Running Title: Sugar metabolism in a chloroplast protease mutant

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Abstract
The crucial role of carbohydrate in plant growth and morphogenesis is widely recognized. In this study we describe the characterization of nana, a dwarf Arabidopsis thaliana mutant impaired in carbohydrate metabolism. We show that the nana dwarf phenotype was accompanied by altered leaf morphology and a delayed flowering time. Our genetic and molecular data indicate that the mutation in nana is due to a T-DNA insertion in the promoter region of a gene encoding a chloroplast-located aspartyl protease that alters its pattern of expression. Overexpression of the gene (oxNANA) phenocopies the mutation. Both nana and oxNANA display alterations in carbohydrate content, and the extent of these changes varies depending on growth light intensity. In particular, in low light soluble sugars levels are lower and do not show the daily fluctuations observed in wild type plants. Moreover, nana and oxNANA are defective in expression of some genes implicated in sugar metabolism and photosynthetic light harvesting. Interestingly, some chloroplast-encoded genes as well as genes, whose products seem to be involved in the retrograde signaling, appear to be down-regulated. These findings suggest that the NANA aspartic protease has an important regulatory function in chloroplasts that not only influences photosynthetic carbon metabolism but also plastid and nuclear gene expression.

Keywords: carbohydrates, aspartyl protease, dwarf mutant, Arabidopsis thaliana
Introduction

Plants synthesize carbohydrates through photosynthesis. The subsequent partitioning of carbohydrates between the site of their production, the chloroplast, and other cellular components is regulated by complex mechanisms to meet the diverse needs of the plant in terms of carbon availability (Rolland et al., 2006; Stitt et al., 2010). Sugars formed during the day are a prerequisite for plant growth, as they represent both cellular building blocks and substrates for mitochondrial respiration. During the night, when sugars are no longer produced by photosynthesis, metabolism and growth are largely dependent on transitory starch reserves that are accumulated during the day and broken down at night (Smith and Stitt, 2007; Graf et al., 2010). This complex time-tuned mechanism of carbon partitioning, among organelles, tissues and organs can be easily disrupted by mutation of genes encoding components of sugar metabolism or regulatory networks. Unless plants are grown under very long photoperiod conditions so that photoassimilates are almost always available, mutations affecting enzymes involved in starch synthesis or degradation result in a phenotype of reduced growth. This demonstrates the importance of leaf carbohydrate reserves for the whole plant’s growth and development (Gibon et al., 2004; Smith and Stitt, 2007; Usadel et al., 2008). Defects in starch metabolism clearly limit the supply of energy and building blocks for growth at night (Yazdanbakhsh and Fisahn, 2011). However, it remains unclear whether the reduced growth of starch mutants is entirely attributable to such limitations or if other factors also play a role. Sugars can indeed also act as osmoregulatory or signaling molecules with effects on plant growth and development throughout the plant’s life cycle, from germination right through to the floral transition and senescence (Koch, 2004; Gibson, 2000, 2004, 2005).

The ability of plants to sense sugars might play an important role in carbon partitioning and allocation in source and sink tissues (Smith and Stitt, 2007). In particular, the expression of genes involved in photosynthate accumulation, mobilization and storage is regulated by glucose and sucrose (Koch, 1996), and by light- and circadian clock-mediated signaling mechanisms (Harmer et al., 2000; Neff et al., 2000). This regulatory network maintains an optimal dynamic carbohydrate status, integrating the synthesis and the consumption of carbohydrates in response to environmental changes, and in response to the availability of other nutrients, such as nitrogen (Coruzzi and Bush, 2001).

Sugar homeostasis is also tightly linked to regulation of photosynthesis and chloroplast physiology, with expression of photosynthesis-related genes being modulated by light intensity, temperature, and CO₂ availability (Jang and Sheen, 1997; Rolland et al., 2002, 2006; Stitt et al., 2010). For example, in conditions of high carbohydrate demand and non-limiting light availability, plants increase production and export of photosynthate by increasing expression of genes involved in
photosynthesis (Koch, 1996). Conversely, when photosynthates are not immediately required, genes involved in starch synthesis are activated to maintain a balance between photosynthate supply, demand, and storage (Rook et al., 2001).

Therefore, chloroplasts play a central role in primary metabolism, supporting the growth and differentiation of plant cells. Chloroplasts are thought to have originated from a cyanobacterium-like endosymbiont, but during evolution most genes of cyanobacterial origin have been transferred from the chloroplast genome to the nucleus (Mayfield et al., 1995; Martin et al, 2002; Dyall et al., 2004; Ajjawi et al., 2010; Myouga et al., 2010). Relatively few photosynthesis-related genes remain in the chloroplast genome, and chloroplast development and functionality are dependent on the import of the nuclear-encoded plastid proteins (Woodson and Chory, 2008; Inaba, 2010).

Most of our knowledge of chloroplastic proteins is focused on the enzymes and transporters involved in photosynthesis and starch metabolism, but other biochemical processes in the chloroplast require diverse enzymatic activities, such as proteases. Proteolysis has several important functions in chloroplasts (Adam, 1996; Andersson and Aro, 1997; Adam et al., 2001), such as processing of precursor proteins, degradation of incomplete proteins, and removal of damaged proteins (Nair and Ramaswamy, 2004). Aspartic proteinases (APs; EC 3.4.23) are one of the major catalytic classes of protease in plants, as well as in vertebrates, nematode parasites, bacteria, fungi and viruses (Timotijevic et al., 2010), and are involved in many aspects of plant physiology and development. APs contain two aspartic acid residues that are crucial for catalytic activity. They are most active under acidic conditions (pH 2-6) and are specifically inhibited by pepstatin A (Mutlu and Gal, 1999; Milisavljevi et al., 2008). Several functions have been proposed for APs, including processing and degradation of storage proteins, protein degradation during organ senescence and cell death, autolysis during formation of tracheary elements, prey digestion by carnivorous plants, and adhesion-mediated proteolytic mechanisms in pollen recognition and growth (Simões and Faro, 2004). In tobacco chloroplasts, an AP named CND41 is involved in degradation of the Rubisco holoprotein during leaf senescence (Murakami et al., 2000; Kato et al., 2004). CND41 homologs have been identified in Arabidopsis and their role in the regulation of Rubisco turnover and senescence has also been confirmed in this species (Kato et al., 2005a, b; Diaz et al., 2008). Moreover, CND41 accumulation is negatively correlated with chloroplast transcript levels in tobacco cells (Nakano et al., 1997), indicating that CND41 may function as a negative regulator of chloroplast gene expression.

