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Identification of traits associated with barley yield performance using contrasting nitrogen fertilizations and genotypes

Rubén Vicente^{a,1}, Omar Vergara-Díaz^a, Samir Kerfal^b, Antonio López^b, James Melichar^c, Jordi Bort^a, María Dolores Serret^a, José Luis Araus^{a,*}, Shawn C. Kefauver^a

^a Section of Plant Physiology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, and AGROTECNIO (Centre for Research in Agrotechnology), Av. Rovira Roure 191, 25198 Lleida, Spain

^b Syngenta España, S.A.U., Calle de la Ribera del Loira 8-10, 28042 Madrid, Spain

^c Syngenta U.K., Hill Farm Road, Whittlesford, Cambridge, CB22 4QT, United Kingdom

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ABSTRACT

Much attention has been paid to understanding the traits associated with crop performance and the associated underlying physiological mechanisms, with less effort done towards combining different plant scales, levels of observation, or including hybrids of autogamous species. We aim to identify mechanisms at canopy, leaf and transcript levels contributing to crop performance under contrasting nitrogen supplies in three barley genotypes, two hybrids and one commercial line. High nitrogen fertilization did not affect photosynthetic capacity on a leaf area basis and lowered nitrogen partial factor productivity past a certain point, but increased leaf area and biomass accumulation, parameters that were closely tracked using various different high throughput remote sensing based phenotyping techniques. These aspects, together with a larger catabolism of leaf nitrogen compounds amenable to sink translocation, contributed to higher crop production. Better crop yield and growth in hybrids compared to the line was linked to a nitrogen-saving strategy in source leaves to the detriment of larger sink size, as indicated by the lower leaf nitrogen content and downregulation of nitrogen metabolism and aquaporin genes. While these changes did not reduce photosynthesis capacity on an area basis, they were related with better nitrogen use in the hybrids compared with the line.

1. Introduction

Due to expected increases in the human population, changes in diet and biofuel consumption, crop production needs to increase by 60–110% worldwide to match the projected global demand by 2050 [1]. However, the rate of production increases for major crops is not keeping in line with the rates required to meet these projected demands. Moreover, and based on a wide range of climate models and emissions scenarios, climate change is forecasted to negatively affect crop yields during coming decades, especially in the Mediterranean

basin [2,3]. Barley (*Hordeum vulgare* L.) is used predominantly for animal feed and malting for alcohol production, with a small proportion used for human food and other industrial applications [4]. In terms of area under cultivation and quantity produced, barley is the most important crop in Spain, and accounted for 10 million tonnes in 2013 according to the Food and Agriculture Organization (FAO; <http://www.fao.org/faostat>) and reached 143.5 million tonnes worldwide. The area of barley harvested has recently decreased globally at a rate of -1.92% per year (0.89 million ha) and its production seems to be limited, with a tendency to decrease by 0.37% per year (0.51 million tonnes) according

Abbreviations: ARI, anthocyanin reflectance index; CRI, carotenoid reflectance index; CSI, crop senescence index; ETR, rate of linear electron transport; GA, relative green area; GGA, relative greener green area; GY, grain yield; MCARI, modified chlorophyll absorption ratio index; NDVI, normalized difference vegetation index; NG, number of grains per area; NUE, nitrogen use efficiency; OSAVI, optimized soil-adjusted vegetation index; PRI, photochemical reflectance index; qRT-PCR, quantitative reverse transcription-PCR; RDVI, renormalized difference vegetation index; RGB, red-green-blue; SAVI, soil adjusted vegetation index; SD, stomatal density; SI, stomatal index; TCARI, transformed chlorophyll absorption ratio index; TGW, thousand grain weight; UAV, unmanned aerial vehicle; VI, vegetation index; WBI, water band index

* Corresponding author.

E-mail addresses: vicenteperez.ruben@gmail.com, vicente@mpimp-golm.mpg.de (R. Vicente), omarvergaradiaz@gmail.com (O. Vergara-Díaz), samir.kerfal@syngenta.com (S. Kerfal), antonio.lopez@syngenta.com (A. López), james.melichar@syngenta.com (J. Melichar), jordi.bort@ub.edu (J. Bort), dserrret@ub.edu (M.D. Serret), jaraus@ub.edu (J.L. Araus), sckefauver@ub.edu (S.C. Kefauver).

¹ Present address: Max-Planck-Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Potsdam-Golm, Germany.

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to the data available from the FAO for the last 20 years (1994–2014). Therefore, in order to sustain the increasing demand for barley in the coming decades under uncertain climate conditions, it will be necessary to improve crop yield per area. Among other alternatives, two possibilities are the optimization of plant mineral nutrition and the yield advantage observed in hybrid plants.

Nitrogen is a major essential element for plants that is made available in agricultural soils mainly as macronutrients in the form of nitrate and ammonium [5] and its demand is greater than for any other mineral element. Nitrogen fertilizer represents a major cost for barley farmers, and a comprehensive knowledge of how barley genotypes respond to nitrogen applications could reap economic benefits while maintaining or even increasing yield [6]. An increase in barley yield and mobilization of nitrogen from vegetative organs to grain is strongly associated with nitrogen application (quantity) and timing [4,7,8]. However, high doses of nitrogen above the optimum can even decrease barley yield [8], while an excessive and continuous application of nitrogen may trigger eutrophication conditions with significant negative environmental consequences [6]. Therefore, it is important to optimize nitrogen application in order to adopt strategies that benefit farmers economically, but that also overcome environmental contamination toward more sustainable agriculture practices.

Arable land is decreasing globally in area each year due to soil erosion and degradation, and as such, any improvement in crop yield per area is inherently linked to our ability to reduce uncertainty and adapt to climate change. The advantages of hybrids relative to line varieties have been exploited extensively in allogamous species (e.g. maize, sunflower, sorghum, sugar beet, rye, cacao, etc.), mainly due to the phenotypically superior performance and higher yield stability of heterozygous hybrid plants (i.e. heterosis), especially under stress conditions [9–12]. The data collected from many experiments clearly indicate that heterosis is the consequence of the action of multiple loci that affect several traits in a way dependent on the hybrid [13]. Araus et al. [9] reported that grain yield was higher in maize hybrids versus inbred lines under optimal and non-optimal water conditions, which was probably associated with an improvement in water use in the hybrids. Chairi et al. [14] also showed that hybrids exhibited greater early vigour than parental lines in maize seedlings including greater root and shoot biomass, net CO₂ assimilation (A_n), and stomatal conductance (g_s), particularly under water-limiting conditions.

On the other hand, improvements reached with hybrids in autogamous cereals have been limited because they exhibit lower degrees of heterosis than allogamous species [10]. Nevertheless, an increasing interest in the generation of barley hybrids started two decades ago, and since then several six-rowed winter barley hybrids have been commercialized by Syngenta and grown across more than 200,000 ha in Europe [10,11]. Recent studies confirmed that barley hybrids also have higher yield stability than lines [11,12], although the physiological and genetic basis conferring higher yield potential and adaptation to unfavourable conditions is still insufficiently understood [9,10,15]. Indeed, studies reporting the integration of agronomic traits with physiological and transcriptional data are still rare, and even scarcer when considering hybrid autogamous crop species under different environmental factors [16,17]. Therefore, although hybrids have been reported to increase grain yield in allogamous and autogamous species with different success, further studies are necessary in order to elucidate the underlying physiological and molecular mechanisms present in barley hybrids.

Hybrid cereals with a higher nitrogen use efficiency or tolerance to low-nitrogen supplies might minimize expenses on fertilizers and the environmental pollution associated with their excessive use. Furthermore, the development of nitrogen-efficient genotypes is a known strategy to improve dry matter production and grain yield [18]. Therefore, the selection of hybrids with an efficient use of nitrogen and an improved understanding of how this metabolism operates are targets for crop improvement, and particularly relevant to autogamous crops.

The nitrogen in the soil is mainly taken up in the form of nitrate and ammonium by high- and low-affinity transport systems in the roots. Later, nitrogen assimilation comprises the reduction of nitrate to ammonium, catalysed by nitrate reductase (NR; EC 1.7.1.1 and EC 1.7.1.2) and nitrite reductase (NIR; 1.7.7.1), and its subsequent incorporation into amino acids mediated by plastidial glutamine synthetase (GS2; EC 6.3.1.2) and ferredoxin-dependent glutamate synthase (GOGAT; EC 1.4.7.1) in the first instance [5,19]. Glutamate dehydrogenase (GDH; EC 1.4.1.2), which occupied a central position between carbon and nitrogen metabolism, is involved in glutamate deamination and thus the release of NH₄⁺ and 2-oxoglutarate. This occurs mainly during carbon shortages [20,21] or leaf senescence. Nitrogen metabolism is highly regulated at transcriptional and posttranslational levels and is finely integrated with carbon metabolism for efficient nitrogen compound synthesis [19]. Primary carbon metabolism and subsequent pathways are highly influenced by the first step in carbon fixation, which involves a limiting reaction in which one CO₂ molecule is incorporated into ribulose-1,5-bisphosphate by ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco; EC 4.1.1.39). Rubisco also plays a key role in the nitrogen economy of the plant as it accounts for 15–30% of total leaf nitrogen and might act as a nitrogen storage protein [19,22]. Furthermore, interest in the water channel proteins called aquaporins is increasing because besides water they can also transport other physiologically important molecules such as CO₂, urea, ammonia, H₂O₂, glycerol, boron, silicon and arsenite [23]. This feature makes these proteins relevant for multiple functions, including nutrient acquisition, carbon fixation, cell signalling, and stress responses [23,24].

