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WHIRLY1 functions in the control of responses to N-deficiency but not aphid infestation in barley (*Hordeum vulgare*)

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Tables: 2

Supplemental Tables: 1

Supplemental Figures: 3

ABSTRACT

WHIRLY1 is largely targeted to plastids, where it is a major constituent of the nucleoids. To explore WHIRLY1 functions in barley, RNAi-knockdown lines (W1-1, W1-7 and W1-9) that have very low levels of *HvWHIRLY1* transcripts were characterized in plants grown under optimal and stress conditions. The W1-1, W1-7 and W1-9 plants were phenotypically similar to the wild type but produced fewer tillers and seeds. Photosynthesis rates were similar in all lines but W1-1, W1-7 and W1-9 leaves had significantly more chlorophyll and less sucrose than the wild type. Transcripts encoding specific sub-sets of chloroplast-localised proteins such as ribosomal proteins, subunits of the RNA polymerase and the thylakoid NADH and cytochrome b_6/f complexes were much more abundant in the W1-7 leaves than the wild type. While susceptibility of aphid infestation was similar in all lines, the WHIRLY1-deficient plants showed altered responses to nitrogen deficiency maintaining higher photosynthetic CO_2 assimilation rates than the wild type under limiting nitrogen. While all lines showed globally similar low nitrogen-dependent changes in transcripts and metabolites, the increased abundance of FAR-RED IMPAIRED RESPONSE1-like transcripts in nitrogen-deficient W1-7 leaves infers that WHIRLY1 has a role in communication between plastid and nuclear genes encoding photosynthetic proteins during abiotic stress.

INTRODUCTION

Plastids contain up to 200 copies of the plastid genome that is associated with non-histone proteins and arranged into discrete protein-DNA complexes, called nucleoids, which are associated with the inner envelope membrane (Kruprinska et al., 2013; Powikrowska et al., 2014). Within these structures, single-stranded DNA (ssDNA) binding proteins are responsible for binding and stabilising the ssDNA until it is utilized by DNA polymerase or other proteins involved in DNA recombination and repair. Since ssDNA binding proteins must bind all available ssDNA as it becomes accessible, they are highly abundant and fulfil essential roles in DNA replication, recombination and repair. However, relatively few ssDNA binding proteins have been characterised in plants.

The WHIRLY family of ssDNA binding proteins have a quaternary structure with a whirligig appearance. Like WHIRLY 2 (Tarasenko, et al., 2012), the WHIRLY 1 protein has a preference for binding ssDNA, without sequence specificity. It was first characterised as a binding subunit of the nuclear transcriptional activator, PBF2 that is involved in plant defence gene expression in *Solanum tubersoum* (Desveaux et al., 2002; Desveaux et al., 2004). WHIRLY proteins have been considered to function as transcription factors in the nucleus because PBF2 regulates the expression of *PATHOGENESIS-RELATED (PR) PROTEIN 10a* by binding to the single strand form of the elicitor response element (Desveaux et al., 2005). The DNA binding activity of WHIRLY 1 is induced by pathogen elicitors and by salicylic acid (SA). It is required both for expression of SA-regulated genes and for associated disease resistance, although this pathway is independent of NON-EXPRESSOR OF PATHOGENESIS-RELATED (PR) PROTEINS (NPR1; Desveaux et al., 2004). The barley WHIRLY1 protein binds to the promoter of the senescence associated gene *HvS40,* which is induced in natural and stress-induced senescence (Krupinska et al., 2002; 2014a).

WHIRLY proteins are found in the chloroplasts and mitochondria as well as in the nuclei (Krause et al., 2005; Grabowski et al., 2008). Most plant species such as barley have two *WHIRLY* genes (Desveaux et al., 2005) but there are three *WHIRLY* genes in *A. thaliana*.

AtWHIRLY1 and *AtWHIRLY3* are targeted to plastids while *AtWHIRLY2* is targeted to mitochondria (Krause et al., 2005; Krause and Krupinska, 2009). WHIRLY1 is mainly targeted to plastids, where it is one of the major DNA-binding proteins, and is a major constituent of the plastid nucleoids. In maize, knock-out of *WHIRLY1* (*zmwhy1-1*) by transposon insertion had dramatic effects on plastid gene expression, resulting in albino plants lacking plastid ribosomes, with altered splicing of specific transcripts (Prikryl et al., 2008). However, both knock-out and knock-down maize lines had equivalent amounts of cpDNA, suggesting that in maize *WHIRLY1* is not required for cpDNA replication (Prikryl et al., 2008). In contrast the *why1* Arabidopsis mutants have no apparent phenotype (Yoo et al., 2007) except that the seeds showed less sensitivity toward SA and abscisic acid (ABA) than the wild type during germination (Isemer et al., 2012a). When the WHIRLY1 protein was expressed without a targeting sequence (and targeted to the nucleus) of the *why1* mutants, the seeds were insensitive to ABA. In contrast, when the wild type WHIRLY1 protein was targeted to both plastids and the nucleus, the seeds showed enhanced ABA sensitivity (Isemer et al., 2012a).

The double knockout mutants lacking both WHY1 and WHY3 (*why1why3*) are largely phenotypically similar to the wild type (Maréchal et al., 2009; Cappadocia et al., 2010). In contrast, a *why1why3polIb-1* mutant that is defective in *WHY1 and WHY3* and also PolIB, one of the two type-I chloroplast DNA polymerases exhibited a more extreme yellow-variegated phenotype (Lepage et al., 2013). The *why1why3polIb-1* mutants had a higher level of illegitimate recombination between repeated sequences and greater plastid genome instability than the wild type (Lepage et al., 2013). Moreover, the *why1why3polIb-1* mutants showed lower photosynthetic electron transport efficiencies than the wild type leaves and they had a higher accumulation of reactive oxygen species (ROS; Lepage et al., 2013). The higher level of oxidation observed in the *why1why3polIb-1* triple mutants was linked to chloroplast to nucleus signaling and enhanced adaptation to oxidative stress (Lepage et al., 2013).

The WHIRLY1 protein localises to the stroma and to the thylakoid membranes in maize and barley chloroplasts (Prikryl et al., 2008; Melonek et al., 2010). While early studies

indicated that WHIRLY1 did not associate with plastid nucleoids (Melonek 2010), loss of the WHIRLY1 protein resulted in a slight increase in cpDNA copy number, correlating with increased expression of an organellar DNA polymerase (Krupinska et al 2014b). Recent evidence from studies using a recombinant form of WHIRLY1 suggested that the protein could translocate from transplastomic chloroplasts to the nucleus (Isemer et al., 2012b). Moreover, WHIRLY1 is a thioredoxin-target and is hence a potential target for redox regulation (Foyer et al., 2014).

Current crop yields are highly dependent on significant levels of inorganic nitrogen fertilization, such that chemical nitrogen fixation for fertilizer production is now the largest fraction of total global nitrogen reduction surpassing both natural biotic and abiotic nitrogen fixation. Thus, a broad range of economic and environmental benefits would follow if crop plants could be selected or engineered to have increased nitrogen use efficiency (NUE). A better understanding of the inherent adaptive responses of plants to low nitrogen availability is therefore required in order improve NUE and maintain high productivity on reduced nitrogen inputs. In the following studies, therefore we have explored the functions of WHIRLY1 in chloroplasts using transgenic barley lines (W1-1, W1-7 and W1-9) that have only about 5% or less of the wild type WHIRLY1 protein (Melonek et al., 2010; Kruprinska, et al., 2014b) under different growth nitrogen regimes. We present data showing that the WHIRLY1 protein influences the expression of specific subsets of transcripts encoding chloroplast proteins, resulting in a reduced sensitivity of photosynthesis to low nitrogen.

RESULTS

The phenotype of WHIRLY1-deficient barley seedlings

In these studies, barley seedlings that had been grown in vermiculite with nutrient solution were harvested at time-points up to 27 days after germination, as indicated in the figure legends. The W1-1, W1-7 and W1-9 barley seedlings had a similar phenotype to the wild type (Fig. 1A) even though they had significantly lower levels of *WHIRLY1* transcripts (Fig. 1B). In contrast *to WHIRLY1*, the abundance of *WHIRLY2* transcripts was similar in all lines (Fig. 1B). The W1-1, W1-7 and W1-9 barley seedlings accumulated similar amounts of leaf and root biomass (Fig. 1C) to the wild type with comparable shoot/root ratios (Fig. 1D). Photosynthetic CO_2 assimilation rates (Fig. 2A) were similar in wild-type and the W1-1, W1-7 and W1-9 seedlings, as were stomatal conductance (Fig. 2B), transpiration rates (Fig. 2C) and Ci values (Fig. 2D) four weeks after germination. However, the W1-1, W1-7 and W1-9 leaves had significantly more chlorophyll and carotenoid pigments than the wild type with the exception of W1-9 carotenoid content that was similar to wild type (Fig. 2E).

Line W1-7 has no detectable WHIRLY1 protein and has already been characterised in terms of ptDNA content and nucleoid structure (Kruprinska, et al., 2014b). We therefore performed a more in-depth analysis on the physiology, transcriptome and metabolome profiles of line W1-7 at the early stages of leaf development. Photosynthesis rates were similar in wild type and W1-7 seedlings 8-14 days after germination (Fig. 3A). However, in very young WHIRLY1-deficient seedlings i.e. 7 days after germination and younger, photosynthesis rates were lower in W1-7 leaves than the wild type, as demonstrated by the maximal rates of CO_2 assimilation under high light (Fig. 3B). In contrast, at 14 days the light response curves for photosynthesis were similar in WHIRLY1-deficient and wild type leaves (Fig. 3A, C). Stomatal conductance and transpiration rates were higher in the W1-seedlings than the wild type up to 14 days after germination (Fig. 3D, E). The total nitrogen content of

the shoots and roots were comparable in W1-7 and wild type seedlings (Supplemental Fig. S1A).