In this study, we identified and characterized a dwarf Arabidopsis mutant bearing a T-DNA insertion in a gene encoding a chloroplast-located AP. We named the mutant *nana*, which means “dwarf” in Italian. NANA plays an important role in carbohydrate homeostasis, as demonstrated by
the severe alteration in carbohydrate metabolism resulting from mis-expression or over-expression of this AP.
RESULTS AND DISCUSSION

Isolation and morphological characterization of the nana mutant

The nana mutant was isolated while searching for mutants altered in their sugar sensing abilities. The growth of the hypocotyl and/or root were indeed reduced in nana seedlings compared to the WT on various growth conditions, either in the presence or absence of sucrose (Fig. 1, A and B; Supplemental Table S1). When grown on a media without sucrose, the length of the root in nana seedlings was shorter than the WT, when kept in the presence of light, while, in darkness, it was the hypocotyl elongation to be severely inhibited in nana (Supplemental Table S1). When grown in soil, adult nana plants maintained a dwarf phenotype, with a reduced diameter of the rosette and a diminished height of the inflorescence axis (Fig. 1, C and D; Table I). The nana mutant also exhibited small leaves, which were more lobed than WT leaves and slightly curled, particularly during the early vegetative stages of development (Fig. 1E). We did not observe differences in the leaf number between nana and WT plants (Table 1). At the generative stage, bolting was significantly delayed in nana compared to WT plants (Fig. 1D; Table I). Epidermal cells were substantially smaller in the mutant compared with wild-type plants (Fig. 1F). However, in contrast to other mutants with altered epidermal cell size, the nana mutant did not show any differences in the degree of endoreduplication (Supplemental Fig. S1).

Molecular characterization of the nana mutation

A T-DNA insertion was identified in the nana mutant, 344 nt upstream of the start codon of the coding sequence (cds) of the At3g12700 (NANA) gene, which is annotated to encode a member of the aspartyl protease family. This gene is composed of two exons separated by one intron (Fig. 2A). As a result of the T-DNA insertion, the At3g12700 cds is unchanged, but a series of putative regulatory elements (e.g. GARE, SURE and ABRE elements) in the promoter are displaced away from the 5’UTR and cds (Fig. 2A).

Sucrose slightly induced expression of At3g12700 in WT plants and the effect was enhanced in the mutant (Fig. 2B). In WT adult rosette leaves, At3g12700 was found to be expressed with a pronounced diurnal rhythm characterized by a peak at the end of the day (Fig. 2C). In nana plants the level of expression of the gene was not only higher than in WT plants at almost all times of day, but also peaked earlier in the light period (8 h instead of 12 h) and showed a second peak in the middle of the night (Fig. 2C). This indicates that the T-DNA insertion in the promoter region leads to misexpression of At3g12700 in nana plants.
Since \textit{At3g12700} is expressed at higher levels in \textit{nana}, an overexpressor line with the gene under the control of the CaMV 35S constitutive promoter was produced for comparison. \textit{35S::At3g12700 (oxNANA)} plants exhibited a dwarf phenotype that was very similar to that of the \textit{nana} mutants in terms of reduced rosette diameter (Fig. 3A), leaf morphology (Fig. 3B) and delayed time of flowering (data not shown).

Next, we examined the accumulation of \textit{At3g12700} protein (hereafter called NANA) in the wild-type, in the \textit{nana} mutant and in the \textit{oxNANA} line during a 24 h time-course. Immunoblot analysis of leaves harvested from 12-h photoperiod grown WT plants at different times of the day showed that NANA was accumulated late during the day and during the early night phase, and partially degraded during the last 4 h of the night (Fig. 3C). In contrast, the \textit{nana} mutant and the NANA-overproducing line showed a higher accumulation of NANA in the early part of the light period (Fig. 3C). Remarkably, the level of NANA decreased during the night in the \textit{oxNANA} line, suggesting that a post-transcriptional mechanism operates to prevent excessive NANA accumulation during the night (Fig. 3C). Overall, these results suggested that under normal conditions NANA would be regulated by a day/night rhythm, but the \textit{nana} mutation alters this regulation.

\textbf{NANA encodes a chloroplast-located aspartyl protease}

Bioinformatic analysis (http://suba.plantenergy.uwa.edu.au/; Heazlewood et al., 2007) did not predict a predominant subcellular localization for NANA, indicating the lack of a canonical subcellular localization signaling sequence in \textit{At3g12700}. We examined the NANA localization of an in-frame fusion construct with the enhanced green fluorescent protein (EGFP). Confocal microscopy showed that GFP fluorescence perfectly overlapped with chlorophyll autofluorescence both in transiently transformed Arabidopsis protoplasts (Fig. 4A) and in stably transformed leaf cells (Fig. 4B). To further confirm the localization of NANA, we performed an immunoblot analysis that showed a clear enrichment of NANA in isolated chloroplasts. The signal arising from the NANA antisera was strongly enriched in the chloroplast fraction. (Fig. 4C). Evidently, even without an identifiable chloroplast transit peptide, NANA was targeted to chloroplasts. Most of the chloroplast proteins do present a recognizable N-terminal transit peptide; however, the chloroplast proteome also contains many other nuclear-encoded proteins not previously predicted as chloroplast-localized (Klefmann et al, 2004; Li and Chiu, 2010). Different and not always completely clarified pathways of sorting of proteins without identifiable plastid transit peptides in chloroplasts seem to take place in plant cells (Asatsuma et al, 2005; Li and Chiu, 2010).
The Plant Proteome Data Base (ppdb.tc.cornell.edu/) classified NANA as a “eukaryotic aspartyl protease family protein”. NANA possesses a highly conserved aspartyl protease domain (ASP, PFAM PF00026) located between aa 104 and 459 (Fig. 5A) (“SMART6 bioinformatic tool”. Letunic et al., 2009). Phylogenetic analysis based on sequenced plant genomes revealed the presence of homologues with sequence similarity to NANA in chlorophyte algae, mosses, lycophytes and both mono- and dicotyledonous flowering plants, including major crop plants (e.g. rice, maize and soybean), indicating their presence throughout the plant kingdom (RefProt, blastp threshold E<10⁻¹⁰, Supplemental Fig. S2A). Although the Solanaceae were not represented in the phylogenetic analysis, a putative homolog of NANA was identified in the *Solanum lycopersicum* draft genome (SL2.40ch05:4111801-4114200; Bombarely et al., 2010) with a high sequence similarity (tblastn E-value 8*10⁻⁵⁰). However, the high sequence similarity between these putative homologues is limited to the ASP domain. The N-terminal portion of *nana* (aa 1-103) appears to be unique to the *Arabidopsis* genus and, apart from *A. thaliana*, was found only in the *Arabidopsis lyrata*: Alyr-NANA ortholog (protein ARALYDRAFT_478632, XP_002882786). *A. thaliana* possesses at least 50 putative paralogs to *nana* (blastp, threshold E<10⁻¹⁰, Fig. S2B), all possessing the ASP domain. Among these, we identified four genes whose transcripts are regulated in a circadian manner (Covington and Harmer, 2007), and several genes (including *nana*) whose expression is significantly different in dark vs. light grown *A. thaliana* 7-day-old seedlings (Dohmann et al., 2008) (Supplemental Fig. S2B, and Supplemental Table S2).