This study compared the performance of three barley genotypes, two hybrids and the commercial line most cultivated in the testing area. Plants were grown in a field trial under different contrasting nitrogen supplies. The objectives were to (i) identify mechanisms regulating growth impact of contrasting nitrogen supplies in barley and (ii) evaluate the processes at canopy, leaf and transcript levels determining high grain yields by using the barley hybrids, Jallon and Smooth, and the line Meseta, a widely cultivated commercial genotype in Spain. First, we analysed agronomic components and traits related to the nitrogen use of the plants at harvest, as well as the nitrogen status in flag leaves and grains by the evaluation of nitrogen content and isotope composition ($\delta^{15}\text{N}$). We then evaluated the canopy biomass and green area of the genotypes by comparing of vegetation indices (VIs) obtained from multispectral aerial imagery using an unmanned aerial vehicle (UAV) and red-green-blue (RGB) images taken from the ground. The water status and the pigment content of the canopy were also inferred from the VIs calculated. To that end, we ran gas exchange and chlorophyll fluorescence measurements in the flag leaves, together with analyses of chlorophyll content, leaf area, stomatal density and index, and carbon content and isotope composition ($\delta^{13}\text{C}$). In addition, $\delta^{13}\text{C}$ was also analysed in mature grains. Finally, we studied the expression of 13 key genes involved in nitrogen metabolism, photosynthesis and nutrient transport in the flag leaves by quantitative reverse transcription-PCR (qRT-PCR).

2. Material and methods

2.1. Plant material and experimental setup

Research was conducted at the Institute of Agrifood Technologies and Infrastructures of Navarra (INTIA), Arazuri Station (42° 48' N, 1° 43' W, 396 m a.s.l.), located near to Pamplona, Spain during the 2015/2016 crop season. This region represents a transition from a Mediterranean to an Atlantic climate where barley was the most cultivated crop in 2016 (Ministry of Agriculture and Fisheries, Food and Environment of Spain; www.mapama.gob.es), usually grown under rainfed conditions. Mean, maximum and minimum daily air temperatures were 10.6 °C, 15.9 °C and 5.6 °C, respectively, with an average relative humidity of 79.1% and accumulated precipitation of 654 mm

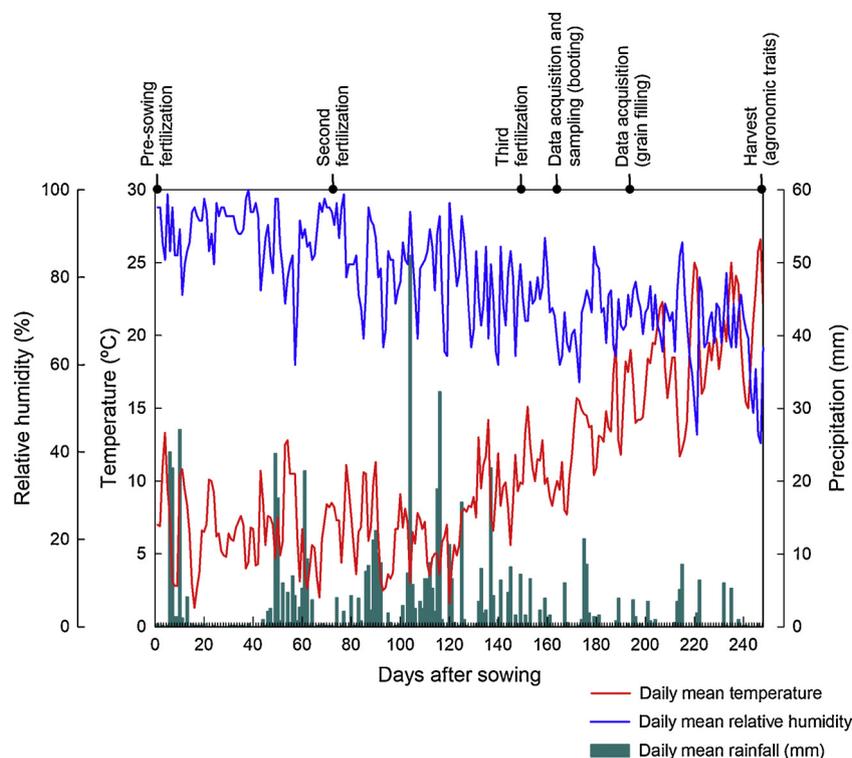


Fig. 1. Daily mean temperature, relative humidity, rainfall pattern, and fertilization and sampling dates in the field trial during the crop season from 16th November 2015 to 20th July 2016.

from the beginning of the experiment to harvest (see Fig. 1). Three barley (*Hordeum vulgare* L.) genotypes were chosen, one conventional two-row genotype (Meseta), and two six-row genotypes (Jallon and Smooth). Meseta is one of the most widely cultivated winter barley genotypes in Spain due to its high adaptability and yield potential in most winter barley cropping areas. On the other hand, Jallon and Smooth are two winter barley hybrids released by Syngenta S.A.U. using their Hyvido™ technology. Barley seeds were sown on 16th November 2015 in 12 m²-plots with eight lines separated by 15 cm, using three plots per genotype and nitrogen treatment. Initially, ten different nitrogen treatments differentiated by application dates and doses were established per genotype in the trial (Supplementary Table 1). We selected three of them with the most contrasting nitrogen supplies for the determination of physiological traits and sampling for further analyses: N₀, with no application of nitrogen; N₁₃₀, with three nitrogen applications of 40, 50 and 40 kg ha⁻¹ during pre-sowing (urea), 25th January (mix of urea and ammonium sulfate) and 11th April (urea), respectively; and N₁₇₀, with 40, 50 and 80 kg ha⁻¹ during the same dates. Weeds, insect pests and diseases were controlled via recommended agrochemicals.

Direct measurement parameters such as vegetation indices (VIs) from the ground, gas exchange and chlorophyll fluorescence were recorded at the booting stage (Zadoks 41–43) in 163-day-old plants [25], as well as image data acquisition from a UAV for the calculation of canopy temperature and several multispectral VIs for each plot. All these measurements were carried out during the morning and early afternoon between 10:00–15:00 (except the canopy temperature, which was recorded also in the afternoon) in clear sky conditions. Epidermal impressions of the leaves were performed *in situ* to estimate the stomatal density and index. Leaf samples were harvested to calculate leaf area and to analyse the isotope composition and gene expression. For the latter, ten flag leaves per plot were cut and immediately plunged into liquid nitrogen, transported to our laboratory and stored at –80 °C until analysis. In a second visit to the field experiment during the mid grain filling stage (Zadoks 77–83), VIs from the ground were also

recorded in 192-day-old plants. Agronomic traits and carbon and nitrogen content and isotope composition of the grain were determined at physiological maturity. The following parameters related to N-use efficiency (NUE) were calculated according to Chenet al. [26] and Lü et al. [27]:

$$N \text{ grain yield (or } N \text{ uptake)} (NGY, \text{ kg ha}^{-1}) \\ = (\text{Grain yield} \times \text{Grain } N \text{ conc.})/100$$

$$N \text{ recovery efficiency (NRE, \%)} = (NGY - NGY \text{ for zero } N \text{ plot})/N_{\text{supply}} \\ \times 100$$

$$\text{Agronomic NUE (ANUE, g grain/g } N_{\text{supply}}) = (\text{GY} - \text{GY for zero } N \text{ plot}) \\ /N_{\text{supply}}$$

$$\text{Physiological NUE (PNUE, g grain/g } N_{\text{supply}}) = (\text{GY} - \text{GY for zero } N \text{ plot}) \\ /(\text{NGY} - \text{NGY for zero } N \text{ plot})$$

$$N \text{ partial factor productivity (NFPF, g grain/g } N_{\text{supply}}) \\ = \text{GY for } N \text{ treated plot}/N_{\text{supply}}$$

2.2. Ground-measured vegetation indices

The Normalized Difference Vegetation Index (NDVI) was evaluated for each plot with a hand-held portable spectroradiometer (GreenSeeker, NTech Industries, Ukiah, USA) as described in Vergara-Diaz et al. [28]. For each plot, one RGB picture was taken by holding an Olympus E-M10 (Olympus Corporation, Tokyo, Japan) camera at one m above the canopy in a zenithal plane and focusing near the center of the plot, with 14 mm focal length, 1/250 shutter speed and automatic aperture. The pictures (4608 × 3072 pixels) were analysed with BreedPix 0.2 software [29] for the calculation of RGB indices in the canopy based on the sum of frequencies of the histogram classes included in a certain range of hue in the image: the relative green area

Table 1

Vegetation indices calculated from the 11 multispectral reflectance bands obtained from aerial images. NDVI_{ai}, normalised difference vegetation index from aerial images; SAVI, soil adjusted vegetation index; RDVI, renormalized difference vegetation index; OSAVI, optimized soil-adjusted vegetation index; PRI, photochemical reflectance index; MCARI, modified chlorophyll absorption ratio index; TCARI, transformed chlorophyll absorption ratio index; CRI, carotenoid reflectance index; ARI, anthocyanin reflectance index; WBI, water band index. In the equations, R indicates reflectance and the subscripts indicate wavelengths in nm. In the SAVI equation, L is a canopy background adjustment factor with an optimal value of L = 0.5.