The WHIRLY1-deficient lines were visibly similar to the wild type plants throughout development (Supplemental Fig. S2). Seed yields were significantly lower in the WHIRLY1-deficient lines than the wild type with W1-7 plants exhibiting fewer fertile tillers (Table 1). However, seed yield per fertile tiller was similar in W1-7 and wild type plants (Table 1).

The levels of ascorbate, pyridine nucleotides and glutathione were similar in line W1-7 and wild type seedlings (Fig. 4). The metabolite profile of the W1-7 leaves was very similar to that of the wild type seedlings (Fig. 5), indicating a significant difference only in the amount of leaf sucrose and a trend towards a lower abundance of reducing sugars and TCA cycle intermediates but no differences in the leaf amino acid pools.

The transcript profile is modified in WHIRLY1-deficient leaves

The transcript profile of the W1-7 leaves was characterized by increases in the abundance of large numbers of transcripts encoding proteins involved in photosynthesis and protein synthesis (Fig. 6A, Supplemental table S1). The differences in transcript abundance between W1-7 and wild type leaves were confirmed by qPCR (Fig. 6B).

Transcripts that were much more abundant in W1-7 leaves include a component of the DNA polymerase type 1 complex (MLOC 54735.1) that functions in the replication and repair of plastid DNA. This transcript is identical to the barley organelle DNA polymerase described recently (Krupinska et al., 2014) and is homologous to the AtPol1B (At3g20540) sequence. Similarly, a microtubule plus-end binding protein EB1A (MLOC 52339.1) and a transcript encoding a protein containing a B3 DNA binding domain (AK251585.1) that is found exclusively in transcription factors were significantly more abundant in W1-7 leaves relative to wild type (Fig. 6C, Supplemental table 1). The

transcript profile of the W1-7 leaves showed no changes in the innate immune responses of the plants. To confirm the absence of effects on the innate immune responses, we compared the susceptibility of the W1-1, W1-7 and W1-9 lines to aphid infestation and found that that aphid fecundity was similar in the WHIRLY1-deficient and the wild type lines (Supplemental Fig. S3).

A large number of transcripts encoding chloroplast-associated proteins were significantly increased in abundance in W1-7 leaves relative to the wild type (Table 2). These include a number of components associated with the thylakoid NADH dehydrogenase complex (NDHA, NDHC, NDHD, NDHF, NDHB.2, NDHH, NDHJ, NDHI and NDHG), the chloroplast RNA polymerase (RPOC2, RPOB and RPOC1), the cytochrome b/f complex (*PETA*, *PETD* and *YCF5*), chloroplast ribosomes (RPL20, RPL23.2, RPL33 and RPS2) and minor components associated with the PSII (PSBF, PSBB and PSBJ) and PSI (PSAC, PSAJ) reaction centres (Table 2).

WHIRLY1-deficient seedlings show enhanced resistance to nitrogen deficiency

The wild type and W1-1, W1-7 and W1-9 lines showed similar low nitrogen-dependent decreases in shoot (Fig. 7A) and root (Fig. 7B) biomass. The W1-1, W1-7 and W1-9 leaves had three-fold more chlorophyll than the wild type under nitrogen deficiency (Fig. 7C). In contrast to the wild type leaves, which were only capable of respiration after 22 days of nitrogen deficiency, the W1-1, W1-7 and W1-9 leaves were still able to undertake photosynthetic CO_2 assimilation (Fig. 7D). The carbon contents of the wild-type and W1-7 lines were similar irrespective of nitrogen supply (Supplemental fig. S1 B, E) while both lines exhibited a similar decrease in nitrogen content and increase in C:N ratio under nitrogen limitation (Supplemental Fig. S1 A, C, D, F).

The transcript profile of WHIRLY1-deficient leaves exhibits a distinct response to low nitrogen

WHIRLY1 transcripts were increased in the leaves of the wild type plants grown with low nitrogen (Fig. 8A). In contrast, the levels of *WHIRLY1* transcripts were much lower in W1-1, W1-7 and W1-9 lines than the wild type under both nutrition regimes (Fig. 8A). However, *WHIRLY2* transcripts were increased to a similar extent under the low growth nitrogen regimes in all lines (Fig. 8B). The levels of psbA, psbJ, ndhA, ndhJ and were significantly lower in the W1-1, W1-7 and W1-9 lines than the wild type when expressed relative to the chloroplast 16S ribosomal transcripts under optimal nitrogen (Fig. 8C). However, the levels of these transcripts relative to the chloroplast 16S ribosomal transcripts were similar in all lines under the low growth nitrogen regime (Fig. 8D).

To analyse the effects of nitrogen regime on the leaf transcript profile W1-7 and wild type seedlings were grown for 15 days under either optimal nitrogen conditions or under nitrogen deficiency. About 50% the transcripts that were more abundant in W1-7 leaves under nitrogen-replete conditions were also more abundant in conditions of nitrogen deficiency (Fig. 9A). A further 40 transcripts were induced in W1-7 leaves under conditions of nitrogen deficiency (Fig. 9A). A marked difference between the leaf transcriptome profiles in seedlings grown under nitrogen deficiency compared to those grown under nitrogen-replete conditions was the high number of transcripts involved in RNA processing and signaling that were increased in W1-7 leaves relative to the wild type (Fig. 9C-D). Of note was the large decrease in the abundance of transcripts encoding Eukaryotic Initiation Factor 4A (Supplemental table S1), a component of the Eukaryotic Initiation Factor 4F (eIF4F) complex, which recognizes the 7-methylguanosine cap of messenger RNA and is involved in the initiation phase of eukaryotic translation (Aitken and Lorsch, 2012). Together with eIF4G, which serves as a scaffold to recruit other translation initiation factors, eIF4F ultimately assembles the 80S ribosome. In addition, the levels of transcripts encoding a FAR-RED IMPAIRED RESPONSE1 (FAR1) like protein were greatly increased in the W1-7 compared to the wild type under conditions of nitrogen deficiency, as were transcripts encoding a putative histidine kinase and a leucine-rich repeat

transmembrane receptor-like kinase (LRR-RLK) of the *STRUBBELIG* family (Supplemental table S1).

DISCUSSION

The WHIRLY1 protein is mainly targeted to plastids, where it is one of the major DNA-binding proteins and constituent of the plastid nucleoids. In these studies we have characterised WHIRLY1 functions in independent transgenic barley lines (W1-1, W1-7 and W1-9) that have very low levels of *HvWHIRLY1* transcripts. The loss of WHIRLY1-dependent controls had little effect on photosynthetic CO_2 assimilation rates, except at the early stages of seedling development. However, leaves lacking WHIRLY1 accumulate more chlorophyll under optimal growth conditions and under conditions of nitrogen limitation. The differences between the results presented here and those obtained in chloroplasts isolated from these RNAi lines (Malonek et al. 2010), may be explained by differences in the growth irradiances used in the two studies. The data presented here demonstrate that barley leaves that have low expression of *WHIRLY1* accumulate more chlorophyll, even under conditions of nitrogen deficiency.

The role of WHIRLY 1 in expression of SA-regulated genes and in pathogen resistance (Desveaux et al., 2004) led us to address the question of whether WHIRLY1-deficient barley seedlings showed altered responses to aphid infestation. However, the data shown in Supplemental Fig S3 demonstrate that aphid fecundity was similar in all lines. This observation is consistent with the absence of changes in transcripts encoding pathogen-related signalling pathways in the W1-7 leaf profile. Moreover, the significantly lower sucrose content of the W1-7 leaves relative to the wild type did not affect the ability of aphids to infest the leaves.

The transcript profile analysis of the WHIRLY-deficient leaves showed that genes encoding discrete subsets of photosynthetic proteins were markedly changed relative to the wild type. These findings are consistent with the observation that barley leaves appear to be less able to regulate cpDNA copy number in the absence of WHIRLY1 (Krupinska et al 2014b). Transcripts encoding photosynthetic proteins including the thylakoid NADH dehydrogenase and cytochrome b/f complexes, and chloroplast ribosomes were more abundant in the WHIRLY-deficient leaves than the wild type. Plastid DNA contains 11

ndh genes, which are significantly represented in the W1-7 profile (Table 2). The chloroplast NADH complex, which is composed of chloroplast- and nuclear-encoded subunits, functions in cyclic electron flow around PSI (Peng et al., 2009; 2011) and in protection against stress (Casano et al., 2001). The data shown in Table 2 suggest that WHIRLY1 functions to suppress the expression of genes encoding intersystem carriers such as the cytochrome b/f and NDH complexes and may therefore be important in the regulation of cyclic electron flow (Foyer et al., 2012; Miyake 2010). While these changes may be linked to a failure to regulate cpDNA copy number (Krupinska et al 2014b), it is interesting that the observed increases in these transcripts are not accompanied by similar changes in the expression of nuclear genes encoding photosynthetic proteins. The expression of nuclear genes encoding photosynthetic proteins therefore appears to be uncoupled to some extent from plastid gene expression in the WHIRLY-deficient leaves.