To test whether NANA encodes a catalytically active aspartic protease, we expressed the protein as an N-terminal His₆-tagged fusion protein in *Escherichia coli*. The crude lysate from induced *E. coli* cells contained a prominent 53-kDa protein band when analysed by SDS-PAGE (Fig. 5B), which was recognised by an anti-NANA antibody (Fig. 5C). The 53-kD immunoreactive protein was affinity-purified and enzymatic activity was assayed using azocasein as the substrate, which is specific for endo-proteases. Aspartyl protease activity was significantly higher in both the induced cell lysate and purified protein fraction than in extract from non-IPTG treated *E. coli* cells (NTC) (Fig. 5D). The recombinant NANA protein also displayed a pH optimum of 6 (Fig. 5E), a value compatible with the chloroplast environment, likely closer to that of the stroma at night than during day, when illumination induces a stromal alkalization (Song et al., 2004). This correlates well with the expression data (Fig. 3C) that indicated accumulation of NANA proteins in WT during the night. Interestingly, this pH optimum of NANA appears quite different from that reported for the previously characterized CND41 chloroplast aspartyl protease of tobacco, whose strongest activity was found at an acidic pH (Murakami et al., 2000). Aspartyl protease activity was also measured in crude extracts from rosette leaves. Both *nana* and oxNANA plants showed a significantly higher (2
to 6-fold) activity than the WT (Supplemental Fig. S3A). All these results confirmed that NANA is an aspartyl protease and that its activity was increased in the nana and oxNANA lines.

**nana affects endogenous sugar levels**

Dwarf phenotypes have previously been observed in several mutants with defective chloroplasts or chloroplast function (Nakano et al., 2003; Yabe et al., 2004; Qin et al., 2007; Huang et al., 2009; Myouga et al., 2010). Such observations, together with the chloroplast localization of NANA (Fig. 4, A and B), suggest that higher NANA perturbs chloroplast function in some way. We therefore measured several chloroplast-related physiological and biochemical parameters in wild-type and mutant plants. The chlorophyll content of nana leaves was about 50% lower than in WT (Fig. 6A) and the electron transport rate (ETR) was similarly decreased, being about 30% lower in nana plants (Fig. 6B). Conversely, CO₂ assimilation rate was not significantly affected by the mutation (Fig. 6C). In the oxNANA line chlorophyll content, ETR and assimilation rate of CO₂ were very similar to those of nana plants (Fig. 6A, B and C). In accordance with the unchanged CO₂ assimilation rate, Rubisco protein levels seemed to be unaffected by the mutation, with WT, nana and oxNANA plants having similar amounts of RbcL (Rubisco large subunit) protein to each other, both at the end of the night and at the end of the day (Fig. 6D).

Sugar levels were measured in wild-type and mutant plants. As shown in Figure 7A, the levels of soluble sugars such as sucrose, glucose and fructose were significantly reduced in nana compared to the WT when the plants were grown at an irradiance of 80 µmol m⁻² sec⁻¹. Under these conditions, soluble sugar levels in nana leaves were dramatically lower than in WT, not only during the day, but also at night (Fig. 7A). The oxNANA plants showed the same behavior as the nana mutant (Fig. 7A).

In contrast to these observations, growing the plants with a higher irradiance (150 µmol m⁻² sec⁻¹) led to increased carbohydrate levels in the nana mutant compared to WT (Fig. 7B), but did not change the characteristic dwarf phenotype of these plants (data not shown). In addition to the higher sucrose content of the nana plants, compared to WT, the mutant also contained elevated levels of sucrose 6-P, ADP-glucose, UDP-glucose, 3-PGA and glucose 1-P (Fig. 7B). Trehalose 6-phosphate (T6P) has been postulated to act as a signal of sucrose status, and was found to be higher in the pgm mutant than in WT plants (Lunn et al., 2006). Actually, pgm plants, due to a mutation in the gene encoding the plastidial isoform of the phosphoglucomutase enzyme, show a strongly impaired starch synthesis, thus accumulating high levels of sucrose during the day (Caspar et al., 1985). Interestingly, the levels of T6P were very similar in the nana and WT plants, despite the higher
sucrose content in the mutant (Fig. 7B). However, within the individual genotypes, the level of T6P showed a similar pattern of diurnal changes to sucrose, rising to a peak at the end of the day and then falling at night. This suggests that the molecular mechanism linking T6P to sucrose, which is as yet unknown, is still operating in the nana mutant, but that the dynamic range of the response is tuned down in the mutant.

In an attempt to understand whether the alteration in carbohydrate metabolism could be a direct consequence of the slightly lower photosynthetic activity (Fig. 6, A, B and C), nana plants were crossed with the pgm mutant (Caspar et al., 1985). The pgm plants (growth irradiance: 80 μmol m\(^{-2}\) sec\(^{-1}\)) were smaller than WT, but still clearly distinguishable from nana plants (Supplemental Fig. S4). The double mutant nanaxpgm showed the same dwarf phenotype as nana, in terms of both reduced leaf size and morphology (Supplemental Fig. S4). Interestingly, in nanaxpgm the levels of soluble sugars, such as glucose, sucrose, and fructose, perfectly matched the levels found in pgm plants (Fig. 7C), supporting the idea that the metabolic defects in the nana mutant are not directly due to the lower photosynthetic activity but are related rather to differences in the flow of carbon into starch.