Index	Equation	Estimated biological parameter	Reference
NDVI _{ai}	$(R_{840}-R_{670})/(R_{840}+R_{670})$	Biomass, healthy and green vegetation	[63]
SAVI	$(R_{840}-R_{670})/(R_{840}+R_{670}+L) \times (1+L)$	Biomass, healthy and green vegetation	[64]
RDVI	$(R_{840}-R_{670})/(R_{840}+R_{670})^{1/2}$	Biomass, healthy and green vegetation	[65]
OSAVI	$(1+0.16) \times (R_{780}-R_{670})/(R_{780}+R_{670}+0.16)$	Biomass, healthy and green vegetation	[66]
PRI	$(R_{550}-R_{570})/(R_{550}+R_{570})$	Photosynthetic light- and radiation-use efficiency, xanthophyll cycle activity, chlorophyll/carotenoids ratio (or its inverse)	[67]
MCARI	$[(R_{700}-R_{670})-0.2 \times (R_{700}-R_{550})] \times (R_{700}/R_{670})$	Chlorophyll content	[68]
TCARI	$3 \times [(R_{700}-R_{670})-0.2 \times (R_{700}-R_{550})] \times (R_{700}/R_{670})$	Chlorophyll content	[69]
CRI	$(1/R_{510})-(1/R_{700})$	Carotenoid content	[70]
ARI	$R_{840} \times (1/R_{550})-(1/R_{700})$	Anthocyanin content	[71]
WBI	(R_{900}/R_{950})	Water status	[45]

(GA) and greener green area (GGA) are the percentage of pixels in the image (values between 0 and 1) in the hue range from 60 to 180° (from yellow to bluish green) and from 80 to 180° (from yellowish-green to bluish green), respectively [28,29]. GGA is more restrictive than GA by excluding yellowish-green tones and therefore describes more accurately the photosynthetically active biomass. Additionally, the crop senescence index (CSI) was calculated as follows [30]:

$$CSI = 100 \times (GA - GGA) / GA$$

Chlorophyll content was measured *in vivo* using a SPAD-502 portable chlorophyll meter (Minolta, Tokyo, Japan) in the central segment of the flag leaf lamina (adaxial surface), using ten plants per plot selected randomly and averaged. Leaf area was calculated after scanning two flag leaves per plot by using ImageJ software (National Institutes of Health, USA).

2.3. Vegetation indices and canopy temperature from aerial data

Aerial images were acquired using a UAV for the calculation of 11 multispectral VIs (see Table 1) and canopy temperature. The aerial platform consisted in a Mikrokopter Oktokopter 6S12 XL (HiSystems GmbH, Germany) equipped with thermal (FLIR Tau2 640; FLIR Systems, USA) and multispectral (Tetracam mini MCA 11 + ILS; Tetracam, Inc., USA) cameras. The flights were controlled manually at a steady velocity of 5 m s⁻¹ and an a.g.l. altitude of 50 m in continuous capture for the duration of each flight with image acquisition rates of 1/20 s and 5 s for the thermal and multispectral cameras, respectively. The multispectral camera was used to obtain 11 band 8-bit TIFF images (1280 × 1024 pixels) with an estimated ground spatial resolution of 27 mm per pixel [30] within approximately 3 h of solar noon (10:00–15:00) for the determination of VIs. The full resolution thermal video (640 × 520 pixels at 20 frames s⁻¹) obtained had an estimated ground spatial resolution of 54 mm per pixel and was used for the determination of canopy temperature both at midday (T_{mid}, 13:00) and in the afternoon (T_{aft}, 17:00) by using the TeAx ThermoViewer v1.3.12 software [31]. The images from each sensor were combined into orthomosaics to correct for terrain and flight movement using Agisoft Photoscan Professional (Agisoft LLC, Russia), then cut for each individual plot using FIJI (ImageJ) and batch processed using custom macro functions for index calculations and simultaneous data export for each sensor. The remote sensing image data acquired from the UAV was all calibrated and corrected to at-sensor reflectance in real-time using an advanced incident light sensor (ILS), which includes a matching microarray of 11 miniature spectral filters that correspond to the exact same wavelengths of the 11 spectral bands of the image sensors. At 50 m distance between the sensor (UAV) and the target (crop), atmospheric correction was deemed unnecessary for the calculation of

broad-band reflectance indexes.

2.4. Photosynthesis, stomata and isotope composition

Gas exchange measurements were conducted with an air flow rate of 350 ml min⁻¹, saturating 1500 μmol m⁻² s⁻¹ irradiance, 25 °C, 60% relative humidity and 400 μmol CO₂ mol⁻¹ using a LI-6400 Portable Photosynthesis System infrared gas analyser (LI-COR Inc., Lincoln, NE, USA) in the central segment of fully expanded flag leaves for each treatment combination. Chlorophyll fluorescence in the light-adapted state was determined in parallel with a 6400-40 Leaf Chamber Fluorometer coupled to the LI-6400 system. Chlorophyll fluorescence parameters were determined according to Baker [32].

The stomatal density (SD) and index (SI) were determined in at least two flag leaves per plot. First, the central leaf segments of the adaxial and abaxial sides were smeared with nail polish. The dried layers were carefully peeled off the leaves using adhesive tape and placed onto a glass slide. The stomata and epidermal cell numbers were counted under a microscope in three fields of view per leaf replica. The stomatal density was calculated as the number of stoma per unit area, while the stomatal index was calculated as the ratio of stomata to epidermal cells.

The flag leaves collected at the booting stage and the grains at harvest were used to determine the carbon and nitrogen content and the ratio of stable isotopes of carbon (¹³C/¹²C) and nitrogen (¹⁵N/¹⁴N). After drying the samples in an oven at 70 °C until constant weight, they were homogenized to a fine powder using a Mixer Mill MM400 (Retsch GmbH, Haan, Germany). One mg of each sample was weighed into tin capsules for the measurements at the Scientific Facilities of the University of Barcelona as described in Vergara-Diaz et al. [28].

2.5. Real-time PCR analysis

RNA isolation, cDNA synthesis and qRT-PCR assays were as described for wheat in Medina et al. [33]. Total RNA was extracted from 100 mg of flag leaf samples after grinding plant material (ten leaves per plot) with a mortar and pestle using liquid N. Ribozol RNA Extraction Reagents (Amresco, Solon, OH, USA) were used for RNA isolation according to the manufacturer's instructions. RNA concentration and quality were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and by agarose gel electrophoresis. A total of 2 μg of RNA per sample was treated with PerfeCTa DNase 1 RNase-free (Quanta Biosciences, Gaithersburg, MD, USA) to remove any genomic DNA contamination and used subsequently for double-strand cDNA synthesis with the qScript cDNA Synthesis Kit (Quanta Biosciences).

Genes selected for the expression analyses were related to photosynthesis (Rubisco subunits), nitrogen metabolism (enzymes involved

Table 2
Primer pairs for barley sequences associated with photosynthesis, nitrogen assimilation and aquaporins.