The data presented here demonstrate that transcripts encoding core PSI and PSII reaction center complexes and light harvesting components were unaffected by WHIRLY1. This finding is surprising given the sensitivity of PSI and PSII gene expression to light and metabolic controls (Foyer et al., 2012). The absence of any effects of WHIRLY1-deficiency on PSI and PSII gene expression may explain why there were few detectable differences in photosynthetic CO_2 assimilation rates between the WHIRLY1-deficient and wild type leaves in the absence of stress. However, the W1-7 leaves had significantly less sucrose than the wild type suggesting that the influence of WHIRLY1 extends to carbon metabolism.

The *why1why3polIb-1* mutants of *A. thaliana* exhibited higher plastid genome instability and enhanced oxidative stress (Lepage et al., 2013). The data presented here show that transcripts encoding a chloroplast-targeted Cu, Zn superoxide dismutase 2 (SOD2) were much more abundant in WHIRLY1-deficient leaves than the wild type controls, as were transcripts encoding phytochelatin synthase that functions in xenobiotic metabolism and the catabolism of glutathione-conjugates (Fig. 9, Supplemental table S1). However, the levels of the reduced and oxidized forms of the major leaf antioxidants ascorbic acid and

glutathione, and also pyridine nucleotides were not changed in the WHIRLY1-deficient leaves, suggesting that if WHIRLY1-deficiency leads to enhanced production of reactive oxygen species, it does not cause a global change in the cellular redox state but rather a localized increase in oxidation, for example in the plastids. The increase in the levels of transcripts encoding chloroplast-targeted Cu, Zn superoxide dismutase 2 (SOD2), which is a nuclear gene, lends support to the hypothesis that WHIRLY1 is involved in chloroplast to nucleus signaling (Lepage et al., 2013).

The data presented here demonstrate that the regulation of plastid DNA replication by WHIRLY1 has wide reaching implications for other pathways and processes influenced by chloroplast-derived signals. However, transcripts encoding transcription factors and stress signaling proteins were not significantly altered in abundance by WHIRLY1-deficiency. The absence of a changed stress response was confirmed by the similar levels of aphid infestation observed in wild type and WHIRLY1-deficient leaves. Transcripts encoding histidine kinases and *STRUBBELIG* (*SUB*), which is a receptor-like kinase involved in intercellular signal transduction (Eyüboglu et al., 2007) were increased in WHIRLY1-deficient leaves. A transcript that shows homology to an *Oryza sativa* plant-specific type II MIKC MADS box gene was increased in abundance in W1-7 leaves relative to the wild type. A transcription factor of this type that has been most intensively studied, the *Triticum aestivum* AGAMOUS-like 33 (TaAGL33) is a key regulator of developmental processes, such as meristem identity, flowering time, and fruit and seed development (Winfield et al., 2009). The expression of TaAGL33 represses flowering and cell elongation by down-regulation of a group of genes related to the Arabidopsis FLOWERING PROMOTING FACTOR 1 (Greenup et al., 2011). The enhanced abundance of the type II MIKC MADS box transcripts in W1-7 plants may explain, at least in part, why the WHIRLY1-deficient barley plants show altered development withfewer fertile tillers than the wild type (Table 1).

The WHIRLY1-deficient barley seedlings were more resistant to nitrogen deficiency in terms of decreased loss of leaf chlorophyll and less inhibition of photosynthetic CO_2

assimilation rates, suggesting that the failure to control cpDNA copy number and repress plastid DNA replication exerts an influence on the systems that decrease photosynthesis in response to nitrogen deficiency. While the data presented here do not give many insights into the mechanisms that underpin this response, it is interesting to note that FAR1 like protein transcripts were much more abundant in the W1-7 leaves than the wild type under conditions of nitrogen deficiency. Although the molecular nature of plastid-to-nucleus signalling pathways remains poorly understood, the transcriptional control in response to light is closely tied to the primary signaling function of the phytochrome system. The *FAR1* gene encodes a transposase-related transcription factor that activates the expression of *FHY1* and *FHL,* which promote the nuclear translocation of phytochrome A, resulting in the activation of phytochrome A-mediated gene expression such as chloroplast division and chlorophyll biosynthesis (Tang et al., 2012). The FAR1 protein also acts as a positive regulator of abscisic acid (ABA) signaling in *A. thaliana*, enabling adaptation to environmental stresses (Tang et al, 2013). WHIRLY1 has been shown to influence the responsiveness of seedlings to ABA (Isemer et al, 2012a). The high abundance of transcripts encoding a FAR1 like protein in the leaves of W1-7 grown under low nitrogen may explain the higher chlorophyll levels observed in WHIRLY1-deficient leaves, if they are linked to increased chlorophyll synthesis through modulation of *HEMB1* as described previously (Tang et al., 2012).

In conclusion, the data presented here shows that WHIRLY1 is an important regulator of cpDNA and plastid gene expression. Severely reduced expression of WHIRLY1 leads to a disruption of the communication between the plastid and nuclear genes encoding photosynthetic proteins, leading to an accumulation of chlorophyll in leaves that persists even under nitrogen limitation. Moreover, photosynthesis was less susceptible to inhibition in plants grown under low nitrogen, suggesting that WHIRLY1 is a potential new target for improvement of nitrogen use efficiency.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

Seeds of three independent transgenic barley (*Hordeum vulgare* L. cv. Golden Promise) lines (W1-1, W1-7 and W1-9) with RNAi knockdown of the *WHIRLY1* gene and wild type controls were produced as described previously (Melonek et al., 2010; Krupinska et al., 2014b). Seeds were allowed to germinate for 7 days in the absence of added nitrogen. They were then sown in pots in vermiculite in controlled environment chambers with a 16h light/8h dark photoperiod (irradiance 450µmol $m^{-2}s^{-1}$), 21ºC/16ºC day/night temperature regime and 60% relative humidity. The pots were arranged in trays (16 pots per tray). Every 2 days each tray was provided with 2L of the nutrient solution described by Møller et al. (2011) consisting of 0.2 mM KH_2PO_4, 0.2 mM K_2SO_4, 0.3 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM NaCl, 0.1 μM $MnCl_2$, 0.8 μM $Na_2MoO_4 \cdot 2H_2O$, 0.7 μM $ZnCl_2$, 0.8 μM $CuSO_4 \cdot 5H_2O$, 2 μM H_2BO_3, 50 μM Fe(III)-ethylenediaminetetraacetic acid (EDTA)-Na and either 5mM KNO_3 (nitrogen replete) or 0.1mM KNO_3 (nitrogen deficient). Plants were harvested 7, 9, 17, 22 or 27 days after the initiation of the low and optimal nitrogen treatment regimes.

For analysis of seed yield, plants were grown to maturity in compost in a standard heated greenhouse at 22°C under 16-h photoperiod where supplementary lighting was provided by high-pressure sodium vapor lamps (Powertone SON-T AGRO 400W; Philips ElectronicUK).

Shoot and Root Biomass

Whole plants were harvested and separated into shoots and roots. These were weighed immediately and then dried in an oven at 80ºC for 2 days after which the tissues were weighed again.

Photosynthesis Measurements

Photosynthetic gas exchange measurements were performed using a portable system Ciras-2 Infrared Gas Analyser (model ADC 225 Mark 3, The Analytical development Co

Ltd, Hoddesdon, UK) set at 300 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 40–50% relative humidity in the leaf chamber, leaf chamber CO_2 and O_2 concentrations maintained respectively at 400 ± 10 μmol mol^{-1} and 210 μmol mol^{-1}. The temperature of the leaf chambers was set at 20 ± 0.5°C. Calculations of CO_2 assimilation rate and stomatal closure were performed as described previously (Von Caemmerer & Farquhar, 1981).

Aphid Fecundity Assays

Barley plants were grown for 15 days under nitrogen-replete conditions as described above. At the end of this period a single one-day nymph of *Myzus persicae* was applied to the lamina of the oldest leaf and plants were individually caged inside clear plastic containers (10 cm internal diameter x 20 cm height) and capped with a 200 μm mesh. Plants were provided with nutrient solution weekly and after 15 days were carefully removed from the cages and aphids present were counted under a hand lens.

Leaf Pigment Content

Leaf pigments were extracted and analysed according to the method of Lichtenthaler (1987).

Carbon and Nitrogen Content

The carbon and nitrogen contents were determined on the dried leaf and root material from 5 biological replicates per genotype per treatment, using a LECO Trumac combustion analyser (Yara UK Limited Company, York, UK).

Ascorbate, Glutathione and Pyridine Nucleotide Assays

Ascorbate, glutathione and pyridine nucleotides were extracted and analysed as described by Queval and Noctor (2007).

Metabolite analysis by Gas Chromatography-Mass Spectrometry (GC/MS)

GC/MS analysis was performed on extracts from 4 biological replicates per genotype per treatment. Leaf samples were lyophilized, polar and non-polar extracts were prepared,

derivatized and analysed by GC/MS (Dobson et al. 2008). Data was then processed using Xcalibur software.

Microarray processing and analysis

Microarray processing was performed on leaf RNA extracts from 4 biological replicates per genotype per treatment, using a custom-designed barley Agilent microarray (A-MEXP-2357; www.ebi.ac.uk/arrayexpress). The microarray contains *c*. 61,000 60-mer probes derived from predicted barley transcripts and full-length cDNAs (IBGSC, 2012). These probes were selected from a total of ~80,000 predicted genes by prioritising them according to their annotation (see section S7.1.4 and Figure S18 in supplementary material file of IBGSC, 2012). The high-confidence gene set was used in its entirety (n = 26,159). This set of predicted genes is based on being supported by homology to at least one closely related species (*Brachypodium distachyon, Sorghum bicolor, Oryza sativa* and *Arabidopsis thaliana*). Next, 14,481 genes were added that had been annotated as "remote homologs", based on a lack of homology to monocot proteins. We also added 7,999 genes from the "Triticeae-specific" category, which is defined as having significant BLASTN hits to the wheat fl-cDNA library but no significant BLASTX hit to angiosperm reference protein sequences. The remainder of sequences on the chip (n = 12,848) derive from genes that had no homology to any of the databases used and are assumed to be specific to barley. This resulted in a total of 61,487 genes represented on the chip by a single probe sequence each.

