Characterization of the Arabidopsis *glutamine dumper1* mutant reveals connections between amino acid homeostasis and plant stress responses

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ABSTRACT

Amino acids constitute the major organic form of transported nitrogen in plants, elements for protein synthesis, and precursors of many plant secondary metabolites, such as lignin, hormones, and flavonoids. Furthermore, amino acid metabolism lies at the crossroad of carbon and nitrogen metabolism. The Arabidopsis *glutamine dumper1* (*gdu1*) mutant secretes glutamine from hydathodes, a phenotype caused by the overexpression of *Glutamine Dumper1* (*GDU1*). GDU1 is a small transmembrane protein presents only in higher plants. The *gdu1-1D* mutant shows a pleiotropic phenotype: perturbed amino acid metabolism, tolerance to exogenous toxic concentrations of amino acids, elevated amino acid export, and activated stress/defense responses, lesions, and smaller rosettes. The biochemical function of GDU1 remains elusive. To better elucidate the biological processes leading to the complex Gdu1D phenotype, two approaches were conducted: (1) An ethyl methanesulfonate suppressor screening of the Gdu1D phenotype, which led to the isolation of intragenic mutations in GDU1 and mutations in the ubiquitin ligase LOG2 (Loss Of Gdu1D 2). Study of the intragenic mutations in GDU1 helped to characterize its structure-function relationships. Characterization of LOG2 showed that LOG2 interacts with GDU1 and is

necessary for the Gdu1D phenotype. (2) The responses of the plant to the dexamethasone-induced expression of *GDU1* were studied over time. This experiment identified major signaling pathways contributing to different components of the Gdu1D phenotype and the early events triggered by the perturbation of amino acid homeostasis. Our results showed that *GDU1* overexpression first increases amino acid export, which is followed by amino acid imbalance and stress responses. This study sheds light on how amino acid imbalance interacts with various plant signaling pathways and stress responses, and suggests that LOG2 is involved in this process.

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Attribution

Several colleagues have assisted with the completion of the research reported in this dissertation. The following is a brief description of their contributions.

Guillaume Pilot, Department of Plant Pathology, Physiology and Weed Science, Virginia Tech

Dr. Pilot served as the major advisor and committee chair for this project. He assisted in editing of this dissertation, providing funding, as well as advising throughout the entire project.

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Dr. Callis assisted in experimental design, providing funding, and writing the paper. Dr. Callis is listed as a co-author of the publication.

Wolf Frommer, Department of Plant Biology, Carnegie Institution for Science

Dr. Frommer assisted in providing funding and writing the paper. Dr. Frommer is listed as a co-author of the publication.

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Dr. Guerra performed the in vitro precipitation assays and the corresponding clonings, membrane washes and purification in Arabidopsis, Leu tolerance assays,

myristoylation assays, experimental design and writing the paper. Dr. Guerra is listed as a co-author of the publication.

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Dr. Kraft assisted in the in vitro ubiquitination assays and the corresponding cloning. Dr. Kraft is listed as a co-author of the publication.

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Dr. Pratelli performed the yeast two hybrid screening, the EMS suppressor screening, the GUS assays, amino acid analyses, and half of the clonings. Dr. Pratelli is listed as a co-author of the publication.

Mark Wogulis, Department of Molecular and Cellular Biology, University of California, Davis

Dr. Wogulis assisted in the in vitro ubiquitination assays and the corresponding cloning, experimental design, providing funding and writing the paper. Dr. Wogulis is listed as a co-author of the publication.

Shi Yu, Department of Plant Pathology, Physiology and Weed Science, Virginia Tech

Shi Yu performed *in planta* co-immunoprecipitation assay, subcellular fractionation, localization of expression and imaging, nucleic acid isolation and qRT-PCR, as well as cloning and experimental design, which generated Figure 2-1 C, Figure 2-4 E and G, Figure 2-5, Figure 2-7 C and D. Shi Yu is listed as a co-author of the publication.

Chapter 3,

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Shi Yu performed the experiments, assisted in experimental design, and writing the paper. Shi Yu is listed as a co-author of the publication.

Chapter 4,

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Dr. Denbow assisted in growing the plants and western blotting. Dr. Denbow will be listed as a co-author of the publication.

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Dr. Pilot assisted in experimental design, providing funding, and writing the paper. Dr. Pilot will be listed as a co-author of the publication.

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Chapter 5,

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Kasia Dinkeloo performed *Hyaloperonospora arabidopsidis* inoculations and sampling. Kasia Dinkeloo will be listed as a co-author of the publication.

John McDowell, Department of Plant Pathology, Physiology and Weed Science, Virginia Tech

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Dr. Pilot assisted in experimental design, providing funding, and writing the paper. Dr. Pilot will be listed as a co-author of the publication.

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Shi Yu, Department of Plant Pathology, Physiology and Weed Science, Virginia Tech

Shi Yu performed the experiments, assisted in experimental design, and writing the paper. Shi Yu will be listed as a co-author of the publication.

Bingyu Zhao, Department of Horticulture, Virginia Tech

Dr. Zhao assisted in experimental design. Dr. Zhao will be listed as a co-author of the publication.

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Chapter I

Introduction

Nitrogen acquisition by plants

Plants have to cope with rapidly changing environmental factors, including temperature, light, water availability, and nutrient levels (Barneix and Causin, 1996). Nitrogen is an essential macronutrient, and it is quantitatively the most important for plants (Krapp et al., 2011). Nitrogen comprises approximately 2% of plant dry matter and 16% of total plant protein (Alvarez et al., 2012). It is a key component for macromolecules, such as proteins, nucleic acids, and secondary metabolites (Crawford and Forde, 2002). Because it is quantitatively and metabolically highly needed for plant growth, nitrogen availability is usually the most limiting factors for crop production (Frink et al., 1999).

Though nitrogen is abundant in the atmosphere, only a few plant species can utilize gaseous nitrogen, and only by symbiotic associations. Other plants depend on nitrogen present in the soil, including inorganic nitrogen, mainly nitrate and ammonium, and organic nitrogen, such as amino acids and peptides. The abundance of nitrogen in the soil can vary over several orders of magnitude; for example, soil nitrate concentration can vary from 10 μM to 100 mM (Crawford, 1995). Although the organic nitrogen pool in soil is relatively larger than the

inorganic nitrogen pool, most of organic nitrogen is found as high molecular weight compounds that plants cannot absorb. On the other hand, organic nitrogen as low molecular weight molecules, mainly amino acids, can be abundant in some specific soils and conditions. Though plant roots have the potential to absorb amino acids, direct evidence that root uptake of amino acids is a major acquisition pathway for nitrogen is still lacking. This is partly due to problems linked to the complexity of the soil and the inability of current experimental techniques to measure amino acid uptake from soil (Jones et al., 2005). Because ammonium is toxic to plants and the diffusion rate of nitrate in soil is about five-times greater than either ammonium or amino acids (Owen and Jones, 2001), it is accepted that, in most cases, plants utilize nitrate as the primary nitrogen source. However, a main criticism to this theory is that in some ecosystems, there is a large discrepancy between inorganic nitrogen production rate and annual plant nitrogen uptake. This suggests that plants must acquire nitrogen from organic forms of nitrogen, probably amino acids (Näsholm et al., 2009).

Once nitrate has been taken up into the cytosol by roots, it is 1) loaded into xylem and transported to the shoots, 2) stored in the vacuole, 3) exported out of the cell to the apoplasm, 4) reduced to nitrite, then ammonium, and incorporated into amino acids. Nitrate is reduced by nitrate reductase (NR), which is primarily located in the cytosol of root epidermal and cortical cells, and shoot mesophyll

cells (Crawford, 1995). Then nitrite is quickly reduced to ammonia by nitrite reductase (NiR) located in chloroplasts in green tissues (plastids in non-green tissues). Ammonia is incorporated into glutamine by the combined actions of glutamine synthetase (GS) and glutamate synthase (GOGAT). This pathway produces substrates for biosynthesis of all other amino acids, and is the only point of entry of nitrogen in the whole metabolism (Lam et al., 1995; Lam et al., 1996; Crawford and Forde, 2002).

Amino acids play critical roles in plant growth

Since, amino acids are the building blocks of proteins, their content needs to be adjusted to protein synthesis needs. The carbon skeletons of amino acids come from the intermediates of the glycolysis, TCA cycle and pentose phosphate pathways. Amino acids thus lie at the crossroads of carbon and nitrogen metabolism. In addition, amino acids participate in many important physiological processes in plants: for example, glycine and glutamate are two precursors for chlorophyll biosynthesis (Klein and Senger, 1978), and lignin is synthesized from phenylalanine (Vanholme et al., 2010). Some amino acids are important for plant hormone biosynthesis: methionine is the precursor of ethylene (Wang et al., 2002), and tryptophan is the precursor for tryptophan-dependent auxin biosynthesis (Mano and Nemoto, 2012). Also, transport of micronutrients, such as Fe and Cu, is facilitated by chelation with amino acids (Grusak et al., 1999). Histidine is crucial

to Ni tolerance because of its high affinity to Ni (Ingle, 2011). Importantly, many amino acids are precursors of plant secondary metabolites, such as flavonoids (Falcone Ferreyra et al., 2012), alkaloids (Ziegler and Facchini, 2008), and glucosinolates (Kraker and Gershenzon, 2011). Since many secondary metabolites are involved in plant stress response, amino acid metabolism indirectly plays an important role in plant stress resistance. For instance, proline and glycine betaine accumulate during drought and salt stresses and are thought to help the cell to cope with dehydration (Ashraf and Foolad, 2007).

Finally, amino acids are the predominant form of transported nitrogen in plants. Amino acids are mainly synthesized in roots and mature leaves (sources) and transported to sinks, such as root tips, flowers, and seeds, through the xylem and phloem (Tegeder, 2014). The location of nitrogen assimilation strongly depends on the plant species and is also influenced by diurnal rhythm (Lam et al., 1995; Tegeder, 2014). Changing concentrations of amino acids in the saps are also deemed to transduce signals of nitrogen availability and demand from source and sink (Imsande and Touraine, 1994).

Because of these numerous roles in plants, the concentration of amino acids needs to be tightly adjusted to carbon and nitrogen availability, and the needs for other metabolic pathways, growth and development. Complex regulatory

mechanisms are expected to exist, that enable amino acid homeostasis by controlling both amino acid synthesis and catabolism.

Amino acid biosynthesis in plants

Unlike animals, which obtain amino acids from diet, plants can synthesize all 20 proteogenic amino acids. Amino acid biosynthesis can be divided into different pathways. Since the amino acids, lysine, methionine, threonine, isoleucine, leucine, valine, histidine, tryptophan, and phenylalanine, are essential amino acids in the human diet, their synthesis pathways have been the most studied and are also the most complexly regulated.

Aspartate-derived amino acid pathway

The aspartate-derived amino acid pathway produces lysine, methionine, threonine and isoleucine (Fig. 1-1). Aspartate modification to aspartate-4-semialdehyde is catalyzed by aspartate kinase (AK) and aspartate semialdehyde dehydrogenase, which are the first two steps of the aspartate-derived amino acid pathway. AK is a highly regulated enzyme: lysine, threonine and S-adenosylmethionine, produced from methionine, directly inhibit AK, while isoleucine activates AK. Enzymes for the first step of each biosynthetic branch are usually feedback-inhibited by the end products of the branch. For instance, dihydrodipicolinate synthase (DHDPS), the first step leading to lysine biosynthesis from aspartate-4-semialdehyde, is inhibited by lysine. Interestingly, S-

adenosylmethionine, produced from methionine, not only inhibits cystathionine γ-synthase (CGS), the first step leading to methionine biosynthesis from O-phosphohomoserine, precursor for both methionine and threonine biosynthesis, but also activates threonine synthase (TS), the first step leading to threonine biosynthesis from O-phosphohomoserine. So when S-adenosylmethionine accumulates, it not only blocks methionine biosynthesis, but stimulates threonine biosynthesis as well (Azevedo et al., 2006; Jander and Joshi, 2009).

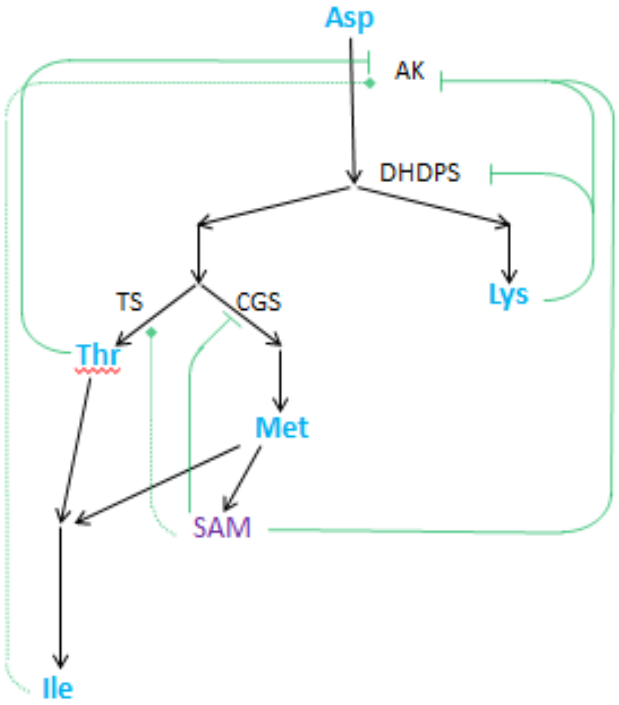

Figure 1-1. Simplified aspartate-derived amino acid synthesis pathway.
Amino acids are in blue, other metabolites in purple. Feedback regulations are in green: inhibitions are represented by solid lines and activations by dotted lines. AK, aspartate kinase. DHDPS, dihydrodipicolinate synthase. CGS, cystathionine γ-synthase. TS, threonine synthase. SAM, S-adenosylmethionine.

Branched-chain amino acid pathway

Valine, leucine and isoleucine are named branched-chain amino acids (BCAAs), because of the small branched hydrocarbon residues. A unique feature of BCAA biosynthesis is that valine/leucine and isoleucine are synthesized by parallel pathways, using the same set of enzymes, but from different substrates (Fig. 1-2). Threonine deaminase (TD) catalyzes the first reaction of isoleucine biosynthesis. 2-oxobutanoate is produced by this reaction, and together with pyruvate, are substrates for acetohydroxyacid synthase (AHAS). An alternative way to make 2-oxobutanoate in plants is from methionine by methionine γ-lyase (MGL). AHAS catalyzes the first step in the parallel pathways: two pyruvates are used to synthesize 2-acetolactate, which is further used for valine and leucine synthesis; a pyruvate and a 2-oxobutanoate molecules are used to synthesize 2-aceto-2-hydroxy-butanoate, which is further used for isoleucine synthesis. Isopropylmalate synthase (IPMS) catalyzes the committed step towards leucine biosynthesis. In term of regulation, TD is feedback-inhibited by isoleucine, but this inhibition can be relieved by valine, AHAS is inhibited by each of the BCAAs, and IPMS is inhibited by leucine (Binder, 2010).

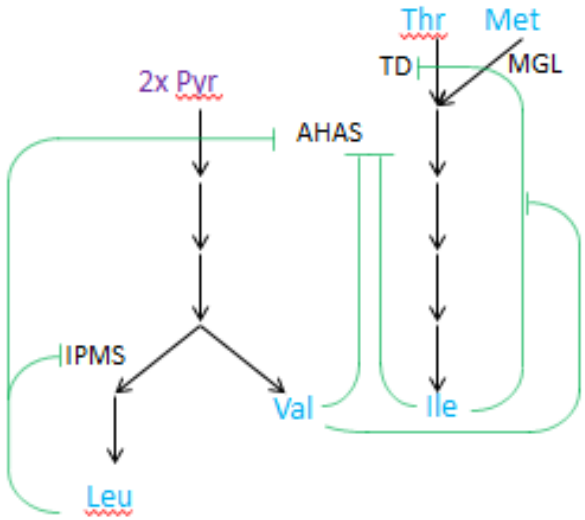

Figure 1-2. Simplified branched-chain amino acid synthesis pathway.
Amino acids are in blue, other metabolites in purple. Feedback regulations are in green: inhibitions are represented by solid lines. TD, threonine deaminase. AHAS, acetohydroxyacid synthase. MGL, methionine γ-lyase. IPMS, isopropylmalate synthase. Pyr, pyruvate.

Histidine pathway

Synthesis of histidine requires a lot of energy, since 31-41 ATP molecules are required for each histidine molecule produced. A series of 11 reactions beginning with the condensation of ATP and phosphoribosylpyrophosphate (PRPP) by ATP-phosphoribosyl transferase (ATP-PRT) leads to histidine synthesis (Fig. 1-3). This very first step in histidine biosynthesis is also the committed step, with ATP-PRT feedback inhibited by histidine itself (Ingle, 2011).

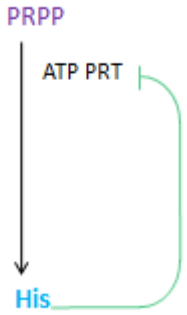

Figure 1-3. Simplified histidine synthesis pathway.
Amino acids are in blue, other metabolites in purple. Feedback regulations are in green: inhibition is represented by a solid line. ATP PRT, ATP-phosphoribosyl transferase. PRPP, phosphoribosylpyrophosphate.

Shikimate pathway and aromatic amino acids biosynthesis

Tryptophan, phenylalanine and tyrosine are aromatic amino acids (AAAs) that are critical for all living cells. AAAs are precursors of many plant secondary metabolites that are crucial for plant growth, development, defense and environmental responses. Though all three AAAs are synthesized from the final product from the shikimate pathway, chorismate, most of the carbon flux is directed to phenylalanine synthesis, which is used as a precursor for flavonoid synthesis, in particular lignin. The shikimate pathway is a seven step pathway that connects central carbon metabolism and the AAA pathway (Fig. 1-4). It utilizes phosphoenolpyruvate and erythrose-4-phosphate, intermediates from glycolysis and the pentose phosphate pathway, to make chorismate. In microbes, the first committed step in shikimate pathway, DAHP synthase, is tightly regulated by cellular AAAs level, which plays a major role in controlling carbon flux into the

pathway. However, the effects of AAAs on DAHP synthase in plants are unclear. Instead, in plants, many transcription factors control the expression of several genes of the shikimate pathway and AAAs biosynthesis in response to environmental stimuli. Besides transcriptional regulation, AAA biosynthesis is under complex posttranscriptional regulation as well: Anthranilate synthase (AS) and chorismate mutase (CM) are feedback-inhibited by tryptophan and phenylalanine/tyrosine, respectively. Interestingly, tryptophan and tyrosine activate CM and arogenate dehydratase (ADT), respectively, to ensure that the carbon flux from the shikimate pathway is directed to phenylalanine biosynthesis (Maeda and Dudareva, 2012).

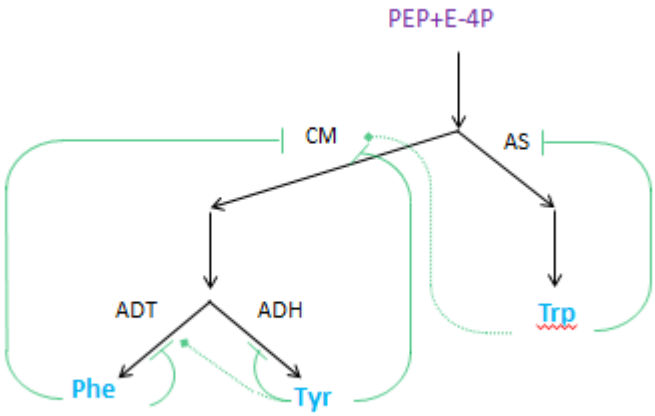

Figure 1-4. Simplified aromatic amino acid synthesis pathway.
Amino acids are in blue, other metabolites in purple. Feedback regulations are in green: inhibitions are represented by solid lines and activations by dotted lines. AS, anthranilate synthase. CM, chorismate mutase. ADT, arogenate dehydratase. ADH, arogenate dehydrogenase. PEP, phosphoenolpyruvate. E-4P, erythrose-4-phosphate.

Proline metabolism

Proline is probably the most important amino acid related to plant adaptation or resistance to environmental stresses, such as salt, drought, and freezing. Proline also plays a role in cell redox buffering and signal transduction. Proline biosynthesis is a two-step reaction from glutamate in the cytoplasm or chloroplast. Proline can be also degraded back to glutamate in mitochondria. Pyrroline-5-carboxylate synthetase (P5CS) catalyzes the first step leading to proline synthesis while proline dehydrogenase (ProDH) catalyzes the first step in proline catabolism (Fig. 1-5). P5CS is feedback-inhibited by proline. Dehydration strongly induces P5CS1 gene expression, and represses ProDH expression. In this way, proline accumulates during drought (Verslues and Sharma, 2010).

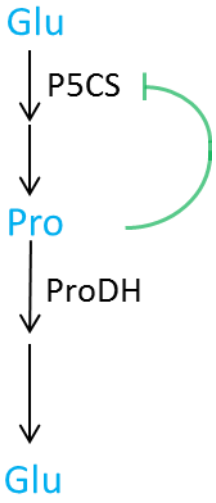

Figure 1-5. Simplified proline synthesis pathway.
Amino acids are in blue. Feedback regulations are in green: inhibition is represented by a solid line. P5CS, pyrroline-5-carboxylate synthetase. ProDH, proline dehydrogenase.

Amino acid transport in plants

Amino acids synthesized in source tissues are transported to sink tissues which do not have the required energy to make them. Such an efficient partitioning between different tissues and organs is critical to maintain amino acid homeostasis. Transfers from one compartment to another in the cell, and from one cell to another require membrane crossing, mediated by transporters. So far, three distinct amino acid transporter families have been identified, with multiple subfamilies in each of them. However, out of about 100 putative amino acid transporters, only a dozen of them have been functionally characterized (Tegeder and Rentsch, 2010; Pratelli and Pilot, 2014). They belong to two superfamilies: the amino acid-polyamine-choline (APC) transporter superfamily (Vastermark et al., 2014) and the drug/metabolite transporter (DMT) superfamily (Jack et al., 2001). Most of the characterized amino acid transporters belong to the APC superfamily and show import activity (Tegeder and Rentsch, 2010). Recently, a member of the UMAMIT family (part of the DMT superfamily) were found to display bidirectional transport activity (Ladwig et al., 2012).

Amino acid import

Depending on soil conditions, free amino acids could be abundant in the soil (Näsholm et al., 2009), but plants need to compete with microorganism to acquire this nitrogen source (Jones et al., 2005). Several amino acid transporter sub-

families play critical roles in amino acid uptake from the soil. Amino acid permease 1 (AAP1), a member of the AAP sub-family, and lysine-histidine transporter 1 (LHT1), a member of the LHT sub-family are important for uptake of acidic and neutral amino acids (Hirner et al., 2006; Lee et al., 2007; Svennerstam et al., 2007). Another member of the AAP sub-family, AAP5, is responsible for cationic amino acid uptake, such as lysine and arginine (Svennerstam et al., 2008). A more detailed study found out that LHT1 and AAP5, but not AAP1, are crucial for amino acid uptake at naturally occurring conditions (Svennerstam et al., 2011). However, a recent report suggested that AAP1 could take up neutral amino acids and glutamate at soil concentrations, and that LHT6 is involved in root uptake of acidic amino acids and a few neutral amino acids (Perchlik et al., 2014).

Amino acids absorbed and synthesized in roots are loaded into the xylem and transported to the shoots, where transpiration is high. There, amino acids could be utilized or stored in the vacuole. Amino acids synthesized in the leaf mesophyll from nitrate are loaded in the phloem and are transported to the sink organs (apex, seeds). Active amino acid exchange between xylem and phloem along the transport pathway necessitates amino acid transport activities born by AAP2 and AAP6. Mutants in *AAP2* show reduced transport activity, resulting in decreased amino acid supply to the seeds and lower total seed nitrogen and protein levels (Zhang et

al., 2010). The *aap6* mutants display reduced amino acid levels in phloem (Okumoto et al., 2002; Hunt et al., 2010).

During reproductive growth, flowers and seeds represent major nitrogen sinks. Many amino acid transporter sub-families (*AAP*, *CAT*, *LHT*) are expressed in flowers (Frommer et al., 1995; Okumoto et al., 2002; Su et al., 2004; Hammes et al., 2006; Hirner et al., 2006; Foster et al., 2008). Among those, members of the LHT sub-family likely play an important role in pollen nourishment and successful fertilization (Foster et al., 2008). Amino acids are unloaded symplastically from the phloem into the seed coat, but have to be transported into the endosperm and embryo via the seed apoplasm. The fact that about half of the fertilized ovules of *aap8* mutants abort suggested that AAP8 plays a crucial role in amino acid uptake into the endosperm during early embryogenesis (Schmidt et al., 2007). AAP1 was found to be essential for amino acid loading into embryo, since both amino acid levels and protein levels were reduced in *aap1* mutant embryos (Sanders et al., 2009).

Amino acid export

Amino acid export is an indispensable process in proper nitrogen distribution in the plant. However, little is known about the molecular characteristics of amino acid exporters and the amino acid export process (Okumoto and Pilot, 2011). So far, only three amino acid transporters have been characterized with bidirectional

transport activity, and a transporter with exclusive export activity is still missing. BAT1 is the first characterized bidirectional amino acid transporter (Dundar and Bush, 2009). The greatly divergent sequence with the other APC superfamily members suggests that its function may not be on par in the other members of the family. Recently, a member of the UMAMIT family, SIAR1 (UMAMIT18) was identified as bidirectional amino acid transporters (Ladwig et al., 2012). UMAMIT18 is supposed to be involved in the transport of amino acids to the developing embryo (Ladwig et al., 2012). It would be interesting to test if other members in the family show similar properties, or even strict export activity. While little is known about regulation of amino acid export, Glutamine Dumper 1 (GDU1) is supposed to be a regulator of amino acid export (See below 1.7).

Relationship between amino acid homeostasis and stress response

In addition to coping with abiotic stresses, plants use sophisticated strategies to defend against invaders, such as bacteria, fungi, viruses, nematodes and herbivores. Depending on their lifestyles, plant pathogens are divided into biotrophs, necrotrophs, and hemibiotrophs: Biotrophs extracts nutrients (mainly sugars and amino acids) from living host tissues, either by feeding on the metabolites present in the apoplasm or through specialized feeding structures (haustoria) that invaginate host cells without disrupting it. Necrotrophs first kill host cells, after

which feed on the contents. Hemibiotrophs display both lifestyles, depending on the stage of their life cycle (Pieterse et al., 2009).