Acc. No.	Description	Forward and reverse primer (5'-3')		Reference
Reference gene				
AY145451	Actin	Fw	GGCACACTGGTGCATGG	Córdoba et al. [35]
		Rv	CTCCATGTATCCAGTT	
AJ508228	ADP-ribosylation factor 1-like protein	Fw	GCTCTCCAACAACATTGCCAAC	Ferdous et al. [36]
		Rv	GAGACATCCAGCATCATTCATCC	
Photosynthesis				
AGP50919	Rubisco large subunit (RbL)	Fw	AGTGCTCTACGTTTGGAGG	Córdoba et al. [35]
		Rv	GCGGGCCTTGAAAGTTTT	
U43493	Rubisco small subunit (RbS)	Fw	ACCAACATGCTCGAGAAAGCA	Córdoba et al. [35]
		Rv	GTGTGGCGTGCAAAGATGT	
N metabolism				
X57845	NADH-dependent nitrate reductase (NR)	Fw	ACCAACTGCGTCATACCAC	Méndez et al. (unpublished)
		Rv	GGATGGATGGATGGAGGAGGA	
X57844	NADH-dependent nitrate reductase (NR)	Fw	ACACCAACTCCGTATCACC	This study
		Rv	TCTTGCCACCGCTGAGTA	
S78730	Nitrite reductase (NIR)	Fw	CACCGGGGTGTACAAGAAGG	This study
		Rv	TACTCTCGTCTCCTCCCT	
X53580	Glutamine synthetase, plastidial (GS2)	Fw	AACTACGACGGATCGAGCAC	This study
		Rv	CCCTGTGGTGTGTAGGTGTC	
S58774	Ferredoxin-dependent glutamate synthase (GOGAT)	Fw	GGCATGGTCTTCTCCACA	This study
		Rv	GGTCTCCAAGCAAGAACCTC	
AK366146	Glutamate dehydrogenase (GDH)	Fw	AATGCTGGTGGTGTGATCGT	This study
		Rv	ATGGCCTTGATGTGCTGGAA	
Aquaporins				
AB219366	Aquaporin (PIP2;1)	Fw	GTGGACTTCGAGGAGCTGAC	This study
		Rv	TGGTCTGTACCCGATGAC	
AK367251	Aquaporin (TIP1;2)	Fw	TCCTCTCAAGATCGCCACT	This study
		Rv	GACGATCTCGAACACCACCG	
AK248215	Aquaporin (TIP2;3)	Fw	TCCATGTGAAGGGTCTGTCG	This study
		Rv	AGTGCTACAACCTTACTGGGCT	
AB447482	Aquaporin (NIP2;1)	Fw	TTCTCCAGCGAGATCCACGACATC	This study
		Rv	ACCTCCGACACCACCTTCTTGAGG	
AB447484	Aquaporin (NIP2;2)	Fw	TACTCGAACGAGATCCACGACCTG	This study
		Rv	CGGGCAATGACTTCTCTGGTAG	

in primary nitrogen assimilation) and aquaporins (Table 2). Primer pairs were designed in the Primer-BLAST tool following the selection criteria described in Vicente et al. [34]. The specificity of PCR amplification was confirmed by the presence of unique amplicons of the expected length on 3.5% (w/v) agarose gels and by dissociation curves with single peaks. The qRT-PCR assays were performed in 384-well optical plates containing per well 5 μ l PerfeCTa SYBR Green FastMix (Quanta Biosciences), 200 nM of each primer pair and 1 μ l of cDNA diluted 1:5 in a LightCycler 480 System (Roche Applied Science, Penzberg, Germany) at the CCITUB (Scientific and Technological Centres of the University of Barcelona). Thermal conditions for qRT-PCR were as detailed in Medina et al. [33]. The relative gene expression was quantified using the comparative threshold cycle method, $2^{-\Delta\Delta C_t}$ [35]. After testing actin [36] and ADP-ribosylation factor 1 [37] as reference genes in our study (Table 2), we decided to use only the latter for the normalization of the gene expression data ($C_T = 23.19 \pm 0.58$ for all the biological replicates).

2.6. Statistical analysis

Two-way analysis of variance (ANOVA) was conducted to calculate differences between the three nitrogen treatments (N_0 , N_{130} and N_{170}), the three genotypes (Meseta, Jallon and Smooth), and their interaction. Both nitrogen supply and the genotype factors were treated as fixed independent variables. All ANOVAs were performed with GenStat 6.2 (VSN International Ltd., Hemel Hempstead, UK). The transcript abundance was presented as logarithmic values (\log_2), which are normally distributed. Differences between treatments means were considered significant at $P < 0.05$ after using the Fisher's least significant difference (LSD) test. Principal component analysis (PCA) for all data was carried out with CANOCO 4.5 (Microcomputer Power, Ithaca NY, USA). Stepwise regressions were performed in IBM SPSS Statistics 23.0 to

develop prediction models for grain yield. All data were normalized and used to construct a correlation network for each genotype according to Vicente et al. [21].

3. Results

The experimental field trial was performed with ten different nitrogen treatments considering the application dates and doses for each genotype, Meseta, Jallon and Smooth. The nitrogen treatments consisted of four different total nitrogen levels (0, 130, 150 and 170 kg ha⁻¹) supplied at different dates and quantities along the experiment (Supplementary Table 1). The nitrogen fertilization improved significantly GY in each genotype compared to N_0 , although there were not statistical differences between nitrogen treatments from 130 to 170 kg ha⁻¹ due, at least in part, to the large differences between N_0 and the rest of the treatments when all the data was used for the statistical analysis. Considering all the genotypes together, we showed some differences in GY between N_{130} and N_{170} . We chose three of these ten treatments for further physiological and molecular analyses as a representation of different categories of GY achieved by the nitrogen fertilization: N_0 , N_{130} and N_{170} , selecting the last two nitrogen levels with no differences in the fertilization timing.

3.1. Effects of nitrogen fertilization on yield, plant growth and nitrogen-related traits

A nitrogen supply of 130 kg ha⁻¹ (N_{130}) improved grain yield (GY) in barley by 65% compared to the non-nitrogen fertilized (N_0) plants; a further increase in N fertilization from 130 to 170 kg ha⁻¹ (N_{170}), only increased yield by an additional 10% (Table 3). The yield increase was associated with a higher number of grains per area (NG): 82% in N_{130} compared to N_0 , and 16% in N_{170} compared to N_{130} . However,

Table 3

Agronomic and nitrogen-related traits at harvest. Nitrogen content and isotope composition were measured in leaves at the booting stage and in grains at harvest. For each comparison of means, letters are significantly different according to ANOVA ($P < 0.05$), while they were omitted with non-significant differences among means. Significant P values are marked in bold.

	N ₀	N ₁₃₀	N ₁₇₀	Meseta	Jallon	Smooth	P _N	P _G	P _{NxG}
GY	4.09 a	6.76 b	7.45 c	5.41 a	6.15 b	6.74 b	< 0.001	0.001	0.531
TGW	44.9 c	41.1 b	38.8 a	40.6 b	39.0 a	41.2 c	< 0.001	< 0.001	0.104
NG	9.1 a	16.6 b	19.2 c	13.5 a	16.1 b	15.3 b	< 0.001	0.012	0.368
NGY	51.1 a	107.1 b	123.5 c	85.0	97.5	99.2	< 0.001	0.115	0.738
NRE	–	43.0	42.6	41.5	44.3	42.6	0.937	0.915	0.538
ANUE	–	20.6	19.8	19.5	18.9	22.2	0.447	0.454	0.721
PNUE	–	49.1	46.8	47.2 ab	43.0 a	53.6 b	0.446	0.034	0.938
NPPF	–	52.0 b	43.8 a	43.1 a	47.8 ab	52.8 b	0.003	0.013	0.646
N _{leaf}	2.58 a	4.04 b	4.45 b	4.08 b	3.46 a	3.54 a	< 0.001	0.002	0.074
N _{grain}	1.25 a	1.58 b	1.66 b	1.53	1.54	1.43	< 0.001	0.117	0.727
δ ¹⁵ N _{leaf}	4.51 c	1.44 b	0.31 a	2.40	1.97	1.89	< 0.001	0.416	0.532
δ ¹⁵ N _{grain}	4.43 c	3.65 b	2.76 a	3.66	3.43	3.75	< 0.001	0.338	0.841

GY, grain yield (t ha⁻¹); TGW, thousand grain weight (g); NG, number of grains per area (grains x 10³ m⁻²); N_{leaf} and N_{grain}, leaf and grain nitrogen content (% of total dry weight); δ¹⁵N_{leaf} and δ¹⁵N_{grain}, leaf and grain nitrogen isotope composition (‰); NGY, nitrogen grain yield (kg ha⁻¹); NRE, nitrogen recovery efficiency (‰); ANUE, agronomic NUE (g grain g N_{supply}⁻¹); PNUE, physiological NUE (g grain g N_{supply}⁻¹); NPPF, nitrogen partial factor productivity (g grain g N_{supply}⁻¹).

Table 4

Multispectral vegetation indices related to biomass and plant health from ground and aerial images at booting and mid grain filling. For each comparison of means, letters are significantly different according to ANOVA ($P < 0.05$), while they were omitted with non-significant differences among means. Significant P values are marked in bold.