Microarray processing was performed according to the 'One-Color Microarray-Based Gene Expression Analysis' protocol (v. 6.5; Agilent Technologies). Data were extracted using Feature Extraction (FE) software (v. 10.7.3.1; Agilent Technologies) with default settings, and subsequently analysed using GeneSpring GX (v. 7.3 and v.12; Agilent Technologies) software. Data were normalised using default Agilent FE one-colour settings in GeneSpring and filtered to remove inconsistent probe data flagged as absent in more than one replicate per sample. Probes were identified as significantly changing

between genotype & nitrogen treatment using 2-way Analysis Of VAriance (ANOVA) with a p-value of <0.05 with Bonferroni multiple-testing correction. Probes were identified as significantly changing between WHIRLY and wild type under sufficient or deficient nitrogen treatments by a Moderate t-test and Benajmini-Hochberg FDR multiple testing correction ($p<0.05$, fold change >2). Raw data can be accessed via the array express website (www.ebi.ac.uk/arrayexpress) using accession number E-MTAB-2242.

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Figure Legends

Figure 1 Phenotypic comparison of wild type seedlings and three independent WHIRLY-1 deficient barley lines (W1-1, W1-7 and W1-9). (A) Representative appearance of lines 22 days after germination. (B) Relative transcript abundance of transcripts encoding Whirly1 and Whirly2 in leaves. (C) Shoot and root biomass, expressed as fresh weight (FW). (D) Shoot/root ratios. Bars in B - D represent mean ± SE (n = 3).

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Figure 7 Impact of nitrogen availability on growth and photosynthesis in wild type seedlings and three independent WHIRLY-1 deficient barley lines (W1-1, W1-7 and W1-9). Plants were grown for 22 days under either optimal (N replete) or low nitrogen (N deficient) conditions. Shoot (A) and root (B) biomass was estimated after destructive harvesting of plants and values are represented as mean ± SE (n = 3). Chlorophyll content (C) was estimated after extraction from harvested shoots and CO_2 assimilation rate (D) was estimated by gas exchange prior to harvest; values are represented as mean ± SE (n = 4). Asterisks indicate significant differences between WHIRLY1 deficient and wild-type plants estimated by the students T-test ($P<0.05$).

Figure 8 Abundance of transcripts encoding whirly and several chloroplast encoded proteins in leaves of wild type and three independent WHIRLY-1 deficient barley lines (W1-1, W1-7 and W1-9). Plants were grown for 15 days under either optimal (N replete) or low nitrogen (N deficient) conditions. The abundance of transcripts encoding WHIRLY-1 (A) and WHIRLY-2 (B) were estimated relative to their abundance in wild type plants under N-replete conditions by the $\Delta\Delta C_T$ method using actin as a reference.

The abundance of chloroplast encoded transcripts were estimated relative to their abundance in wild type plants under N-replete (C) or N-deficient (D) conditions using 16S ribosomal RNA as a reference. All data are presented as mean values ± SD, n = 3.

Figure 9 Transcript profile comparison of 15 day-old wild type leaves and the WHIRLY-1 deficient barley W1-7 line grown under either optimal (N replete) or low nitrogen (N deficient) conditions. (A) Venn diagram illustrating the number of differentially abundant transcripts under each nitrogen regime. (B) Classification of transcripts that showed differential abundance only under nitrogen replete conditions. (C) Classification of transcripts that showed differential abundance under both nitrogen conditions. (D) Classification of transcripts that showed differential abundance only under conditions of nitrogen deficiency. The differentially expressed genes were identified using a moderated T-test with Benjamini-Hochberg multiple testing correction ($p<0.05$, FC>2) (Genespring 12, Aligent Technologies).

Supplemental Figure S1 Comparisons of shoot and root carbon and nitrogen contents in wild-type and WHIRLY-1 deficient W1-7 seedlings grown for 15 days under either optimal (A-C) or limiting nitrogen (D-F) conditions. Data are represented as mean ± SE, values (n = 5) and indicate total nitrogen (A, D), total carbon (B, E) and carbon to nitrogen ratios (C, F). Significant differences between lines were estimated using the students T-test as indicated by asterisks ($P<0.05$).

Supplemental Figure S2 Appearance of wild-type and WHIRLY1-deficient barley lines grown under optimal nitrogen for 18 (A), 27 (B), 81 (C) and 130 (D) days.

Supplemental Figure S3 Aphid fecundity on wild-type and WHIRLY1-deficient barley lines. Data indicate the number of aphids recovered from whole plants 15 days after the

transfer of a single one day old nymph of *Myzus persicae* and are represented as mean ± SE, n = 7.

Supplemental Table S1 Transcripts with significantly different abundance in W1-7 and wild type barley leaves under differing nitrogen availability

Table 1 Seed yield in W1-7 and wild type barley plants

	Wild type	W1-7
Number of fertile tillers	10.80 ± 1.02*	4.80 ± 1.15*
Total seed yield (g)	13.96 ± 1.68*	5.86 ± 1.36*
Seed yield per fertile tiller (g)	1.28 ± 0.05	0.97 ± 0.24

The numbers of fertile tillers were counted and total seed yield quantified in plants grown to maturity in controlled environment glasshouses. Data are presented as the mean values ± SE (n = 5). Values that were significantly different between wild type and W1-7 plants determined using the students T-test are indicated by asterisks ($P<0.05$).

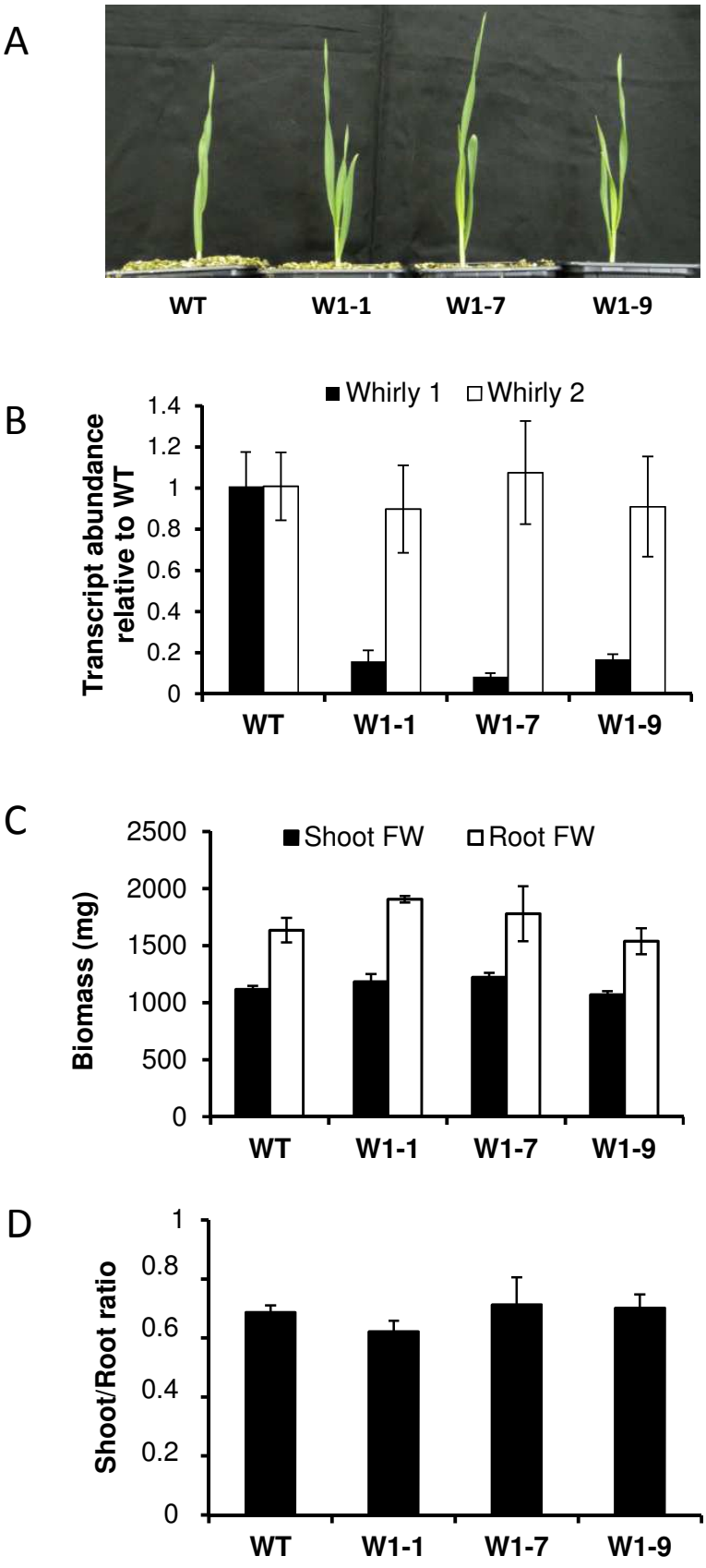
Table 2 Barley Transcripts Homologous to Plastid Encoded Genes in Arabidopsis that Exhibit Significant Differences in Abundance in WT and W1-7 Seedlings