Plants have two levels of immune system. One exploits transmembrane pattern recognition receptors that recognize microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs), to initiate PAMP-triggered immunity (PTI). The other uses the polymorphic NB-LRR (named after their characteristic nucleotide binding (NB) and leucine rich repeat (LRR) domains) protein products encoded by most *Resistance (R)* genes to specifically recognize corresponding pathogen effectors, resulting in effector-triggered immunity (ETI). ETI is an amplified and accelerated PTI response, usually associated with a hypersensitive cell death (HR) and a massive burst of reactive oxygen species (ROS) at the infection site (Jones and Dangl, 2006; Tsuda and Katagiri, 2010). The phytohormone salicylic acid (SA) is a small phenolic compound that plays important roles in both PTI and ETI. So far, it is believed that two major SA biosynthesis pathways exist in plants: the isochorismate (IC) and the phenylalanine ammonia-lyase (PAL) pathways (Dempsey et al., 2011; Seyfferth and Tsuda, 2014). Another important plant stress hormone is jasmonic acid (JA), which is derived from linolenic acid (Schaller and Stintzi, 2009). It usually acts in concert with ethylene (ET), a methionine-derived plant hormone, to protect plants against necrotrophic pathogens (Lin et al., 2009; Pieterse et al., 2009). The SA- and JA-signaling pathways usually act

antagonistically (Pieterse et al., 2009). Many plant defense compounds are derived from amino acid precursors, implying that amino acid homeostasis is important for plant immunity (Bednarek et al., 2009; Zeier, 2013).

Amino acid catabolism and plant immunity

In search of stress-inducible Arabidopsis UDP-dependent glycosyltransferase genes, von Saint Paul *et al.* found that *ugt76b1* knockout lines are more resistant to hemibiotrophic bacteria *P. syringae*, while less resistant to necrotrophic fungus *A. brassicicola*. Further analysis indicated that *ugt76b1* knockout lines accumulate more SA, while JA signaling pathway was repressed. *In vitro* assay showed that isoleucic acid (ILA) is the substrate of UGT76B1, and in animals, ILA originates from α-keto acids formed by transamination of isoleucine and valine by branched-chain amino acid transaminases (BCATs) (Mamer and Reimer, 1992). Though direct evidence showing that free ILA accumulates in plants is lacking, exogenous application of ILA on plants recapitulated the *ugt76b1* knockout mutant phenotype. Therefore, the authors proposed that UGT76B1 modulate plant immunity by glycosylation of BCAA derivatives (von Saint Paul et al., 2011).

Stomata are one of the most important entry sites for foliar bacteria pathogens, like *P. syringae* (Melotto et al., 2008). Stomata closure is mainly regulated by abscisic acid (ABA) and PTI (Melotto et al., 2006). When studying *flagellin*-

insensitve (*fin*) mutants, Macho *et al.* isolated *fin4*, which is a knock-down mutant of *ASPARTATE OXIDASE* (*AO*). AO catalyzes the oxidation of aspartate to iminosuccinate, the committed step in the *de novo* biosynthesis of nicotinamide adenine dinucleotide (NAD). The authors proposed that impaired AO activity reduces NAD biosynthesis. More importantly, it was found that AO is required for the PAMP-induced NADPH oxidase RBOHD-dependent ROS burst and stomata closure, which affects plant stomata immunity to foliar pathogens (Macho et al., 2012).

Amino acid anabolism and plant immunity

Many reports also support that perturbations in amino acid anabolism also modify plant defense. Knockdown mutants of *DOWNY MILDEW RESISTANT1 (DMR1)* were found resistance to the downy mildew *Hyaloperonospora arabidopsidis* (*Hpa*). *DMR1* encodes a homoserine kinase (HSK) which catalyzes the conversion of homoserine, leading to threonine and methionine synthesis in the aspartate-derived biosynthesis pathway. *Dmr1* mutants over-accumulate homoserine, which is undetectable in wild-type plants, while downstream amino acids are not affected in the mutant. Exogenous application of homoserine triggered plant resistance to *Hpa*, but the pathogen growth was not directly affected, suggesting that homoserine provides a novel form of downy mildew resistance (van Damme et al., 2009).

Mutants in *ASPARTATE KINASE2 (AK2)* and *DIHYDRODIPICOLINATE SYNTHASE2 (DHDPS2)*, critical enzymes in aspartate-derived biosynthesis pathway were found less susceptible to *Hpa*. The corresponding mutants over-accumulate the aspartate-derived amino acids methionine, threonine, and isoleucine and pretreatment of plants with threonine renders plant resistance to *Hpa*. The authors conclude that amino acid imbalance caused threonine accumulation, which is unfavorable for *Hpa* infection (Stuttmann et al., 2011).

Amino acid transport and plant immunity

Amino acid transporters also play a role in plant immunity. Liu *et al.* found that *lysine histidine transporter1* (*lht1*) knockout mutant are resistant to the hemibiotrophic fungus *Colletotrichum higginsianum* and the biotrophic fungus *Erysiphe cichoracearum* in a SA-dependent manner (Liu et al., 2010). LHT1 is a broad-spectrum, high affinity amino acid transporter important for root amino acid uptake (Hirner et al., 2006; Svennerstam et al., 2007). Based on amino acid sensitivity assays and the finding that glutamine accumulation is reduced in *lht1* mutant, Liu *et al.* hypothesized that glutamine is the main physiological substrate of LHT1. The authors further proposed that LHT1 and glutamine play a crucial role in plant immunity by regulating the cellular redox status (Liu et al., 2010).

Yang *et al.* discovered that overexpression of *CATIONIC AMINO ACID TRANSPORTER1* (*CAT1*) in Arabidopsis enhanced plant disease resistance to the

hemibiotrophic bacteria *Pseudomonas syringae* by activating the SA-signaling pathway. Plants overexpressing CAT1 also accumulate more lysine. Interestingly, *CAT1* expression was up-regulated upon *P. syringae* infection. Yang *et al.* suggested that overexpression of *CAT1* improved disease resistance by augmenting plant systemic acquired resistance (Yang et al., 2014).

A lysine catabolite, pipecolic acid, regulates plant systemic acquired resistance

Recent reports suggested that the lysine catabolite pipecolic acid (Pip) is a critical regulator of plant systemic acquired resistance (SAR) (Song et al., 2004a; Song et al., 2004b; Návarová et al., 2012; Vogel-Adghough et al., 2013). A metabolomics analysis showed that branched-chain amino acids, aromatic amino acids, histidine and lysine levels significantly increased in Arabidopsis leaves inoculated with *Pseudomonas syringae.* Additionally, lysine catabolites, Pip and α-aminoadipic acid (Aad) strongly accumulate in infected leaves. However, metabolic changes were minimal in un-inoculated distal leaves, with the only exception that Pip content increased substantially. Song et al. suggested that Pip is made from lysine by AGD2-like defense response protein1 (ALD1) (Song et al., 2004a; Song et al., 2004b). The *ald1* mutant did not accumulate Pip and showed impaired SAR. When Pip was exogenously applied before pathogen infection, *ald1* mutant regained SAR and systemic SA accumulation, indicating a crucial role of

Pip in SAR activation upstream from SA biosynthesis. More importantly, Pip was specifically enriched in petiole exudates collected from inoculated leaves, suggesting that Pip can be mobilized from the infection site to distal plant parts. In summary, Návarová *et al.* found that Pip is a pivotal regulator for plant SAR (Návarová et al., 2012).

Regulation of amino acid metabolism and transport

A multi-level regulation of amino acid homeostasis

As detailed above (see Chapter 1.3), amino acid biosynthesis is a highly regulated process. Very often, the first anabolic enzyme in each pathway is feedback inhibited by the end-product, which makes it the committed step in the biosynthetic pathway. When the pathway branches, one end-product may also activates enzymes on the other branch(es) to redirect the flux to other end-products. Amino acid biosynthesis is also regulated at the transcript and post-transcriptional/translational levels. Numerous studies have shown changes in transcripts of genes encoding amino acid metabolic enzymes in response to stimuli (see review, Pratelli and Pilot, 2014). For example, the *ANTHRANILATE SYNTHASE* (*AS*) gene, which is involved in tryptophan biosynthesis, is induced by wounding, drought, free radicals, elicitors, *Pseudomonas syringae*, and $AgNO_3$ (Zhao and Last, 1996; Zhao et al., 1998). A comprehensive study using public Arabidopsis microarray data revealed that genes of the aromatic amino acid

pathway were the most responsive to stresses (Less and Galili, 2008). Less and Galili also suggested that the transcription of catabolic genes is usually more sensitive to stresses, compared to that of biosynthetic genes (Less and Galili, 2008). Besides regulations at the transcript level, protein modifications also play an important role: studies have found that phosphorylation/dephosphorylation regulates the activity of the lysine ketoglutarate reductase/saccaropine dehydrogenase (LKR-SDH) enzyme, involved in lysine degradation (Karchi et al., 1995; Miron et al., 2002; Zhu et al., 2002). In addition, PAL1 and PAL2, two phenylalanine ammonia-lyase catalyzing the first rate-limiting step in the phenylpropanoid pathway, are ubiquitinated and degraded by Kelch repeat F-box (KFB) proteins (Zhang et al., 2013).

Apart from the regulations of the amino acid metabolism, it is important to note that both amino acid metabolism and transport activity are coordinated in response to stimuli. One example of coordination is that ProTs and P5CS, involved in proline transport and biosynthesis, respectively, are both induced during drought and salt treatments (Hare et al., 1999). Modifications of amino acid transport also change amino acid profile: Arabidopsis mutants for the amino acid transporters LHT1 (Liu et al., 2010) and AAP1 (Sanders et al., 2009), as well as CAT1 over-expressor (Yang et al., 2014) showed altered amino acid profiles compared to the wild type.

Understanding the regulation of amino acid metabolism at multiple levels is the prerequisite of crop improvement and manipulations of plant secondary metabolism. For instance it can provide the potential to genetically engineer crops to get a better or more balanced nutrient level for consumption and livestock feeding. It can also help to understand the mechanism of herbicide action, since three enzymes of amino acid biosynthesis pathway are targets of herbicides (GS; AHAS; and 5-Enolpyruvylshikimate 3-Phosphate (EPSP) Synthase in the AAA pathway).

Amino acid sensing

Though it seems that amino acid metabolism and transport are highly regulated and coordinated, how plants sense amino acid status remains unclear. Walch-Liu *et al.* reported that exogenous micromolar concentrations of glutamate were able to modify root architecture through a sensing pathway active in the root tip (Walch-Liu et al., 2006). An integrated transcript and metabolite profiling on Arabidopsis in response to perturbations revealed that leucine plays an important role in regulating many stress-response genes (Hannah et al., 2010). These reports indicate that plants can sense external amino acids and respond accordingly.

While no amino acid sensor in plants has been reported to date, several candidates have been suggested, plant glutamate-like receptors (GLRs) for instance. Unlike their homologs in mammalian, ionotropic glutamate receptors

(iGluRs), plant GLR receptors show broad ligand specificities (Price et al., 2012; Forde and Roberts, 2014): at least 12 of the 20 proteogenic amino acids could act as plant GLR agonists, triggering membrane depolarization and calcium influx (Qi et al., 2006; Vincill et al., 2012; Tapken et al., 2013). While mammalian iGluRs are well known for their important roles in neurotransmission, the biological role of plant GLRs remained unclear until reports suggested their roles in plant defense response. *Glr3.3* mutants were more susceptible to the hemibiotrophic bacteria, *Pseudomonas syringae,* and the biotrophic oomycete pathogen, *Hyaloperonospora arabidopsidis*, suggesting that GLR3.3 plays a role in plant innate immunity (Li et al., 2013; Manzoor et al., 2013). Moreover, a screening of membrane protein mutants impaired in leaf-to-leaf wound signaling led to isolation of *GLRs 3.2*, *3.3*, *3.6* mutants. These mutants showed attenuated wound-induced surface potential changes, it thus appeared that at least some GLRs mediate leaf-to-leaf would signaling and long-distance jasmonate signaling (Mousavi et al., 2013). Summarizing the current literature, Forde and Roberts suggested that plant GLRs may sense amino acids and regulate plant defense response (Forde and Roberts, 2014).

While most nutrient sensors in mammals are G-protein coupled receptors (Wauson et al., 2013), some sensors appear to also mediate transport, thus called transceptors. The plant nitrate transporter NRT1.1 was characterized as a sensor

regulated by phosphorylation and dephosphrylation (Ho et al., 2009). The yeast amino acid permease, Gap1, also acts as a transceptor, mediating signaling to the protein kinase A pathway, without requiring a complete transport process (Van Zeebroeck et al., 2009). It may be interesting to see if any plant amino acid transporter may also act as sensor.

Is GLUTAMINE DUMPER1 involved in the regulation of amino acid transport?

Analysis of GDU1 over-expressors suggests a role in amino acid export

The *gdu1-1D* mutant was discovered by isolation of an *Arabidopsis thaliana* activation-tagged mutant that secreted crystals at the hydathodes. Analyzing the crystals revealed that the secretion is mainly composed of glutamine. Amino acid contents of the apoplasm wash fluid, the phloem and xylem saps were increased in *gdu1-1D*. This Gdu1D phenotype is due to the overexpression of *Glutamine Dumper1* (*GDU1*), encoding a 17-kD protein with homologs only in plants. In addition, *GDU1* is expressed in the vascular tissues of the root and stem. These data prompted the authors to hypothesize that *GDU1* affects an amino acid transport process (Pilot et al., 2004), but the biochemical function of GDU1 remained elusive.

Later studies showed that the *gdu1-1D* mutant was more tolerant to toxic levels of exogenous amino acids. For example, 10 mM of leucine, methionine, or

phenylalanine showed strong inhibitory effects on the growth of the wild type plants, but the *gdu1-1D* mutant grew normally in these conditions (Pratelli and Pilot, 2007; Pratelli et al., 2010). Whole plantlet amino acid uptake assays found that the uptake of several amino acids was reduced in the *gdu1-1D* mutant compared to wild type plants. More complete analyses showed that the reduced uptake was caused by increased amino acid export, but not modifications in import (Pratelli et al., 2010). *GDU1* over-expression leads thus to increase cellular amino acid export, suggesting that GDU1 is involved in this process. One possibility is that GDU1 directly mediates amino acid export. This hypothesis is unlikely, since GDU1 is a small protein with only one transmembrane domain, and amino acid transporters typically consist of several transmembrane domains to form a channel, allowing amino acids to go through. The other possibility is that GDU1 is somehow involved in the regulation of amino acid exporters. GDU1-GFP mainly localized in endosomes and at the plasma membrane, in agreement with a putative role in facilitating endosomal trafficking of a transporter to/from the plasma membrane (Pratelli et al., 2012).

GDU1 may be involved in other processes

The Gdu1D phenotype is pleiotropic: in addition to defects in amino acid content in saps, mutant plants are smaller compared to wild type plants; leaves are curled and darker green and tend to senescence early; and besides glutamine, the

content of several amino acids was increased in leaves. It is not clear at present if these modifications are directly related to GDU1 function or secondary effects of the disturbance in amino acid transport.

Besides *GDU1*, six Arabidopsis genes encode GDU1-like proteins with similar functions. The GDUs share two conserved domains, a transmembrane domain and a VIMAG domain. In most situations, overexpression of these *GDUs* increased amino acid content and reduced plant size. Similarly, the root growth of different *GDU* over-expressors was more tolerant to exogenously supplied amino acids at toxic concentrations (Pratelli et al., 2010).

The *gdu1-1D* mutant also shows lesions in leaves, early senescence, and activated defense response, such as ROS accumulation and increased *PR1* expression. The *gdu1-1D* mutant showed higher resistance to the hemibiotrophic fungus *Colletotrichum higginsianum* and the biotrophic fungus *Erysiphe cichoracearum*. Liu *et al.* speculated that glutamine deficiency in the cell may activate plant defense response via a SA-dependent manner (Liu et al., 2010). Similarly, overexpression of *GDU3*, a member of the GDU family, also boosted plant defense to viruses by activating SA pathway (Chen et al., 2010). These data suggest that GDU1 is not only involved in the regulation of amino acid export. The increased plant resistance in GDU over-expressors may be directly or indirectly

caused by augmented amino acid export. However, the mechanism of elevated plant immunity caused by perturbation in amino acid homeostasis is unclear. A comprehensive analysis of pathways that are involved in this process may provide some hints about GDU1's function.

An E3 ubiquitin ligase, LOG2, interacts with GDU1

In order to find genes involved in the same process as GDU1, an Ethylmethane sulfonate (EMS) suppressor screening of the Gdu1D phenotype was carried out. A mutation leading to a glycine to arginine (G100R) substitution in the VIMAG domain of GDU1, one of the two conserved domains, suppressed the Gdu1D phenotype. Contrary to the wild type GDU1, neither over-expression of G100R GDU1 nor ΔVIMAG GDU1 (in which the VIMAG domain had been deleted) was able to trigger the Gdu1D phenotype. These results suggest that the VIMAG domain in GDU1 is necessary for its function (Pratelli and Pilot, 2006). In parallel, a yeast-two-hybrid assay was also utilized to identify proteins that interact with GDU1. Coincidently, both EMS and yeast-two-hybrid screenings led to the isolation of the same gene, *LOSS OF GDU2* (*LOG2*). Further characterization of the interaction between LOG2 and GDU1 is described in Chapter 2.

Surprisingly, the *log2* mutant was also isolated from a screening of mutants with impaired ABA signaling during the germination stage (Kim and Kim, 2013). Transcript levels of *LOG2* were up-regulated by dehydration, high salinity and

ABA, suggesting a role in the response to these abiotic stresses. The *log2* mutant was less sensitive to ABA during germination and the ABA-mediated stomata closure was affected. Suppression of LOG2 led to hypersensitivity to drought and high salinity conditions, compared to wild type plants. A yeast-two-hybrid screening identified the membrane protein RESPONSIVE TO DEHYDRATION21 (RD21), which may be involved in the ABA-mediated drought stress response, as a LOG2 interactor (Kim and Kim, 2013). In agreement with its role in ABA-mediated drought stress response, another report found that LOG2 interacted with VrUBC1, a mung bean E2 Ubiquitin-Conjugating Enzyme, regulating ABA-related genes during osmotic stress (Chung et al., 2013). Therefore, LOG2 appears to be a dual-function player, involved in the regulation of amino acid export and ABA-mediated drought stress response.

Research objectives

Though LOG2 was identified as a GDU1 interactor by a yeast-two-hybrid assay, it was unclear whether GDU1 interacts with LOG2 *in planta*. Since LOG2 is an E3 ubiquitin ligase, it was expected that GDU1 is ubiquitylated by LOG2, but whether the ubiquitination is necessary for the Gdu1D phenotype needed to be addressed. Based on the increased plant immunity response in GDU1 over-expressors and the dual functions of LOG2 in mediating amino acid export and stress response, two hypotheses could be generated: the first is that over-expression

of *GDU1* increases amino acid export, which directly enhances plant defense response; the other is that overexpression of *GDU1* increases LOG2 activity, which triggers defense responses and elevated amino acid export.

The first objective of my doctoral work was to find the role of LOG2 in GDU1 over-expressors, and test whether *LOG2* is necessary for the Gdu1D phenotype.

The second objective was to better understand the biochemical function of GDU1. Since *GDU1* homologs are only present in plants and the sequence of GDU1 does not bring any information about its function, I tried to understand its structural function by characterizing mutations in GDU1 that suppress the Gdu1D phenotype. Doing so would provide us with clues about the structure-function relationship of GDU1, and possibly post-translational modifications.

The third objective of my work was to characterize the primary function of GDU1 in a phenotypic approach, by monitoring over time the responses of the plant to chemically-induced expression of *GDU1*. Analyzing the different aspects of the Gdu1D phenotype, such as enhanced amino acid export activity, imbalance in amino acid homeostasis, and increased plant defense response, would help us identify connections between amino acid homeostasis and plant defense responses. Characterization of the GDU1 role could also enrich our understanding of the regulation of amino acid transport and metabolism.

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Chapter II

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The ubiquitin E3 ligase LOSS OF GDU 2 is required for GLUTAMINE DUMPER 1- induced amino secretion in Arabidopsis

Introduction

Amino acids are the main form of organic nitrogen transported in the xylem and the phloem in plants (Peoples and Gifford, 1990). Besides a role in nitrogen transfer between plant organs, amino acids are important to coordinate shoot and root metabolism in response to environmental conditions. Cycling of amino acids between shoots and roots, successively transported by the phloem and the xylem, has been proposed to carry information about nitrogen status of roots or shoots to the other organ. In particular, amino acids have been shown to inhibit root nitrate uptake, adjusting inorganic nitrogen uptake to shoot demand (Miller et al., 2007).

Amino acid cycling involves successive transfers across the plasma membrane from symplasm (phloem and cytosol) to apoplasm (xylem and cell wall) and vice-versa. Because membranes are fairly impermeable to these solutes, both import and export are catalyzed by integral membrane proteins. Identified in early physiological studies (Li and Bush, 1990, 1992), amino acid importers are proton-gradient dependent transporters with broad amino acid specificities (Tegeder and Rentsch, 2010). In stark contrast to importers, molecular mechanisms of plasma membrane amino acid export remain largely unknown. While export across the plasma membrane has been measured at the physiological level (*e.g.* Secor and Schrader, 1984; De Jong et al., 1997; Lesuffleur and Cliquet, 2010), no transporter has yet been identified that mediates this process (reviewed in Okumoto and Pilot, 2011). The first cloned plant amino acid exporter, GABP, mediates GABA transport from the cytosol to the mitochondrion (Dundar and Bush, 2009; Michaeli et al., 2011). Because of its localization at the mitochondrial membrane, GABP is not expected to mediate amino acid export at the plasma membrane.

The first gene suggested to be involved in plasma membrane amino acid export is *GLUTAMINE DUMPER 1* (*GDU1*). The *gdu1-1D* mutant, which over-expresses *GDU1*, was isolated in an activation-tag screen for plants with altered hydathode function. *gdu1-1D* exhibited high free amino acid content in the phloem, xylem, and guttation stream, leading to glutamine crystallization at the hydathodes (Pilot

et al., 2004). The Gdu1D phenotype also entailed plant size reduction, constitutive necrotic lesions, and resistance to toxic concentrations of amino acids (Pilot et al., 2004; Pratelli and Pilot, 2007; Liu et al., 2010). *gdu1-1D* was later found to constitutively export amino acids from plant cells (Pratelli et al., 2010). *GDU1* encodes a small protein with a single transmembrane domain. Paralogs are found in Arabidopsis (named *GDU2-GDU7*), and homologs are present in higher and lower plant genomes (Pratelli and Pilot, 2006). The physiological function of the *GDU* family is unknown, but over-expression of any *GDU* gene causes phenotypes reminiscent of Gdu1D (Pratelli et al., 2010). Apart from the membrane domain, GDU proteins also share a VIMAG domain (representing the amino acids Val-Ile-Met-Ala-Gly). The *log1-1* allele of GDU1 ($GDU1^{G100R}$, in which the VIMAG glycine is mutated to arginine) was shown to suppress the Gdu1D phenotype (Pratelli and Pilot, 2006), suggesting that the VIMAG domain is essential for GDU1 function. The molecular basis for this suppression was not determined.

In spite of its effect on amino acid export, the predicted structure of GDU1 makes it unlikely to be a transporter (Pilot et al., 2004), and it had been suggested that GDU1 could be a transporter subunit (Pratelli et al., 2010), by analogy to mammalian heteromeric amino acid transporters (Palacin et al., 2005). Small, single transmembrane domain proteins have also been shown to be involved in the organization of large membrane protein complexes (reviewed in Zickermann et al.,

2010). Finding interacting partners of GDU1 is therefore a necessary step to gain insight about its role. To this end, we performed yeast-two-hybrid and ethyl-methanesulfonate (EMS) suppression screens. We report here that both of these approaches uncovered the same RING-type ubiquitin E3 ligase, subsequently named LOSS OF GDU 2 (LOG2). In a survey of Arabidopsis RING finger-containing E3s, LOG2 was shown to exhibit *in vitro* E3 activity, but the biological function of LOG2 was not investigated in that study (Stone et al., 2005).

E3 ligases facilitate the covalent attachment of the small protein ubiquitin (Ub) to other proteins (ubiquitination). Ubiquitination is an efficient and highly specific mechanism by which intracellular protein activity, localization, and/or stability are governed in eukaryotes. Following ATP-dependent activation by Ub activating enzyme (UBA or E1) and *trans*-thioesterification to a Ub conjugating enzyme (UBC or E2), substrate ubiquitination is catalyzed by E3 Ub ligases (E3s) (Deshaies and Joazeiro, 2009). E3s bind protein substrates and E2-Ub thioesters in a conformation that facilitates ubiquitin transfer to substrates. In the case of RING-type E3s, the RING domain enables interaction with E2s (Joazeiro et al., 1999). In addition to promoting nuclear and cytosolic protein degradation via the 26S proteasome, ubiquitination by E3s can also regulate plasma membrane protein abundance via lysosomal/vacuolar proteolysis (Komander, 2009; Leon and Haguenauer-Tsapis, 2009).

Here, we report that the ubiquitin ligase LOG2 interacts with GDU1 *in vitro* and *in planta*, and that reduction of *LOG2* expression suppresses the Gdu1D phenotype. In addition, the previously described *log1-1* mutation abolishes GDU1 interaction with LOG2. Altogether these data support a model whereby LOG2 and its interaction with GDU1 are required for the observed increased amino acid export observed upon *GDU1* over-expression.

Results

GDU1 interacts with LOG2, a RING domain-containing protein

The physiological consequences of *GDU1* over-expression have been studied extensively, but the mechanism by which GDU1 activates amino acid efflux remains unclear. GDU1 is unlikely to be a transporter, thus it must interact with other proteins to activate efflux. A screen for interacting proteins was performed using a yeast-two-hybrid strategy, in which the region C-terminal to the putative transmembrane domain of GDU1 (cGDU1, amino acids 61-158) was used as bait against an Arabidopsis cDNA library. Three clones, whose inserts contained partial ORFs of genes *At3g09770* and *At5g03200* (data not shown), were shown to restore yeast prototrophy when co-expressed with cGDU1 in the yeast two-hybrid screen. *At3g09770* and *At5g03200* encode members of the same subfamily of RING finger ubiquitin E3 ligases (Kraft et al., 2005; Stone et al., 2005). *At3g09770* and *At5g03200* were respectively named *LOG2 (LOSS OF GDU 2*, see below) and

LUL1 (*LOG2-LIKE UBIQUITIN LIGASE 1*). The three other paralogs were designated *LUL2* (*At3g53410*), *LUL3* (*At5g19080*) and *LUL4* (*At3g06140*). Full length LOG2 and LUL1 also interacted with cGDU1 in the yeast-two-hybrid assay (Fig. 2-1A). Yeast co-expressing LUL1 and cGDU1 grew slower than yeast co-expressing LOG2 and cGDU1, while the proteins were expressed at similar levels (data not shown). This observation was consistent with the activity of the lacZ reporter gene. β-galactosidase activity of yeast cells was 0.14 ± 0.13 nmol $ONP.h^{-1}.10^{-7}$ cells when cGDU1 was co-expressed with LUL1, and 3.02 ± 0.18 nmol $ONP.h^{-1}.10^{-7}$ cells when co-expressed with LOG2, suggesting that the interaction between cGDU1 with LUL1 was weaker compared to that with LOG2.

In addition to the presence of a C-terminal C3HC4 RING finger domain and a predicted N-terminal myristoylation site (see below), the five LOG2 subfamily members contain a functionally-uncharacterized region N-terminal to the RING finger that was previously designated DAR2 (Domain Associated with RING #2; Stone et al., 2005). LOG2-like genes are present in the genomes of diverse taxa, including monocots, lower plants, and mammals but surprisingly not in fungi. While LOG2 function has not been studied in plants, the mammalian homolog MGRN1 (MAHOGUNIN RING FINGER 1) plays an unclear role in the down-regulation of melanocortin signaling and the maturation of endosomes (He et al., 2003; Kim et al., 2007; Cooray et al., 2011). MGRN1 contains a large C-terminal

domain not found in plant LOG2/LUL proteins (data not shown) that seems to play an important role in endosomal trafficking (Kim et al., 2007) suggesting divergence in function from plant LOG2/LULs.