		N ₀	N ₁₃₀	N ₁₇₀	Meseta	Jallon	Smooth	P _N	P _G	P _{NxG}
Ground indices (booting)	NDVI	0.50 a	0.72 b	0.72 b	0.62	0.66	0.66	< 0.001	0.074	0.897
	GA	0.74 a	0.97 b	0.97 b	0.84 a	0.92 b	0.92 b	< 0.001	0.001	0.790
	GGA	0.40 a	0.85 b	0.87 b	0.64 a	0.73 b	0.75 b	< 0.001	< 0.001	0.810
	CSI	46.4 b	12.1 a	10.2 a	26.3 b	22.0 a	20.4 a	< 0.001	0.023	0.531
Aerial indices (booting)	NDVI _{ai}	0.79 a	0.94 b	0.94 b	0.87	0.90	0.90	< 0.001	0.228	0.955
	SAVI	1.17 a	1.40 b	1.41 b	1.30	1.34	1.34	< 0.001	0.214	0.955
	RDVI	6.44 a	8.63 b	8.62 b	7.38 a	8.17 b	8.14 b	< 0.001	0.008	0.793
	OSAVI	0.64 a	1.00 b	1.01 b	0.91	0.84	0.91	< 0.001	0.165	0.939
Ground indices (mid grain filling)	NDVI	0.47 a	0.72 b	0.73 b	0.56 a	0.66 b	0.69 b	< 0.001	< 0.001	0.214
	GA	0.43 a	0.85 b	0.89 b	0.55 a	0.81 b	0.81 b	< 0.001	< 0.001	0.196
	GGA	0.11 a	0.40 b	0.48 c	0.20 a	0.40 b	0.38 b	< 0.001	< 0.001	0.015
	CSI	77.0 c	54.4 b	47.4 a	69.0 b	54.2 a	55.7 a	< 0.001	< 0.001	0.578

NDVI, normalized difference vegetation index; GA, green area; GGA, greener green area; CSI, crop senescence index; NDVI_{ai}, normalized difference vegetation index from aerial images; SAVI, soil adjusted vegetation index; RDVI, renormalized difference vegetation index; OSAVI, optimized soil-adjusted vegetation index.

thousand grain weight (TGW) decreased slightly with the increase in nitrogen supply. Nitrogen grain yield (NGY) significantly increased in N₁₃₀ (210%) and N₁₇₀ (242%) compared to N₀ plants. The nitrogen use efficiency (NUE) parameters including nitrogen recovery efficiency (NRE), agronomic NUE (ANUE), and physiological NUE (PNUE), did not show differences between N₁₃₀ and N₁₇₀, whereas the nitrogen partial factor productivity (NPPF) was higher in N₁₃₀ compared to N₁₇₀. The nitrogen isotope composition (δ¹⁵N) in flag leaves at booting and grains at harvest decreased as the nitrogen application increased, while leaf (N_{leaf}) and grain (N_{grain}) nitrogen, expressed as a percentage of total dry weight, only increased between N₁₃₀ and N₁₇₀ relative to N₀ treatment. Moreover, N₁₃₀ and N₁₇₀, compared to N₀, increased biomass and/or green area at booting and mid grain filling stages as revealed by ground and aerial VIs, while the crop senescence index decreased in N₁₃₀ and N₁₇₀ (CRI; Table 4).

3.2. Effects of nitrogen fertilization on photosynthesis, pigment content, leaf anatomy and water-related traits

Nitrogen treatments only affected the water use efficiency (WUE, calculated as the ratio of A_n/Tr) and the related ratio of intercellular to atmospheric CO₂ concentration (C_i/C_a), and these increased and decreased, respectively, as the N fertilization increased (Table 5). The other gas exchange and chlorophyll fluorescence parameters were not

modified by nitrogen treatment; namely photosynthesis (A_n), stomatal conductance (g_s), transpiration (Tr), electron transport rate (ETR), photosystem II maximum efficiency (F_v/F_m'), photosystem II operating quantum efficiency (F_q'/F_m') and the fraction of photosystem II centres in the open state (qL). Chlorophyll content in flag leaves increased as result of the N fertilization, while canopy VIs related to pigment content [the transformed chlorophyll absorption ratio index (TCARI), the carotenoid reflectance index (CRI), and the anthocyanin reflectance index (ARI)] and photochemical reflectance index (PRI) decreased. Leaf area increased in N₁₃₀ and N₁₇₀ relative to N₀, while SD_{adaxial}, SD_{abaxial} and SI_{abaxial} decreased. No significant differences were observed for water-related traits of the flag leaves [Tr, WUE and carbon isotope composition (δ¹³C)] between nitrogen treatments. However, the δ¹³C in mature grains together with the canopy water band index (WBI) measured at booting increased while the canopy temperature at booting decreased as response to nitrogen fertilizer (i.e. N₁₃₀ and N₁₇₀ compared to N₀).

3.3. Changes in leaf gene expression and relationships between traits under contrasting nitrogen supplies

We studied the expression of 13 genes related to photosynthesis (RBCL and RBCS; Rubisco large and small subunits, respectively), nitrogen metabolism (NR, NADH-dependent nitrate reductase, two genes; NIR, nitrite reductase; GS2, chloroplastic glutamine synthetase;

Table 5

Parameters related to photosynthesis, transpiration, water and other related traits measured in flag leaves, grains and canopy at booting. Chlorophyll content was measured also at mid grain filling. C content and isotope composition of grains were determined at harvest. For each comparison of means, letters are significantly different according to ANOVA ($P < 0.05$), while they were omitted with non-significant differences among means. Significant P values are marked in bold.

	N ₀	N ₁₃₀	N ₁₇₀	Meseta	Jallon	Smooth	P _N	P _G	P _{NG}
Leaf									
A _n	15.3	16.2	15.9	15.7	15.8	15.9	0.638	0.977	0.059
g _s	184	176	141	166	162	174	0.107	0.830	0.329
Tr	3.21	3.26	2.59	2.90	2.92	3.24	0.116	0.533	0.460
C _i /C _a	0.60 b	0.56 b	0.48 a	0.54	0.53	0.57	0.002	0.422	0.915
WUE	4.87 a	5.04 a	6.48 b	5.80	5.66	4.92	0.014	0.236	0.938
ETR	112	113	116	117	110	113	0.810	0.552	0.250
ETR/A _n	7.36	7.04	7.36	7.51	7.02	7.22	0.724	0.566	0.280
F _v '/F _m '	0.407	0.450	0.439	0.459 b	0.405 a	0.432 ab	0.072	0.029	0.791
F _q '/F _m '	0.175	0.180	0.182	0.185	0.173	0.180	0.788	0.512	0.406
qL	0.316	0.270	0.296	0.278	0.307	0.296	0.421	0.690	0.688
Chl1	36.1 a	44.1 b	44.5 b	43.6 b	39.5 a	41.6 ab	< 0.001	0.013	0.878
Chl2	31.6 a	42.0 b	43.5 b	30.8 a	40.7 b	45.7 c	< 0.001	< 0.001	0.679
SD _{adaxial}	82.5 b	69.8 a	69.8 a	79.4 b	70.9 a	71.6 a	< 0.001	0.016	0.029
SD _{abaxial}	83.7 b	67.8 a	66.9 a	85.3 b	66.5 a	66.6 a	< 0.001	0.016	0.438
SI _{adaxial}	0.184	0.172	0.173	0.170	0.182	0.177	0.106	0.084	0.051
SI _{abaxial}	0.185 b	0.170 a	0.173 a	0.179	0.173	0.175	0.018	0.521	0.534
Leaf area	13.0 a	17.3 b	18.1 b	6.3 a	20.3 b	21.8 b	< 0.001	< 0.001	0.060
C _{leaf}	43.93	43.84	44.24	44.33	44.05	43.62	0.574	0.216	0.256
δ ¹³ C _{leaf}	-29.16	-29.29	-29.48	-29.39	-29.40	-29.14	0.058	0.081	0.193
Grain									
C _{grain}	40.98	41.32	41.35	41.16	41.21	41.27	0.466	0.946	0.121
δ ¹³ C _{grain}	-27.5 a	-27.1 b	-26.9 b	-27.2	-27.1	-27.2	< 0.001	0.598	0.094
Canopy									
T _{mid}	20.2	16.3	16.3	17.7	17.4	17.7	0.056	0.969	0.965
T _{aft}	15.3 b	11.6 a	11.8 a	12.7	12.7	13.4	0.016	0.844	0.956
PRI	0.13 b	-0.17 a	-0.17 a	-0.07	-0.08	-0.07	< 0.001	0.930	0.084
MCARI	34.8	27.5	30.6	28.6	33.7	30.8	0.471	0.688	0.458
TCARI	37.3 b	20.5 a	20.4 a	25.4	27.2	25.7	< 0.001	0.300	0.195
CRI	-0.001 b	-0.019 a	-0.016 a	-0.010	-0.014	-0.012	0.002	0.597	0.978
ARI	-0.09 b	-1.58 a	-1.24 a	-0.72	-1.17	-1.01	0.002	0.483	0.986
WBI	0.90 a	0.98 b	0.98 b	0.92 a	0.96 b	0.98 c	< 0.001	< 0.001	0.327

A_n, CO₂ assimilation rate (μmol m⁻² s⁻¹); g_s, stomatal conductance (mmol m⁻² s⁻¹); Tr, transpiration rate (mmol m⁻² s⁻¹); C_i/C_a, ratio of intercellular to ambient CO₂ concentration; WUE, instantaneous water use efficiency (calculated as A_n/Tr); ETR, rate of linear electron transport (μmol m⁻² s⁻¹); F_v'/F_m', photosystem II maximum efficiency; F_q'/F_m', photosystem II operating quantum efficiency; qL, fraction of photosystem II centres in the open state; Chl, chlorophyll content at booting (1) and mid grain filling (2); SD, stomatal density (stomata mm⁻²); SI, stomatal index (stomata/pavement cells); C, grain C content (% of total dry weight); δ¹³C, grain C isotope composition (‰); T_{mid}, canopy temperature during morning; T_{aft}, canopy temperature during afternoon; PRI, photochemical reflectance index; MCARI, modified chlorophyll absorption ratio index; TCARI, transformed chlorophyll absorption ratio index; CRI, carotenoid reflectance index; ARI, anthocyanin reflectance index; WBI, water band index.