Accession[a]	Transcript abundance W1-7/WT[b]	Top Arabidopsis match[c]	Arabidopsis gene description[d]
MLOC 24854.1	8.59	AtCg01050	NDHD, NADH-Ubiquinone/plastoquinone (complex I) protein
MLOC 24746.1	4.76	AtCg00190	RPOB, RNA polymerase subunit β
MLOC 1704.1	4.74	AtCg00190	RPOB, RNA polymerase subunit β
MLOC 54708.1	4.56	AtCg00170	RPOC2, RNA polymerase family protein
MLOC 24776.1	3.96	AtCg01040	YCF5, Cyt C assembly protein
MLOC 9149.1	3.24	AtCg01010	NDHF, NADH-Ubiquinone oxidoreductase (complex I), chain 5 protein
MLOC 9313.1	3.09	AtCg00040	MATK, Maturase K
MLOC 25280.1	3.047	AtCg01010	NDHF, NADH-Ubiquinone oxidoreductase (complex I), chain 5 protein
MLOC 61567.1	2.92	AtCg01010	NDHF, NADH-Ubiquinone oxidoreductase (complex I), chain 5 protein
MLOC 456.1	2.87	AtCg00170	RPOC2, RNA polymerase family protein
MLOC 32552.1	2.87	AtCg01300	RPL23.2, Ribosomal protein L23
MLOC 34251.1	2.81	AtCg01050	NDHD, NADH-Ubiquinone/plastoquinone (complex I) protein
MLOC 24733.1	2.81	AtCg00180	RPOC1, RNA polymerase family protein
MLOC 24753.1	2.78	AtCg01050	NDHD, NADH-Ubiquinone/plastoquinone (complex I) protein
MLOC24802.1	2.63	AtCg00040	MATK, Maturase K
MLOC 36249.1	2.62	AtCg01050	NDHD, NADH-Ubiquinone/plastoquinone (complex I) protein
MLOC 26369.1	2.61	AtCg01250	NDHB.2, NADH-Ubiquinone/plastoquinone (complex I) protein
MLOC 63387.1	2.58	AtCg01110	NDHH, NAD(P)H dehydrogenase subunit H
MLOC 9538.1	2.53	AtCg00180	RPOC1, RNA polymerase family protein

MLOC 34273.1	2.47	AtCg00040	MATK, Maturase K
MLOC 8394.1	2.47	AtCg00360	YCF3, Tetratricopeptide repeat (TPR)-like superfamily protein
MLOC 33340.1	2.40	AtCg01250	NDHB.2, NADH-Ubiquinone/plastoquinone (complex I) protein
MLOC 365.2	2.34	AtCg01250	NDHB.2, NADH-Ubiquinone/plastoquinone (complex I) protein
MLOC 77504.1	2.23	AtCg01250	NDHB.2, NADH-Ubiquinone/plastoquinone (complex I) protein
MLOC 6335.1	2.16	AtCg00590	ORF31, Electron carriers
MLOC 36200.1	2.11	AtCg00730	PETD, Photosynthetic electron transfer D
MLOC 36158.1	2.11	AtCg00590	ORF31, Electron carriers
MLOC 9573.1	2.10	AtCg00170	RPOC2, RNA polymerase family protein
MLOC 2607.1	2.06	AtCg00570	PSBF, Photosystem II reaction centre protein F
MLOC 24780.1	2.03	AtCg00730	PETD, Photosynthetic electron transfer D
MLOC 24745.1	2.02	AtCg00660	RPL20, Ribosomal protein L20
MLOC 9018.1	2.00	AtCg00640	RPL33, Ribosomal protein L33
MLOC 8945.1	1.95	AtCg00420	NDHJ, NADH dehydrogenase subunit J
MLOC 7873.1	1.95	AtCg01090	NDHI, Subunit of the chloroplast NAD(P)H dehydrogenase complex
MLOC 9520.1	1.93	AtCg00470	ATPE, ATP synthase ε chain
MLOC 3829.1	1.93	AtCg00530	YCF10
MLOC 8957.1	1.91	AtCg00420	NDHJ, NADH dehydrogenase subunit J
MLOC 9641.1	1.90	AtCg00530	YCF10
MLOC 36232.1	1.88	AtCg00160	RPS2, Ribosomal protein S2
MLOC 9681.1	1.87	AtCg00530	YCF10
MLOC 61555.1	1.86	AtCg01080	NDHG, NADH-Ubiquinone/plastoquinone oxidoreductase, chain 6
MLOC 8426.1	1.85	AtCg00530	YCF10
MLOC 12189.1	1.82	AtCg00420	NDHJ, NADH dehydrogenase subunit J
MLOC 65510.1	1.82	AtCg01090	NDHI, Subunit of the chloroplast NAD(P)H dehydrogenase complex
MLOC 24811.1	1.78	AtCg00420	NDHJ, NADH dehydrogenase subunit J
MLOC 41067.1	1.77	AtCg00630	PSAJ, PSI subunit J

MLOC 68023.1	1.76	AtCg00530	YCF10
MLOC 34111.1	1.75	AtCg00540	PETA, Photosynthetic electron transfer A
MLOC 9478.1	1.69	AtCg00160	RPS2, Ribosomal protein S2
MLOC 61315	1.68	AtCg00480	ATPB, ATP synthase subunit β
MLOC 9572.1	1.64	AtCg01100	NDHA, NADH dehydrogenase family protein
MLOC 59993.1	1.59	AtCg00440	NDHC, NADH dehydrogenase D3 subunit
MLOC 63096.1	1.31	AtCg00680	PSBB, PSII reaction centre protein B
MLOC 9455.1	1.30	AtCg00680	PSBB, PSII reaction centre protein B
MLOC 9691.1	1.22	AtCg00550	PSBJ, PSII reaction centre protein J
MLOC 24726.1	0.61	AtCg01060	PSAC, PsaC subunit of PSI
MLOC 9445.1	0.45	AtCg01110	NDHH, NAD(P)H dehydrogenase subunit H

[a] Barley gene model primary accession number (The International Barley Genome Sequencing Consortium, 2012).
[b] Transcript abundance in W1-7 seedlings relative to transcript abundance in WT seedlings.
[c] TAIR accession of top Arabidopsis match based on BLAST e-value.
[d] TAIR annotation of Arabidopsis gene.



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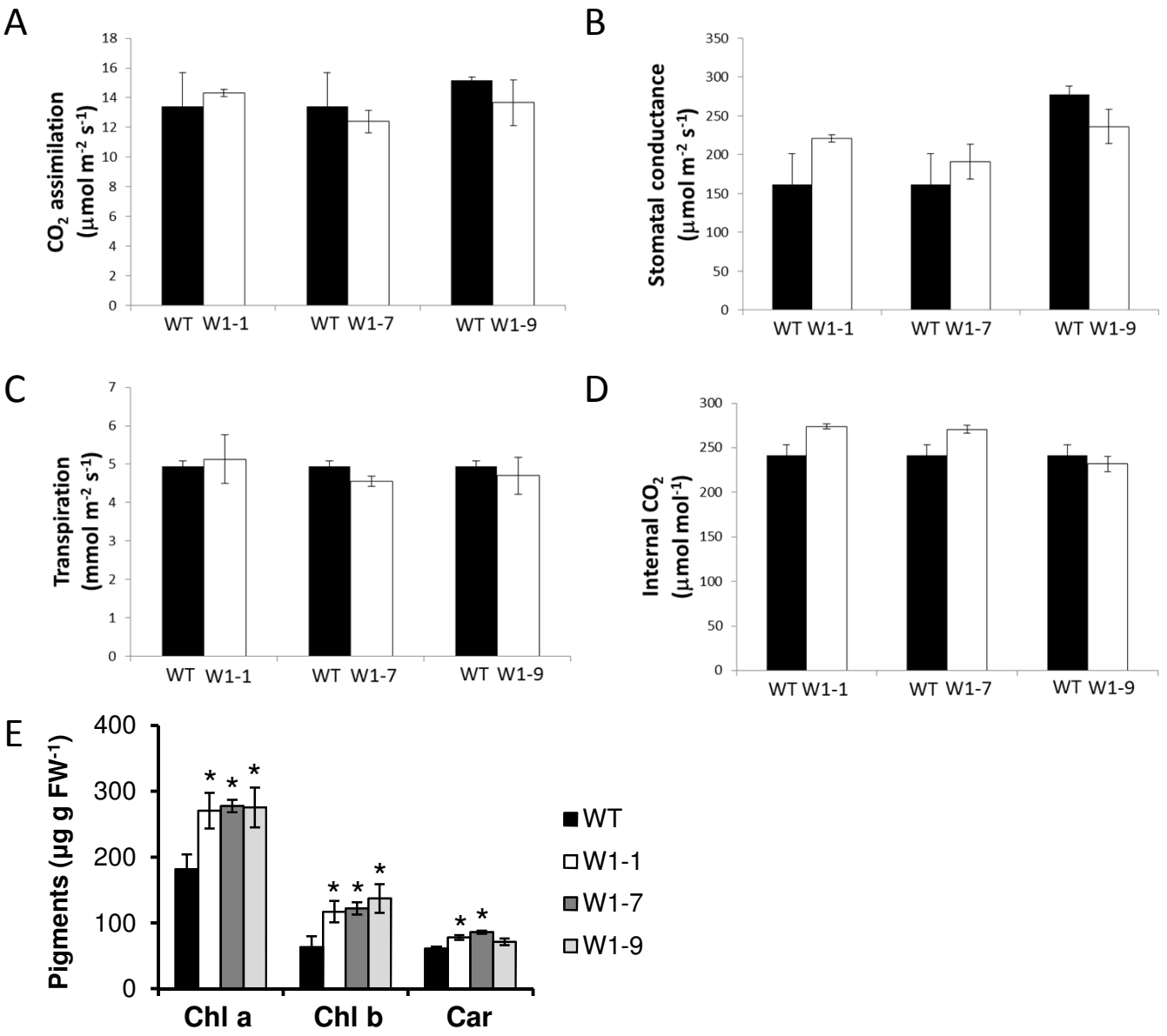