The interaction of cGDU1 with LOG2 and LUL1 was tested by glutathione S-transferase (GST) *in vitro* pull down assays. The interaction of GST-LOG2 with cGDU1 was the only one to be detected (Fig. 2-1B, top panel, lane 2), indicating that cGDU1 binds directly to LOG2 and more strongly to LOG2 than to LUL1 in this assay. The interaction between GDU1 and LOG2 was then tested *in planta* using proteins transiently expressed in *Nicotiana benthamiana* leaves. Wild type LOG2 was expressed at levels too low to be suitable for co-immunoprecipitation experiments (data not shown). Hypothesizing that auto-ubiquitination may contribute to protein instability, we generated a LOG2 mutant with a catalytically-inactive RING domain ($LOG2^{CC354+357AA}$, called mLOG2, see below and Fig. 2-2C), which could successfully be expressed at high levels. Full length GDU1 and mLOG2 with C-terminal Myc or HA tags were then co-expressed in tobacco leaves, and the Myc-fused protein was immunoprecipitated. Independent of which protein contained the Myc epitope tag, GDU1 and mLOG2 co-immunoprecipitated (Fig. 2-1C, lanes 1 and 3), showing that the interactions detected by yeast-two-hybrid and *in vitro* assays are observed *in planta* as well.

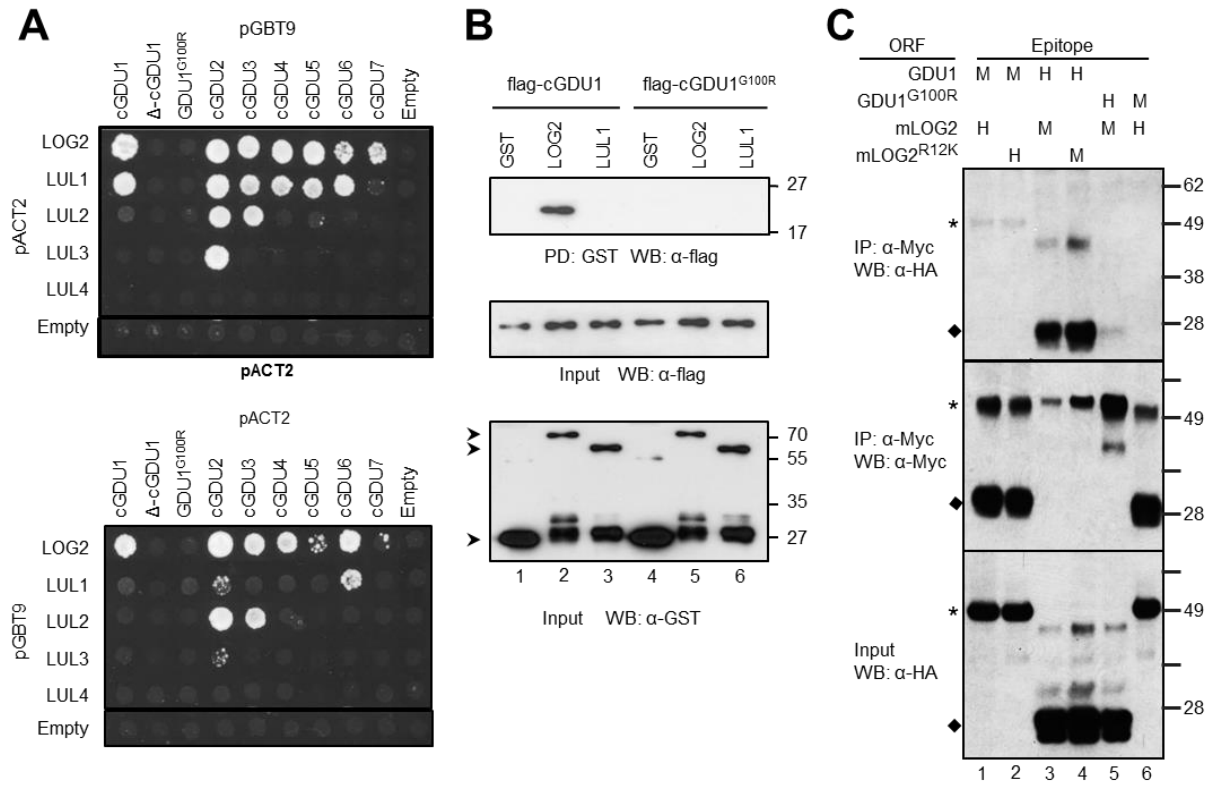

Figure 2-1. Interaction assays between GDU family members and the E3 ubiquitin ligases of the LOG2 family.

A. Yeast two-hybrid interaction of the cytosolic domain of the GDU proteins (cGDU) with LOG2 and LULs. The panels show swapping of inserts between the bait (pGBT9) and prey (pACT2) plasmids. Yeasts co-expressing the protein pairs were grown for four days on medium selecting for protein interaction, lacking leucine, tryptophan, adenine and histidine. All yeasts grew on a medium lacking leucine and tryptophan, selecting for the plasmids only (not shown).

B. GST pull-down (PD) assay of flag-cGDU1 or flag-cGDU1^{G100R} with GST-LOG2, GST-LUL1 or GST alone. Top, pulled-down samples; middle, cGDU input; bottom, GST protein input. Arrowheads indicate GST (~27 kDa) or full-length GST-tagged LOG2 and LUL1.

C. Co-immunoprecipitation (IP) assay after expression of GDU1 or GDU1^{G100R} and mLOG2 or mLOG2^{R12K} in transiently infiltrated *N. benthamiana* leaves. Top, Myc IP samples probed with α-HA; middle, Myc IP samples probed with α-Myc; bottom, HA-protein input. Asterisks and diamonds indicate LOG2 and GDU1 proteins, respectively.

Numbers on the right (B and C) indicate molecular weight in kDa.

The GDU1-LOG2 interaction is affected by a suppressor mutation in GDU1

It had previously been reported that the EMS-generated *log1-1* mutant, corresponding to a G100R substitution in the VIMAG motif of GDU1, suppresses multiple features of the Gdu1D phenotype (Pratelli and Pilot, 2006, 2007).

Although the C-terminal domains of the seven Arabidopsis GDUs show limited sequence conservation outside the VIMAG domain, all cGDUs could interact with LOG2 in yeast, and most could interact with LUL1 (Fig. 2-1A). This suggested that the interaction with LOG2 is mediated through the VIMAG domain. To test the hypothesis that the G100R mutation alters the interaction with LOG2, the interactions of cGDU1^{G100R} (log1-1) and Δ-cGDU1 (in which the whole VIMAG domain has been deleted) with LOG2 and LULs were tested using the yeast two-hybrid system. While these mutations did not alter protein abundance (data not shown), neither log1-1 nor Δ-cGDU1 could interact with LOG2 or LUL1 (Fig. 2-1A). Consistent with these results, flag-tagged cGDU1^{G100R} failed to interact with GST-LOG2 in a GST pull-down assay (Fig. 2-1B, lane 5), and co-immunoprecipitation of GDU1^{G100R} and mLOG2 expressed in *N. benthamiana* was greatly reduced (Fig. 2-1C, lanes 5 and 6). These data indicate that the GDU1-LOG2 interaction requires the conserved VIMAG domain of GDU1 and that suppression of the Gdu1D phenotype observed in *log1-1* plants (Pratelli and Pilot, 2006) may result from loss of interaction with LOG2.

cGDUs interact with LOG2 and LULs proteins

To systematically characterize interactions between the LOG2/LUL and GDU families, yeast-two-hybrid assays were conducted between the C-terminal domains of all Arabidopsis GDUs and LOG2/LUL proteins. In contrast to LOG2, cGDU-

LUL interactions were typically specific for a pair of bait-prey combinations (Fig. 2-1A). LUL2 and LUL3 interactions were observed only for cGDU2 or cGDU3, and LUL4 did not interact with any cGDU. As expected, deletion of the VIMAG motif from cGDU1 abolished all LUL interactions. The interaction between GDU and LOG2-like proteins appears facilitated by the VIMAG motif of the GDUs and a conserved domain among LOG2 homologs (most likely the DAR2, see below).

LOG2/LULs exhibit E3 ligase activity, but cGDU1 is ubiquitinated exclusively by LOG2 *in vitro*

LOG2 had previously been shown to polymerize ubiquitin *in vitro* (Stone et al., 2005). To examine the E3 activities of the LOG2 paralogs, GST fusions of LOG2 and LUL1-4 were expressed in *E. coli*, purified, and assayed with ubiquitin pathway proteins (E2, E1 and ubiquitin). Mirroring previously published results with LOG2 (Kraft et al., 2005; Stone et al., 2005), LUL1-4 were active, forming high molecular weight ubiquitinated proteins in the presence of all ubiquitin pathway components, while omission of the E2 prevented their production (Fig. 2-2A).

Only the proteins demonstrated to interact with GDU1, i.e. LOG2 and LUL1 (Fig. 2-1), were tested for ubiquitination of cGDU1 using *in vitro* assays. Only LOG2 could ubiquitinate cGDU1 above the background of the E3 reaction (Fig. 2-2B). Substitution to alanine of two zinc-coordinating cysteine residues (mLOG2)

or an isoleucine residue in the RING domain (LOG2^{I321A}) were previously described for other RING E3s to respectively abolish E3 activity or hinder association with E2s (Lorick et al., 1999; Brzovic et al., 2003). As predicted, these modifications of LOG2 led to abolished or compromised cGDU1 ubiquitination, respectively (Fig. 2-2C). Interestingly, truncation of the non-conserved 125 amino acid region N-terminal to DAR2 did not impede cGDU1 ubiquitination by LOG2 (Fig. 2-2C). cGDU1^{G100R}, log1-1, was then assayed with or without LOG2-V5-His$_6$ in the presence of ubiquitin pathway components. While cGDU1 formed ladders in the presence of LOG2, only weak monoubiquitination of cGDU1^{G100R} was observed (Fig. 2-2D). These data show that the decreased interaction of cGDU1^{G100R} with LOG2 (Fig. 2-1B) results in decreased *in vitro* ubiquitination of cGDU1^{G100R}, and suggest that the specificity determinants of the GDU1-LOG2 interaction lie in the DAR2 of LOG2.

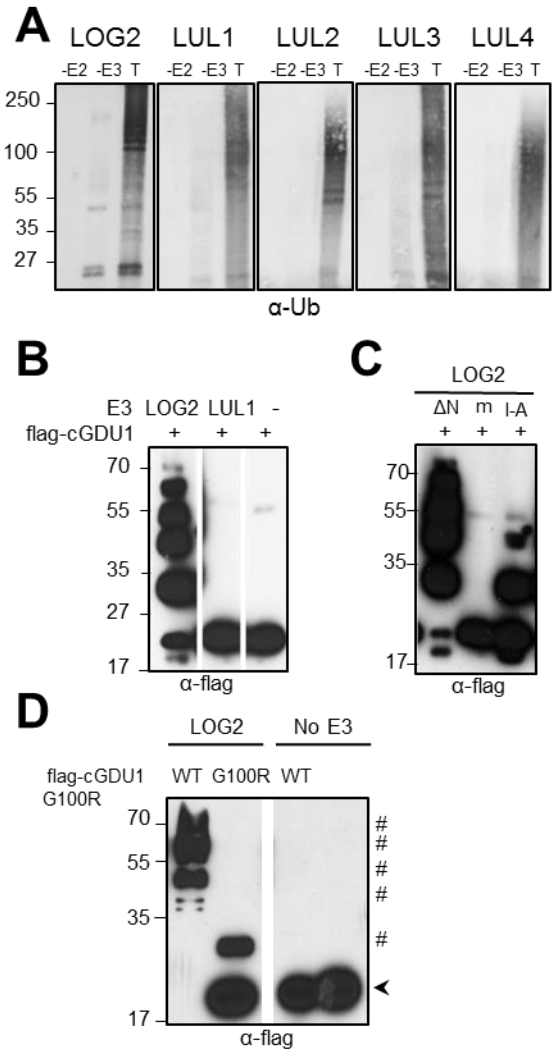

Figure 2-2. Ubiquitin ligase activity tests of LOG2 and LULs.

A. *In vitro* ubiquitination assay of GST-LOG2 and GST-LUL1-4 visualized with an anti-ubiquitin antibody. -E2, -E3, omission of ubiquitin conjugating enzyme (E2) or the indicated ubiquitin ligase (E3) from the assay, respectively; T, all ubiquitin pathway components were present in the assay.

B. *In vitro* ubiquitination assay of flag-cGDU1 by GST-LOG2, GST-LUL1 or no added E3, visualized with anti-flag antibody

C. *In vitro* ubiquitination assay of flag-cGU1 by truncated and mutant forms of GST-LOG2.; ΔN, a LOG2 truncation lacking amino acids N-terminal to the DAR2; m, $LOG2^{CC354/347AA}$ (RING-dead); I-A, $LOG2^{I321A}$ (RING-E2 interaction impaired).

D. *In vitro* ubiquitination assay of either flag-cGDU1 or flag-$cGDU1^{G100R}$ by LOG2-V5. Arrowhead, non-ubiquitinated flag-cGDU1; #, ubiquitinated flag-cGDU1.

Numbers on the left indicate molecular weight in kDa.

Similarly to *GDU1*, *LOG2* is expressed in the vasculature

The interaction between GDU1 and LOG2, confirmed so far *in vitro* and after co-expression *in planta*, is physiologically relevant only if the two proteins are expressed in the same cell types. To investigate LOG2 expression pattern, the *LOG2* promoter region fused to the coding sequence of the *uidA* gene from *E. coli*, encoding β-glucuronidase (GUS), was introduced into the Arabidopsis genome. GUS activity was detected in vascular tissues of roots, leaves and stems (Fig. 2-3A-D), and more precisely in both phloem and xylem parenchyma cells, as revealed by stem cross sections (Fig. 2-3C). The *LOG2* promoter was active in all shoot cells: a light background staining appeared in leaves and in non-vascular parenchyma cells of the stems (Fig. 2-3A and 2-3C). Examination of lightly stained plants showed that this background staining did not result from diffusion of the product of the histochemical reaction out of the vascular tissues (data not shown). Presence of staining in roots up to the division zone indicated that the *LOG2* promoter is active in the root phloem (Fig. 2-3D). Interestingly, the *LOG2* promoter was only active in cells from the root stele and root tip; even with longer reaction time, no staining could be observed in the cortex and epidermis (Fig. 2-3D and 2-3E). In reproductive organs, strong GUS activity was detected in the style, connective tissue, and the base of the flower (Fig. 2-3F and 2-3G), which persisted along the development of the silique (data not shown). Finally, the *LOG2* promoter

was active in pollen grains (Fig. 2-3H). The observed expression pattern is in good agreement with quantitative RT-PCR results (data not shown), the AtGenExpress dataset (Schmid et al., 2005) and the Arabidopsis Gene Expression Database (Cartwright et al., 2009), where stronger expression was detected in vasculature-rich tissues (stems), pollen, columella and phloem and xylem cells (data not shown). The expression pattern of *LOG2* largely overlaps with that of *GDU1*, shown to be expressed in the vasculature of roots, stems and petioles (Pilot et al., 2004).

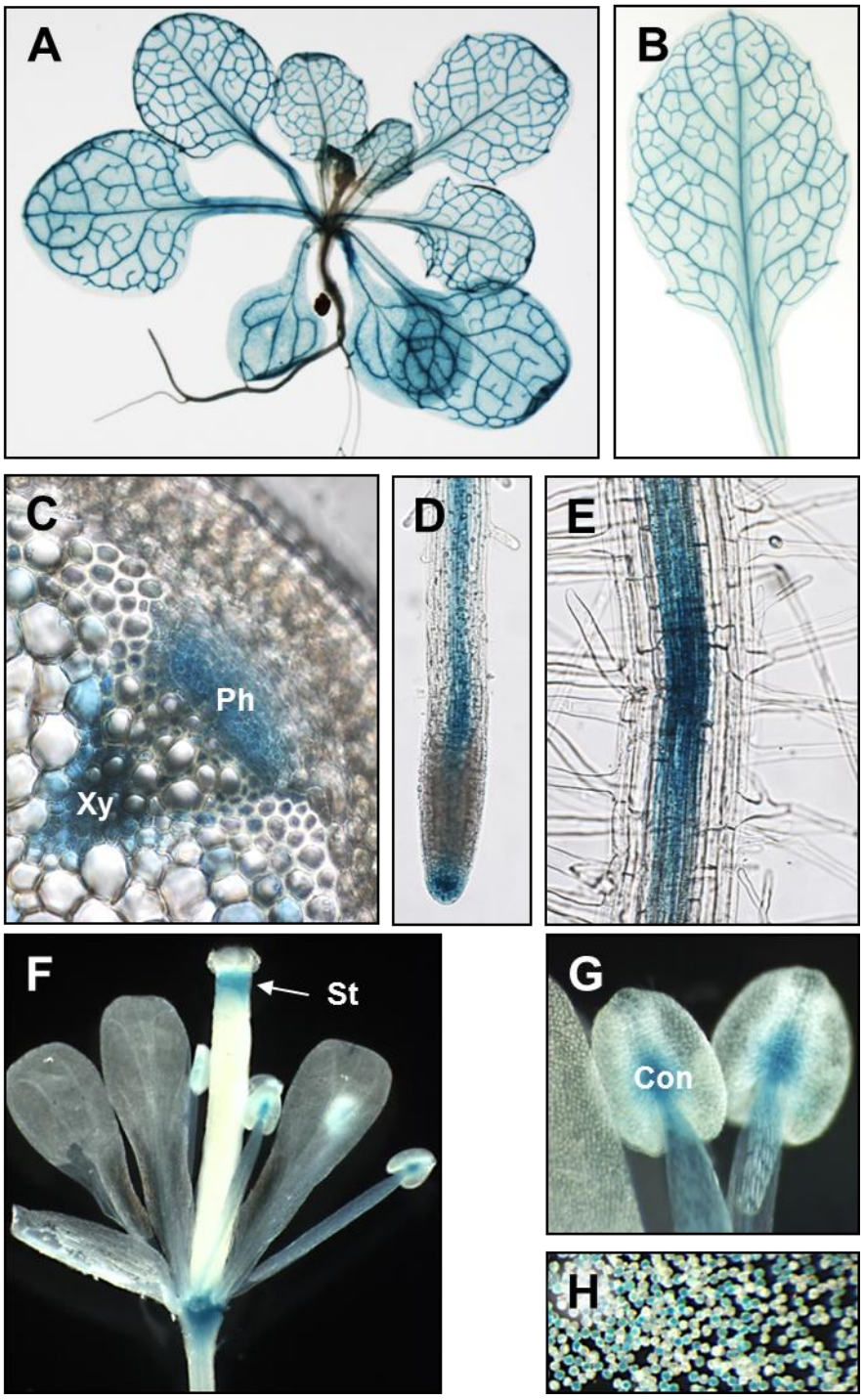

Figure 2-3. *LOG2* promoter-GUS analysis.
A. Rosette. **B.** Leaf. **C.** Stem cross section. **D and E.** Root. **F.** Flower. **G.** Stamens. **H.** Pollen grain. Xy: xylem; Ph: phloem ; St: style; Con: connective.

GDU1 and LOG2 localize to microsomes and are enriched in the plasma membrane

GDU1 is predicted to be a single pass transmembrane domain protein (Pilot et al., 2004). In order to interact with GDU1, LOG2 needs to be recruited to the same membrane(s). Confocal imaging of Arabidopsis protoplasts transiently expressing GDU1-GFP had suggested that GDU1 was targeted to the plasma membrane (Pilot et al., 2004). We created transgenic Arabidopsis that over-expressed Myc-tagged GDU1 under the control of the CaMV 35S promoter. One line, designated 35S-GDU1-Myc, showed stable expression of GDU1-Myc over several generations and recapitulated the smaller leaf size and over-accumulation of amino acids seen in the activation-tagged *gdu1-1D* line (data not shown). To examine the subcellular localization of GDU1, rosette leaf proteins were fractionated into cytosolic and total microsome (TM) fractions, after which TMs were further processed into plasma membrane-depleted vesicles (PDVs) and plasma membrane-enriched vesicles (PEVs). GDU1-Myc was highly enriched in the microsomal fraction compared to the soluble fraction confirming membrane localization (Fig. 2-4A, lanes 1 and 2). Similar to the plasma membrane localized PMA2 used as a control (Dambly and Boutry, 2001; Elmore and Coaker, 2011), GDU1 was enriched in PEVs compared with PDVs (Fig. 2-4A, lanes 3 and 4), indicating enrichment at the plasma membrane. Consistent with these results, GDU1-GFP transiently expressed in tobacco epidermal cells localized at the plasma membrane and in small dots

(Fig. 2-5A), which could be labeled with the endosome marker FYVE-GFP (Voigt et al., 2005; Fig. 2-5B). Limited overlap was found with fluorescent markers specific to the Golgi apparatus, and no localization could be detected in mitochondria, chloroplasts, the endoplasmic reticulum, lysosomes, or the cytosol (data not shown).

To explore the membrane association of GDU1, microsomes from 35S-GDU1-Myc leaves were isolated and treated with sodium chloride, alkaline sodium carbonate, or Triton X-100 detergent, i.e. reagents that extract peripheral, lumenal, or integral membrane proteins, respectively (Santoni et al., 1999; Rolland et al., 2006). GDU1 was retained in the microsomal pellet after incubation with salt or base, whereas it was solubilized by the detergent (Fig. 2-4B). In accordance with earlier characterizations of protein-membrane interactions (Santoni et al., 1999; Rolland et al., 2006), this result indicates that GDU1 is an integral membrane protein.

LOG2 was found to interact directly with cGDU1 *in vitro* (Fig. 2-1B) and to co-immunoprecipitate with GDU1 *in planta* (Fig. 2-1C), but unlike GDU1, LOG2 lacks a predicted transmembrane domain. To determine whether LOG2 is membrane-associated, microsomes, PDVs, and PEVs were prepared from Arabidopsis stably expressing LOG2-HA under the control of the CaMV 35S

promoter (Fig. 2-4C) or after transient expression in tobacco leaves (Fig. 2-4D, left). Mirroring the localization profile of GDU1, LOG2 was found primarily in the total microsomal fraction and enriched in PEVs. In accordance with these data, expression in *N. benthamiana* epidermal cells of LOG2-GFP lead to a continuous fluorescence at the cell periphery, suggesting plasma membrane localization (Fig. 2-5C, left). This pattern was identical to the pattern obtained with BRI1, a known plasma membrane protein (Friedrichsen et al., 2000). Microsomal LOG2-Myc expressed in tobacco was, like GDU1, resistant to NaCl, but some protein was released with alkaline sodium carbonate and Triton X-100 detergent, indicating a weaker association with the membrane than GDU1 (Fig. 2-4E, left).

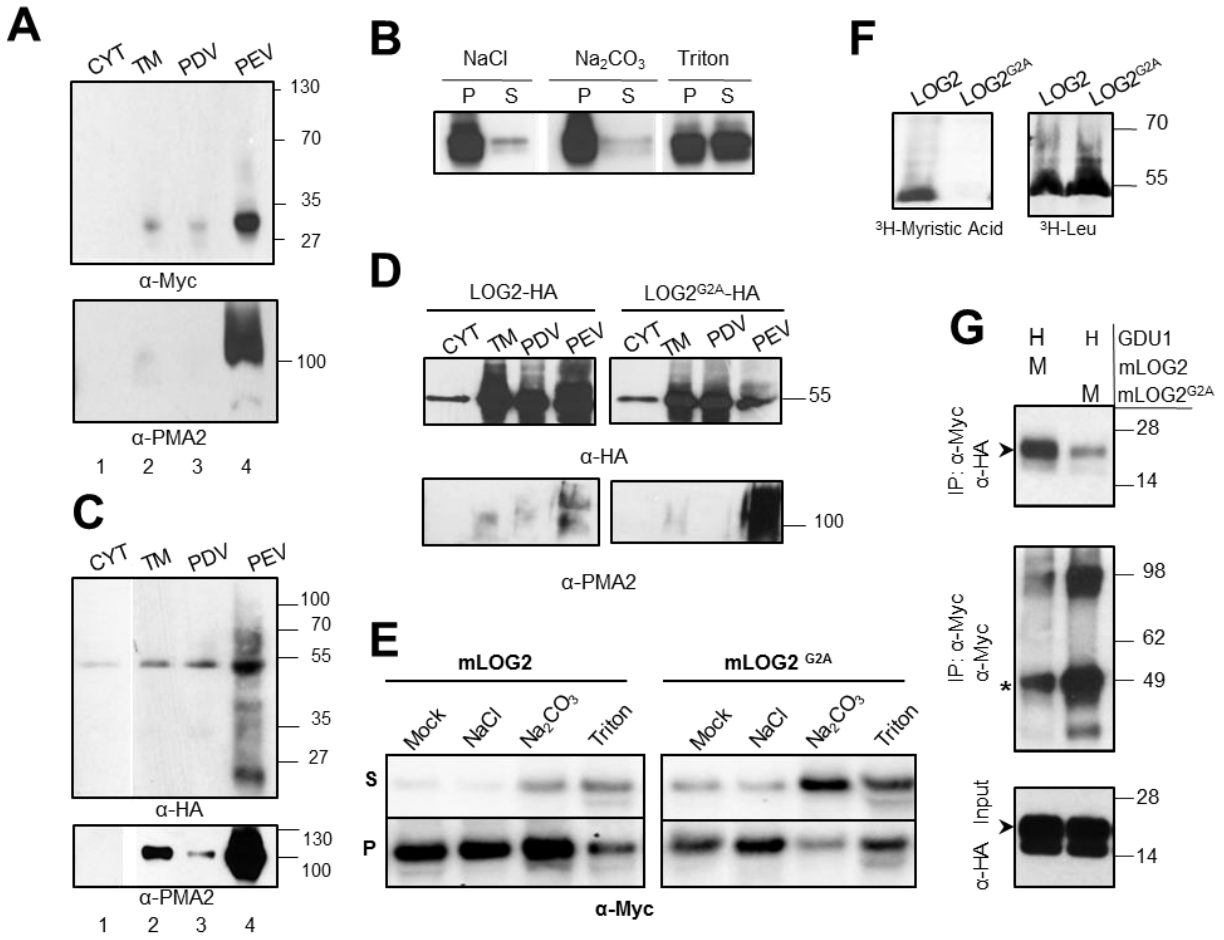

Figure 2-4. Analysis of membrane localization and association of GDU1 and LOG2.

A. and **C**. Subcellular fractionation of transgenic Arabidopsis expressing GDU1-Myc (A) or LOG2-HA (C). Fractionation of plasma-membrane localized PMA2 in respective preparations (bottom). CYT, cytosolic fraction; TM, total microsomes; PDV, plasma membrane depleted vesicles; PEV, plasma membrane enriched vesicles.