**Altered starch accumulation and remobilization in nana mutant**

The impact of NANA on starch metabolism was investigated by staining wild-type, nana and oxNANA rosettes with Lugol’s solution. A strong staining, indicating the presence of starch, was detected in all three genotypes at the end of the day (Fig. 8A). Interestingly, both nana and oxNANA showed starch staining at the end of the night, whereas no staining was present, as expected, at this time-point in the WT samples (Fig. 8A). To further confirm these results, the endogenous starch contents were measured in the WT, nana and oxNANA extracts of 4-week-old plants. When grown under 12-h light/12-h dark cycles (80 μmol m\(^{-2}\) sec\(^{-1}\)), starch levels were significantly lower in nana and oxNANA than in WT at the end of the day (Fig. 8B). Moreover, the level of starch at the end of the night in both the nana and oxNANA mutants was higher than that of the WT respectively by a factor of 4.5 and 4.9 (Fig. 8B), in line with the iodine staining results. These results suggested that starch is not properly synthesized and/or degraded in the nana mutant.

We did not observe any differences in the redox-dependent oligomerisation of the small subunit (APS1) of AGPase in nana when compared to the WT (Supplemental Fig. S5), indicating that an alteration in post-translational activation of this enzyme is not the prime cause of the defects in starch synthesis. The possibility of a defect in starch mobilization was also considered. In Arabidopsis leaves, β-amylases are important enzymes for starch breakdown (Fulton et al, 2008). The time-course of the β-amylolytic activities in 4-week-old wild-type, nana and oxNANA plants
was then compared. Whereas the β-amylases activity in the WT leaf extracts showed a clear diurnal fluctuation with a peak during the dark phase, the nana mutant displayed 3 to 6-fold lower levels of β-amylase activity with no increase in the dark (Fig. 8C). Conversely, the oxNANA leaf extracts showed a constant activity, which was higher than that of the WT during the light period. Judging from the low β-amylases activity shown by nana plants, we initially inferred that chloroplastic β-amylases could represent a possible target for NANA acting as a protease. We thus studied the diurnal expression patterns of some β-amylases more in detail, both at the gene and at the protein levels (Supplemental Fig. S6). β-amylase family members are characterized by an extreme complexity as regards their protein function (Fulton et al., 2008). Among the four chloroplast-targeted β-amylases found in Arabidopsis leaves, only two, BAM1 and BAM3, are considered active in the degradation of starch (Valerio et al., 2011). The BAM1 gene resulted to be more expressed in oxNANA compared to the nana mutant and the WT, while expression of BAM3 was similar in all three genotypes (Supplemental Fig. S6). Despite the differences in transcript levels, we found that the amounts of BAM1 or BAM3 proteins were fairly similar in the WT and both the nana mutant and the oxNANA line (Supplemental Fig. S6). Thus, we did not find clues suggesting that NANA could be active on these β-amylases. On the contrary, the strong differences in the β-amylolytic activities (Fig. 8C) could be attributed to another β-amylase, BAM5 (Supplemental Fig. S6), accounting for most of the β-amylase activity in Arabidopsis leaves (Laby et al, 2001). Actually, the high β-amylase activity observed in the oxNANA line (Fig. 8C) correlates well with the strong induction of the BAM5 gene in this genotype (Supplemental Fig. S6). Moreover, the expression levels of BAM5 in nana are clearly lower than in the WT (Supplemental Fig. S6), in accordance with the lower β-amylolytic activity of nana (Fig. 8C). However, BAM5 encodes an extraplastidic β-amylase (Laby et al., 2001), which thus can not be a direct target of NANA proteolytic activity.

On the whole, all these results appear to indicate a possible involvement of NANA in starch metabolism. On the other hand, whether this involvement can be a direct consequence of NANA proteolytic activity on some enzymes acting in starch synthesis or degradation requires more extensive analyses.

Changes of gene expression in nana

As the nana mutant showed alterations in carbohydrate metabolism and different levels of sugars, we investigated whether the expression of sugar-responsive genes was affected in the mutant. In plants grown in 80 µmol m⁻² sec⁻¹ irradiance, where nana and oxNANA generally have lower levels of soluble sugars, especially during the day (Fig. 7A), we observed differential expression of
several sugar-responsive genes in the three genotypes. Two sugar-induced genes, ApL3
(At4g39210) and a 6-phosphogluconate dehydrogenase family protein encoding gene (PG:
At5g41670) (Wingler et al., 2000; Gonzali et al., 2006; Solfanelli et al., 2006; Veyres et al., 2008),
were expressed at lower levels in nana and oxNANA leaves than in WT (Fig. 9A), consistent with
the lower sugar content of the mutants. In contrast, the GPT2 (At1g61800) gene, which is also
reported to be induced by sugars (Gonzali et al., 2006; Kunz et al., 2010), showed generally higher
expression in nana and oxNANA (Fig. 9A).
Suppression of the close homologue of NANA CND41 (Supplemental Fig. S7) in tobacco led to
marked changes in expression of several chloroplast encoded genes (Nakano et al., 1993, 1997).
Therefore, we compared the expression level of the RbcL (AtCG00490), psbA (AtCG00020), and
petD (AtCG00730) genes, as representative of chloroplast encoded ones, in nana, oxNANA and WT
Arabidopsis. Interestingly, the transcript levels of RbcL, encoding the large subunit of RUBISCO,
and psbA, which encodes a component of the photosystem II reaction center core, were substantially
repressed in both nana and oxNANA (Fig. 9B). In contrast, petD, which encodes the cytochrome
b6/f complex subunit IV involved in photosynthetic electron transfer, was similarly expressed in all
three genotypes (Fig. 9B), suggesting that in nana and oxNANA plants a process of transcriptional
repression of important chloroplast-located genes occurs, although not general.
Most chloroplast proteins, including many that are essential for photosynthesis such as the light-
harvesting chlorophyll-binding proteins (LHC), are encoded by nuclear genes (Jansson et al., 1999;
Martin et al., 2002). Therefore, we also investigated if expression of several nuclear genes linked to
photosynthesis and other chloroplast functions was affected in the nana and oxNANA mutants.
These included: GLK1 (At2g20570), a GOLDEN2-LIKE transcription factor involved in chloroplast
development (Fitter et al., 2002), Lhca1 (At3g54890), Lhcb1.1 (At1g29920), Lhcb1.2 (At1g29910)
and Lhcb1.3 (At1g29930), which encode members of the LHCl and LHClI complexes (Jansson,
1999) and are subject to regulation by plastid-derived signals (Strand et al., 2003; Waters et al.,
2009). Interestingly, transcript levels of all four genes were significantly reduced in the nana and
oxNANA mutants in comparison with WT (Fig. 9C). However, the down-regulation of the
transcripts which are directly involved in photosynthetic processes was not a general trend. For
example, the ribulose-1,5-bisphosphate carboxylase small chain 1A (RbcS1-A) encoding gene
showed similar expression levels in both the mutants and WT plants (Fig. 9C).
Together, these observations indicate that misexpression of NANA results in the down-regulation of
different chloroplast-related or chloroplast-located genes. They also suggest that this defect might
perturb chloroplast homeostasis, inducing the generation of a retrograde plastid signal that
suppresses the expression of chloroplast-related genes in the nucleus.
CONCLUSION