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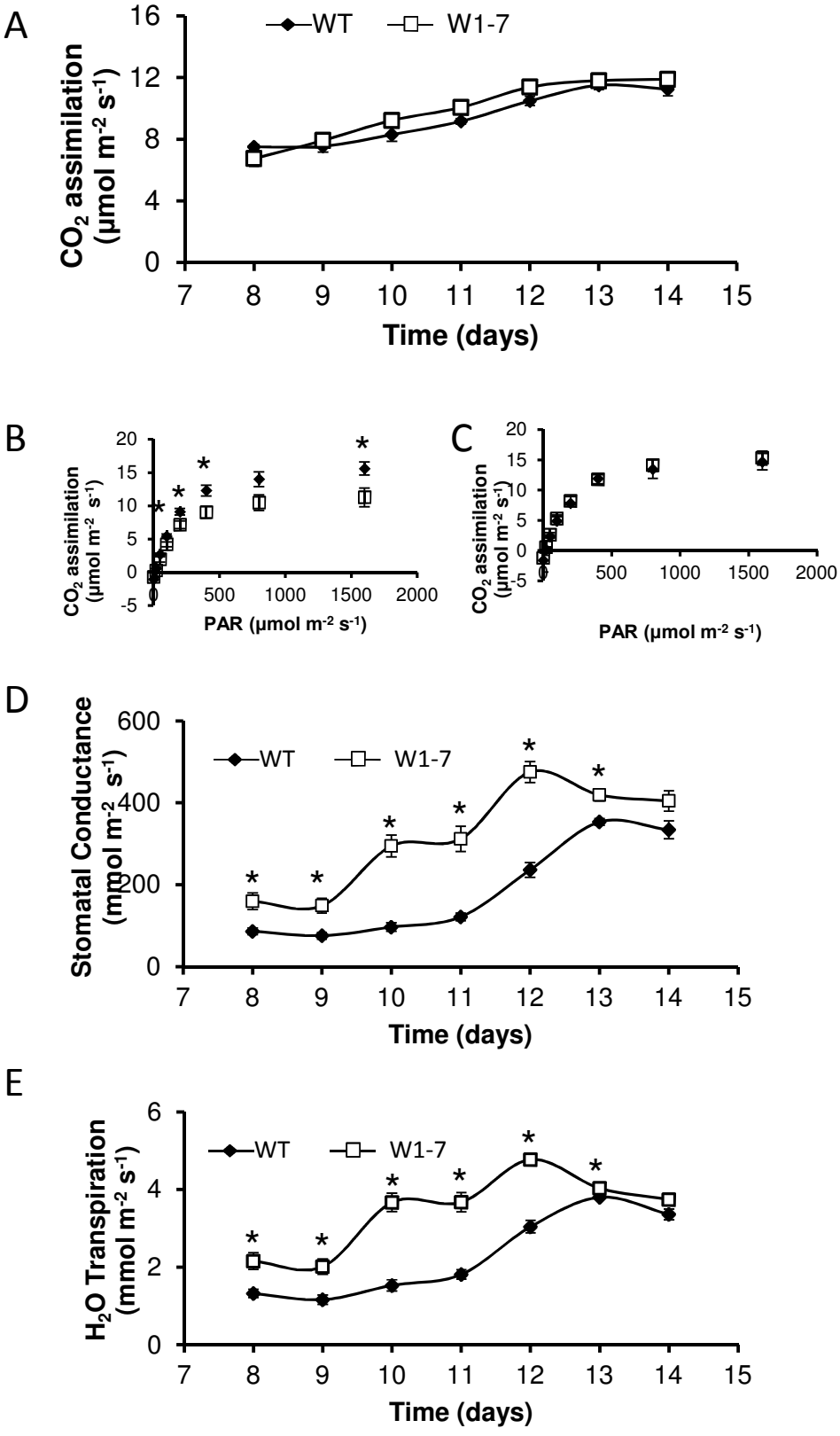

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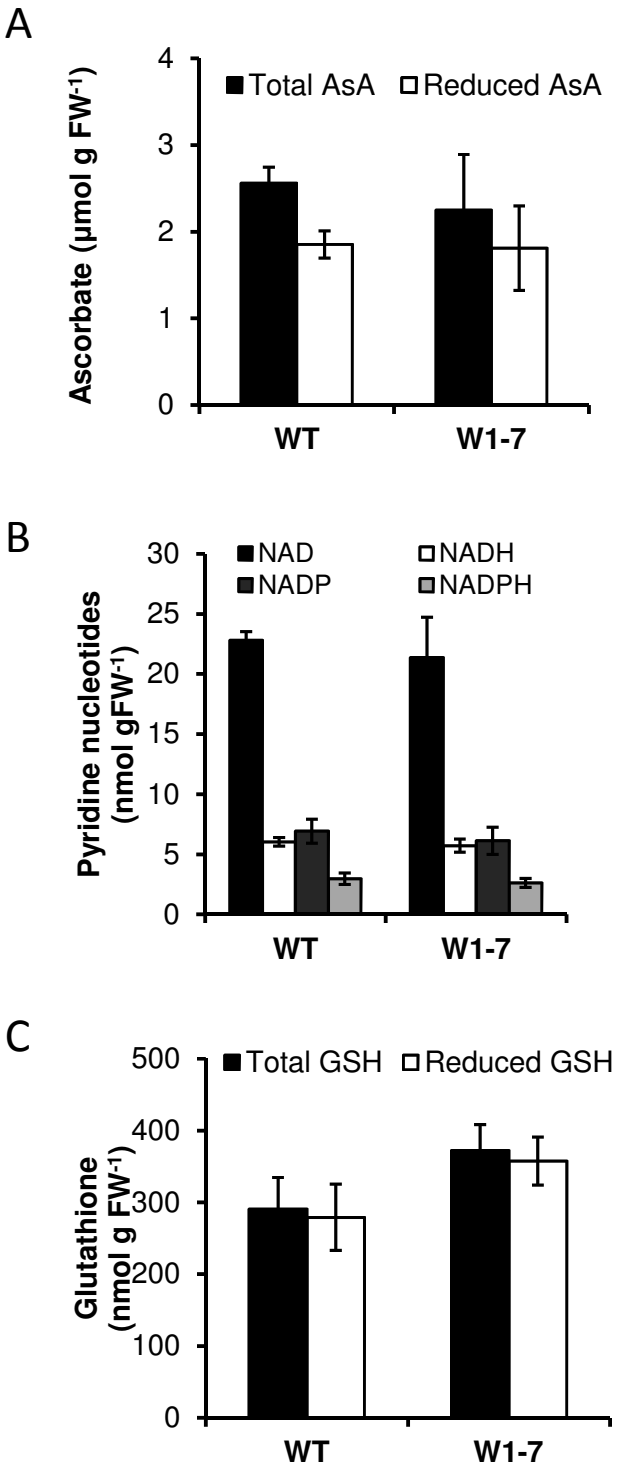

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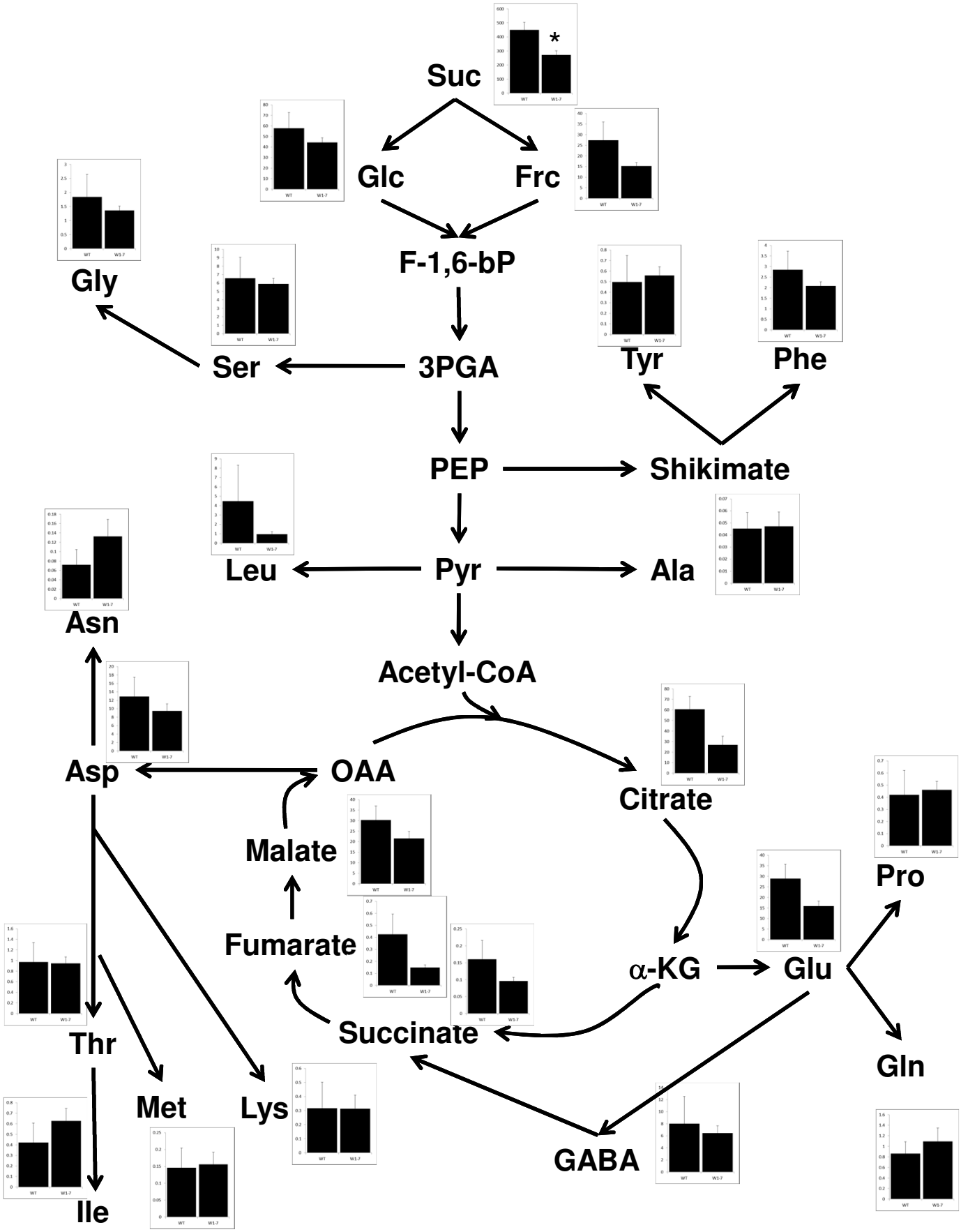