B. Sensitivity of microsomal GDU1-Myc expressed in Arabidopsis to 1 M NaCl, 0.1 M Na_2CO_3 pH 11.5, or 1 % (v/v) Triton X-100. S, supernatant; P, pellet.

D. Subcellular fractionation of LOG2-HA and $LOG2^{G2A}$-HA after transient expression in *N. benthamiana*. Abbreviations : see A and C.

E. Sensitivity of microsomal LOG2 and $LOG2^{G2A}$ to 1 M NaCl, 0.1 M Na_2CO_3 pH 11.5, or 1 % (v/v) Triton X-100 after transient expression in *N. benthamiana*. S: supernatant; P, pellet.

F. *In vitro* transcription/translation assay reactions incubated with either ^{3}H-myristic acid (left) or ^{3}H-leucine (right) using plasmids expressing either wild type LOG2 or LOG^{G2A}.

G. Co-immunoprecipitation (IP) after transient expression of GDU1-HA and mLOG2-Myc or $mLOG2^{G2A}$-Myc in *N. benthamiana* leaves using an anti-Myc antibody. Top, Myc IP samples probed with α-HA; middle, Myc IP samples probed with α-Myc; bottom, HA-protein input. Asterisks and arrowheads indicate LOG2 and GDU1 proteins, respectively.

Numbers on the right indicate molecular weight in kDa.

Myristoylation of LOG2 is important for its membrane localization and the GDU1-LOG2 interaction

We reasoned that membrane association of LOG2 could result from covalent lipid modifications such as myristoylation, prenylation or palmitoylation. Myristoylator (Bologna et al., 2004) and NMT (http://mendel.imp.ac.at/myristate/) predicted N-myristoylation sites for LOG2 and LUL1-4. Moreover, *in vitro* myristoylation of LUL1 was recently demonstrated (Yamauchi et al., 2010). To experimentally verify that LOG2 could be myristoylated, LOG2 and the corresponding G2A mutant (bearing a substitution known to inhibit N-terminal myristoylation (Gordon et al., 1991)) were expressed in rabbit reticulocyte lysate coupled transcription-translation systems in the presence of ^{3}H-myristic acid or ^{3}H-leucine. These lysates contain the enzymes necessary for myristoylation (Heuckeroth et al., 1988). Both proteins were expressed at similar levels, and LOG2 incorporated ^{3}H-myristic acid. As expected, LOG2^{G2A} was not myristoylated (Fig. 2-4F). Similar to LUL1 and LOG2, LUL3 was also myristoylated in a Gly2-dependent manner (data not shown), suggesting that all LOG2-like proteins are myristoylated *in planta*.

To determine whether myristoylation affects LOG2 localization *in planta*, plasma membrane vesicles were prepared from *N. benthamiana* transiently expressing mLOG2-HA or mLOG2^{G2A}-HA. While both proteins were found

primarily in the microsomal fraction, mLOG2^{G2A} was depleted from PEVs compared to wild type mLOG2 (Fig. 2-4D), suggesting that LOG2 myristoylation is important for localization to the plasma membrane. Extraction of mLOG2^{G2A} with sodium carbonate and Triton X100 was markedly enhanced compared to mLOG2 (Fig. 2-4E), showing that the G2A mutation reduced the strength of binding to the membrane. While wild-type LOG2-GFP located exclusively at the plasma membrane in *N. benthamiana*, LOG2^{G2A}-GFP also localized in the cytoplasm (Fig. 2-5C, middle). In accordance with biochemical data (Fig. 2-4D), the fluorescence pattern of LOG2^{G2A}-GFP overlapped extensively with cytosolic mCherry (Fig. 2-5D), suggesting that suppression of myristoylation prevented a fraction of LOG2 from being anchored to the membrane.

To assess the effect of the G2A mutation on the GDU1-LOG2 interaction *in planta*, GDU1-HA was co-expressed with mLOG2-Myc or mLOG2^{G2A}-Myc in *N. benthamiana*, and Myc-tagged proteins were immunoprecipitated as in Fig. 2-1C. GDU1 co-immunoprecipitated with less efficiency with mLOG2^{G2A} than mLOG2 (Fig. 2-4G), indicating that myristoylation enhances LOG2 interaction with GDU1 *in planta*. The localization of the LOG2-GDU1 interaction was further studied by expressing in *N. benthamiana* mLOG2 and GDU1 fused respectively to mCherry and GFP. LOG2-expressing *Agrobacterium* strains were infiltrated at low density, leading to heterogeneous expression in the epidermis, while GDU1-expressing

Agrobacterium was infiltrated at a density enabling expression in all cells. GDU1 localized at the plasma membrane and in endosomes in cells expressing mLOG2 at low levels (middle cell of Fig. 2-5E), but mainly at the plasma membrane in cells expressing mLOG2 at higher levels (lateral cells of Fig. 2-5E). This change in localization pattern suggests that interaction of LOG2 and GDU1 stabilized GDU1 localization at the plasma membrane.

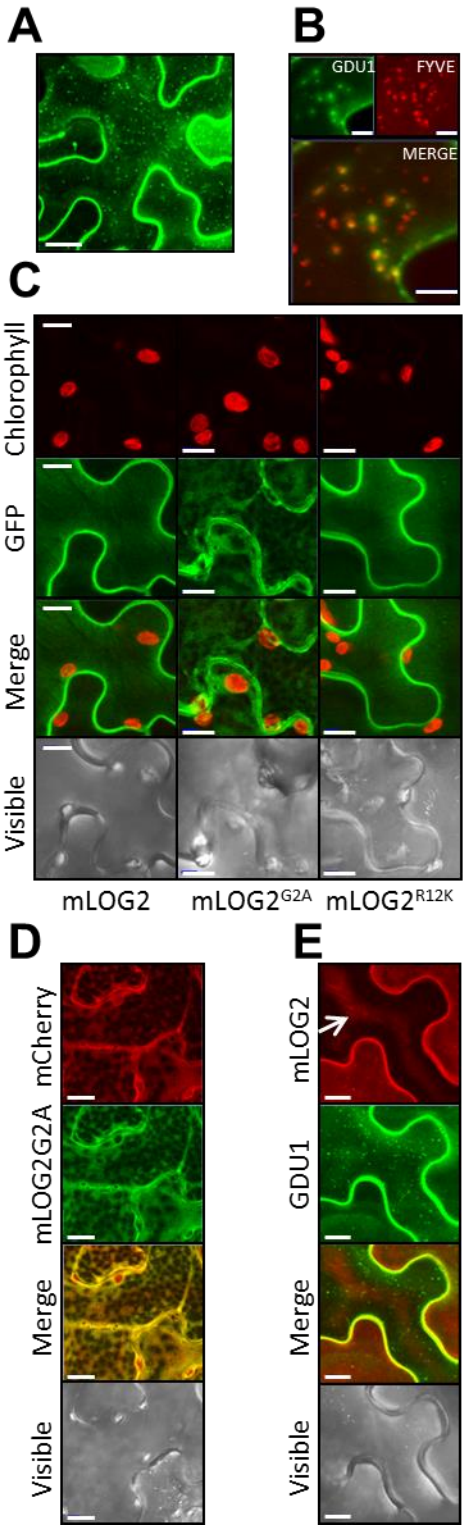

Figure 2-5. Subcellular localization of GDU1, LOG2 and LOG2 variants.

All proteins were transiently expressed in *N. benthamiana* epidermis cells. Images are maximum projections of optical sections of the abaxial side of cells, obtained by confocal microscopy. Bars = 5 µm.
A. Localization of GDU1-GFP protein.
B. Co-localization of GDU1-GFP and FYVE-RFP, an endosome marker.
C. Localization of mLOG2-GFP, mLOG2^{G2A}-GFP and mLOG2^{R12K}-GFP.
D. Co-localization of mLOG^{G2A} with cytosolic mCherry.
E. Change of localization of GDU1-GFP when co-expressed with mLOG2-mCherry. The middle cell (arrow) expressed mLOG2-mCherry at low level while the two lateral cells expressed mLOG2-mCherry at higher levels. All three cells expressed GDU1-GFP. Central fluorescence of the middle cell is potentially due to internal reflection.

T-DNA disruption of *LOG2* suppresses the Gdu1D phenotype conferred by GDU1 over-expression.

The observation that disruption of the LOG2-GDU1 interaction by the *log1-1* mutation suppresses the Gdu1D phenotype (Pratelli and Pilot, 2006) suggested that LOG2 is involved in the pathway altered by *GDU1* over-expression. To test this hypothesis, *gdu1-1D* was crossed with a plant carrying a T-DNA insertion (*log2-2*) in the first intron of *LOG2*. Reduction of *LOG2* wild type mRNA in the homozygous *log2-2* mutant was confirmed by RT-PCR (data not shown). In *log2-2 gdu1-1D* double homozygous plants, *GDU1* mRNA levels remained unaffected by the *log2-2* mutation (data not shown). Five-week old *log2-2* and wild-type plants had similar rosette sizes, while as previously observed, *gdu1-1D* individuals exhibited characteristically smaller rosettes (Fig. 2-6A). In contrast to *gdu1-1D* segregants, *gdu1-1D log2-2* double mutant plants developed rosettes similar in size to wild-type (Fig. 2-6A) and grew to normal height (data not shown) indicating suppression of the Gdu1D growth phenotype in the *log2-2* background.

gdu1-1D plants have been shown to be more tolerant to toxic concentrations of amino acids compared to wild type (Pratelli and Pilot, 2007). To determine whether this other aspect of the Gdu1D phenotype is suppressed in the *gdu-1-D log2-2* double mutant, susceptibilities of wild type, *log2-2*, *gdu1-1D* and *gdu1-1D log2-2* seedlings to 10 mM Phe, Met and Leu were compared. Wild type, *log2-2* and *gdu1-1D log2-2* plants did not grow in the presence of these amino acids, while most *gdu1-1D* plants developed green cotyledons (Fig. 2-6B). The phenotype of the soil-grown plants and amino acid tolerance assays indicate that loss of *LOG2* expression suppresses all characterized aspects of the Gdu1D phenotype.

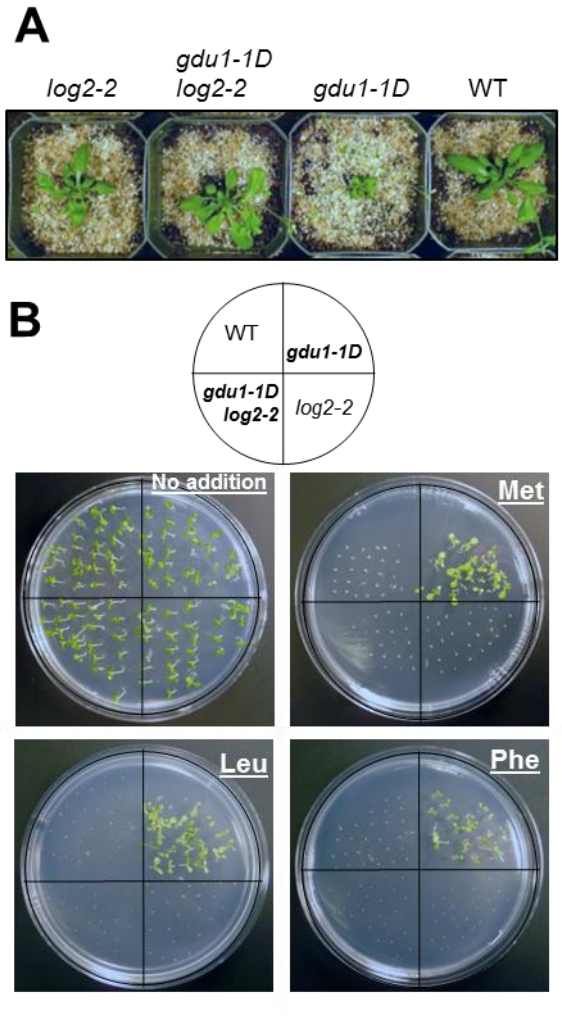

Figure 2-6. Suppression of the *gdu1-1D* phenotype by T-DNA insertion in the *LOG2* gene.
A. *gdu1-1D* was crossed to plants harboring a T-DNA in the first intron of *LOG2* (*log2-2*). Five-week old F3 plants recovered from F2 parents with the indicated genotypes.
B. *gdu1-1D*, *log2-2* and *log2-2 gdu1-1D* seeds were sown on media containing 10 mM of the indicated amino acid or no added amino acid (top left plate). Each plate is oriented with quadrants as shown in the model above. Clockwise from top left: wild-type; *gdu1-1D; log2-2*; and *log2-2 gdu1-1D* double homozygote. Experiments were repeated three or more times with 25 seeds from each line. Representative images are shown.

Expression of artificial miRNA targeting *LOG2* suppressed the Gdu1D phenotype

Of the available T-DNA insertion lines, only the *log2-2* T-DNA was found to repress *LOG2* transcript accumulation. To confirm results obtained with the *log2-2*

line, four artificial microRNAs (amiRNAa, b, c and d) directed against the *LOG2* transcript were created. The amiRNAs were expressed in the *gdu1-1D* line under the control of the CsVMV promoter (Verdaguer et al., 1998), a viral promoter of activity comparable to that of the CaMV 35S. The CsVMV promoter was used to avoid silencing of *GDU1*, driven in *gdu1-1D* by the 35S enhancer. Similar results were obtained with amiRNA expression as seen with the *log2-2* mutation in the *gdu1-1D* background. Transformants created using *LOG2-amiRNAa* and *b* had a phenotype similar to wild type plants in the T2 generation at the expected 75% ratio (Fig. 2-7A). This phenotypic change occurred while *GDU1* transcripts remained at levels similar to the progenitor line (data not shown). Amino acid tolerance to Leu, Phe and Met was tested for five lines (three from *LOG2-amiRNA*a and two from *LOG2-amiRNAb*). Tolerance of the transformants was intermediate between *gdu1-1D* and wild type, with lines a2 and b3 showing almost wild type susceptibility on Leu and Phe (Fig. 2-7B).

While the expression of *LOG2*-directed *amiRNAs* did suppress the Gdu1D phenotype, it was not resolved if the suppression affected GDU1 protein content. The 35S-GDU1-Myc line (see above) was used to probe GDU1 protein accumulation in a parallel experiment. *amiRNAb* was expressed in the 35S-GDU1-Myc line. Similar to the above results, expression of *LOG2-amiRNAb* in 35S-GDU1-Myc suppressed the Gdu1D phenotype in most of the transformants. Four

independent transformation lines (lines 249A-D) were chosen and studied for GDU1-Myc protein accumulation, *LOG2* and *GDU1* mRNA contents, and amino acid levels. Lines 249A and D exhibited a mild Gdu1D phenotype, while lines 249B and C showed wild type morphology (Fig. 2-7C). Expression of *LOG2-amiRNAb* led to an 80% decrease in *LOG2* mRNA in the four lines (Fig. 2-7D). *GDU1* mRNA content was not significantly changed compared to the untransformed control, with about 5,000 fold over-accumulation compared to the wild type (Fig. 2-7D). Amino acid contents were reduced in the suppressed lines, but were not equivalent to those in wild type (data not shown). Significantly, GDU1-Myc protein content was only slightly increased by the expression of *LOG2-amiRNAb* (Fig. 2-7D).

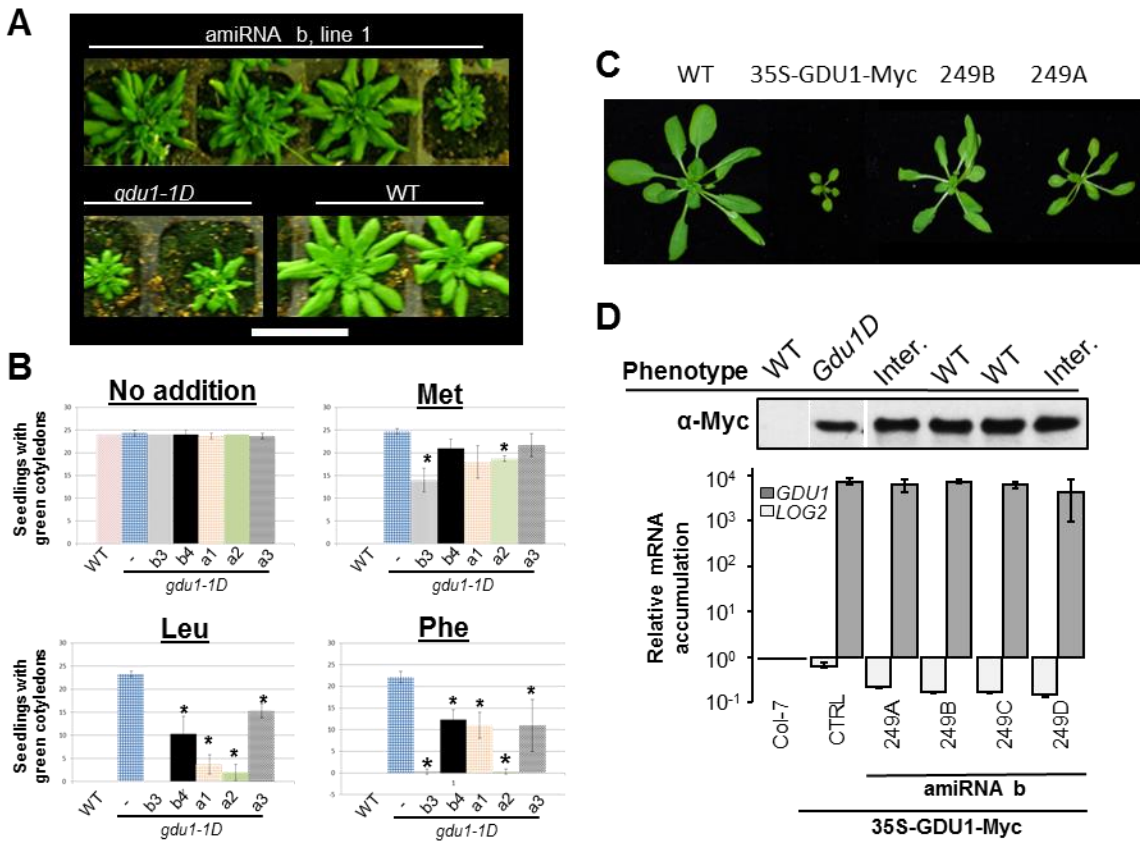

Figure 2-7. Suppression of the *Gdu1D* phenotype by *LOG2*-directed artificial microRNAs in two different Gdu1D over-expression backgrounds.

A. Phenotypic segregation on soil of T2 plants expressing *LOG2-amiRNA* in the *gdu1-1D* background. Scale bar=5 cm.

B. Growth of seedlings expressing one of two *LOG2-amiRNAs* in the *gdu1-1D* background were assessed for presence of green, expanded cotyledons after 14 days growth on 10 mM methionine, leucine, and phenylalanine. Bars represent standard deviation. *: significant difference from *gdu1-1D alone* (-) on same media (t-test with the Bonferroni correction for multiple comparisons; $p<0.01$). Experiments were repeated three or more times with 25 seeds from each line.

C. Phenotype of plants over-expressing *LOG2-miRNAb* in the 35S-GDU1-Myc background.

D. For each line over-expressing *LOG2-miRNAb* in the 35S-GDU1-Myc background, the phenotype of about 10 progeny was recorded (WT, wild type; Inter., intermediate between Gdu1D and WT), GDU1 protein levels were estimated by western blotting using an anti-Myc antibody (top), and *GDU1* and *LOG2* mRNA contents were measured by quantitative RT-PCR (accumulation is expressed relative to the levels in WT (Col-7) and the error bars represent the results of two independent experiments). CTRL: 35S-GDU1-Myc background only, no amiRNA present.

An R12K substitution in LOG2 suppresses the Gdu1D phenotype but not the GDU1-LOG2 interaction

A suppressor mutation, *log2-1*, was isolated in the same screening that led to the isolation of the *log1-1* mutation (Pratelli and Pilot, 2006; Fig. 2-8A). Analyses of phenotypic segregation after crosses of *log2-1* with the wild type and the *gdu1-5D* parental line validated the hypothesis that *log2-1* is a single recessive mutation, independent of the *log1-1* mutation. Positional cloning of *log2-1* showed that the mutation was very close to the *LOG2* gene (data not shown). Sequencing of the *LOG2* gene in *log2-1* revealed the presence of a G to A mutation 35 bp after the ATG, leading to an arginine to lysine mutation (R12K). Transformation of the *log2-1 gdu1-5D* double mutant by a genomic fragment containing the wild type *LOG2* gene complemented the *log2-1* mutation. This complementation proved that suppression of the Gdu1D phenotype in the *log2-1 gdu1-5D* mutant resulted from the R12K mutation in *LOG2* (Fig. 2-8A).

The fact that *log2-1* is recessive suggested a loss-of-function mutation, but, despite extensive trials, no difference in functional properties has been found to date between $LOG2^{R12K}$ and LOG2: the R12K mutation did not impair the ubiquitin ligase activity of the protein (Fig. 2-8B), nor the ability to ubiquitinate cGDU1 *in vitro* (Fig. 2-8E). In addition, yeast-two-hybrid, GST pull-down and *in planta* co-immunoprecipitation assays showed that $LOG2^{R12K}$ interacted with

GDU1 very similarly to LOG2 (Fig. 2-1C; Fig. 2-8C and 2-8D). LOG2^{R12K}-GFP also localized to the plasma membrane as wild-type LOG2-GFP in transiently transformed *N. benthamiana* leaves (Fig. 2-5C, right). The R12K mutation had no effect on GDU1-Myc accumulation when *log2-1* was introduced into the 35S-GDU1-Myc line (Fig. 2-8F). Leading to significant suppression of the Gdu1D phenotype with no detectable effect on the LOG2-GDU1 interaction, the nature of the defect in LOG2^{R12K} is currently unknown.

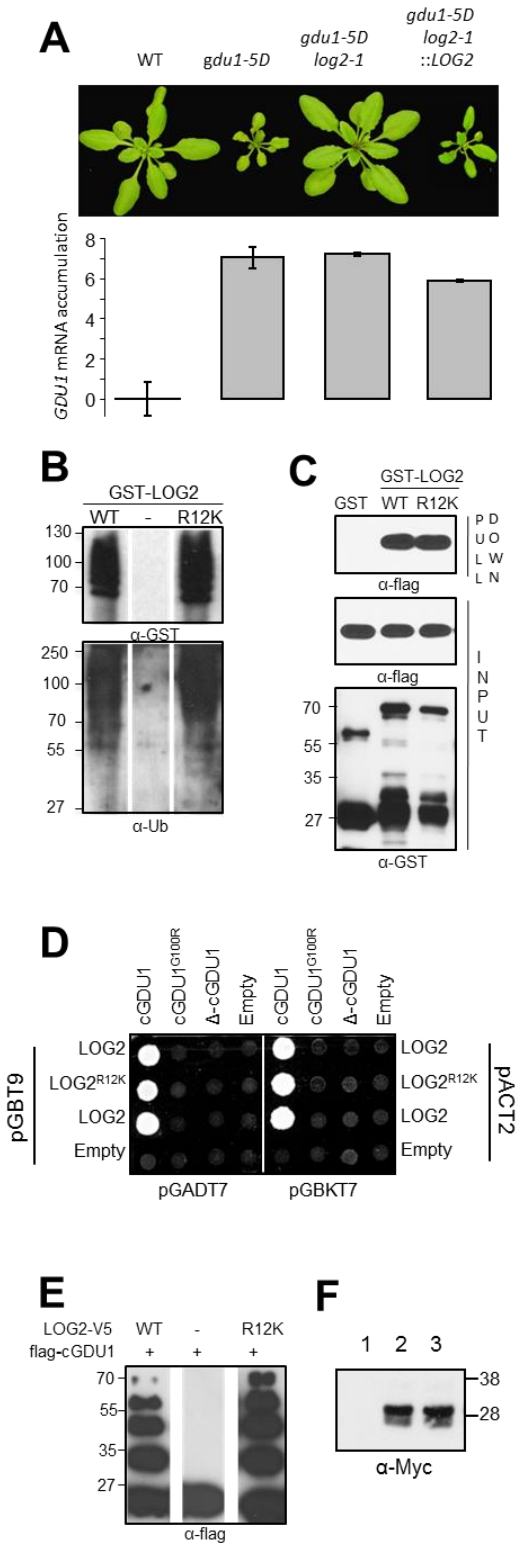

Figure 2-8. Analysis of the effect of the *log2-1* mutation on LOG2 protein properties.

A. Complementation of the *log2-1* mutation. A wild type genomic fragment (8,511 bp *Xho*I-*Pst*I from BAC F11F8) was cloned into a hygromycin resistance-conferring binary vector and inserted into the genome of the *log2-1 gdu1-5D* double mutant. *GDU1* mRNA content was estimated by qRT-PCR and is given as ΔΔCt compared to the wild type and A*ctin2* mRNA content. Errors bars from two biological replicates.
B. GST-LOG2 and GST-LOG2^{R12K} ubiquitination assays without substrate.
C. GST-pull down assay using flag-cGDU1 and GST-LOG2 or GST-LOG2^{R12K}.
D. Yeast-two hybrid interaction assay of LOG2^{R12K} with cGDU1, cGDU1^{G100R} or ΔcGDU1.
E. *In vitro* ubiquitination assay with LOG2-V5 or LOG2^{R12K}-V5 and flag-cGDU1.
F. GDU1-myc accumulation in 35S-GDU1-Myc and in 35S-GDU1-Myc *log2-1* double mutant. Lane 1: Wild type; lane 2: 35S-GDU1-Myc; lane 3: 35S-GDU1-Myc *log2-1*.
Numbers on the side of western blots indicate molecular weight in kDa.

Discussion

LOG2 is necessary for the Gdu1D phenotype

The GDU1 protein has been suggested to be involved directly or indirectly in the control of amino acid export, potentially interacting with an amino acid exporter (Pilot et al., 2004; Pratelli et al., 2010). Parallel attempts at characterizing GDU1 function using EMS suppressor and yeast-two hybrid screens led to the isolation of LOG2, an E3 ubiquitin ligase. Subsequent biochemical analyses showed that LOG2 and GDU1 interacted in *in vitro* pull-down and *in planta* co-immunoprecipitation assays (Fig. 2-1B-C, 2-2B). We also establish here the functional significance of this interaction in multiple assays. Previous characterization of the *log1-1* suppressor allele (GDU1^{G100R}) validated the relevance of the conserved GDU VIMAG motif (Pratelli and Pilot, 2006), but the molecular mechanism of the suppressor effect was not explained. When tested by yeast-two-hybrid, GST-pull down, *in planta* co-immunoprecipitation, and *in vitro* ubiquitination, the *log1-1* mutation diminished the interaction between LOG2 and

GDU1 (Fig. 2-1A-C, and 2-2D, respectively). This indicated that the phenotypic suppression conferred by the *log1-1* allele could result from impaired interaction with LOG2. Genetic interaction assays with plants affected in *LOG2* expression further support this conclusion. Reduction of the Gdu1D phenotype was observed in three independent genetic contexts: in *gdu1-1D* plants homozygous for the *log2-2* T-DNA (Fig. 2-6); upon expression of *LOG2*–directed artificial miRNAs in either *gdu1-1D* or 35S-GDU1-Myc backgrounds (Fig. 2-7); and in *gdu1-5D* plants homozygous for the *log2-1* loss-of-function allele ($LOG2^{R12K}$) (Fig. 2-8). The nature of phenotypic suppression was similar in all three cases: plant size increased (Fig. 2-6A, 2-7A, 2-7C, 2-8A), amino acid sensitivity decreased (Fig. 2-6B, 2-7B), and amino acid accumulation decreased (data not shown). Notably, the suppression effects observed in multiple *LOG2* mutation / *GDU1* over-expressor combinations reinforce the hypothesis that LOG2 is necessary for the development of the Gdu1D phenotype upon *GDU1* over-expression. These findings presage a role for LOG2 in amino acid homeostasis.