We identified a novel chloroplast-located aspartyl protease (NANA) that not only influences photoassimilate partitioning and transitory starch turnover in a complex manner that is dependent on the growth irradiance, but also perturbs both plastid and nuclear gene expression and possibly the crosstalk between the chloroplast and nucleus. NANA misexpression causes several severe morphological alterations, such as dwarfism, small curly leaves, and delayed flowering. The major cause of these distinctive traits could be the alteration of carbohydrate metabolism, and the consequent modification of the expression of genes involved in chloroplast physiology. It is therefore not easy to define the exact function of NANA in the framework of the complexity of chloroplast metabolism. Although the low level of sugars detected in nana and oxNANA grown at 80 μmol m⁻² sec⁻¹ was suggestive of an impaired photosynthetic activity, the nanaxpgm plants contained the same sugar levels as the parental pgm mutant but showed the same dwarf phenotype of nana, suggesting that the slightly lower rates of photosynthetic electron transport are not a major cause of the metabolic defects observed in nana. It appears that the effects of NANA misexpression are highly dependent on growth irradiance, as nana plants grown in higher light (150 μmol m⁻² s⁻¹) accumulated more sucrose than WT, and also had higher levels of phosphorylated intermediates involved in CO₂ fixation, sucrose synthesis or starch synthesis. Together, these observations suggest that NANA misexpression affects fluxes through all the major pathways of photosynthetic carbon metabolism, affecting the availability of sugars for immediate growth as well as the turnover of transitory starch metabolism which is essential to support respiration, sucrose export and growth at night. The expression of a number of nuclear genes encoding for chloroplast proteins was altered in nana and oxNANA, suggesting the activation of a retrograde signal because of the NANA-related defects in chloroplast functionality. The expression of GLK1, identified as a crucial transcription factor in retrograde signaling and as a positive regulator of photosynthesis-related nuclear gene expression (Kakizaki et al., 2009), was indeed affected by the nana mutation. Thus, it seems that, because of altered NANA expression, a strong alteration in a number of processes involved in chloroplast physiology occurred. This, in turn, might generate a plastid retrograde signal, possibly under the control of GLK1, which consequently results in a down-regulation of a series of nuclear genes, also affecting the chloroplast metabolism and/or functionality, and whose result is a strong impairment in plant growth and development.

The strong impact of the nana mutation that leads to a relatively modest alteration in its pattern of expression is suggestive of an important role of this aspartyl protease for chloroplast homeostasis. Our investigation suggested a possible but still unclear involvement of NANA in starch
metabolism, Further studies are required to identify the actual protein target of NANA and to elucidate the specific regulatory role of NANA in the context of chloroplast functionality.
MATERIALS AND METHODS

Plant material and growth conditions

*Arabidopsis thaliana*, accession Columbia glabra (*gl1*), was used in this study. The *nana* mutant was identified by screening T-DNA tagged lines from the Jack T-DNA enhancer trap collection (Campisi et al., 1999), obtained from the Nottingham Arabidopsis Stock Centre (NASC, http://nasc.nott.ac.uk/home.html) (Scholl et al., 2000). For the screening experiment, seeds were sterilized with diluted bleach (10 min incubation in 1.7% v/v sodium hypochlorite, rinsing and washing thoroughly in sterile water) and germinated in horizontal petri dishes, prepared using solid growing media [0.5x Murashige Skoog (MS) solution containing 1% agar and 90mM sucrose].

The *nanaxpgm* double mutant was generated by crossing *nana* with the *pgm* mutant that lacks a functional plastidial phosphoglucomutase (NASC ID N210) and is unable to synthesize starch (Caspar et al., 1985).

In most experiments seeds were sown in moist soil or in a hydroponic system, as described by Gibeaut et al. (1997), stratified at 4°C in the dark for 48 hours and germinated at 22°C day/18°C night with a photoperiod of 12-h-light/12-h-darkness at 80 µmol photons m⁻² sec⁻¹ irradiance, unless otherwise specified. Plants were harvested by transferring rosettes into liquid nitrogen under ambient irradiance.

Identification of the *nana* mutation

For the screening of the insertional mutants, 630 different T-DNA tagged lines from the Jack T-DNA enhancer trap collection (Campisi et al., 1999) were used. The screening was carried out in petri dishes containing 0.5x MS, 1% agar and 90 mM sucrose, and putative sucrose-hypersensitive mutants were collected as previously described (Gonzali et al., 2005). The *nana* mutant described here was initially selected for its sucrose-hypersensitive phenotype.

For the molecular characterization of *nana*, DNA extraction was performed with the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, MO, USA) using approximately 100 mg rosette leaves from 4-week-old *nana* plants. All PCR amplifications were carried out with 20 ng genomic DNA and a PCR Master Mix (Promega, Madison, WI, USA). For the amplification of the T-DNA/plant genome junctions, thermal asymmetric interlaced-PCR (TAIL-PCR) was used, as described by Liu et al. (1995). Primers used were AD1, AD2, AD3 and different TR1, TR2 and TR3 oligos for T-DNA left border (LB) or right border (RB). All primer sequences are reported in Supplemental Table S3.
Cloning

The NANA coding sequence was amplified from a cDNA template using Phusion High Fidelity DNA-polymerase (New England Biolabs) and cloned into the pENTR-D/TOPO Gateway vector (Invitrogen) to obtain pENTR-NANA. The resulting entry vector was recombined into destination vectors pK7WG2 and p7WGF2 (Karimi et al., 2002) using the LR reaction mix II (Invitrogen), to obtain the expression vectors 35S::NANA and 35S::GFP-NANA, respectively. The GFP-NANA in-frame fusion construct has the enhanced green fluorescent protein (EGFP) at the N-terminal of NANA. EGFP at the C-terminal of NANA resulted in the absence of fluorescence and this is the reason for using EGFP at the N-terminal. The CaMV 35S promoter drove expression of the chimeric gene in both the constructs. Primers used for cloning in the pENTR-D/TOPO plasmid are reported in Supplemental Table S4.