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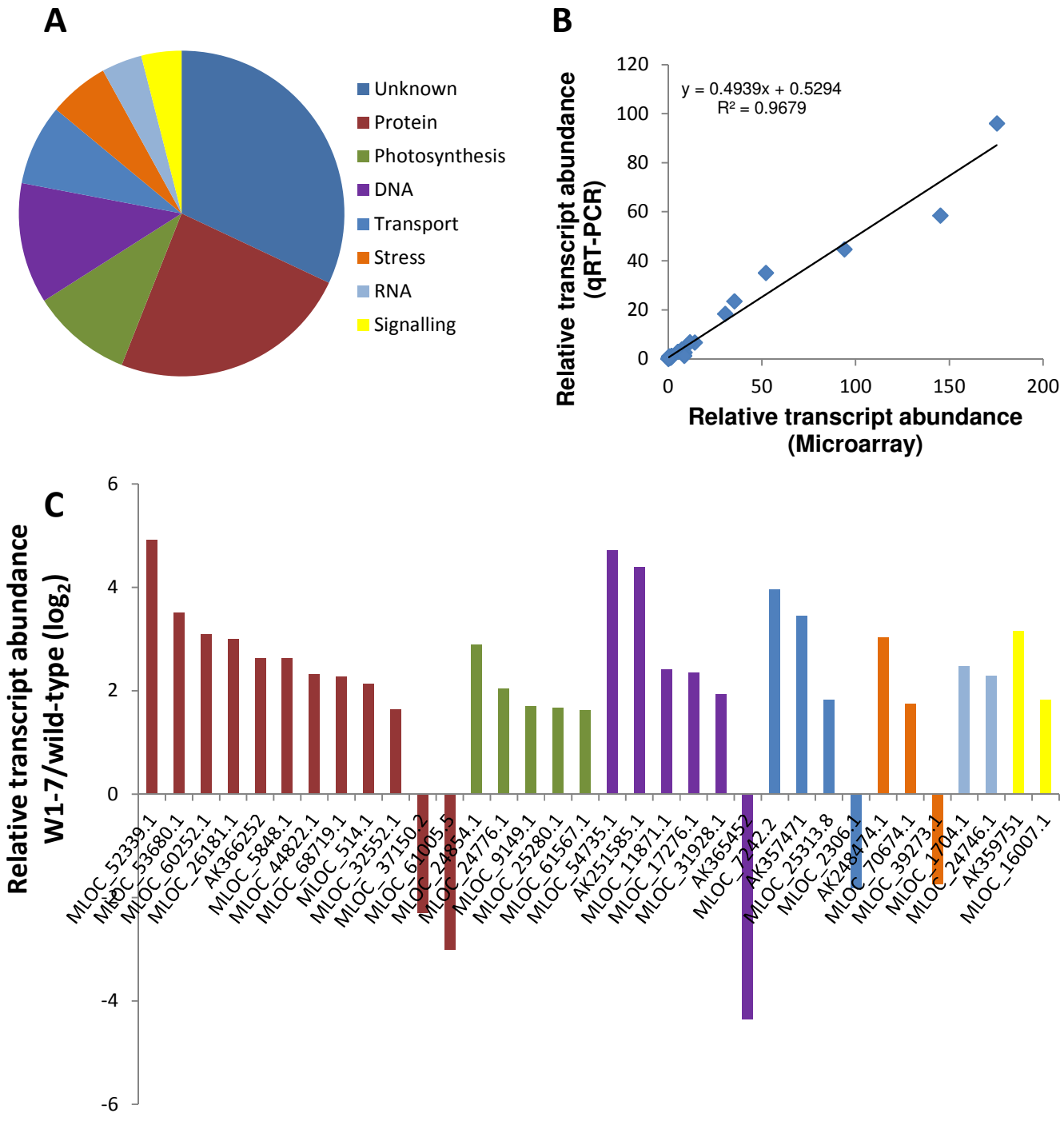

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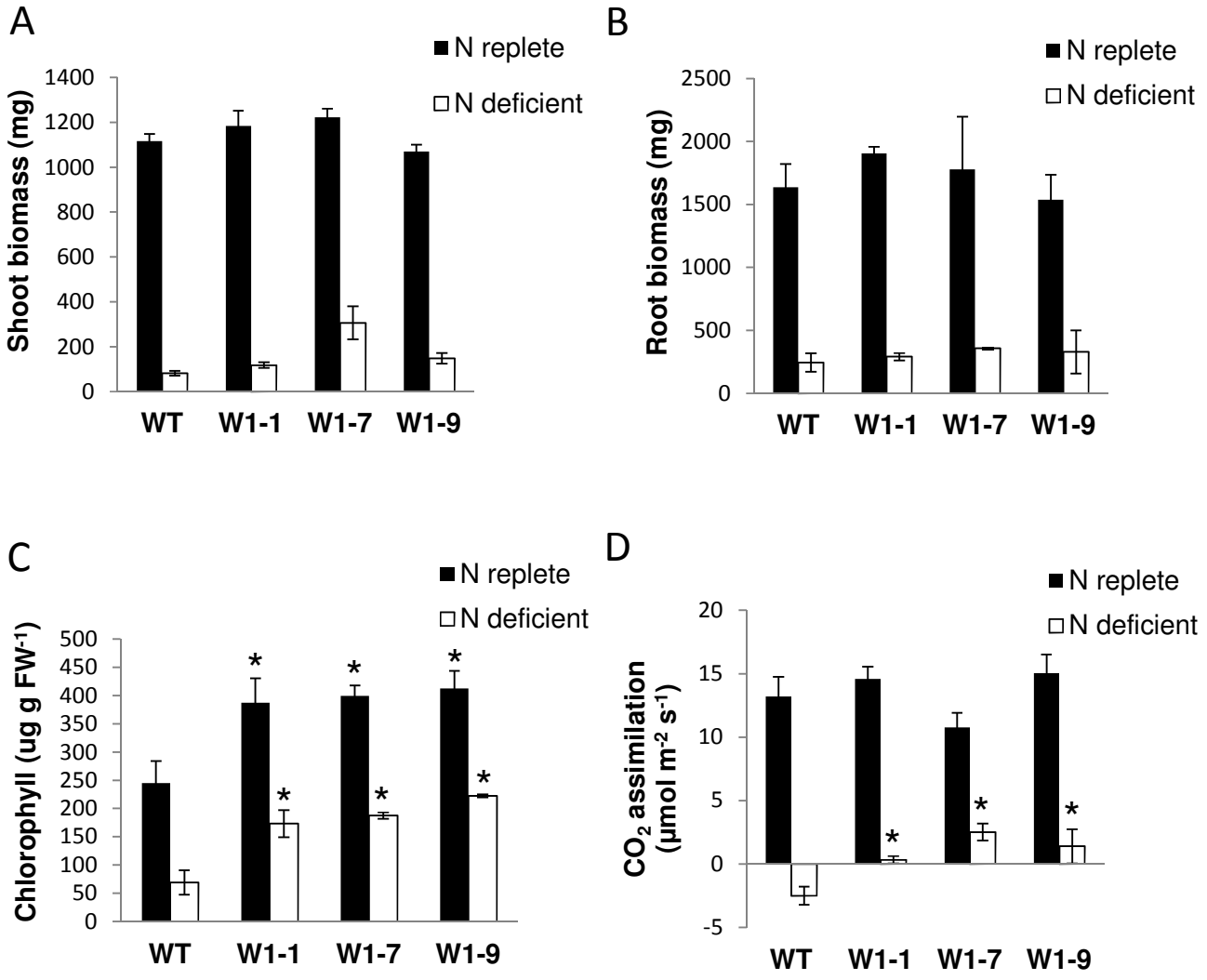

Figure 7 Impact of nitrogen availability on growth and photosynthesis in wild type seedlings and three independent WHIRLY-1 deficient barley lines (W1-1, W1-7 and W1-9). Plants were grown for 22 days under either optimal (N replete) or low nitrogen (N deficient) conditions. Shoot (A) and root (B) biomass was estimated after destructive harvesting of plants and values are represented as mean ± SE (n = 3). Chlorophyll content (C) was estimated after extraction from harvested shoots and CO_2 assimilation rate (D) was estimated by gas exchange prior to harvest; values are represented as mean ± SE (n = 4). Asterisks indicate significant differences between WHIRLY1 deficient and wild-type plants estimated by the students T-test ($P<0.05$).