Localization of LOG2 and GDU1 at the plasma membrane

LOG2 and GDU1 were found almost exclusively in the microsomal membrane fraction and were particularly enriched at the plasma membrane. GDU1 was previously postulated to be an integral membrane protein (Pratelli and Pilot, 2006), and the sensitivity of GDU1 to a high ionic strength buffer, high pH, and detergent,

typical of integral membrane proteins, confirmed this hypothesis (Fig. 2-5B). Although LOG2 is not predicted to contain a membrane-spanning domain, LOG2 could be myristoylated *in vitro* (Fig. 2-4F), a lipid modification that can anchor otherwise cytosolic proteins to membranes (Resh, 1999). Inhibition of LOG2 myristoylation did not completely abolish membrane association *per se*, as most LOG2^{G2A} still partitioned into microsomes (Fig. 2-4D, E). However, LOG2^{G2A} was significantly depleted from plasma membrane enriched vesicles (Fig. 2-4D), its association with microsomes was markedly more sensitive to extraction (Fig. 2-4E), and the fluorescence signal of C-terminally GFP-tagged LOG2 was no longer confined to the plasma membrane (Fig. 2-5C, left and center panels). Possible reasons for the partial membrane retention of LOG2^{G2A} include its ability to bind to integral membrane proteins and/or the presence of an N-terminal poly-arginine tract. Polybasic regions can facilitate binding of soluble proteins to the negatively-charged intracellular leaflets of lipid bilayers via electrostatic interactions (Murray et al., 1998). However, other lipid modifications or undetected reentrant loops could account for persistent membrane anchoring. The observed weakened *in planta* interaction between GDU1 and LOG2^{G2A} (Fig. 2-4G) hints that myristoylation may potentiate the interaction of LOG2 with GDU1, possibly by shifting LOG2 into the same membrane microdomains as GDU1.

Role of LOG2 with respect to GDU1

Multiple tagged forms of LOG2 ubiquitinated cGDU1 (Fig. 2-2B, C, D), an observation consistent with the possibility that GDU1 is a substrate of LOG2 *in vivo*. Polyubiquitination will often destine a substrate protein for degradation, either via the 26S proteasome, or in the case of many mammalian and yeast plasma membrane proteins, the vacuole or lysosome upon endocytosis (Jehn et al., 2002; Korolchuk et al., 2010). Such ubiquitin ligases are thus negative regulators of the activity of the substrate protein. Our data do not support the hypothesis that LOG2 negatively regulates GDU1. Mutations that inhibit GDU1 (*log1-1*) association with LOG2, or eliminate LOG2 (*log2-2*), would be expected to enhance the Gdu1D phenotype if LOG2 were a negative regulator of GDU1. However, the opposite was observed: in a *GDU1* over-expression background, *log1-1* and *log2-1* homozygotes are phenotypically wild-type (Pratelli and Pilot, 2006; and Fig. 2-8A). GDU1 protein over-accumulation does not cause the Gdu1D phenotype in the absence of LOG2 or when GDU1 is unable to interact with LOG2. In conclusion, LOG2 appears to be a required component of the pathway affected by GDU1 over-expression.

Our data are consistent with a model in which either LOG2 activates GDU1 via ubiquitination, or GDU1 acts as an adaptor protein for LOG2 to recognize an unidentified substrate (see Leon and Haguenauer-Tsapis, 2009, and Woelk et al.,

2007, for reviews). Examples for both hypotheses in other systems have been reported. In mammals, activation of a kinase in the inflammatory response requires its monoubiquitination (Hinz et al., 2010). Plasma membrane-associated E3 ligase adaptors have also been described. For instance, the adaptor Grb2 binds to the RING E3 c-Cbl and facilitates its association with activated plasma membrane epidermal growth factor receptor (EGFR) in mammals, resulting in ubiquitination of the latter (Huang and Sorkin, 2005). Similar E3 adaptors have been also described in yeast (Leon and Haguenauer-Tsapis, 2009).

LOG2-associated proteins probably contribute to amino acid export

By analogy to mammalian heteromeric amino acid transporters (Palacin et al., 2005), GDU1 has been hypothesized to be a subunit of an amino acid exporter (Pilot et al., 2004; Pratelli et al., 2010). One viable hypothesis is that GDU1 over-expression alters the localization of an unknown amino exporter through a LOG2-dependent pathway, either by promoting the degradation of a yet-to-be-identified negative regulator of the exporter, or by directly influencing the movement of the transporter to the cell surface. The *log2-1* mutation suppresses the Gdu1D phenotype without discernibly affecting its sub-cellular localization, ubiquitin ligase activity, or interaction with GDU1 (Figs. 2-5C, right panel; and 2-8B-D). It is possible that this mutation alters the recognition/interaction with as-yet undiscovered LOG2 substrate(s). While an arginine to lysine mutation is often

regarded as conservative, lysines are common sites of modification such as acetylation, methylation and ubiquitination. Modification of lysine 12 of LOG2^{R12K} could disrupt interactions between LOG2^{R12K} and its substrate(s) or other interacting proteins, leading to suppression of the Gdu1D phenotype. This hypothesis suggests that all the proteins involved in the GDU1 pathway are not discovered. The identification of these proteins and their role in membrane protein trafficking or amino acid export will be the subject of further investigations.

Materials and methods

Plant lines, transformations, and crosses

Transgenic Arabidopsis thaliana (ecotypes Col-0 or Col-7) were procured via the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* GV3101 (pMP90). Seed harboring the *log2-2* T-DNA (Syngenta Arabidopsis Insertion Library accession 729_A08; Sessions et al., 2002) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH). *gdu1-1D log2-2* double homozygote plants were identified in the F2 generation of a cross between *gdu1-1D* pollen and *log2-2* ovules via PCR of genomic DNA. Phenotypic analyses of *gdu1-1D log2-2* and *gdu1-1D amiRNA-LOG2* double mutant plants were performed on homozygous F3 and T4 individuals, respectively (except for segregation of the *GDU1* over-expression phenotype of *gdu1-1D amiRNA-LOG2* double mutant plants on soil, which was carried out on T2

individuals). 35S-GDU1-Myc *log2-1* double homozygotes were identified in the F2 generation of a cross between the pollen of *log2-1* single mutant, obtained from a cross of *log2-1 gdu1-5D* double mutant with Col-7, and 35S-GDU1-Myc ovules via analysis of antibiotic resistance and phenotype. For transient expression, leaves of five-week old *Nicotiana benthamiana* plants were infiltrated with a suspension of *Agrobacterium tumefaciens* carrying constructs of interest according to Batoko et al., 2000. To assess amino acid sensitivity, surface-sterilized seeds were sown on agar plates comprising 1x MS salts (Murashige and Skoog, 1962), 1% (w/v) sucrose , 2.5 mM MES, 0.5 μg mL^{-1} pyroxidine-Cl, 0.1 μg mL^{-1} myoinositol, 0.8 % (w/v) BactoAgar (BD Biosciences, Franklin Lakes, NJ), pH 5.7, and 10 mM amino acids. Plants were grown for 14 days in an incubator (24 h light, 50 μmol m^{-2} s^{-1}, at 22 ℃).

Cloning and constructs

For the yeast-two hybrid screening, the region encoding the C-terminal domain of GDU1 (residues 61 to 158) was cloned into pGBKT7 or pGBT9 (Clontech, Mountain View, CA) using specific primers and the *Eco*RI and *Pst*I sites. For the yeast-two-hybrid interaction matrix, cDNAs encoding the C-terminal soluble part of the GDUs, and the full sequence of LOG2 and LULs were amplified by PCR with primers containing the *att*B Gateway® sequences, cloned into pDONRZeo (Invitrogen, Carlsbad, CA), sequenced, and transferred into pACT2 and pGBT9

vectors, which were previously made Gateway® compatible by insertion of the Gateway® cassette (Pratelli and Pilot, unpublished). To obtain GST- and V5-tagged LOG2 and LUL1, the coding sequences were amplified by PCR, cloned into pDONR201 (Invitrogen) using the Gateway® technology, and recombined into pDEST15 and pET-DEST42 (Invitrogen), respectively. Flag-cGDU1 was produced similarly with the destination vector pEAK2 (Kraft, 2007). Site-directed mutagenesis was performed with the QuikChange kit (Stratagene) to create RING-dead LOG2 ($LOG2^{C354/C557AA}$, mLOG2) RING-weak LOG2 ($LOG2^{I321A}$), and myristoylation-inhibited LOG2 ($LOG2^{G2A}$). For the *in vitro* myristoylation assay, LOG2 was recombined into pEXP2 and pET-DEST42 (Invitrogen), respectively. GFP and mCherry fusion constructs were created by Gateway® cloning into pPWGTkan and pPWMTkan, derivative of pJHA212K (Yoo et al., 2005), where the Gateway® cassette was inserted between the CaMV35S promoter and the eGFP or mCherry coding sequence (Pratelli and Pilot, unpublished). HA fusion construct used for Arabidopsis transformation was obtained by Gateway® cloning into pGWB14 (Nakagawa et al., 2007). HA and Myc fusions used for transient assays in *N. benthamiana* were obtained using vectors similar to pPWGTkan, where a double HA tag or triple Myc tag replaced the eGFP. The GDU1 VIMAG domain was deleted by cloning next to each other in pBluescript two PCR fragments corresponding to the regions upstream and downstream of the domain

respectively, and sharing an *Eco*RI site at the exact place of the VIMAG domain. The resulting construct was used as a template for a PCR reaction towards a Gateway® cloning into pDONR221 (Invitrogen). Artificial microRNAs were designed following the guidelines found in WMD3 (http://wmd3.weigelworld.org/; Schwab et al., 2010). The primers corresponding to pRS300 (Schwab et al., 2010) used for amplification of the microRNAs contained the Gateway® *att*B sites. The final PCR fragment was cloned into pDONRZeo (Invitrogen), sequenced, and transferred into the pSWsNkan binary vector, another derivative of pJHA212K (Yoo et al., 2005, Pratelli and Pilot, unpublished), between the CsVMV promoter (Verdaguer et al., 1998) and the terminator of the small subunit of the rubisco from pea (Accession: X00806). For the *log2-1* complementation, a *Xho*I-*Pst*I 8.5 kb genomic DNA fragment from BAC F11F8 containing the wild type *LOG2* gene was cloned into the pTkan binary vector, derivative of pJHA212K.

Yeast-two-hybrid screening and interaction assays

For screening, the bait plasmids were co-introduced along with an Arabidopsis cell cDNA library (Németh et al., 1998) into yeast strain AH109 (Clontech) using the TRAFO protocol (Gietz and Schiestl, 2007). About 2 million transformants were selected on SC medium lacking leucine, tryptophan, histidine and adenine (2% glucose, 6.7 $g.L^{-1}$ Yeast Nitrogen Base without amino acid [BD Biosciences] pH 6.3, and dropout amino acid mix). Plasmids were extracted from the colonies

displaying auxotrophy for the four amino acids and introduced back into yeast together with the cGDU1 construct. The inserts of the eight plasmids restoring yeast growth on selective media were then sequenced. For interaction matrices, yeast strains AH109 (MATa) and Y187 (MATα) were transformed as described above with the prey and bait vectors, respectively, and selected on media lacking tryptophan or leucine. Several colonies were scrapped and resuspended together in 250 µL water. Prey-bait pairs were mixed together (10 µL of each suspension and 50 µL of water), and 5 µL were spotted on YPDA (1% (w/v) yeast extract, 2% (w/v) bacto peptone, 2% (w/v) glucose, 80 mg L^{-1} adenine, 1.5% agar) for mating. After overnight growth at 30 ℃, the cell spots were scrapped, resuspended in 100 µL water, and 5 µL were spotted on SC medium lacking tryptophan and leucine. After growth at 30 ℃ for 2 days, the spots were scrapped, resuspended in 100 µL water and 5 µL were spotted on SC medium lacking tryptophan, leucine, adenine and histidine. Growth was assessed after 2, 3 and 6 days to identify positive interactions. β-galactosidase activity of yeast was measured using a protocol obtained from Clontech (Manual #PT3024-1). Briefly, cells grown to OD_{600}=0.5 were harvested by centrifugation, and resuspended in 100 mM Na_2HPO_4 pH 7, 10 mM KCl, 1 mM $MgSO_4$, 5 mM β-mercaptoethanol (Z-buffer), and subjected to three freeze-thaw cycles in liquid N_2 and at 37 ℃. The broken cell suspension (100 µL) was added to 900 µL of 800 µg.mL^{-1} ortho-nitrophenyl-β-galactoside in Z-

buffer, and incubated for several hours at 37 ℃. Reaction was stopped by addition of 400 µL 1 M Na_2CO_3 and OD_{420} was measured.

Recombinant protein expression and purification

GST-, V5-His_6-, and His_6-flag- fusion proteins were expressed in BL21-pLysS *E. coli* essentially as in Kraft et al., (2005) with a few modifications. The lysis buffer comprised 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM DTT, 0.1% (v/v) Nonidet P-40, 0.5X Complete® Protease Inhibitors (Roche Diagnostics, Indianapolis, IN) for GST-tagged proteins, and was supplemented with 20 mM imidazole for His-tagged proteins. Cells were lysed by sonication. For GST proteins, glutathione bead slurries were brought to 20 % (v/v) glycerol after the final wash, flash-frozen in liquid nitrogen, and stored at -80^{o}C. His-tagged proteins were eluted from nickel sepharose beads, brought to 20% glycerol and flash-frozen. His_6-flag-cGDU1 was purified likewise, except buffers contained 50 mM K_2HPO_4-KH_2PO_4 pH 5.75 in place of Tris-HCl. To further purify cGDU1, eluted protein was centrifuged through a 30 kDa NMWL Amicon concentrator (Millipore, Billerica, MA), and the eluate was concentrated and buffer exchanged in a 5 kDa NMWL Amicon concentrator with 50 mM mM K_2HPO_4-KH_2PO_4 pH 5.75, 150 mM KCl, 1 mM DTT, 0.1 % Nonidet P-40, 0.5X Complete® Protease Inhibitors. The final retentate was brought to 20 % glycerol and flash-frozen.

GST pull-down assays

In vitro pull-downs were performed as in Gilkerson et al., (2009) with the following differences: GST proteins-on-beads were washed once in 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 1 mM DTT, 1% (v/v) Nonidet P-40, 0.5X Complete® Protease Inhibitors (wash buffer) prior to being mixed with a soluble prey protein in 400 µL wash buffer and incubated at 4^{o}C for 2 h. Proteins were eluted by suspending beads in 60 µL elution buffer (wash buffer + 50 mM glutathione) and shaking at 4^{o}C for 15 minutes.

Ubiquitination assays

In vitro ubiquitination assays were conducted essentially according to Hsia and Callis, (2010) with slight modifications: 4 µg bovine ubiquitin (Sigma-Aldrich, St. Louis, MO), ~2 µg GST- or V5-His_6-LOG2; and ~1.5 µg His_6-flag-cGDU1were used. Reactions were quenched with 10 µL 5x Laemmli sample buffer (200 mM Tris pH 6.8, 32% (v/v) glycerol, 6.4% (w/v) SDS, 0.32% (w/v) Bromophenol Blue, 200 mM DTT), boiled for 5 min, and separated via SDS-PAGE. Proteins were visualized by Western blot using anti-flag linked HRP (Sigma-Aldrich) anti-GST (Santa Cruz Biotechnology; Santa Cruz, CA) according to manufacturers' recommedations or anti-ubiquitin antibodies. Anti-ubiquitin antibodies were raised against bovine full length ubiquitin prepared according to Haas (1985) by Aves Labs (Tigard, OR), affinity purified and used at 1:5,000 dilution.

In planta co-immunoprecipitation assay

Proteins were transiently expressed in *N. benthamiana* leaves. Three days after infiltration, 500 mg leaves were ground with 1.5 ml extraction buffer on ice (50 mM Tris-HCl pH 7.3, 150 mM NaCl, 10 mM $MgCl_2$, 0.5% Nonidet P-40, 10 mM DTT, 1X Complete®). Homogenate was centrifuged at 10,000 g, 4℃ for 15 min. The supernatant was filtered through several layers of Miracloth (EMD Biochemicals, Rockland, MA) and quantitated using the Bradford assay (Fermentas, Glen Burnie, MD). Proteins were co-immunoprecipitated use the ProFound c-Myc Tag IP/Co-IP Kit (Pierce, Rockford, IL): 3 mg of proteins were placed on a rotary wheel overnight at 4℃ in a Co-IP spin column with the cMyc-agarose; after washing, the proteins were eluted three times with 10 µL of the supplied elution buffer and neutralized by 1.5 µL of 1 M Tris pH 9.5. One µL of Co-IP elutate and 10 µg of total proteins were analyzed by SDS-PAGE (4-12% polyacrylamide MES gel, Invitrogen) and western blotting. Proteins were transferred on a nitrocellulose membrane (GE Healthcare), and detected using an anti-cMyc (Clone A-14, Santa Cruz; 1:10,000) or anti-HA (Clone 3F10, Roche Diagnostics; 1:5,000) primary antibodies, anti-rabbit or anti-rat (Thermo Scientific, Waltham, MA) secondary antibodies, and the ECL-Plus western blotting detection system (GE Healthcare).

Subcellular fractionation

Endomembranes were prepared from Arabidopsis and *N. benthamiana* according to Liu et al., (2009) with the following modifications: 5-30 g of 8-day Arabidopsis seedlings, 5-week Arabidopsis rosette leaves, or tobacco leaves 3 days after infiltration were homogenized with a Waring blender in 60 mL homogenization buffer (50 mM MOPS-KOH pH 6.8, 5 mM EDTA, 0.33 M sucrose, 2 mM ascorbic acid, 1.5 mM DTT, 0.5 mM PMSF, 0.2% (w/v) polyvinylpolypyrrolidone). Sensitivity of GDU1 or LOG2 membrane association to detergent, salt, and pH was examined as in Phan et al., (2008), with a 1 h incubation on ice. Plasma membrane-enriched vesicles (PEVs) and plasma membrane-depleted vesicles (PDVs) were purified from total microsomes according to Larsson et al., (1994): upper phases 4-5 were combined to afford PEVs, while lower phase 1 was five-times extracted with fresh upper phase to afford PDVs. Plasma membrane enrichment and depletion were qualitatively assessed with the α-PMA2 antibody (M. Boutry, Université Catholique de Louvain, Belgium). Protein concentration was assessed by the Bradford or BCA assays.

Localization of expression and imaging

About 3 kb of the region upstream from LOG2 ATG was amplified by PCR and cloned using *Bam*HI and *Pst*I in pUTkan (Pratelli et al., 2010). GUS

histochemical staining was performed as described in Pratelli et al., 2010). *N. benthamiana* epidermis cells were visualized on a Zeiss LSM510 META confocal system on an Axio Observer.Z1 microscope using a C-Apochromat 40X water-immersion N.A. 1.2 objective (Carl Zeiss, Thornwood, NY), a 488nm Argon multi-line gas laser, and a 543nm Helium Neon gas laser, with BP 505-550 and LP 560 emission filters, respectively. Serial images were captured and processed by the Zen 2009 software (Carl Zeiss) using maximal projection.

Nucleic acid isolation and PCR

Genomic DNA was extracted from Arabidopsis plants using the CTAB method (Murray and Thompson, 1980). Total RNA was extracted either with the RNeasy kit (Qiagen, Valencia, CA), or by 1 mL of TRI REAGENT (Sigma-Aldrich). cDNAs were synthesized using the SuperScript III system (Invitrogen) according to the manufacturer's instructions. For quantitative PCR, the efficiency-calibrated method was implemented (Pfaffl, 2001). Five µl of primer mix (1 µM each) and 5 µl of the reverse transcription product (made from 2 µg total RNA) diluted 50 times were mixed with 10 µl of 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and subjected to the following cycles: 50 ℃, 2min, 95 ℃, 10 min, 40 times of 95 ℃, 15 sec, 55 ℃, 15 sec, 72 ℃, 1 min (in a 7300 Real Time PCR System, Applied Biosystems).

Amino acid quantitation

Tissues were frozen in liquid nitrogen, freeze dried, weighed, and ground with three 3-mm glass beads in an Ultramat Amalgamator (SDI, Bensenville, IL). Amino acids were extracted from the dry powder by 200 µL of 10 mM HCl and 200 µL of chloroform. After vortexing for 2 min, the solution was centrifuged 5 min at 16,000 g and 150 µL of the supernatant was dried under vacuum. The dried extract was solubilized in 500 µL of 50 % acetonitrile in water, 0.05 % heptafluorobutyric acid and the metabolites were separated by ion paring liquid chromatography and analyzed by mass spectrometry.

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Chapter III

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Testing the efficiency of plant artificial microRNAs by transient expression in *Nicotiana benthamiana* reveals additional action at the translational level

Introduction

MicroRNAs (miRNAs) are a class of small noncoding RNAs of 20 to 24 nucleotides in length that regulate target gene expression at the post-transcriptional level in eukaryotes (Brodersen and Voinnet, 2009; Rogers and Chen, 2013). miRNAs are processed from longer precursor transcripts to a stable hairpin structure with two complementary short RNA strands, which are further processed to miRNA:miRNA* duplexes, by RNaseIII enzymes (miRNA* being the passenger strand). The miRNA:miRNA* duplexes are then transported out of the nucleus to the cytoplasm, where the miRNA* are degraded. Mature miRNAs are

bound by ARGONAUTE proteins to form the RNA-induced silencing complex (RISC). miRNAs serve as guides for RISC to bind target mRNA(s) and silence gene expression (Brodersen and Voinnet, 2009; Meng et al., 2011; Sun, 2012). It is believed that perfect or near-perfect complementarity favors RISC-catalyzed endonucleolytic mRNA cleavage, while central mismatches promote translational repression (Brodersen and Voinnet, 2009; Huntzinger and Izaurralde, 2011). In contrast to most animal miRNAs, most plant miRNAs show perfect or near-perfect complementarity to their targets, hence mRNA cleavage is deemed to be the dominant mode of action in plants (Brodersen and Voinnet, 2009; Huntzinger and Izaurralde, 2011). In disagreement with this postulate, evidence from recent reports suggests that translational repression plays a vital role in regulating target gene expression in plants (Aukerman and Sakai, 2003; Chen, 2004; Gandikota et al., 2007; Brodersen et al., 2008; Dugas and Bartel, 2008; Lanet et al., 2009; Zhu et al., 2009; Beauclair et al., 2010; Zhu and Helliwell, 2011; Alonso-Peral et al., 2012; Li et al., 2013; Ma et al., 2013; Meijer et al., 2013). It has also recently been shown that miRNAs affect target gene DNA methylation in plants (Wu et al., 2010; Wu et al., 2012). Thus, the action mode of miRNAs on gene expression appears more diverse than initially thought, with effects on target mRNA cleavage, translation inhibition and DNA methylation, possibly exerted concomitantly (Pillai et al., 2007; Voinnet, 2009).

Plant artificial microRNAs (amiRNAs) are produced by expression of a miRNA gene genetically modified by replacing the original miRNA:miRNA* duplex region with customized sequences to silence one or more genes of interest in various plant species (Schwab et al., 2006; Ossowski et al., 2008). Plant amiRNA technology utilizes the high degree of complementarity between the miRNAs and their mRNA targets to ensure the silencing specificity for the amiRNAs. Compared to RNA interference (RNAi) and virus-induced gene silencing (VIGS), plant amiRNAs have several advantages such as minimal predicted off-target effects and ability for multigene silencing (Schwab et al., 2006; Ossowski et al., 2008). Use of amiRNA is also a potential strategy for engineering plant resistance to viruses (Sablok et al., 2011; Jelly et al., 2012). The Web-based amiRNA designer, developed by the Weigel lab, (WMD; Ossowski, Fitz, Schwab, Riester and Weigel, personal communication) provides a platform to design gene-specific amiRNA candidates for more than 100 plant species. While the candidates are ranked by an algorithm according to the canonical sequence complementarity and hybridization energy, WMD does not consider factors that may elicit translational repression, such as target mRNA structure and mRNA binding proteins (Schwab et al., 2005; Fabian et al., 2010; Pasquinelli, 2012). The evidence that translational control plays an important part in the effect of endogenous plant miRNAs questions the validity of the prediction for a given amiRNA to silence its

target gene solely based on the cleavage efficiency, often measured by decreases in target mRNA abundance. The possible variation in efficacy between different amiRNA candidates to the same target gene might thus hinder the broad application of amiRNA technology for gene silencing. Two articles reported the testing of amiRNAs by co-expression of the tagged target gene and the candidate amiRNAs in Arabidopsis protoplasts (Kim and Somers, 2010; Li et al., 2013). Interestingly, the authors noticed that, similar to miRNAs, many amiRNAs exert translational inhibition of the target gene while leading to a small decrease in the target mRNA abundance.

Our previous work with amiRNAs targeting the *LOSS OF GDU 2* (*LOG2*, AT3G09770) gene showed that these amiRNAs led to a reduction of less than 80 % of *LOG2* mRNA content while effectively triggering a LOG2-dependent phenotype (Pratelli et al., 2012). Here, we report a more in depth analysis of the effect of these four amiRNAs on the *LOG2* mRNA and protein accumulation in both Arabidopsis and *Nicotiana benthamiana*. To expand our analysis, the effects of twelve amiRNAs targeting three additional genes were studied. While assays aiming at testing amiRNA efficacy have been developed, they rely on the use of Arabidopsis protoplasts (Kim and Somers, 2010; Li et al., 2013), a technique not available in every laboratory. Thus, we tested whether *N. benthamiana* could be substituted to Arabidopsis protoplasts for testing amiRNA efficacy, and compared

the results obtained by expression of the amiRNAs in *N. benthamiana* and Arabidopsis.

Results

Analysis of amiRNAs targeting *LOG2*

In an earlier report, we found that the LOG2 ubiquitin ligase interacted with the GLUTAMINE DUMPER 1 (GDU1) protein, and that knockdown or knockout of *LOG2* in Arabidopsis suppressed the phenotype caused by the over-expression of *GDU1* (Pratelli et al., 2012). *GDU1* over-expression, as in the *gdu1-1D* mutant, leads to reduced-size plants secreting glutamine at the leaf margin, and displaying curled and darker green leaves in addition to several metabolic phenotypes (Gdu1D phenotype, Pilot et al., 2004). Expression in the *gdu1-1D* of amiRNA^{LOG2}-A and -B targeting *LOG2* suppressed endogenous *LOG2* gene activity (three out of 24 and six out of 21 transformants, respectively): a wild type phenotype indicates a suppression of *LOG2* activity and the Gdu1D phenotype indicates a lack of suppression (Fig. 3-1A). In none of the lines, wild type phenotype was due to loss of GDU1 over-expression (data not shown). Some lines displayed an intermediary phenotype, probably due to partial suppression of the *LOG2* gene activity (data not shown). In these lines, *LOG2* mRNA accumulation was reduced by less than 80 % in the 35S-GDU1-cMyc (Pratelli et al., 2012) or *gdu1-1D* (Fig. 3-1A) backgrounds, while the strength of wild type phenotype (suppression of *LOG2*

activity) did not directly correlate with the reduction in *LOG2* mRNA accumulation (compare lines 230D and 230F; Fig. 3-1A).