Plant Transformation

Stable transgenic plants were obtained using the floral dip method (Clough and Bent, 1998; Zhang et al., 2006). T0 seeds were screened for kanamycin resistance and single-insertion lines were identified by real-time PCR. Homozygous T3 or subsequent generations were used in the following experiments. Arabidopsis mesophyll protoplasts were prepared and transiently transformed with 10 µg plasmid DNA according to Yoo et al. (2007).

Microscopy

For GFP imaging, fluorescence was observed with a Nikon ViCo videoconfocal microscope (http://www.nikon.com/) using a GFP filter. To observe epidermal cells size, leaves of WT and nana plants were plunged in liquid N2 and analyzed in a frozen-hydrated state by Cryo-SEM, as described by Di Baccio et al. (2009).

Total RNA extraction and qPCR

Total RNA was extracted from Arabidopsis rosettes as previously described (Perata et al., 1997) with a minor modification (omission of aurintricarboxylic acid) to make the protocol compatible with the subsequent PCR procedures. Electrophoresis using a 1% agarose gel was performed for all RNA samples to check for RNA integrity, followed by spectrophotometric quantification. Contaminating DNA was removed using a TURBO DNA-free kit (Ambion). RNA was then reverse-transcribed using an iScript™ cDNA Synthesis kit (BioRad Laboratories). Gene expression analysis was performed by real-time PCR using an ABI Prism 7300 sequence detection system (Applied Biosystems). Quantitative PCR was performed using 40 ng cDNA (nuclear genes) or
0.005 ng cDNA (chloroplastic genes) and \( iQ^{TM} SYBR^{®} \) Green Supermix (BioRad Laboratories), according to the manufacturer’s instructions. Transcripts of \( GAPDH \) and \( 40SrRNA \) for nuclear genes and of \( 16SrRNA \) and \( accD \) for plastid genes were used as reference genes. Relative expression levels were calculated using Genorm (http://medgen.ugent.be/~jvdesomp/genorm). For a list of the primers used see Supplemental Table S5.

**SDS-PAGE and western blots**

Plant samples (about 500 mg) were ground in liquid nitrogen. The extraction buffer, as described by Siddique et al. (2008), was added at a 1:2 ratio (plant tissue:buffer). Total protein content was quantified using BCA Protein Assay Reagent (Pierce). SDS-PAGE was performed on a 10% Criterion polyacrylamide gel (BioRad Laboratories). Blotting on an Amersham Hybond-P polyvinylidene difluoride membrane was performed with a Novablot electrophoretic transfer system (Amersham Pharmacia Biotech). After blocking and challenge with appropriate antibodies, immunoreactive proteins were detected using an Immun-Star HRP Chemiluminescent Detection Kit (BioRad Laboratories). An antibody against NANA was produced by Agrisera (www.agrisera.com) using a synthetic peptide (CLFWKQNPTGDKKNQ) based on amino acid residues 6-19 (underlined) from the deduced sequence of the NANA protein. An antibody against the large subunit of Rubisco (AS03037) was purchased from Agrisera. The antibody against Actin-11 (AS10702, Agrisera) was used to confirm even loading and transfer. Antisera specific for the BAM1 and BAM3 proteins were kindly provided by Prof. Samuel C. Zeeman (Institute of Plant Sciences, ETH Zurich, Switzerland). Densitometric analysis of the protein signals on the western blots was performed with the software package UVP VisionWork LS (Ultra-Violet Products). Normalization was carried out using Actin-11 signal and setting to 100 the relative protein signal value for each “end of the day” time-point. Extraction of AGPase, separation by non-reducing SDS-PAGE and electroblotting onto nitrocellulose membrane were performed as in Hendriks et al. (2003). After blocking, the membrane was incubated with anti-potato AGPB antiserum (Tiessen et al., 2002) for 1 h at 20°C. After washing, the membrane was incubated with IRDye800-conjugated anti-rabbit IgG F(c) antibody from goat (Biomol, http://www.biomol.de/). The fluorescent infrared signal from the dye-labelled secondary antibody was detected using an Odyssey Infrared Imaging System (LI-COR, http://www.licor.com/).

**Chloroplasts isolation and protein extraction**

Rosette leaves of Arabidopsis (20 g fresh weight), previously kept in dark for 14-16 hours, were homogenized in 400 mL cold grinding buffer (GB) (330 mM sorbitol, 50 mM HEPES/KOH pH 8.0,
2 mM EDTA, 1 mM MnCl₂, 1 mM MgCl₂) using a Waring blender. The homogenate was filtered through 4 layers of miracloth. The filtered samples were centrifuged at 6,000 × g for 30 min. The precipitate was gently resuspended in 5 mL of GB and separated by a step-gradient with 80% and 40% Percoll. After centrifugation at 3,600 rpm for 30 min intact chloroplasts were collected, resuspended in 2 volumes of GB and centrifuged at 10,000 × g for 1 min. The supernatant was removed and the pellet was vigorously resuspended in 1 ml of a hypotonic buffer, containing 10 mM HEPES/KOH pH 8.0, 5 mM DTT, 1 mM EDTA. All procedures were carried out at 4°C. Chloroplast proteins were extracted according to Yang et al. (2007), with some modifications. Chloroplasts were ground in liquid nitrogen and homogenized with 1 mL of extraction buffer (5 M Urea, 2 M Thiourea, 40 mM Tris-Cl, 2% CHAPS, 50 mM DTT). The homogenate was centrifuged at 15,000 × g for 15 min. Supernatant was precipitated using TCA (15% v/v) containing 0.007% β-mercaptoethanol in acetone at -20°C for 2 h and then at 4°C for a minimum of 2 h. Samples were centrifuged at 4°C at 14,000 × g for 15 min, supernatant was discarded and pellets were washed twice with ice cold acetone containing 0.007% β-mercaptoethanol. Pellets were dissolved in a rehydration buffer (5 M Urea, 2 M Thiourea, 2% CHAPS, 50 mM DTT). The immunoblot analysis was performed as described above, loading 30 µg of protein for each fraction.

Expression of recombinant protein in Escherichia coli
The NANA coding sequence was recombined from pENTR-NANA into the pDEST17 plasmid (Invitrogen) and transformed into the Escherichia coli Rosetta2 strain (Novagen). The expression of the His₆-tagged NANA fusion protein was induced by addition of isopropylthio-b-D-galactoside (IPTG, final concentration 0.5 mM) to early log phase cultures and incubation over-night at 20°C. The overexpressed protein was purified using TALON immobilized metal ion affinity chromatography resin (Clontech). The purified protein was eluted in a buffer containing 50 mM HEPes, 300 mM NaCl, 150 mM imidazole, 5 mM MgCl₂, pH 7.0, and the identity of the protein was confirmed by SDS-PAGE and immunoblot analysis with anti-NANA antibody.