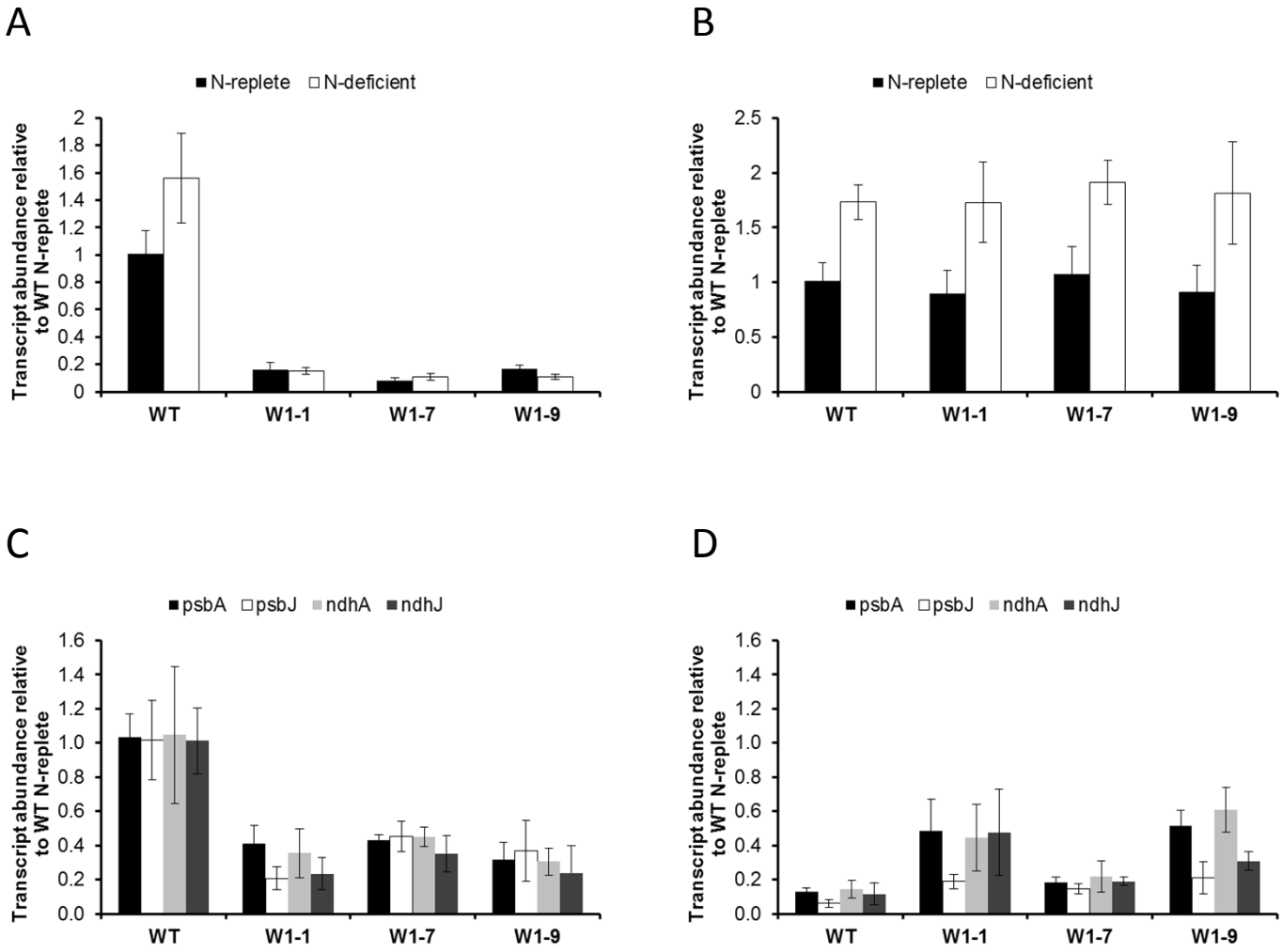

Figure 8 Abundance of transcripts encoding whirly and several chloroplast encoded proteins in leaves of wild type and three independent WHIRLY-1 deficient barley lines (W1-1, W1-7 and W1-9). Plants were grown for 15 days under either optimal (N replete) or low nitrogen (N deficient) conditions. The abundance of transcripts encoding WHIRLY-1 (A) and WHIRLY-2 (B) were estimated relative to their abundance in wild type plants under N-replete conditions by the $\Delta\Delta C_T$ method using actin as a reference. The abundance of chloroplast encoded transcripts were estimated relative to their abundance in wild type plants under N-replete (C) or N-deficient (D) conditions using 16S ribosomal RNA as a reference. All data are presented as mean values ± SD, n = 3.

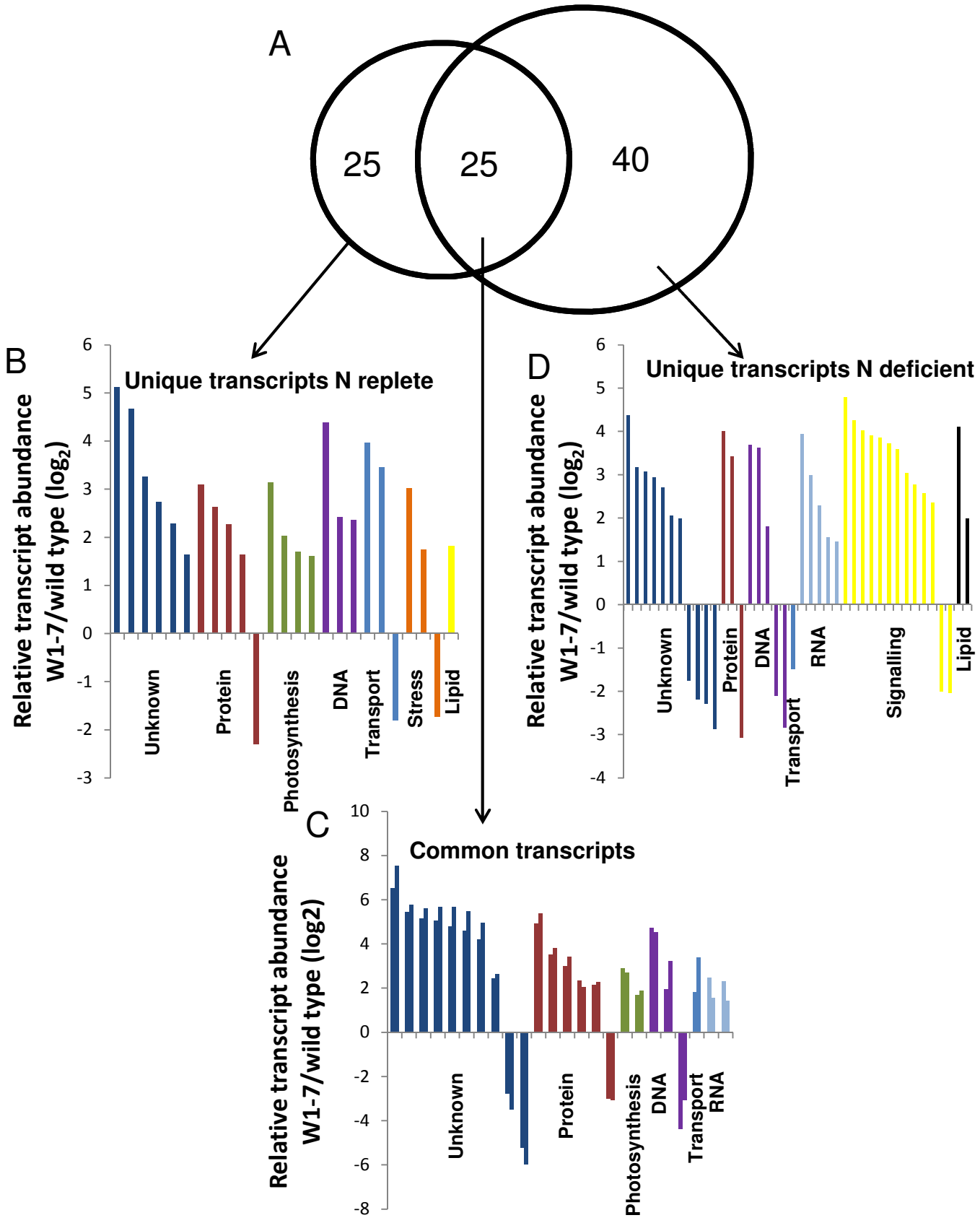

Figure 9 Transcript profile comparison of 15 day-old wild type leaves and the WHIRLY-1 deficient barley W1-7 line grown under either optimal (N replete) or low nitrogen (N deficient) conditions. (A) Venn diagram illustrating the number of differentially abundant transcripts under each nitrogen regime. (B) Classification of transcripts that showed differential abundance only under nitrogen replete conditions. (C) Classification of transcripts that showed differential abundance under both nitrogen conditions. (D) Classification of transcripts that showed differential abundance only under conditions of nitrogen deficiency. The differentially expressed genes were identified using a moderated T-test with Benjamini-Hochberg multiple testing correction ($p<0.05$, FC>2) (Genespring 12, Aligent Technologies).