Two amiRNAs (amiRNA^{LOG2}-C and -D) could not lead to loss of the Gdu1D phenotype in any of 22 and 21 independent transformants, respectively, suggesting that these amiRNAs are not as efficient as amiRNA^{LOG2}-A and -B to suppress *LOG2* expression (Fig. 3-1A). This hypothesis was tested by transiently co-expressing *mLOG2* (ubiquitination defective LOG2, fused to the HA tag or the GFP) and the four amiRNAs in *N. benthamiana* and testing for *LOG2* expression, both at the mRNA and protein levels. *Agrobacterium*-mediated transient expression in leaves of *N. benthamiana* is a widely used technique to study proteins (*e.g.* Voinnet et al., 2003; Popescu et al., 2007; Liu et al., 2010; Shah et al., 2013), and has been recently used for expressing and testing miRNAs and amiRNAs (Bhagwat et al., 2013; Liu et al., 2014). The control for this experiment consisted in the co-expression of the target protein mLOG2-HA with LUL1, a LOG2 paralog (Pratelli et al., 2012), to establish mLOG2-HA protein accumulation baseline when co-expressed with an unrelated construct. Co-expression of the unrelated amiRNA$^{SnRK1.1}$-C (see below) showed that the effect on mLOG2-HA protein is specific to the amiRNA targeting this transgene (Fig. 3-1C). *mLOG2* mRNA levels were reduced by 75-80 % by co-expression with amiRNA^{LOG2}-A and -B (Fig. 3-1B), while no mLOG2 protein accumulation could be detected (Fig. 3-

1C). In contrast, amiRNA^{LOG2}-C and -D slightly increased *mLOG2* mRNA and protein contents (Fig. 3-1B and 3-1C). Observing the fluorescence of mLOG2-GFP co-expressed with amiRNA^{LOG2} confirmed that amiRNA^{LOG2}-A and -B were the most efficient at reducing the accumulation of mLOG2 protein (Fig. 3-1D).

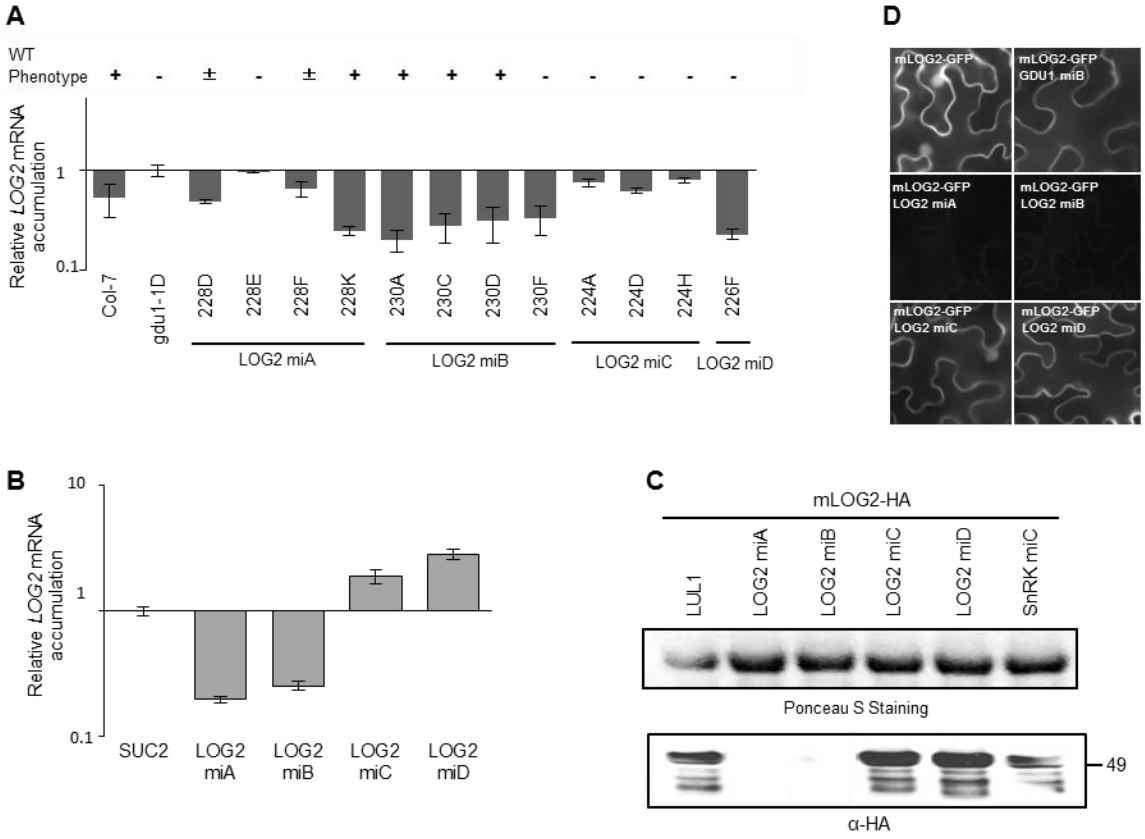

Figure 3-1. Effects of artificial miRNAs targeting *LOG2* in stably transformed Arabidopsis and in transiently transformed *N. benthamiana*.

(A) amiRNAs targeting *LOG2* (LOG2 miA, miB, miC and miD) were stably introduced into the *gdu1-1D* Arabidopsis mutant. The phenotype of about 20 progenies of each transformant line was recorded ("+", wild type; "±", intermediate between Gdu1D and WT; "-", Gdu1D). mRNA level of *LOG2* in each line was measured by qRT-PCR, normalized with *ACT2* and expressed relative to the level in *gdu1-1D*. (B) Relative *LOG2* mRNA levels in transiently transformed *N. benthamiana*. *mLOG2* (ubiquitination inactive LOG2) was co-expressed with *SUC2* or amiRNA^{LOG2} (see main text). *LOG2* mRNA levels were measured by qRT-PCR and expressed relative to levels in leaves transformed with *mLOG2* and *SUC2*. Co-expression of *SUC2* was used as co-expression control. (C) Western blot detection of the accumulation of the mLOG2 protein (ubiquitination inactive LOG2), when co-expressed transiently with four amiRNA^{LOG2} in *N. benthamiana*. amiRNA$^{SnRK1.1}$-C (SnRK miC) was used as non-targeting amiRNA negative

control. Numbers on the right indicate molecular weight in kDa. Co-expression of LUL1 (see main text) was used as co-expression control. (D) Analysis of amiRNAs effect on mLOG2-GFP fluorescence in transiently transformed *N. benthamiana* epidermis cells. Fluorescence microscope pictures were all taken with the same settings. Error bars represent standard error of at least three biological replicates.

These data indicate that amiRNA^{LOG2}-C and -D could not suppress LOG2 protein accumulation in *N. benthamiana*, and were unable to suppress the Gdu1D phonotype in Arabidopsis. In Arabidopsis, amiRNA^{LOG2}-A and -B were able to suppress the Gdu1D phenotype similar to *LOG2* knock-out lines, suggesting that *LOG2* expression is suppressed, but with still 20-25 % of *LOG2* mRNA present in the cells. These data suggest that amiRNA^{LOG2}-A and -B also act at the translational level to suppress *LOG2* expression.

Design of an approach to compare amiRNA efficiency in Arabidopsis and *N. benthamiana*

Based on this encouraging correlation of the results in Arabidopsis and *N. benthamiana*, we decided to develop an approach to help determining the efficiency of amiRNAs to suppress the expression of their target genes before Arabidopsis transformation and plantlet selection. We extended this analysis to three additional genes, to parallel the article from *Li et al.* (Li et al., 2013), but instead of expressing the amiRNAs and their target genes in Arabidopsis protoplasts, we tested the co-expression in *N. benthamiana* leaves. We further tested the reliability of the prediction of amiRNA efficacy from transient expression assays into stably transformed Arabidopsis.

Three genes (*GDU1*, AT4G31730; *SnRK1.1*, AT3G01090; and *MIPS1*, AT4G39800) were selected as amiRNA targets because of interests in our laboratory or alteration of the expression of these genes had been shown to lead to phenotypic changes. Suppression of *MIPS1* reduces inositol synthesis and causes spontaneous cell death (Donahue et al., 2010). Overexpression of *SnRK1.1* in Landsberg *erecta* leads to glucose sensitivity, late flowering and delayed senescence (Baena-Gonzalez et al., 2007). For each of these three genes, four amiRNAs targeting different regions within the genes were designed using WMD (http://wmd3.weigelworld.org/): the criteria were that they should bind different regions of the target mRNAs and displayed the best scores (data not shown).

amiRNA constructs were introduced into Arabidopsis lines expressing the corresponding target genes cloned in frame with the HA tag, cMyc tag or the GFP coding sequences, to ease protein detection. These constructs are under the control of the viral CaMV 35S promoter, and expressing the amiRNA under the control of the CaMV 35S promoter would likely trigger suppression (G. Pilot, unpublished results; Daxinger et al., 2008). The amiRNAs were thus expressed under the control of another viral promoter, the CsVMV (Verdaguer et al., 1998), which has been shown to be compatible with the CaMV 35S promoter (see above, and Pratelli et al., 2012, for LOG2). Phenotypes of the transformants were recorded, and both target mRNA and protein levels were measured in the progenies.

In parallel, the amiRNAs were transiently co-expressed in *N. benthamiana* leaves with the target genes fused to the coding sequence of protein tags, and the corresponding target mRNA and protein levels were assessed. The viral RNAi inhibitor p19 has been shown to enhance protein production in *N. benthamiana* (Voinnet et al., 2003), and is routinely used to express genes in this system because it prevents suppression of the expression of the transgene. While Ahn *et al.* suggested that p19 would not affect miRNA-mediated silencing (Ahn et al., 2011), we found that p19 strongly suppressed the amiRNA^{LOG2}-A and -B-induced silencing in *N. benthamiana* (data not shown). Yu et al. also showed that miRNA methylation, which is a critical step in miRNA biogenesis, is interfered by RNA silencing suppressor (Yu et al., 2005; Yu et al., 2006). Consequently, all assays in *N. benthamiana* were performed without co-infiltration of p19 and measuring target protein and mRNA content two days after infiltration, to prevent any silencing of the constructs. The first control for these experiments corresponded to a co-expression of the target construct with a construct available in the laboratory encoding an unrelated protein under the control of CsVMV promoter: LOG2-HA; LUL1 (AT5G03200), and LUL4-HA (AT5G19080), paralogs of LOG2; the sucrose transporter SUC2 (AT1G22710). The second control was an amiRNA targeting another gene from the present study (amiRNA$^{SnRK1.1}$-C and amiRNA^{LOG2}-B). These controls ensured that the expression of the amiRNAs did not perturb

protein accumulation and that the effect of the amiRNA is specific to the target gene. We finally compared the results of transient expression in *N. benthamiana* with stable expression in Arabidopsis.

Comparison of the effect of amiRNAs to suppress the expression of *MIPS1*

Four different amiRNA^{MIPS1} were introduced into a 35S-MIPS1-GFP/*mips1-2* Arabidopsis line (provided by Dr. G. Gillaspy). For each amiRNA, about 20 transformants were selected and screened for lower MIPS1-GFP fluorescence at the seedling stage. Five transformants that showed lowest GFP fluorescence (highest suppression of *MIPS1* expression) were transferred to soil and their seeds were collected. GFP fluorescence of the T2 plants was screened again at the seedling stage, and, for each amiRNA, six progeny of two lines that showed the strongest reduction in GFP fluorescence were transferred to soil and used for the study of target mRNA and protein levels. A total of seven transformant lines were studied for *MIPS1*, corresponding to the four amiRNAs (plant sample for line 278D was lost). Two of these seven lines (279B and 280A) showed a phenotype similar to the *mips1-2* mutant in the T2 generation, and one (281E) showed an intermediate phenotype, despite the fact that they all displayed the lowest MIPS1-GFP fluorescence during the screening (Fig. 3-2A). Lines 279B, 280A and 281E accumulated less *MIPS1* transcript and MIPS1-GFP protein was reduced by more

than 90 %, contrary to the other lines (Fig. 3-2A). Lines 278E, 279E, 280B and 281D showed little reduction in *MIPS1* mRNA and protein accumulation, and displayed the wild type phenotype. Overall, the four different amiRNA^{MIPS1} could lead to similar efficiency to silence *MIPS1* gene, and MIPS1 protein accumulation correlated to *MIPS1* mRNA accumulation and the phenotype.

The efficacy of amiRNA^{MIPS1} was estimated by transient assay in *N. benthamiana* leaves. In this system, expression of amiRNA^{MIPS1}-A, -B and -D reduced *MIPS1* transcript abundance by 65-75 %, less than in Arabidopsis (Fig. 3-2B), while amiRNA^{MIPS1}-C could not reduce *MIPS1* mRNA level by more than 20 %. In parallel, the reduction of MIPS1 protein accumulation was moderate (~50 %) and similar for the four amiRNA^{MIPS1} (Fig. 3-2C). The effects of the various amiRNA^{MIPS1} at the target mRNA and the protein levels were different, with amiRNA^{MIPS1}-C being potent at reducing MIPS1 protein but not *MIPS1* mRNA accumulation.

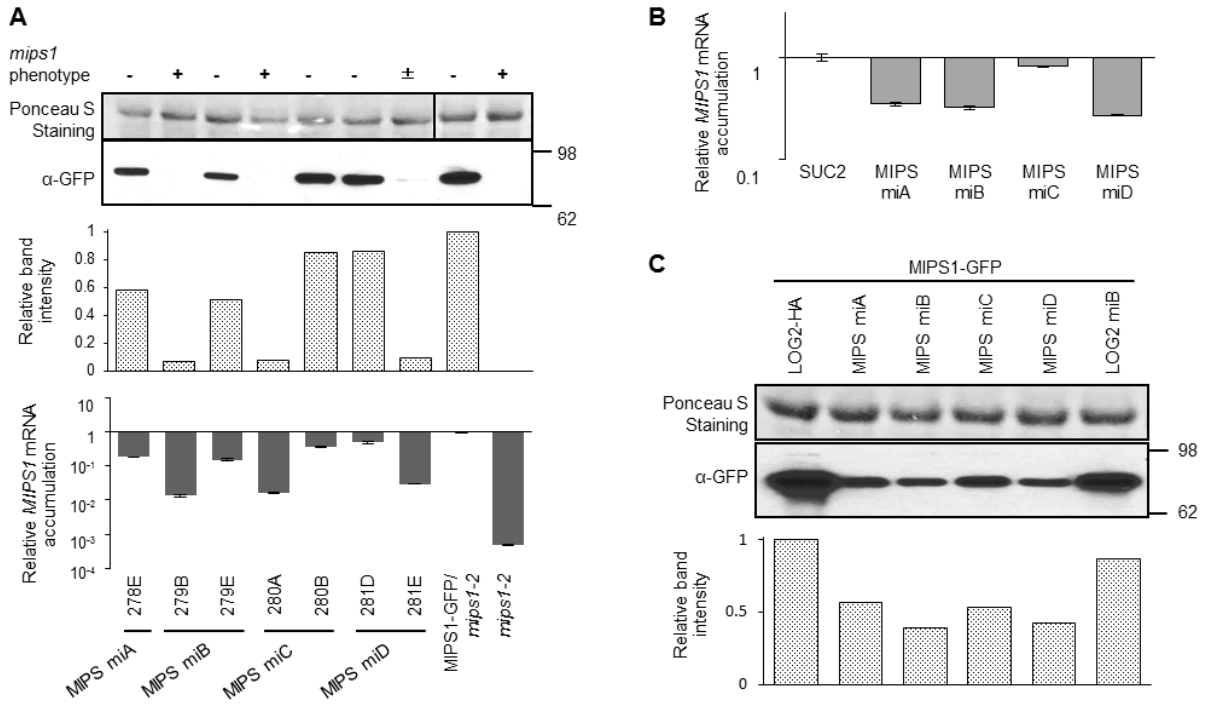

Figure 3-2. Analysis of amiRNAs targeting *MIPS1* in stably transformed Arabidopsis and in transiently transformed *N. benthamiana*.
(A) amiRNAs targeting *MIPS1* (MIPS miA, miB, miC and miD) were stably introduced into the MIPS1-GFP/*mips1-2* background. The phenotype of about 10 progeny of each line was recorded ("-", wild type; "±", intermediate between *mips1* and WT; "+", *mips1*). MIPS1 protein levels were determined by western blot with an anti-GFP antibody; band intensities are expressed relative to the maximum (line MIPS1-GFP). *MIPS1* relative mRNA levels were measured by qRT-PCR (accumulation is expressed relative to the mRNA levels in MIPS1-GFP/*mips1-2*). Error bars represent standard error processed by qbase^{PLUS}. (B) Relative *MIPS1* mRNA levels in transiently transformed *N. benthamiana*. *MIPS1* was co-expressed with SUC2 or amiRNA^{MIPS1}. *MIPS1* mRNA levels were estimated by qRT-PCR and expressed relative to levels in leaves transformed with *MIPS1* and *SUC2*. Error bars represent standard error of three biological replicates. (C) Western blot showing the effects of amiRNA^{MIPS1} on MIPS1 protein accumulation in transiently transformed *N. benthamiana;* band intensities are expressed relative to the maximum (sample LOG2-HA). amiRNA^{LOG2} (LOG2 miB) was used as negative control, and co-expression of LOG2-HA was used as co-expression control. Numbers on the right indicate molecular weight in kDa.

Comparison of the effect of amiRNAs to suppress the expression of S*nRK1.1*

The four amiRNA$^{SnRK1.1}$ were introduced into a 35S-SnRK1.1-GFP/WT (Landsberg) line, and transformants were selected similarly as the MIPS1 amiRNA

lines above. The amiRNA$^{SnRK1.1}$ reduced *SnRK1.1* transcripts abundance by 80-95 % of SnRK1.1-GFP/WT levels, paralleled by a complete suppression of SnRK1.1-GFP protein accumulation (Fig. 3-3A). Because the SnRK1.1-GFP construct was introduced into a wild type background and an anti-SnRK1.1 antibody was used, endogenous SnRK1.1 protein levels could be monitored. Contrary to the transgene, endogenous SnRK1.1 protein was reduced in four out of the eight lines studied, with 299A and 299D being the most potent, recapitulating the protein levels found in *snrk1.1* knock-down mutant (Fig. 3-3A). It should be noted here that the suppression of the wild type SnRK1.1 protein accumulation could result from siRNAs generated from the degradation of the SnRK1.1-GFP mRNAs. In the transient assay in *N. benthamiana*, all amiRNA$^{SnRK1.1}$ reduced *SnRK1.1* transcripts level equally, by 70 %, less than in Arabidopsis, while SnRK1.1 protein accumulation was almost completely suppressed (Fig. 3-3B and 3-3C). In conclusion, SnRK1.1 protein accumulation correlated with mRNA levels upon action of the four amiRNAs targeting *SnRK1.1* in *N. benthamiana*. In Arabidopsis, although the four amiRNA$^{SnRK1.1}$ were equally sufficient to suppress SnRK1.1-GFP protein accumulation, amiRNA$^{SnRK1.1}$-D was the most potent.

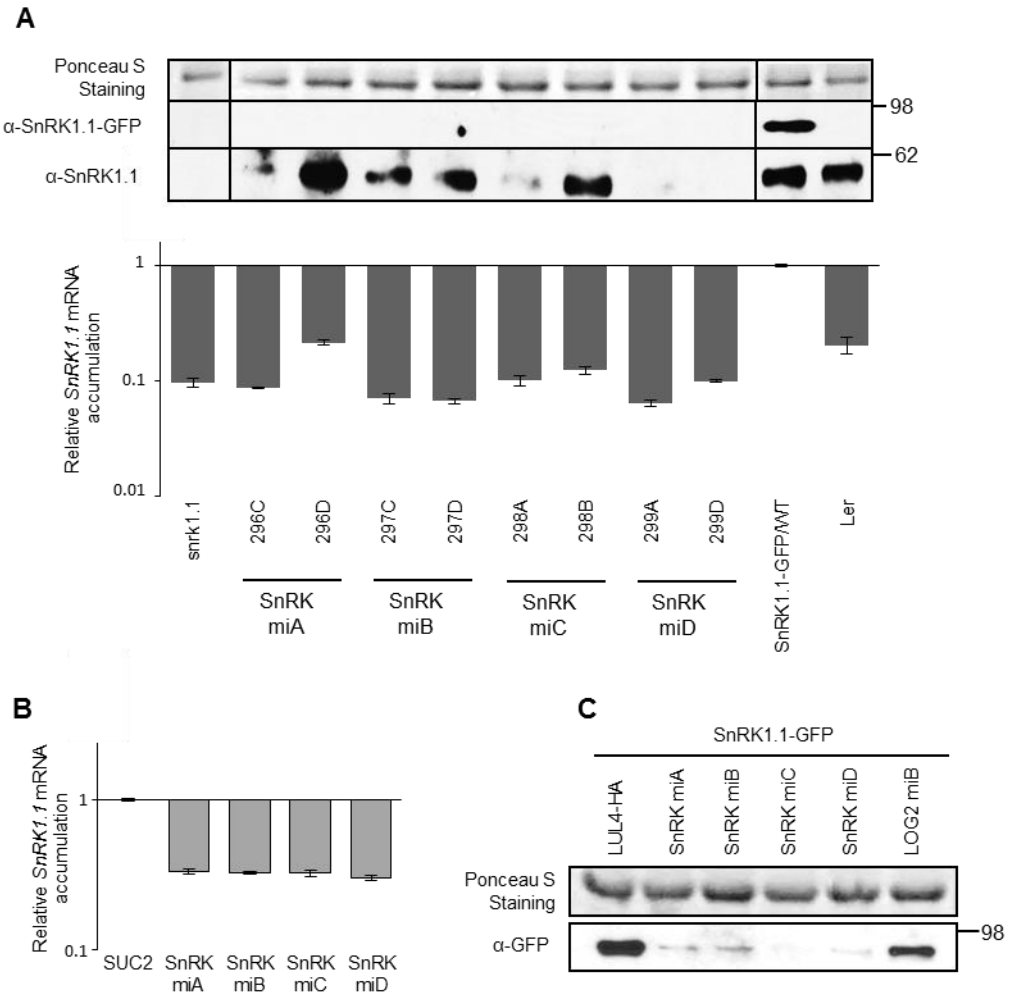

Figure 3-3. Analysis of amiRNAs targeting *SnRK1.1* in stably transformed Arabidopsis and transiently transformed *N. benthamiana.*

(A) amiRNAs targeting *SnRK1.1* (SnRK miA, miB, miC, miD) were stably introduced into the SnRK1.1-GFP/WT(L*er*) background. About 10 progeny of each line were analyzed. SnRK1.1 protein levels were determined by western blot using anti-SnRK1.1 antibodies. Endogenous SnRK1.1 and SnRK1.1-GFP blots are shown with different exposure time. *SnRK1.1* relative mRNA levels were measured by qRT-PCR (accumulation is expressed relative to the level of *SnRK1.1* in SnRK1.1-GFP/WT). Error bars represent standard error processed by qbase^{PLUS}. (B) Relative *SnRK1.1* mRNA levels in transiently transformed *N. benthamiana*. *SnRK1.1* was co-expressed with *SUC2 or* amiRNA$^{SnRK1.1}$. *SnRK1.1* mRNA level were estimated by qRT-PCR and expressed relative to levels in leaves transformed with *SnRK1.1* and *SUC2*. Errors bars represent standard error of at least two biological replicates. (C) Western blot showing effects of amiRNA$^{SnRK1.1}$ on SnRK1.1 protein accumulation in transiently transformed *N. benthamiana*. amiRNA^{LOG2} (LOG2 miB) was used as a negative control, and co-expression of LUL4-HA (a paralog from LOG2; Pratelli et al., 2012) was used as co-expression control. Numbers on the right indicate molecular weight in kDa.

Comparison of the effect of amiRNAs to suppress the expression of *GDU1*

amiRNA^{GDU1} were introduced into a line expressing GDU1-cMyc under the control of its own promoter (Pilot et al., 2004). Since the parental line did not display any visible phenotype, no simple visualization of the efficiency of each amiRNA could be obtained in T1, contrary to *LOG2*, *MIPS* and *SnRK1* studies above. Thus, four independent transformants from each transformation were randomly selected and studied. Two of the four lines expressing amiRNA^{GDU1}-A, -B and -C showed >98 % suppression of GDU1 protein accumulation while none of the lines expressing amiRNA^{GDU1}-D did (Fig. 3-4A). Surprisingly, lines accumulating GDU1 protein at the same level as the parental lines (lines 293A, 294A, 294D, 295A and 295C) showed an apparent increase in *GDU1* mRNA accumulation, reminiscent of the effect of amiRNA^{LOG2}-C and -D on *LOG2* mRNA in *N. benthamiana* (see Fig. 3-1B). Reduction of GDU1-cMyc protein was always accompanied by reduction in *GDU1* mRNA: the strongest decrease in *GDU1* mRNA accumulation always corresponded to the strongest reduction in GDU1 protein content (Fig. 3-4A). In the *N. benthamiana* transient assay, amiRNA^{GDU1}-A, -B and -C reduced *GDU1* transcript levels by 80, 95 and 75 %, respectively, but amiRNA^{GDU1}-D had no effect. Similar to Arabidopsis, a strong correlation between *GDU1* mRNA and protein contents could be observed in this system (Fig. 3-4B and 3-4C). The different silencing efficiency of the four amiRNA^{GDU1} was also

observed by fluorescence microscopy when co-expressing amiRNA^{GDU1} with GDU1-GFP in *N. benthamiana* leaves (data not shown), and was in accordance with the above results.

