefore have contributed to the absence of a growth promoting effect of elevated CO_2 in Col-0 but not in *ein2-5*. An imbalance in the C/N ratio as a consequence of the exposure to elevated CO_2 could be the driving factor leading to the ethylene-mediated acceleration of leaf senescence (Aguera and De la Haba, 2018). Furthermore, leaf senescence has been shown to be regulated by sugars (Paul and Foyer, 2001; Wingler et al., 2006; van Doorn, 2008; Aguera and De La Haba, 2018). Starch excess plants exhibited signs of accelerated leaf senescence (Jauregui et al., 2018). The promotion of leaf senescence by elevated CO_2 could contribute to the downregulation of photosynthesis (Miller et al., 1997; Ludewig and Sonnewald, 2000). The inhibitory role of ethylene in leaf longevity (Fig. 5) (Jing et al., 2005) was reflected in the enrichment of leaf senescence genes among the downregulated genes in *ein2-5* compared to Col-0 in response to elevated CO_2 (Table 3; SData 3). The absence of the previously observed growth-promoting effect of ethylene-insensitivity on *Arabidopsis* rosettes (Fig. 1D-F and Fig. S2D-F), measured during generative growth in phytotrons (Bleecker et al., 1988; Van Der Straeten et al., 1993; Grbic and Bleecker, 1995; Hua et al., 1995), could be related to leaf senescence. The promotion of leaf growth upon ethylene-insensitivity was postulated to be caused by a delay in leaf senescence (Tholen et al., 2004) and because leaf senescence only commences during generative growth, ethylene-insensitivity could fail to promote leaf expansion during vegetative growth (Fig. 1E; Fig. S2E). The delay in leaf senescence could also explain the higher transcript abundance of some photosynthesis-related genes in *ein2-5* compared to Col-0 in response to elevated CO_2 (Fig. 5).

4.2.4. Plant stress

Among the differentially expressed genes in elevated compared to ambient CO_2 an important part were stress responsive genes, although the plants were not showing any visible symptom of stress (Table 3; Fig. 4). Consistent with the findings of previous studies (Miyazaki et al., 2004; Li et al., 2006) our results indicate plant stress as a consequence of elevated CO_2. Although many stress responsive genes were differentially expressed in both wild-type and ethylene-insensitive plants, there were some interesting differences (Fig. 4; Fig. 5). The upregulation of genes involved in the response to water deprivation in *ein2-5* compared to Col-0 under elevated CO_2, could be linked to the aforementioned upregulation of stomatal movement (Fig. 5). Furthermore, genes encoding *GSTs,* peroxidases and redox enzymes as well as genes involved in the response to oxidative stress were downregulated in *ein2-5* compared to Col-0 in response to elevated CO_2 (Table 3; Fig. 5). Consistently, under elevated compared to ambient CO_2, more of these genes where upregulated in Col-0 but downregulated in *ein2-5*. Hence, the involvement of ethylene in the regulation of cell redox homeostasis and oxidative stress in response to elevated CO_2 is suggested. Leaf senescence has been shown to cause oxidative stress (Aguera and De La Haba, 2018). Senescence-associated genes were induced in response to oxidative stress and senescence mutants are affected in their oxidative stress response (Ding et al., 2016 and references therein). The delayed leaf senescence of ethylene-insensitive plants compared to the wild-type in response to elevated CO_2 (Table 4 and 5), could therefore explain the difference in expression of genes related to oxidative stress and cell redox homeostasis. For example, *GLUTATHIONE REDUCTASE 2* (*GR2*), which was shown to delay leaf senescence by regulating glutathione signaling (Ding et al., 2016), was downregulated in Col-0 but not in *ein2-5* in response to elevated CO_2 (SData 2). Furthermore, several *GSTs* were shown to be ethylene-responsive (Itzhaki and Woodson, 1993; Zhou and Goldsbrough, 1993; Lieberherr et al., 2003; Smith et al., 2003).

5. Conclusion

In contrast with most phytotron studies no increase in vegetative growth of *Arabidopsis* plants in response to elevated CO_2 was found. Also in ethylene-insensitive and constitutive ethylene signaling mutants no enhanced rosette growth was observed. In both Col-0 and *ein2-5* photosynthesis was downregulated whereas respiration was upregulated in response to elevated CO_2. Stress as a consequence of growth under increased atmospheric CO_2 was suggested for both Col-0 and *ein2-5*. Our results suggest a function for ethylene in the regulation of starch biosynthesis and turnover, lipid metabolism, stomatal movement and leaf senescence in response to elevated CO_2. Furthermore, the consequences of ethylene-insensitivity were considerably more pronounced under elevated compared to ambient CO_2. Consistent with

other plant species, an increase in ethylene biosynthesis in response to elevated CO_2 was suggested.

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CRediT authorship contribution statement

Dajo Smet: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Thomas Depaepe:** Methodology, Investigation, Visualization, Writing - review & editing. **Filip Vandenbussche:** Conceptualization, Methodology, Investigation. **Pieter Callebert:** Investigation. **Ivan Nijs:** Conceptualization, Methodology. **Reinhart Ceulemans:** Conceptualization, Methodology, Validation, Supervision, Resources, Funding acquisition. **Dominique Van Der Straeten:** Conceptualization, Methodology, Validation, Writing - original draft, Supervision, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2020.104128.

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