Detection of enzymatic activity
An azocasein assay was performed for the determination of the aspartic proteolytic activity of NANA. Protease activity was determined by measuring the release of acid-soluble material from azocasein (Sigma Aldrich). Soluble protein were extracted from leaves by homogenization in 100 mM Na-acetate (pH 5.0) buffer, and incubated for 30 min at 4°C. The reactions were performed in 50 mM Na-acetate (pH 5.0) and 0.8% (w/v) Azocasein reaction buffer, with or without 100 µM Pepstatin A (Promega), as aspartyl proteases inhibitor. The mixture was incubated for 3 h at 30°C
and the reaction was stopped by addition of TCA to a final concentration of 10%. After incubation on ice for 15 min, the reaction mixture was centrifuged (5,000 × g, 5 min) to remove precipitated protein and unreacted azocasein. Absorbance of the azopeptide-containing supernatant was measured at 330 nm. Aspartyl-protease activity units were calculated as ΔOD_{330} h^{-1} mg protein^{-1}.

Aspartyl protease activity units were calculated as the difference between the total protease activities in the presence and absence of pepstatin A. The activity of the recombinant NANA protein was assayed as above using a 50 mM Na-acetate (pH 6.0) buffer. The pH optimum for both the recombinant protein and the crude extract was determined within a pH range of 3.6 – 8.0, using 50 mM Na-acetate (pH 3.6 – 5.6) and 100 mM Na-phosphate (pH 6.0 – 8.0) buffers.

Activities of β-amylase were measured using the Betamyl assay kit from Megazyme, according to Zeeman et al. (1998).

**Extraction and measurement of metabolites**

Analyses of sucrose, fructose and glucose were carried out as previously described (Guglielminetti et al., 1995). Samples (0.05–0.30 g fresh weight) were rapidly frozen in liquid nitrogen and ground to a powder. Samples were then extracted as described by Tobias et al. (1992). After centrifugation, the supernatant was used for analysis of sucrose, fructose and glucose. The starch-containing pellet was extracted with 10% KOH, centrifuged, and neutralized with 18% (v/v) acetic acid. The resulting suspension was treated with 2.5 units amyloglucosidase (from *Rhizopus niger*) for 3 h to release glucose. Samples were assayed by coupled enzymatic assay methods, measuring the increase in absorbance at A_{340}. Starch was quantified on the basis of the glucose units released after the amyloglucosidase treatment. For qualitative assay of starch, leaves were stained using an iodine solution after first decolorizing them in 80% (v/v) ethanol. T6P and phosphorylated intermediates were extracted and measured by anion exchange liquid chromatography coupled to tandem mass spectrometry as described by Lunn et al. (2006).

**Photosynthesis measurements**

Fluorescence emission was measured *in vivo* using a PAM fluorometer (Walz) on 5-week-old plants maintained at fixed irradiance (250 and 700 μmol photons m^{-2} sec^{-1}) for 30 min prior to measurement of chlorophyll fluorescence yield and relative ETR at 330 μmol photons m^{-2} sec^{-1}, which were calculated using the WinControl software package (Walz). Chlorophyll quantification was performed as described by Lichtenthaler (1987) and Porra (2002). Gas-exchange measurements were performed in a special custom-designed open system at 200 μmol photons m^{-2} sec^{-1} irradiance (Lytovchenko et al., 2002).
Sequence and phylogenetic gene analysis

BLASTP 2.2.26+ (Altschul et al., 1990) was used to identify protein sequences similar to NANA (Supplemental Fig. S2A), using the BLOSUM62 scoring matrix and applying an E-value threshold of 10^-10, against the RefProt database (NCBI, release 11-01-2012). The Solanum lycopersicum homologous sequences were obtained from the SGN draft genome (Bombarely et al., 2010). A. thaliana putative paralogous protein sequences were taken within the same threshold (E<10^-10), and aligned using MUSCLE (Edgar, 2004). The subsequent maximum likelihood phylogenetic tree (Supplemental Fig. S2B) was built using MEGA5 (Tamura et al., 2011) with default parameters and 100 bootstraps for branch point validation (branch points with less than 50 bootstrap concordance are collapsed). In the tree in Supplemental Figure S2B, gene expression alterations are indicated in a dataset from 7-day-old A. thaliana wild-type Col-0 dark grown seedlings compared to light grown seedlings (Dohmann et al., 2008, GEO entry GSE9728); the p-value significance threshold for differential expression was set to 0.05 using the Genevestigator software (Hruz et al., 2008). “Clock-regulated genes” in the phylogenetic tree (with respect to the transcript quantity oscillation) are indicated according to Covington and Harmer (2007). A list of all these genes is in Supplemental Table S2.
Acknowledgements

We would like to acknowledge Prof. Samuel C. Zeeman (ETH, Zurich, Switzerland) for kindly providing us with the BAMs antibodies. We also thank Dr. Antonio Minnocci (Scuola Superiore Sant’Anna, Italy) for Crio-SEM analysis, Dr. Paola Collecchi (University of Pisa, Italy) for help with the endoreduplication experiment, and Dr. Valentina Lucarotti (University of Pisa, Italy) for chloroplasts isolation. We furthermore are particularly grateful to Dr. Joachim Fisahn and Dr. Alexander Ivakov (Max Plank Institute, Golm, Germany) for helping in the measurement of photosynthetic parameters.
LITERATURE CITED


FIGURE LEGENDS

Figure 1. Phenotypes of nana. A, Effects of treatments with sucrose on seedling growth. Seedlings of nana and wild-type (WT) grown in vitro for 10 days on agar plates containing Murashige-Skoog medium in absence (control) or presence of sucrose (90 mM). B, Effects of treatments with sucrose on hypocotyl and root growth. Detail of one seedling from the plates in A. C, Representative growth phenotypes of nana and wild-type (WT) plants grown on soil for 30 days. D, Phenotypes of 6-week-old mutant and wild-type plants. E, Photographs of 4-week-old leaves from WT and nana plants. Scale bar = 1 cm. F, Cryo-scanning electron microscopy images of frozen-hydrated leaf freeze-fractures in WT and nana plants. A representative leaf cell is highlighted in yellow. Scale bar = 50 μm.