GOGAT, ferredoxin-dependent glutamate synthase; and *GDH*, glutamate dehydrogenase) and transport of water and other substrates (aquaporins *PIP2;1*, *TIP1;2*, *TIP2;3*, *NIP2;1* and *NIP2;2*). The nitrogen supplied (N₁₃₀ and N₁₇₀) to field-grown barley plants induced expression of *RBCL* and *NIR* (especially in N₁₇₀), *GOGAT*, *GDH*, *PIP2;1* and *TIP2;3* genes in the flag leaves relative to N₀ and repressed the *GOGAT* gene (Fig. 2). The expression of two genes encoding NR showed different patterns, one (X57845) being repressed in N₁₃₀ (especially in the hybrids) and the other (X57844) being induced in N₁₇₀ relative to the other nitrogen treatments.

The PCA analysis (Supplementary Fig. 1) showed that the higher nitrogen availability in N₁₃₀ and N₁₇₀ relative to N₀ was associated with better crop productivity (GY and NGY) and performance (NG, canopy VIs associated with plant biomass and green area, δ¹³C_{grain}, aquaporin *TIP2;3* expression and the nitrogen content of grains at harvest and leaves at booting). The traits associated with the N₀ treatment were CSI, ARI, CRI, PRI, δ¹⁵N in leaves and grains, canopy temperature, TGW, and SI and SD in both leaf surfaces. Few differences were observed between N₁₃₀ and N₁₇₀ treatments.

3.4. Genotypic variability in grain yield, plant growth and nitrogen-related traits

We compare here the differences between three genotypes; Meseta, the most widely cultivated commercial line in the region where the

study was performed, and two recently released barley hybrids, Jallon and Smooth. GY was higher in both Jallon and Smooth barley hybrids than in the line Meseta by 14% and 25%, respectively (Table 3). While TGW was lower in Jallon and higher in Smooth relative to Meseta, both hybrids had greater NG than the line. NGY was increased in the hybrids compared to the line (15–17%), although it did not reach statistical significance, probably due to the heterogeneity of error variances when N₀, N₁₃₀ and N₁₇₀ were averaged for each genotype. Other NUE parameters, i.e. NRE and ANUE, were not statistically different between genotypes, while PNUE and NPFP clearly improved in Smooth compared to Jallon and Meseta. N_{leaf} was lower in Jallon (15%) and Smooth (13%) compared to Meseta. Some ground (GA and GGA) and aerial (RDVI) VIs indicated that green area and biomass were higher in both hybrids relative to Meseta at booting (Table 4). At mid grain filling, the NDVI and RGB indices indicated the same shifts. CSI was lower in the hybrids compared to the line at booting and mid grain filling. Additionally, and regardless of the nitrogen treatment, the start of stem elongation (Zadoks 30) and heading date (Zadoks 59) were earlier in Meseta (102 and 169 days from planting to heading, respectively) compared with the hybrids Smooth (118 and 177) and Jallon (106 and 178 days) for every plot of each variety.

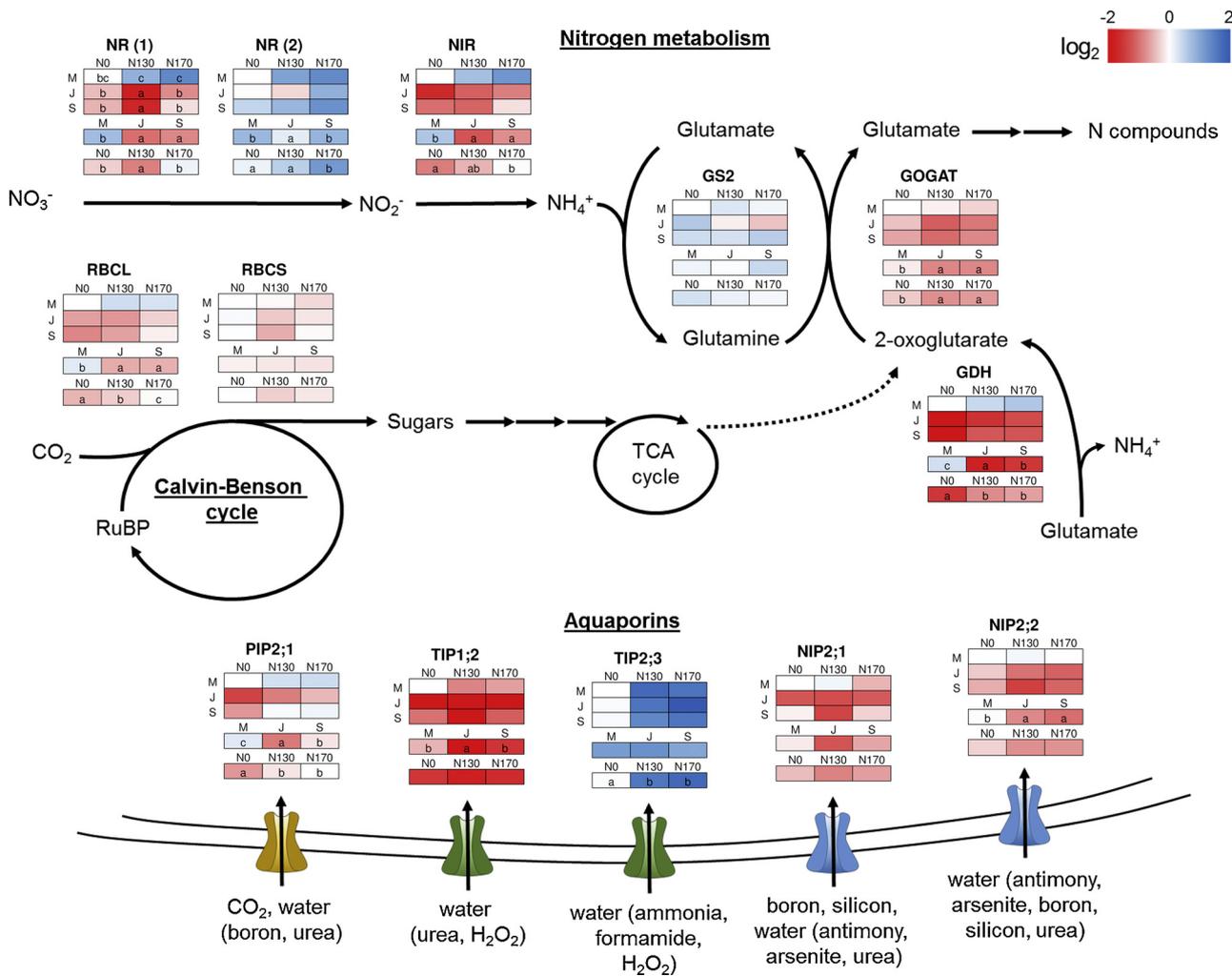


Fig. 2. Changes in transcript abundances for genes involved in the Calvin-Benson cycle, nitrogen metabolism and substrate transport (aquaporins) in flag leaves at booting. Transcript levels with different letters are significantly different at $P < 0.05$ for nitrogen supply (N_0 , N_{130} , N_{170}), genotypic variability (M, Meseta; J, Jallon; S, Smooth) and their interaction. White indicates no change, red down-regulation and blue up-regulation as shown in the colour scale bar for each factor, considering Meseta and N_0 as control treatments. Abbreviations for transcripts are shown in Table 2. The substrates transported for every aquaporin are shown according to Hove et al., while potential substrates are between brackets (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.5. Genotypic variability in photosynthesis, water-related traits and gene expression

Except for F_v/F_m' , where Meseta exhibited a higher value than the hybrids, genotypic variability did not have any effect on the same set of gas exchange and fluorescence parameters (Table 5). Chlorophyll content in flag leaves was higher in Meseta relative to Jallon and Smooth at booting, while the opposite trend was observed for chlorophyll content at mid grain filling. Leaf area was notably higher in the hybrids compared to Meseta, while SD in the adaxial and abaxial surfaces was reduced, but not the SI. Also, canopy WBI was higher in the hybrids.

Several transcript levels were notably lower in Jallon and Smooth than in Meseta, such as the *RBCL*, *NIR*, *GOGAT*, *NIP2;2* and one of the *NR* (*X57845*) genes, as well as *PIP2;1* and *GDH* (especially in Jallon) genes (Fig. 2). The expression of the other *NR* (*X57844*) and *TIP1;2* genes were reduced in Jallon relative to the other genotypes.