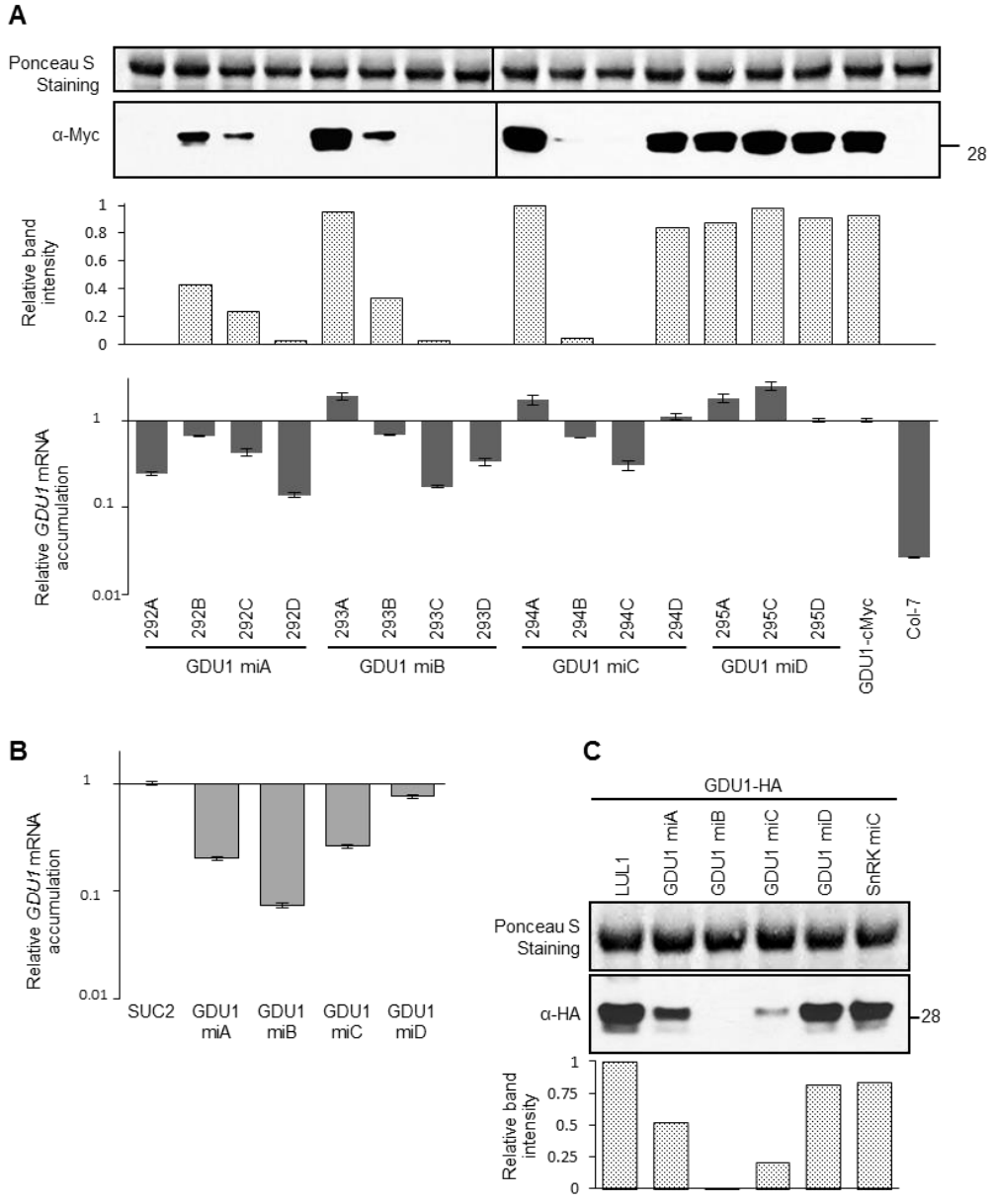

Figure 3-4. Analysis of amiRNAs targeting *GDU1* in stably transformed Arabidopsis and transiently transformed *N. benthamiana.*

(A) amiRNAs targeting *GDU1* (GDU1 miA, miB, miC and miD) were stably introduced into Arabidopsis line expressing GDU1-cMyc under the control of the *GDU1* promoter. About four progeny of each line were analyzed. GDU1 protein levels in stems were determined by western blot using an anti-cMyc antibody, band intensities are expressed relative to the maximum (line 294A). *GDU1* relative mRNA levels were measured by qRT-PCR (expressed relative to the *GDU1-cMyc* line). Error bars represent standard error processed by qbase^{PLUS}. (B) Relative *GDU1* mRNA levels in transiently transformed *N. benthamiana*. *GDU1* was co-expressed with *SUC2* or amiRNA^{GDU1}. *GDU1* mRNA level were estimated by qRT-PCR and expressed relative to levels in leaves transformed with *GDU1* and *SUC2*. Errors bars represent standard error of at least two biological replicates. (C) Western blot showing effects of amiRNA^{GDU1} on GDU1 protein accumulation in transiently transformed *N. benthamiana*, band intensities are expressed relative to the maximum (sample LUL1). amiRNA$^{SnRK1.1}$ (SnRK miC) was used as a negative control, and co-expression of LUL1 (a paralog from LOG2; Pratelli et al., 2012) was used as co-expression control. Numbers on the right indicate molecular weight in kDa.

Testing the relationship between amiRNA, target mRNA and protein accumulations.

These results suggested that the efficacy of amiRNA varies greatly from one amiRNAs to another, and that even amiRNAs found poorly efficient in *N. benthamiana* are sometimes able to suppress the expression of the target genes when stably expressed in Arabidopsis.

To test whether different expression of the amiRNAs in different Arabidopsis lines could explain this latter observation, amiRNA accumulation was determined by quantitative RT-PCR (Chen et al., 2005; Schmittgen et al., 2008). A slight decrease in *LOG2* mRNA accompanied increase in amiRNA^{LOG2} B abundance (Fig. 3-5A). Nevertheless, the change in LOG2 mRNA was modest (from 65 to 80 % reduction compared to the *gdu1-1D* parent), while the change in amiRNA^{LOG2}-B expression varied over 20 folds. The phenotype of the plants correlated well with amiRNA^{LOG2}-B accumulations but not with the reduction in *LOG2* mRNA level

(compare Fig. 3-1A and Fig. 3-5A): phenotype suppression was absent in line 230F which expressed amiRNA^{LOG2}-A ten times less than 230D, yet *LOG2* mRNA abundance was reduced by 65 and 75 % from the *gdu1-1D* parent in these lines, respectively. For GDU1, a clear negative correlation was observed between the expression of both amiRNA^{GDU1}-A and -B and *GDU1* mRNA accumulation (Fig. 3-5B and 3-5C) in Arabidopsis. The only discrepancy is the low GDU1 mRNA accumulation in line 293D while amiRNA^{GDU1} B is not expressed at very high levels, which remains unexplained. The accumulation of amiRNA^{GDU1}-A (2 to 50 times more than the ACT2 mRNA levels, as measured by q-RT-PCR) seemed higher than the accumulation of amiRNA^{GDU1}-B (0.01 to 5 times the ACT2 mRNA levels). It should be noted that the amplification efficiency of each amiRNA could be different, and the above results might reflect this difference. As a comparison, the levels of the efficient amiRNA^{LOG2}-B were estimated as 0.005 to 0.1 times the *ACT2* mRNA accumulation (data not shown). These data support the above observation that amiRNA^{LOG2}-B and amiRNA^{GDU1}-A and -B act differently on the stability of the target mRNA.

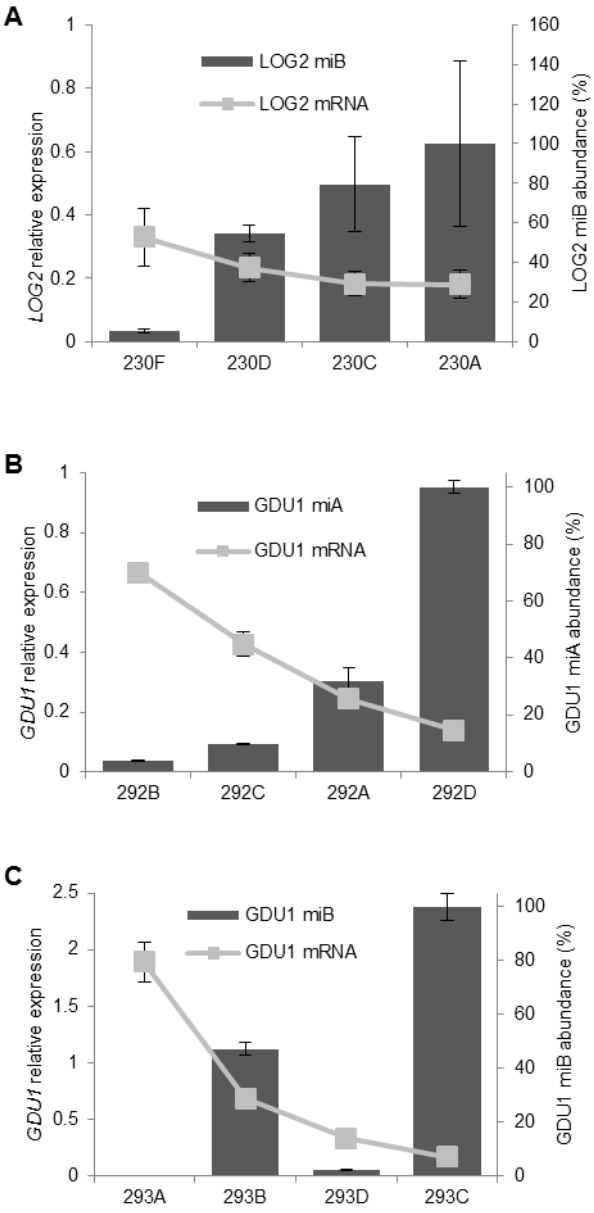

Figure 3-5: Analysis of the relationship between abundance of amiRNA and target mRNAs. (A) amiRNALOG2-B abundance determined by qRT-PCR for the same lines as in Fig. 3-1A. Accumulation is expressed relative to the highest expression (line 230A). LOG2 mRNA level is expressed relative to levels in gdu1-1D. (B and C) amiRNA abundance determined by qRT-PCR for amiRNAGDU1-A (B) and amiRNAGDU1-B (C) for the same lines as in Fig. 3-4A. Accumulations are expressed relative to the highest expression (lines 292D and 293C respectively). GDU1 mRNA level is expressed relative to levels in the GDU1-cMyc line.

To test whether the difference in amiRNA^{LOG2}-B abundance could cause differences in *LOG2* mRNA cleavage at the binding site, primers flanking

amiRNA^{LOG2} B binding site were designed. *LOG2* mRNA levels were quantitated by the flanking primer pair and a non-flanking primer pair, and no difference was found between the two quantities in any of the four tested lines (data not shown). This result indicates that the cleaved mRNA does not accumulate, otherwise the non-flanking pair should have detected more *LOG2* mRNA than the flanking pair, suggesting that the cleavage products are quickly degraded. The *LOG2* mRNA species quantitated by the qPCR correspond thus to the full length, translatable, *LOG2* mRNA.

The effect of the intensity the expression of various amiRNAs on protein accumulation was tested in *N. benthamiana* by varying the ratios of *Agrobacterium* strains delivering the various constructs in the plant cells. The amount of target construct being kept constant, the amount of amiRNA used varied from 1/10 to four times the amount of target construct. amiRNA^{LOG2}-A and -B affected LOG2 mRNA and protein content is a very similar way, in good agreement with their similar efficacy noted earlier (Fig. 3-6A). With increasing amiRNA^{LOG2} / LOG2 ratio, the effect on mRNA levelled off to about 90% reduction, while protein content decreased down to 1% of the control (Fig. 3-6A), suggesting an greater effect of the amiRNA on protein rather than mRNA content, especially at higher amiRNA expression. Similarly, amiRNA^{GDU1}-A and -B affected more strongly protein than mRNA content (Fig. 3-6B), but the efficacy of these amiRNAs was

different. As noted previously, amiRNA^{GDU1}-B could suppress more efficiently *GDU1* expression than amiRNA^{GDU1}-A. While increasing amiRNA^{GDU1}-A expression led to a paralleled decrease in both GDU1 mRNA and protein accumulation, increase in amiRNA^{GDU1}-B expression led to levelling off of *GDU1* mRNA suppression while GDU1 protein content further decreased (Fig. 3-6B). amiRNA^{GDU1}-A and -B seem to have different modes of action, amiRNA^{GDU1}-B possibly affecting more translation than amiRNA^{GDU1}-A. This hypothesis could explain the complete suppression of GDU1 protein accumulation by amiRNA^{GDU1}-B in *N. benthamiana* (Fig. 3-4C), compared to amiRNA^{GDU1}-A.

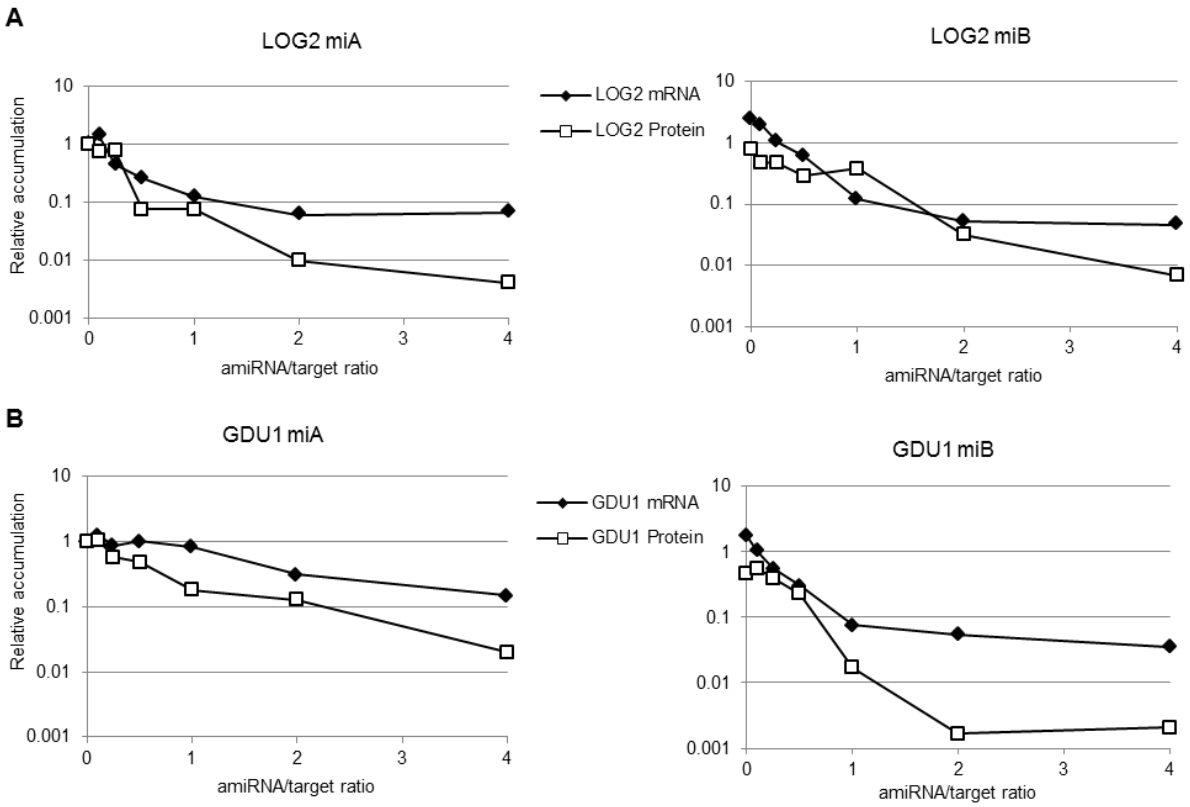

Figure 3-6. Analysis of the relationship between amiRNA expression, target mRNA and protein accumulations.
Two amiRNAs targeting (A) LOG2 and (B) GDU1. Leaves of N. benthamiana were infiltrated with various ratios of amiRNA / target gene (mLOG2-HA or GDU1-HA), ranging from 0.1 to 4. Control (ratio 0, left panels) corresponds to co-expression of the target gene with SUC2. The ratio 0 for the right panels corresponds to co-expression of the target genes with amiRNASnRK1.1. The same amount of the target constructs was infiltrated for each sample. Variable amounts of amiRNAGDU1 (or amiRNALOG2) and amiRNASnRK1.1-C were co-infiltrated to keep constant the total amount of amiRNA expressed in the leaf. Target mRNA and protein levels were determined by qRT-PCR and western blot respectively. Error bars represent standard error processed by qbasePLUS. Levels are expressed relative to the 0 ratio of the left panels.

Discussion

Strong activity of amiRNAs at the translational level

We found that in *N. benthamiana* leaves, a decrease in mRNA content parallels a decrease in protein content (for 15 out of 16 amiRNAs tested, the exception being amiRNA^{MIPS1}-C). Nevertheless, the reduction of the target mRNA by

amiRNA is most of the time modest (less than five times), while the decrease in target protein accumulation is much stronger (more than 10 times). For *MIPS1* and *SnRK1.1*, no linear relationship could be observed by comparing mRNA content and protein accumulation, and it rather appeared that protein accumulation was suppressed when a threshold in mRNA decrease was met (Fig. 3-2 and 3-3). The relationship was much more linear for *GDU1* (Fig. 3-4). This suggests that measuring the silencing efficacy of amiRNAs based on the target mRNA level would not perfectly reflect their ability at suppressing the target protein accumulation or activity.

The discrepancy between the intensity of the decreases in target mRNA and protein accumulations observed for *LOG2*, *MIPS1* and *SnRK1.1* suggests that the decrease in target mRNA level is not the only reason for decrease in target protein content. *N. benthamiana* assays for amiRNA^{LOG2}-A and -B and amiRNA^{GDU1}-B support this hypothesis (Fig. 3-6). Reports by Li *et al.* for amiRNAs (Li et al., 2013) and several recent articles for endogenous miRNAs (Aukerman and Sakai, 2003; Chen, 2004; Gandikota et al., 2007; Brodersen et al., 2008; Dugas and Bartel, 2008; Lanet et al., 2009; Zhu et al., 2009; Beauclair et al., 2010; Zhu and Helliwell, 2011; Alonso-Peral et al., 2012; Li et al., 2013; Ma et al., 2013; Meijer et al., 2013) support the fact that miRNAs in plants can act at the translation level, in addition to initiating mRNA cleavage. The expression of the target gene is likely

being repressed by a combination of mRNA cleavage and translational inhibition. Our results support this observation, and agree with previous results for amiRNAs (Li et al., 2013), which demonstrated that assessing amiRNA efficiency at the level of the target protein accumulation is more appropriate than at the target mRNA level.

Use of *N. benthamiana* to test amiRNA efficiency is valid

By transiently expressing amiRNAs with the corresponding target gene in Arabidopsis mesophyll protoplasts, Kim and Somers were able to rapidly find amiRNAs efficiently silencing known genes that recapitulates loss-of-function mutant phenotype of the circadian clock (Kim and Somers, 2010). In a more comprehensive assay, Li *et al*. used Arabidopsis protoplasts to transiently express a protein and the corresponding amiRNAs to be tested and developed epitope-tagged protein-based amiRNA (ETPamir) screens to facilitate validation of optimal amiRNAs (Li et al., 2013). While well described and used, we found that preparation of protoplasts requires some experience, and might pose troubles in laboratories where this technique is not established. Transient gene expression in *N. benthamiana* leaves is often viewed as a simpler method, used by many groups. *N. benthamiana* has been used to test for amiRNA expression before stable transformation of potato and *N. benthamiana* (Bhagwat et al., 2013). The authors of this report found a trend between the strength of expression of amiRNA in the

transient assay and stable transformation, suggesting that amiRNA could be selected for higher expression in *N. benthamiana* before stable transformation. Nevertheless, the efficacy at suppressing target gene expression, neither at the target mRNA level, nor at the target protein level was tested in this study (Bhagwat et al., 2013). More recently, transient expression in *N. benthamiana* has been used to study complementarity requirement of miRNAs for their target to efficiently induce suppression (Liu et al., 2014). The *N. benthamiana* system offers several advantages: 1) It directly assesses amiRNA silencing efficacy by measuring target protein accumulation, which circumvents the complexity of the mode of action of amiRNA at the target mRNA and/or the target protein level. Fusion of a tag, such as HA and cMyc, to target proteins enables measuring protein accumulation in plants, with minimal interference with target protein function. The use of GFP can facilitate selection of efficient amiRNAs at the seedling stage and provides direct visualization by microscopy, but may interfere with target protein function and stability. 2) Expressing target protein and amiRNA candidates at the same time is simple and fast. As long as amiRNA candidates and their tagged target protein constructs are prepared and introduced into *Agrobacterium*, transient expression of amiRNAs and their target in *N. benthamiana* requires minimum preparation. Two days after infiltration, the target mRNA and/or protein levels can be measured and the most efficient amiRNA can be determined. 3) Many amiRNA candidates can

be tested in parallel, with various target construct/amiRNA ratios if necessary. 4) Inefficient amiRNAs (*e.g.* amiRNA^{LOG2}-C and -D; amiRNA^{GDU1}-D) can be discarded before considering stable plant transformation.

In addition to testing amiRNA efficiency in *N. benthamiana* by transient assay, we expressed all of the 16 amiRNAs in Arabidopsis and tested their effect on target protein levels and corresponding phenotypic changes to confirm the reliability of results from the transient assay. Indeed, amiRNAs that are more potent to suppress target protein accumulation in the transient assay were more likely to suppress target protein accumulation in stably transformed Arabidopsis and cause expected phenotypic changes (*e.g.* amiRNA^{LOG2}-A and -B; amiRNA^{GDU1}-B an- C). amiRNAs that suppressed target protein accumulation with equal efficiency in the transient assay exhibited similar equal efficiency to suppress target protein accumulation in stably transformed Arabidopsis (*e.g.* amiRNA^{MIPS1} and amiRNA$^{SnRK1.1}$). Li *et al.* expressed only six of 63 amiRNAs in Arabidopsis and observed phenotypic change of four lines (another one was modest; Li et al., 2013). Surprisingly, even our less efficient amiRNA in *N. benthamiana* (amiRNA^{MIPS1}-C) was able to reduce target protein accumulation and cause the corresponding phenotypic changes in stably transformed Arabidopsis (Fig. 3-2). On the other hand, amiRNAs found efficient in *N. benthamiana* did not always lead to strong decrease in protein accumulation in every transformed Arabidopsis

line (see amiRNA^{MIPS1}-A, -B and -D, and amiRNA^{GDU1}-B, Fig. 3-2 and 3-4). This observation can be attributed to the different expression level of the amiRNAs amongst different transformants, as observed for amiRNA^{LOG2}-B, and amiRNA^{GDU1}-A and -B (Fig. 3-5). This indicates that expression levels of the amiRNA are important for its efficacy in stably transformed plants, and likely depend on number of T-DNA copies inserted in the genome and localization of the insertion(s). In conclusion, this assay will enable discarding non-efficient amiRNAs, but cannot determine the most efficient ones.

In the transient assay, the target proteins are driven by CaMV 35S promoter and epitope tagged to allow estimation of amiRNA silencing efficiency by measuring the target protein accumulation. However, in most cases, the real target of those amiRNAs is an endogenous gene. In this study, we have verified the efficiency of amiRNA to suppress target expression under various genetic backgrounds in Arabidopsis. amiRNAs targeting *LOG2* were introduced into *gdu1-1D* background. amiRNA^{LOG2}-A and -B, which were shown potent to suppress *LOG2* expression in the transient assay, successfully reduced LOG2 activity and abolished the LOG2-dependent Gdu1D phenotype in Arabidopsis. amiRNA^{GDU1} were introduced into a line expressing GDU1-cMyc under GDU1 promoter, where *GDU1* mRNA accumulation is only slightly increased compared to the wild type (data not shown). amiRNA^{GDU1}-D, which was found inefficient in the transient

assay, could not suppress GDU1 protein accumulation in Arabidopsis. The four amiRNA^{MIPS1} showed similar modest efficiency to suppress MIPS1 protein accumulation in the transient assay. In the 35S-MIPS1-GFP/*mips1-2* background, one out of the two lines selected for each amiRNA^{MIPS1} showed reduced MIPS1 protein accumulation and the corresponding phenotypic changes. In summary, the silencing efficiency of amiRNAs measured by the transient assay in *N. benthamiana* represents well their efficiency in stably transformed Arabidopsis, no matter if the target is a transgene or an endogenous gene.

Analysis of free energy and target site location of amiRNAs

Li *et al.* proposed a new amiRNA selection criteria, in which optimal amiRNAs should have no predicted off-target genes, complementary sequence within the first 5' 200 nucleotides of the coding sequence, displaying up to two mismatches in position 1 or 15-21, and with an hybridization energy larger than 80 % of the perfect match (Li et al., 2013). The hybridization energy of our optimal amiRNAs varied from 78 % to 99 %, in good agreement with the published results. However, we found several amiRNAs not meeting these criteria that also efficiently silenced target gene expression, with no clear preference for the target site location on the target transcript (data not shown). Our results indicate that the mode of action of an efficient amiRNA involves many different factors, making necessary to test experimentally the amiRNAs to be selected for stable plant

transformation. Similar to Li *et al.*, we could not detect any mRNA cleavage product by using a quantitative RT-PCR approach, suggesting that the cleavage products are quickly degraded (Li et al., 2013). The reduction of target mRNA level does not always correlate with decreases in target protein accumulation, and is thus an insufficient measure to determine optimal amiRNAs. As also concluded by Li *et al.*, we found that measuring target protein level by transient co-expression of amiRNAs with the target in heterologous system (*N. benthamiana* leaves or in protoplasts) is a better way to find optimal amiRNAs (Li et al., 2013).

Material and methods

Plant lines and transformations

Arabidopsis thaliana and *Nicotiana benthamiana* were grown under 120 $\mu E/m^2/s$, 22 ℃, 16 h light /8 h dark, or 40 $\mu E/m^2/s$ (low light condition) when needed. Plant lines 35S-MIPS1-GFP in the *mips1-2* mutant (ecotype Columbia; Donahue et al., 2010), 35S-SnRK1.1-GFP in wild type (Landsberg *erecta*) and *mips1-2* were obtained from Dr. Glenda Gillaspy (Virginia Tech). *snrk1.1* knock-down line (Baena-Gonzalez et al., 2007) was obtained from Dr. Glenda Gillaspy with permission from Dr. Filip Rolland. *Arabidopsis thaliana* were transformed by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* GV3101 (pMP90). Phenotypes were recorded about a month after germination. For transient expression of proteins in *N. benthamiana*, young leaves of five-week old

plants were infiltrated with a suspension of *Agrobacterium tumefaciens* carrying the constructs of interest according to (Batoko et al.), with the following modifications. The bacteria were grown overnight in LB supplemented with appropriate antibiotics, washed twice in 10 mM $MgCl_2$, 100 µM acetosyringone, and diluted to final OD_{600} of 0.05 (target constructs) and 0.1 (amiRNA constructs) in the same solution before infiltration in *N. benthamiana* leaves.

Cloning and constructs

Sequences of the artificial microRNAs were obtained from WMD (http://wmd3.weigelworld.org/; Schwab et al., 2006; Ossowski et al., 2008) following the guidelines of the website, and were created by single overlap extension PCR of three fragments as described previously (Pratelli et al., 2012). The primers corresponding to pRS300 (Schwab et al., 2010) used for amplification of the amiRNAs contained the Gateway attB sites. The final PCR fragment was cloned into pDONRZeo (Life Technologies), sequenced, and transferred into the pSWsNkan or pSWSNhyg binary vector, derivatives of pJHA212K (Yoo et al., 2005, R. Pratelli and G. Pilot, unpublished data), between the CsVMV promoter (Verdaguer et al., 1998) and the terminator of the small subunit of the Rubisco from pea (Pisum sativum; accession no. X00806). The MIPS1-GFP construct has been previously described (Donahue et al., 2010). The SnRK1.1-GFP construct was obtained from Dr. Gillaspy, Virginia Tech (unpublished). mLOG2-HA (whose

ubiquitin ligase activity has been abolished by mutagenesis), GDU1-cMyc and GDU1-HA constructs were previously described (Pratelli et al., 2012).