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**Figure 9.** Diurnal changes in the transcript levels measured in leaves of 4-week-old plants during a 48 h time-course. A, Transcript levels for sugar-modulated genes, *ApL3* (*At4g39210*), *PG* (*At5g41670*), and *GPT2* (*At1g61800*). B, Transcript levels for chloroplast encoded genes linked to photosynthesis, *RbcL* (*AtCG00490*), *psbA* (*AtCG00020*), and *petD* (*AtCG00730*). C, Transcript levels for nuclear-encoded photosynthetic genes, *GLK1* (*At2g20570*), *Lhca1* (*At3g54890*), *Lhcb1.3* (*At1g29930*), *Lhcb1.2* (*At1g29910*), *Lhcb1.1* (*At1g29920*), *RbcS1-A* (*At1g67090*). WT (open circles), *nana* (filled circles) and *oxNANA* (grey circles) rosettes (80 µmol photons m⁻² sec⁻¹). Transcript levels are expressed as relative units, with the value of WT at the beginning of the day (light-input) set to one. Each value is the mean (±SD) of three independent measurements. Background shading: white and dark grey show the light period and the night, respectively.
Supplemental Table S1 Hypocotyl and primary root length in WT and nana seedlings, grown in presence or absence of 90 mM sucrose either in light or dark conditions.

Supplemental Table S2 List of putative Arabidopsis paralogs of the NANA (At3g12700) gene.

Supplemental Table S3 List of primers used in TAIL-PCR and to test T-DNA insertion.

Supplemental Table S4 List of primers used for cloning in the pENTR-D/TOPO plasmid.

Supplemental Table S5 List of primers used for gene expression analysis using real-time quantitative RT-PCR.

Supplemental Figure S1 DNA ploidy levels in the WT and nana mutant determined by flow cytometric analysis.

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Supplemental Figure S3 Aspartyl-proteolytic activities in WT, nana, and oxNANA rosette extracts using the azocasein assay.

Supplemental Figure S4 Phenotype of the double mutant nanaxpgm, in comparison with the WT and the nana and pgm parental single mutants.

Supplemental Figure S5 Dimerization of the APS1 subunit of ADPglucose pyrophosphorylase WT and nana plants, resolved by non-reducing SDS-PAGE and detected by immunoblotting.

Supplemental Figure S6. Diurnal changes in transcript and protein levels of genes encoding β-amylase enzymes in leaves of 4-week-old plants during a time-course of 48 hours.

Supplemental Figure S7. Clustal alignment of NANA and tobacco CND41 (Nakano et al., 2003) polypeptide sequences.
**Table I.** Comparison of wild-type (WT) and *nana* mutant phenotypes. Morphological parameters were measured in 4-week-old adult plants.

<table>
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<tr>
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<th>WT (±)</th>
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<tr>
<td><strong>Leaf number</strong></td>
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| **Leaf length (mm)** | 18.6 (±1.85) | 7.4 (±0.55)
| **Leaf width (mm)** | 12.5 (±1.52) | 6.7 (±1.06)
| **Petiole length (mm)** | 13.8 (±1.70) | 6.2 (±1.15)
| **Rosette size (cm)** | 8.74 (±0.28) | 4.02 (±0.37)
| **Bolting time (DAG)** | 29.6 (±1.70) | 36.7 (±1.55)
| **Inflorescence height (cm)** | 34.1 (±4.51) | 8.3 (±2.31)
| **Inflorescence number** | 10.2 (±1.58) | 5.1 (±1.25)
| **Silique number** | 50.3 (±9.24) | 16.2 (±1.52)

*Significantly different (P < 0.001) as calculated using T test*

*DAG, days after germination.*
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Figure 7. Changes in the level of metabolites during the diurnal cycle in wild-type (WT) and *nana* mutant plants, grown in a 12-h-light/12-h-dark cycle. The whole rosette was harvested at the end of the night, 4, 8, and 12 h into the light period, and 4, 8, and 12 h into the dark period. A, Sucrose, glucose and fructose content determined for WT (open circles), *nana* (filled circles) and *oxNANA* (grey circles) grown at a light intensity of 80 μmol photons m⁻² sec⁻¹. B, Sucrose, glucose, fructose, sucrose-6P, ADPG, UDPG, glucose-1P, 3-PGA, and trehalose-6P measured in WT and *nana* grown at an irradiance of 150 μmol photons m⁻² sec⁻¹. C, Sucrose, glucose and fructose levels determined for WT (open circles), *nana* (filled circles), *pgm* (filled squares), and the double mutant *nana*/*pgm* (triangles) grown at an irradiance of 80 μmol photons m⁻² sec⁻¹. The results are given as mean (±SD; n = 4 independent samples, each consisting of five rosettes). Background shading: white and dark grey show the light period and the night, respectively. FW, fresh weight.
Figure 8. Alterations in starch accumulation and remobilization due to the nana mutation. A, Iodine staining showing the presence of starch in the wild-type (WT), nana and oxNANA mutants at the end of the night and the day, respectively. B, Changes in starch content in WT and mutant plants during the diurnal cycle at an irradiance of 80 μmol photons m⁻² sec⁻¹. C, Diurnal changes in β-amylase activity in leaves of WT and mutants were determined in vitro using the Betamyl assay. In B and C, leaves of 4-week-old WT (open circles), nana (filled circles) and oxNANA (grey circles) were used. Each point represents the mean (±SD) from duplicate experiments. Background shading: white and dark grey show the light period and the night, respectively.
Figure 9. Diurnal changes in the transcript levels measured in leaves of 4-week-old plants during a 48 h time-course. A, Transcript levels for sugar-modulated genes, ApL3 (At4g39210), PG (At5g41670), and GPT2 (At1g61800). B, Transcript levels for chloroplast encoded genes linked to photosynthesis, RbcL (AtCG00490), psbA (AtCG00020), and petD (AtCG00730). C, Transcript levels for nuclear-encoded photosynthetic genes, GLK1 (At2g20570), Lhca1 (At3g54890), Lhcb1.3 (At1g29930), Lhcb1.2 (At1g29910), Lhcb1.1 (At1g29920), RbcS1-A (At1g67090). WT (open circles), nana (filled circles) and αNANA (grey circles) rosettes (80 μmol photons m⁻² sec⁻¹). Transcript levels are expressed as relative units, with the WT at the beginning of the day (light-input) set to one. Each value is the mean of three independent measurements. Background shading: white and dark grey show the light period and the night, respectively.