3.6. Relationships between traits at canopy, leaf and transcript levels across genotypes

As it was introduced above, the distance-based method of PCA separated the line Meseta from the hybrids Jallon and Smooth regardless

of the nitrogen levels (Supplementary Fig. 1). To deepen the integration of the traits studied at canopy, leaf and transcript levels, we constructed a correlation network for each genotype based on strong significant correlations (Pearson's $r > 0.9$ for positive correlations and $r < -0.9$ for negative correlations, with $P < 0.001$ in both cases). An overview of the correlation networks showed that the three genotypes presented different associations, but the two hybrids, Jallon and Smooth, had a more similar pattern compared to the line Meseta (Supplementary Fig. 2). In every genotype, the agronomic traits, particularly GY, were positively associated with the RGB and aerial VIs, indicating that the canopy biomass and greenness was directly related with the final production of each genotype. In Meseta, several transcript levels (some aquaporins and nitrogen metabolism genes) and leaf physiological traits were positively associated with the agronomic traits and VIs. However, in general the transcript abundances and leaf traits in Jallon and Smooth were not significantly associated with agronomic traits and other plant-growth related traits, including few cases with negative correlations. Furthermore, we calculated multivariate linear models via a forward stepwise method for the prediction of GY (dependent variable) and using the rest of the traits, except the agronomic components, as the independent parameters. The models proposed for each genotype were significant at $P < 0.001$ with a high degree of the variation in

yield explained (Supplementary Table 2). We observed that GY in Meseta could be assessed using leaf traits as N_{leaf} , SI_{abaxial} , chl content at booting, and *GS2* expression, together with canopy VIs (ARI and OSAVI) and N_{grain} (Supplementary Table 2). By contrast, using this approach the GY in the hybrids was correlated similarly with the index RDVI, N_{grain} , water-related traits WUE and Tr, as well as some transcript levels (NR and GDH).

4. Discussion

4.1. Nitrogen fertilization increases grain yield and nitrogen assimilation with small differences between the N_{130} and N_{170} treatments

We investigated the benefits of fertilizing with nitrogen in three barley genotypes, Meseta, Jallon and Smooth. As expected, nitrogen supply strongly increased GY as well as NG in barley plants relative to N_0 , although this increase was lower but still significant between the N_{130} and N_{170} treatments (Table 3) as reported elsewhere [7,8]. Also, NGY and grain nitrogen content data highlighted that the nitrogen assimilated into the grains throughout the life cycle increased in N_{130} and even more so in N_{170} compared to N_0 , while other NUE parameters did not clearly differ between N_{130} and N_{170} (Table 3). In this sense, the higher NGY in N_{170} compared to N_{130} was due to the higher NG, and probably related to more extensive tillering and larger numbers of ears per plant as the nitrogen supply increases [38], rather than a greater NUE or grain nitrogen. VIs assessed from the ground or using the UAV platform revealed that canopy biomass and green area were higher with the application of nitrogen at the booting stage, but this increase was negligible between N_{130} and N_{170} (Table 4), probably because these indices were saturated at these nitrogen doses [39]. Leaf and $\delta^{15}N_{\text{grain}}$ decreased with fertilization, as reported in wheat [40,41], probably because the $\delta^{15}N$ of the chemical fertilizer (urea in our case) is lower than the natural nitrogen (i.e. mostly from mineralization) available in the soil. Moreover, other VIs related to pigment content measured from the UAV platform indicated relatively higher carotenoid and anthocyanin contents under N_0 , which are related to protective functions under stress conditions and plant senescence [42]. Multispectral VIs at mid grain filling also revealed higher biomass and green area with the application of nitrogen but indicated some differences in GGA and CSI between N_{130} and N_{170} (Table 4). Overall, these results suggest that nitrogen fertilization favours nitrogen assimilation and grain productivity and quality (i.e. higher N content) in barley plants, which is likely associated with higher biomass, NG, nitrogen content per grain and a delay in senescence. Although this increase is reduced between N_{130} and N_{170} , an extra nitrogen application late in plant development could have helped to delay plant senescence and explain, at least in part, the differences in GY, NGY and VIs between these treatments (Tables 3 and 4).

4.2. Higher leaf nitrogen and chlorophyll content does not increase photosynthesis on a leaf area basis in well-fertilized treatments, probably due to a long-term stomatal limitation to CO_2 diffusion

Increased nitrogen availability frequently stimulates the photosynthetic capacity because the majority of leaf nitrogen content is present in Calvin-Benson enzymes and thylakoid proteins [34,43]. However, the photosynthetic rate, stomatal conductance or transpiration per unit area were not modified and canopy photosynthetic light-use efficiency decreased in fertilized treatments compared to N_0 even when nitrogen content in flag leaves was higher (Tables 3 and 5). Under high nitrogen supply, Zeng et al. [43] observed a decrease in these parameters in barley, likely due to an excessive accumulation of starch that disrupted chloroplast structure and inhibited CO_2 assimilation. In this sense, the ratio of C_i/C_a decreased in N_{170} with a concomitant increase in WUE, indicating a limited CO_2 diffusion on a leaf area basis. Additionally, SD in the adaxial and abaxial surfaces was reduced with

nitrogen fertilization, and in the case of the abaxial surface it led to a decrease in SI (Table 5). Mature $\delta^{13}C_{\text{grain}}$ provides information relative to photosynthetic performance and WUE on a long-term basis [40,44]. In our study, $\delta^{13}C_{\text{grain}}$ as well as WBI highlighted poorer water status [45] in N_{130} and N_{170} compared to N_0 , in concordance with the higher WUE observed in N_{170} . The lower canopy temperatures measured in the canopies more fertilized with nitrogen compared with those less fertilized may be just an artefact due to the low canopy of the N_0 treatments which exposes naked soil. As the temperature of the bare soil is higher than that of the canopy, the resulting measured temperature is higher than that of well-developed canopies; the WBI and canopy temperature values may be less closely correlated to crop water status in the presence of other confounding factors due to soil background effects or canopy architecture [46,47]. Our results highlight an adjustment in the photosynthetic rate to plant demand when the nitrogen supply is modified. The increase in grain $\delta^{13}C_{\text{grain}}$ (less negative) with fertilization may be caused by stomatal limitation on photosynthesis, which would be the result of the larger transpiring surface area of the fertilized treatments [40,48], in agreement with a decrease in SD, SI, C_i/C_a and a tendency towards stomatal closure, as mainly observed in N_{170} . In fact, the increase in WBI also suggests a poorer water status in the nitrogen fertilized plots [45]. Nevertheless, together with the larger nitrogen and chlorophyll content observed in flag leaves, the increase in plant biomass and leaf area in fertilized plots could indicate an improvement of canopy photosynthesis in agreement with Cabrera-Bosquet et al. [49].

4.3. Nitrogen fertilization increased the expression of genes associated with CO_2 and nitrogen assimilation and nutrient transport, which indicates enhanced biosynthetic processes as nitrogen supply increases

Traits with agronomical relevance are complex and quantitative, determined by multiple genes and environmental signals that define the plant phenotype [50]. We selected several genes in order to obtain an overview of key processes (CO_2 and nitrogen assimilation and nutrient transport) affecting plant growth and development (Table 2). Imai et al. [51] reported that nitrogen influx is strongly related to the amount of Rubisco synthesizes in rice leaves. Indeed, nitrogen fertilization increased *RBCL* expression in N_{130} and more in N_{170} compared to N_0 (Fig. 2). It is important to mention that the differences in the relative transcript abundances are calculated using the same amount of mRNA. Therefore, although photosynthesis did not increase with nitrogen fertilization on a leaf area basis, the higher Rubisco expression might reinforce the enhanced whole plant and canopy photosynthesis hypothesized previously based on the larger leaf area and canopy biomass. Furthermore, nitrogen fertilization induced the expression of *NIR*, *GDH* and one *NR* genes, but downregulated *GOGAT* and one *NR* gene in N_{130} and did not affect *GS2* expression (Fig. 2). These results indicate that despite a low-level induction of some nitrogen metabolism genes, nitrogen was probably in excess. This was because the GS-GOGAT pathway, which is at the core of amino acid and nitrogen compound synthesis [52], was not upregulated or even downregulated at the transcript level, probably due to an accumulation of end-products. Recently, it was shown that the GDH enzyme is mainly involved in the release of ammonium and carbon skeletons for respiration and oxidative phosphorylation, and its overexpression is associated with plant biomass and grain production [20,52]. Thus, the change in *GDH* expression suggests that nitrogen fertilization induced catabolism of flag leaf glutamate. Subsequent use of glutamate products in the sink tissues may have promoted plant biomass and might be linked to a block in the GS-GOGAT pathway. Another finding was aquaporin upregulation (PIP2;1 and specially TIP2;3) in response to nitrogen fertilization (Fig. 2), as reported elsewhere [53]. These water channel proteins are known to transport water and other physiologically important molecules, implicating them in multiple functions [23,24]. Considering the putative substrates described in barley for PIP2;1 and TIP2;3 by Hove

et al. [54], the upregulation of these enzymes could be associated with greater transport of nutrients within the plant (water, CO₂, and specially the urea and ammonium from the applied fertilizers) and they thus contribute to the larger plant biomass and GY observed in fertilized plots.

In summary, nitrogen fertilization improved relevant agronomic and physiological traits in barley. Although the increase of the total leaf area per plant under high nitrogen fertilization may be partially offset by a lower photosynthetic capacity on a leaf area basis related with a lower leaf N content, the increase in total plant photosynthesis in response to high fertilization may contribute to greater biomass accumulation. In addition, nitrogen fertilization improved catabolism of nitrogen compounds in the leaf for their transport to sink tissues contributing to crop production. Nevertheless, there were only small differences in GY between the N₁₃₀ and N₁₇₀ treatments, including a decrease in NUE (i.e. NFPF) in N₁₇₀. This could suggest that although an over application of fertilizer could lead to 10% greater GY in N₁₇₀ relative to N₁₃₀, the higher cost of inputs and the risk of environmental contamination should be considered when planning fertilizer applications.

4.4. Barley hybrids improve grain productivity, plant biomass, green area and nitrogen use efficiency compared to the line, regardless of nitrogen fertilization level

The GY of the barley hybrids Jallon and Smooth was notably improved relative to the Meseta line (Table 3), which is widely cultivated in Spain due to its productivity [55]. This increase was associated with higher NG in Jallon and increases in both NG and TGW in Smooth. NGY tended to increase in the hybrids compared to the line due to the increase in GY with similar grain nitrogen content, indicating a greater uptake and assimilation of nitrogen in the canopy. Other NUE parameters were not statistically different between genotypes. However, NFPF, which did not account for the zero nitrogen plots (which were particularly low in Meseta), and which adjusts for yield in reference to the nitrogen supply, indicated higher NUE in the hybrids (especially in Smooth) than in the line. Akintoye et al. [56] also reported that maize hybrids exhibited superior traits for nitrogen uptake and NUE, while Miersch et al. [57] showed a high nitrogen harvest index and NUE in oilseed rape hybrids.

Lower nitrogen content in leaves of Jallon and Smooth genotypes, expressed as a percentage of total dry weight, might reflect a nitrogen dilution effect due to the larger leaf area relative to the line and/or switches in nitrogen distribution within the plant (Tables 3 and 5). Additionally, canopy biomass and green area, which are generally associated with higher nitrogen acquisition [38,58], were higher in the hybrids than in the line at booting and even more at mid grain filling (Table 4). This fact allowed us to hypothesize that nitrogen allocation was optimized within the plant in both hybrids to maximize crop productivity under every nitrogen treatment. Moreover, changes in the stem elongation and heading dates, CSI and leaf chlorophyll indicated early senescence in Meseta relative to Jallon and Smooth (Tables 4 and 5). Interestingly, Martin et al. [59] confirmed the ability of late-senescent maize hybrids to improve nitrogen absorption and storage. Overall, these findings indicate that crop performance during the whole life cycle and productivity were significantly improved in the hybrids, maybe due to higher canopy nitrogen assimilation, NUE and a delay in senescence.

4.5. Barley hybrids do not show significant changes in photosynthesis on a leaf area basis, although changes in leaf chlorophyll and area and biomass could indicate greater canopy photosynthetic capacity

Although hybrids frequently exhibit higher photosynthetic rates, this is not always the case [15], e.g. in maize under optimal growth conditions [14]. Even with lower leaf nitrogen content and slightly

smaller chlorophyll content at booting, the hybrids showed similar photosynthetic rates per unit area than the line (Tables 3 and 5). The SD in both the adaxial and abaxial surfaces was reduced in the hybrids, but not the SI (ratio of stomata to epidermal cells), indicating larger leaf cells in the hybrids, which is in agreement with the greater leaf area. Although canopy WBI changed between genotypes, which may be related with fractional canopy cover as detailed above, other water traits (Tr, WUE and $\delta^{13}\text{C}$) did not reveal significant changes (Table 5). The larger leaf area and the smaller SD could have contributed to the non-significant changes in photosynthesis on a leaf area basis between genotypes. These findings also demonstrate a better nitrogen use in the flag leaves and plant nitrogen allocation of the hybrids than the line in order to maintain key photosynthetic processes in spite of low leaf nitrogen content, as also pointed out above. Additionally, it is important to note that canopy green area and leaf area were significantly higher in the hybrids, frequently beginning with seedling vigour [15], which could suggest greater photosynthetic capacity per organ or per plant due to a better nitrogen distribution. Nevertheless, delayed leaf senescence could be a greater advantage for grain production than improvement in the photosynthetic rate [15,60].

4.6. The gene expression pattern in the hybrids differs greatly from the line, indicating a lower demand of nitrogen compounds in the flag leaves, including Rubisco, and suggesting a better nitrogen distribution towards other organs that benefits grain production

The gene expression analysis undoubtedly showed that both Jallon and Smooth presented a similar pattern and, in most cases, it differed from Meseta (Fig. 2). Rubisco protein accounts for 15–30% of total leaf nitrogen, which under optimal growth conditions is partly used for nitrogen storage [22]. In order to improve NUE, an optimization of Rubisco content in the plant is required. In this sense, Kanno et al. [22] observed that a small decrease in Rubisco content was associated with a greater photosynthesis and biomass in rice. Our results showed lower Rubisco transcription, which could also indicate lower Rubisco protein synthesis because both processes are coordinated as reported in tobacco [61] and rice [62]. Moreover, the primary nitrogen assimilation in flag leaves might be reduced in the hybrids, as speculated by the lower transcript abundance of genes involved in this pathway (NR, NIR and GOGAT; Fig. 2). This could suggest (i) inhibition of nitrogen assimilation by the accumulation of nitrogen end-products or (ii) lower demand of nitrogen compounds in this organ due to greater sink strength. Due to the significantly lower leaf nitrogen content in the hybrids, the latter hypothesis is more reasonable. On the other hand, the GDH enzyme is involved in nitrogen remobilization, particularly in the release of ammonium during senescence [52]. The transcript levels for GDH in the hybrids were dramatically downregulated compared to the line, which alongside changes in canopy senescence and water status could suggest a longer growth cycle in the hybrids (Fig. 2 and Tables 4 and 5). The hybrids clearly downregulated aquaporin genes (PIP2;1, TIP1;2 and NIP2;2), which are proteins that play an important role in nitrogen metabolism [53]. This might indicate that the transport of the fertilizers urea and ammonium, which are putative molecules transported by these aquaporins [54], were diminished in flag leaves. Lastly, some small differences in gene expression were observed between the hybrids, such as higher expression of NR, GDH and PIP2;1 in Smooth (Fig. 2), which could have contributed to the trend of higher GY and chlorophyll content relative to Jallon.

The multivariate analyses (PCA and correlation networks) and linear models (stepwise regression) showed that the genotypes with higher GY, the hybrids Jallon and Smooth, were different at canopy, leaf and transcript levels from Meseta, highlighting that the better crop productivity of hybrids was observed regardless of the nitrogen fertilization level. The integration of the data revealed that the hybrids were characterized by lower expression of genes related to photosynthesis, nitrogen assimilation and aquaporins and by higher leaf area, as well as

by higher chlorophyll content during late growth stages (Supplementary Fig. 1). Moreover, the multispectral VIs, measured at ground and aerial scale, were, in all the genotypes, a good indicator of GY (Supplementary Fig. 2). In this sense, some VIs were present in all the models for the prediction of GY (Supplementary Table 2), constituting an attractive and efficient low-cost strategy for crop breeding. These models, together with the correlation networks, highlight that the agronomic and plant growth components were correlated with leaf traits, including transcript levels for nitrogen assimilation and transport. Weaker correlations were observed for the high-yielding genotypes, Jallon and Smooth, indicating that the leaf metabolism was less relevant in these plants.

In summary, these results related the better crop performance of hybrids, including GY, to a lower demand for nitrogen in leaves (nitrogen-saving strategy) to the detriment of the larger sink size, as indicated by the lower leaf nitrogen content and the downregulation of nitrogen metabolism and aquaporin genes. We highlight the likely key role of GDH and aquaporin TIP2;3 in the source-sink coordination. However, these changes did not reduce photosynthesis on a leaf area basis but increased biomass, green area, NPPF, GY, NGY (non-significantly), and delayed senescence, indicating again a better NUE in the genotypes with high yield via an improvement in nitrogen assimilation and allocation at the whole plant level.

Our study of different parameters related to plant growth, photosynthesis and water and nitrogen use allowed us hypothesize about the mechanisms contributing to grain yield under contrasting nitrogen supplies or using genotypes with different crop performance at canopy, leaf and transcript levels. Fertilization in barley plants improved nitrogen assimilation and crop performance, although it is important to regulate an adequate nitrogen dose. Hybrids exhibited higher GY regardless of the nitrogen treatment associated to a delayed senescence and a better use of nitrogen at the whole plant level. Therefore, the use of hybrids and management of nitrogen application doses are important factors to consider for the economics of barley production.

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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.plantsci.2018.10.002>.

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