RNA extraction and quantitative RT-PCR

Total RNA were extracted from leaves with TRI Reagent® (Sigma-Aldrich) following manufacturer's instructions. The integrity of the RNA was confirmed by agarose gel electrophoresis before reverse transcription. cDNAs were synthesized from 2 µg of total RNA using the SuperScript III (LifeTechnologies) according to the manufacturer's instructions, in a 10 µl reaction volume. Five µl of primer mix (1 µM each) and 5 µl of the reverse transcription products diluted 50 times in water were mixed with 10 µl of 2X SYBR® Green PCR Master Mix (LifeTechnologies) and subjected to the following cycles: 2min 50 °C, 10 min 95 °C, 40 times of 15 sec 95 °C, 15 sec 55 °C, 1 min 72 °C in a 7300 Real Time PCR System, Applied Biosystems. Three reference genes (Actin2 - AT3G18780, UBC9 - AT4G27960 and PP2A - AT1G13320) were tested in the same experimental conditions. Results were processed with qbase^{PLUS} software (Biogazelle; Vandesompele et al., 2002; Hellemans et al., 2007). qRT-PCR results were normalized and processed by the reference gene(s) selected from qbase^{PLUS} (Hellemans et al., 2007). amiRNA analysis by qPCR was performed following a previously described method (Chen et al., 2005; Schmittgen et al., 2008).

Protein extraction and western blot

Proteins were extracted from infiltrated *N. benthamiana* leaves by homogenization in extraction buffer in a mortar on ice, or from stably transformed *Arabidopsis* tissues, ground in liquid nitrogen. Samples (300 mg fresh weight) were mixed with 1 ml extraction buffer (50 mM Tris-HCl pH 7.3, 150 mM NaCl, 10 mM $MgCl_2$, 0.5% Nonidet P-40, 10 mM DTT, 1X Complete® (Roche Diagnostics)). Homogenates were centrifuged at 10,000 g, at 4℃ for 15 min. Protein in the resulting supernatants were quantitated using the Bradford assay (Thermo Scientific), and 10 µg were analyzed by SDS-PAGE (4-12% polyacrylamide NuPAGE® MES gel, Life Technologies). Proteins were transferred on a nitrocellulose membrane (GE Healthcare), and detected using anti-cMyc (Clone A-14, Santa Cruz; 1:10,000), anti-HA (Clone 3F10, Roche Diagnostics; 1:5,000), anti-GFP (Clone FL, Santa Cruz; 1:2,000), or anti-SnRK1.1 (Dr. Gillaspy, Virginia Tech, unpublished; 1:1,500) primary antibodies, anti-rabbit, anti-rat or anti-goat (1:2,000-1:10,000; Thermo Scientific) secondary antibodies, and the ECL-Plus western blotting detection system (GE Healthcare), and recorded on X-ray films or CCD camera. Intensity of the bands on the film was measured after film scanning, and may not accurately reflect the dynamic range of the signal intensities.

Fluorescence microscope imaging

GFP-labeled proteins expressed in *N. benthamiana* epidermis cells were visualized on a Zeiss HBO 100 microscope illuminating system on an Axio Imager.M1 microscope using an EC plan-NEOFLUAR 20X N.A. 0.5 objective (Carl Zeiss), with BP 515-565 emission filter. All images were captured with the same light intensity and the same configurations.

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Chapter IV

Suppressor mutations in the GDU1 protein dissociate size reduction and amino acid export enhancement

Introduction

Amino acids are critical metabolites in plants which fulfill several roles, in addition to being the constituting blocks of proteins. Amino acids are used a precursors for the synthesis of many secondary metabolites, like flavonoids (Falcone Ferreyra et al., 2012), alkaloids (Ziegler and Facchini, 2008), and glucosinolates (Kraker and Gershenzon, 2011), which are critical for interaction of the plant with the environment (attraction, defense and protection). Amino acids also serve as essential carriers for organic nitrogen throughout the plant, being transported through the xylem and phloem saps between leaves, roots, storage organs and meristems (Tegeder, 2014). Transport in the plant is mediated at the cell level by membrane proteins with specialized functions. Amino acid importers and exporters mediate transport of amino acids in opposite directions, either towards or from the cytosol, and respectively from or to the apoplasm, vacuoles or intracellular vesicles.

Characterized amino acid importers belong to the APC superfamily (Vastermark et al., 2014), composed of 63 members in Arabidopsis (Tegeder and Rentsch, 2010). Importers use energy of the proton gradient to import amino acids

against their concentration gradient, and are involved in many roles in the plant, like uptake from the soil, import into the phloem, phloem-xylem exchange, and transport into the embryo (Tegeder, 2012). Much less is known about amino acid exporters. Some APC members have been described as possible amino acid exporters (e.g. CAT8, (Yang et al., 2010); BAT1, (Dundar and Bush, 2009)), and one gene belonging to the DMT superfamily (Jack et al., 2001), SiAR1, has been shown to unequivocally mediate amino acid export from plant cells (Ladwig et al., 2012). The family to whom SiAR1 belongs to contains 47 members with only one other gene characterized, the auxin transporter WAT1 (Ranocha et al., 2013).

The existence of processes controlling the activity of amino acid transporter was evidenced by the discovery of the *GDU1* gene, encoding a 158 amino acid protein, with a single transmembrane domain (Pilot et al., 2004). Two domains are conserved among GDU proteins: the membrane domain, and a cytosolic 19 amino acid long region, called the VIMAG domain (Pilot et al., 2004; see Fig. 4-1). Over-expression of *GDU1* leads to a complex phenotype characterized by reduced plant size; early leaf senescence; crystallization of glutamine at the leaf margins; increased amino acid content in leaves, apoplasm, and xylem and phloem saps; and notably enhanced amino acid export from cells, while amino acid import unaffected (Pilot et al., 2004; Pratelli et al., 2010). GDU1 over-expressors are also tolerant to toxic concentrations of amino acids when grown on synthetic medium

(Pratelli and Pilot, 2007). This complex phenotype can almost entirely be explained by enhanced amino acid export from cells: this phenomenon would increase amino acid content in the apoplasm and phloem and xylem saps, and prevent absorption of Gln from the xylem in the leaf, which then is excreted by the hydathodes (Pilot et al., 2004). Size reduction likely comes from the induced disturbance in nitrogen metabolism.

Overexpression of the seven Arabidopsis *GDU* genes leads to a phenotype similar to *GDU1* over-expressing plants, such as enhanced amino acid efflux, increased amino acid content in leaves and very often, reduced plant size. Differences in the expression profiles of the *GDU* gene suggest that they fulfil different roles in specific developmental stages and organs. The fact that over-expression of *GDU1* in *Nicotiana tabacum* and GDU1-homologs in Arabidopsis leads to a Gdu1D-similar phenotype suggests that this family of proteins have a conserved function in plants, related to the regulation of amino acid export (Pratelli and Pilot, 2006; Pratelli et al., 2010).

The ubiquitin ligase LOG2 was identified in a yeast-two-hybrid screening as a GDU1 interactor, and the two proteins were found to co-immunoprecipitate when expressed in *Nicotiana benthamiana*. Decrease in *LOG2* expression suppresses the Gdu1D phenotype, indicating that LOG2 activity is necessary for the development

of the phenotype (Pratelli et al., 2012). LOG2 is similar to the mammalian MGRN1 protein, involved in the regulation of the trafficking membrane proteins through the endosome. MGRN1 and LOG2 were found to have several overlapping properties, despite coming from different organisms (Guerra et al., 2013). Like *GDU1*, *LOG2* is expressed at the plasma membrane. It is possible that *LOG2* and *GDU1* are involved in the regulation of the activity or trafficking of amino acid exporters, such as, when *GDU1* is over-expressed, the exporter is more active at the plasma membrane (Pratelli et al., 2012; Guerra et al., 2013).

An ethyl methanesulfonate (EMS) screening aiming at isolating mutation suppressor of the Gdu1D phenotype led to the isolation of two mutants: *log1-1* and *log2-1* (*loss of gdu*; Pratelli and Pilot, 2006; Pratelli et al., 2012). *log1-1* is a G100R substitution in the conserved VIMAG domain of GDU1, which abolishes the interaction with LOG2 (Pratelli et al., 2012). The *log2-1* is a R12K substitution in LOG2, whose biochemical effect has not been determined (Pratelli et al., 2012). Another mutation, *log2-3*, was isolated from the same screening, and is a non-sense mutation in LOG2 (R15X; Pratelli and Pilot, unpublished). These three *log* mutations led to plants that are phenotypically indistinguishable from the wild type when grown on soil and on amino acid containing media.

From the same screening, we isolated three additional *log1* mutations, whose characterization is reported here. Surprisingly, while the size of *log1* mutants was similar to the wild type, the amino acid export activity of those mutants showed intermediate between *gdu1-1D* and the wild type, which indicates that *log1* mutations do not completely suppress the Gdu1D phenotype. Mutations that abolished the GDU1-LOG2 interaction suppressed the Gdu1D phenotype. The function of GDU1 is likely regulated by protein phosphorylation and ubiquitination, which increases the complexity of regulation of GDU1 protein.

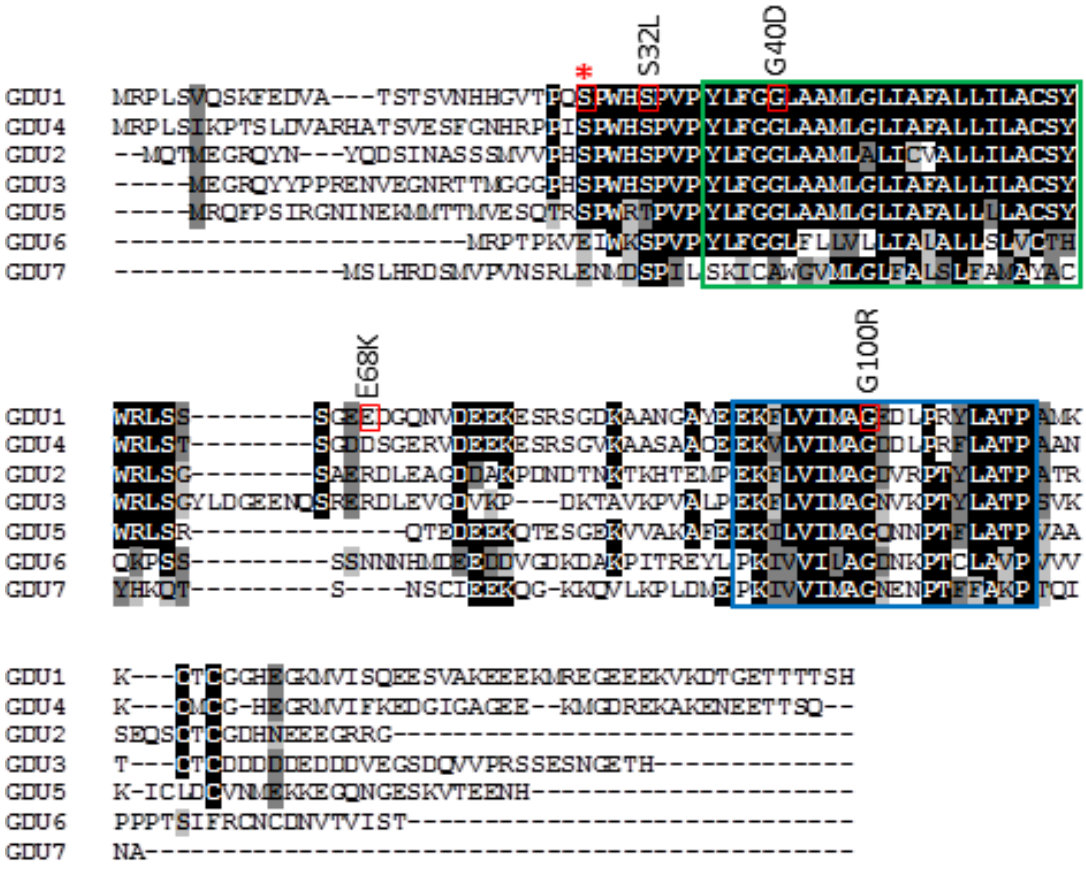

Figure 4-1. Localization of the suppressor mutations in GDU1.
Residues of interest are boxed in red, the conserved membrane and VIMAG domains are boxed in green and blue, respectively. Suppressor mutations are indicated above the corresponding boxes (*log1-2*, *log1-3* and *log1-4* correspond to E68K, G40D, S32L respectively). Star indicates GDU1 S28, a predicted phosphorylation site located in the extracellular side of GDU1.

Results

Identification of three mutations suppressor of the Gdu1D phenotype

The same pipeline as previously described was followed to isolate Gdu1D suppressor mutants: visual screening of M2 plants from the EMS mutagenesis of the GDU1 over-expressors *gdu1-5D* and *gdu1-6D*, confirmation of the consistence

of *GDU1* mRNA over-accumulation, and analysis of segregation of the mutation in the progeny from a cross between the suppressor mutant and the parents (Pratelli and Pilot, 2006). Out of a total of 110,000 M2 seeds screened, three additional suppressor mutations were isolated that segregated in accordance with an intragenic mutation in GDU1. Similar to *log1-1*, the corresponding mutants came from the mutagenesis of the over-expressor *gdu1-6D* (Pratelli and Pilot, 2006), and were named *log1-2*, *log1-3* and *log1-4*. Sequencing of the GDU1 locus in the T-DNA leading to the over-expression revealed that *log1-2*, *log1-3* and *log1-4* corresponded respectively to G202A, G119A and C95T mutations in GDU1 DNA sequence (numbered from the ATG), leading respectively to E68K, G40D and S32L substitutions in GDU1 protein sequence (Fig. 4-1). Because these mutation suppressed the Gdu1D visual phenotype (plant size and early senescence), it was hypothesized that they affected GDU1 protein function. Their characterization could thus lead to better understanding of GDU1's structure-function relationships in relation to LOG2 role.

Gln uptake of the suppressors was analyzed and compared to the parental line *gdu1-6D*. The *log1-1*, *log1-2*, *log1-3* and *log1-4* suppressor mutants showed uptake and efflux similar to one-another, but surprisingly different from both the wild type and *gdu1-6D*. The *log1* mutants had an uptake about 30% of the wild type (Fig. 4-2A) and an efflux twice as large as the wild type (Fig. 4-2B), while the uptake of

gdu1-6D was 25% and the efflux was three times as large as the wild type. This intermediate phenotype was clearly visible when the uptake and efflux were plotted on the same graph (Fig. 4-2C).

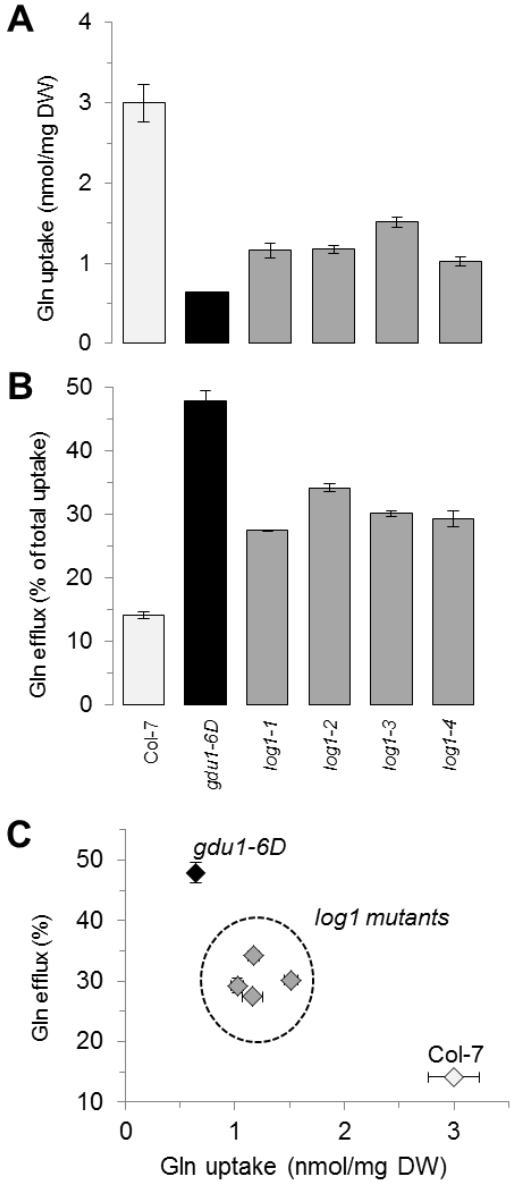

Figure 4-2. Gln uptake and efflux analyses of the log suppressor mutants.
(A) Gln uptake of 2-week old plantlet. (B) Gln efflux of 2-week old plantlet, expressed a percentage of total Gln taken up. (C) A 2-D plot of results shown in (A) and (B). Error bars are SEM (n=3).

Characterization of plants over-expressing the GDU1 variant proteins

The mutagenized *gdu1-5D* and *gdu1-6D* plants expressed a wild type GDU1 protein, preventing any detection of the accumulation of the protein (despite two different attempts, no suitable antibody could be raised against GDU1). In order to test for any effect of the suppressor mutations on the stability of the GDU1 protein, the wild type and the three *log1* GDU1 variants were placed in fusion with the HA tag under the control of the CaMV 35S promoter, and expressed in *N. benthamiana*. Mutations E68K and S32L did not affect protein accumulation, while mutation G40D led to a consistent reduction of about 30% in protein accumulation in this system (Fig. 4-3). The sub-cellular localization of the GDU1 variants was determined by expression of the GFP-tagged proteins (GFP positioned in C-terminal) in *N. benthamiana* leaves and observation by confocal microscopy. Similar to GDU1, the proteins localized to the plasma membrane and in vesicles that could correspond to endosomes. Interestingly, G100R and G40D GDU1 located more in tiny punctuated structures than the other proteins (data not shown).

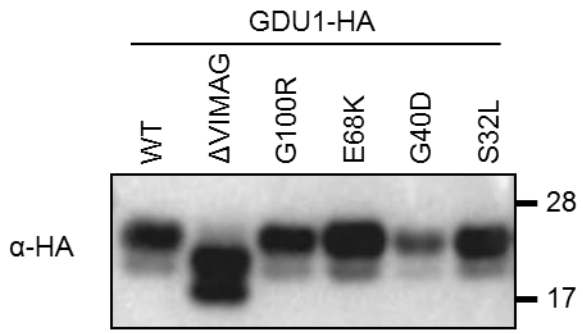

Figure 4-3. Accumulation of the GDU1 variant proteins in *N. benthamiana* leaves.
Analysis of plants over-expressing the GDU1 variants. Accumulation of the HA-tagged GDU proteins in each line was estimated by western blot with an anti-HA antibody. The same amount of Agrobacterium was infiltrated per leaf.

To test for the functional properties of the proteins in Arabidopsis, the HA-fusion constructs were used to transform Arabidopsis. The accumulation of the proteins in lines that segregated 3:1 for the kanamycin resistance for each construct was tested by western blot (Fig. 4-4B). In all cases, the size of the plant expressing the GDU1 suppressor variants was identical to the wild type, while the plants over-expressing GDU1-HA showed a ~45% reduction in rosette diameter compared to the wild type and the empty vector-transformed plants (Fig. 4-4A). In addition, only the GDU1-HA over-expressors displayed Gln secretion crystals, typical of the Gdu1D phenotype. The size reduction of these lines was lower than for the original *gdu1-1D* over-expressor (~60%; Fig. 4-4A), and could be attributed to the difference in the construct used or the presence of the HA tag, which might slightly interfere with the protein stability or activity. Analysis of Gln uptake and efflux from these over-expressors showed that G40D, S32L GDU1-HA over-expressing

plants behaved similarly to the *log1* mutants: the uptake was reduced by ~50% while the efflux was increased by ~50-100%. On the contrary, E68K GDU1-HA over-expressors transported Gln similar to the GDU1-HA plants, and different from the *log1-2* plants that they were supposed to recapitulate (Fig. 4-5A and B). The discrepancy between the Gln uptake and efflux of *log1-2* and E68K GDU1-HA over-expressors remains unexplained.

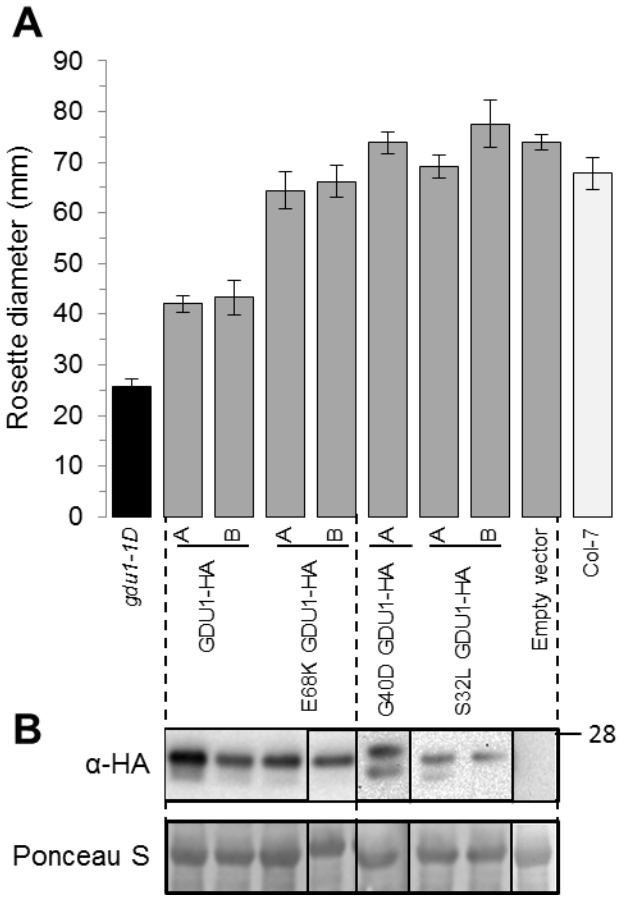

Figure 4-4. Analysis of plants over-expressing the GDU1 variants.
(A) Rosette diameter of four week-old Arabidopsis. Error bars are SEM (8 plants). (B) Accumulation of the HA-tagged GDU proteins in each line was estimated by western blot with an anti-HA antibody. Col-7 and *gdu1-1D* do not express any tagged protein and were not tested.

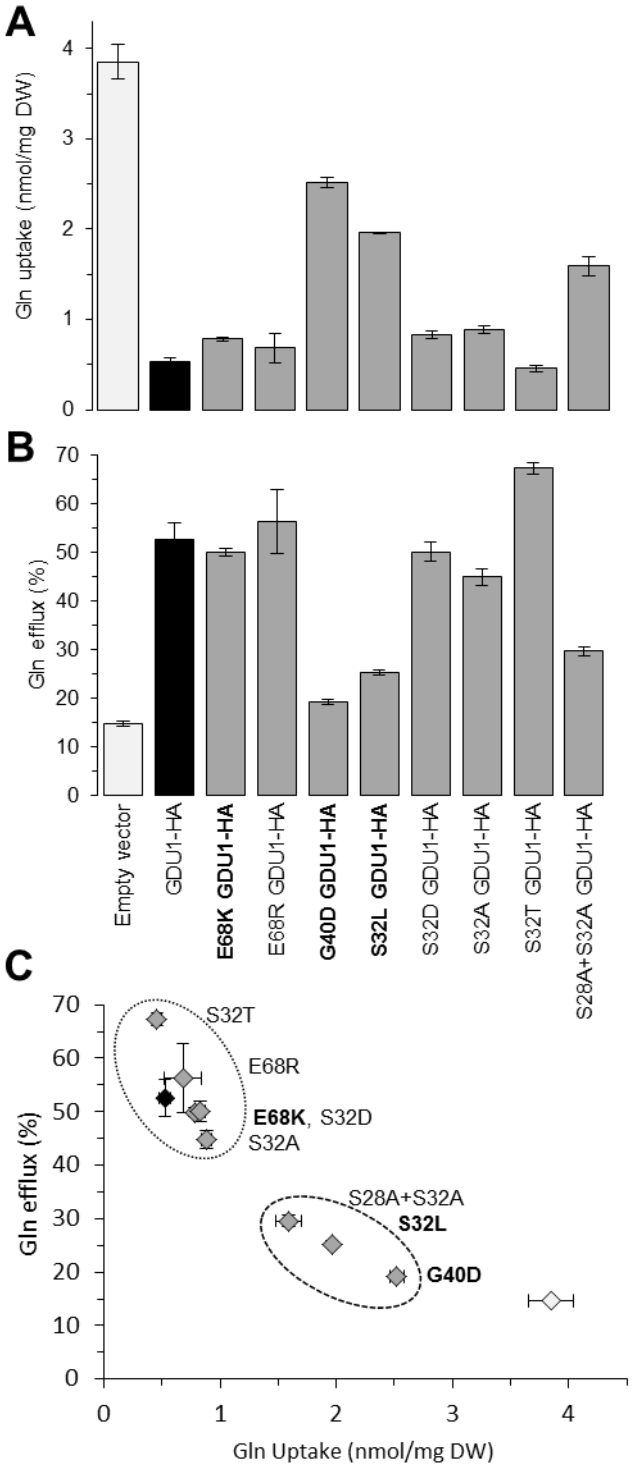

Figure 4-5. Gln uptake and efflux analyses of plant over-expressing the GDU1 variants.
(A) Gln uptake of 2-week old plantlet. (B) Gln efflux of 2-week old plantlet, expressed a percentage of total Gln taken up. (C) A 2-D plot of results shown in (A) and (B). The mutations corresponding to the *log1-2*, *log1-3* and *log1-4* mutants are indicated in bold. Error bars are SEM (n=3).

These results showed that the over-expression of the GDU1 suppressor variants led to plants visually indistinguishable from the wild type, with increased amino acid export and reduced import, similar to the original *log1* suppressors. The intermediate phenotype of the *log1* mutants is thus caused by the over-expression of variant GDU1 proteins, endowed with reduced functionality.

Testing of the interaction of the GDU1 variants for the interaction with LOG2

The suppression of the Gdu1D phenotype by the G100R (*log1-1*) mutation was explained by the loss of interaction with LOG2, the ubiquitin ligase necessary for the development of the Gdu1D phenotype (Pratelli et al., 2012). The G100R mutation affects the Gly residue of the VIMAG domain found in every GDU proteins tested so far, and it is supposed that it either affects the folding of the VIMAG domain or the Arg creates a steric clash at the interface surface between GDU1 and LOG2. We tested if any of the three other *log1* mutations would similarly affect the GDU1-LOG2 interaction, and would thus explain the phenotype suppression.

The tagged proteins were expressed in *N. benthamiana* leaves as Myc- or HA-fusions and immunoprecipitated using cMyc agarose beads. A GDU1 protein lacking the VIMAG domain (ΔVIMAG GDU1) and previously shown not to interact with LOG2 (Pratelli et al., 2012), was used as a negative control (Fig. 4-6).

GDU1-HA, E68K GDU1-HA and S32L GDU1-HA could be co-purified, but G40D GDU1-HA did not co-immunoprecipitate with LOG2 (Fig. 4-6). The G40D mutation, in addition to affecting the protein abundance, thus seems to prevent the interaction of GDU1 with LOG2, which could explain the suppressor effect.

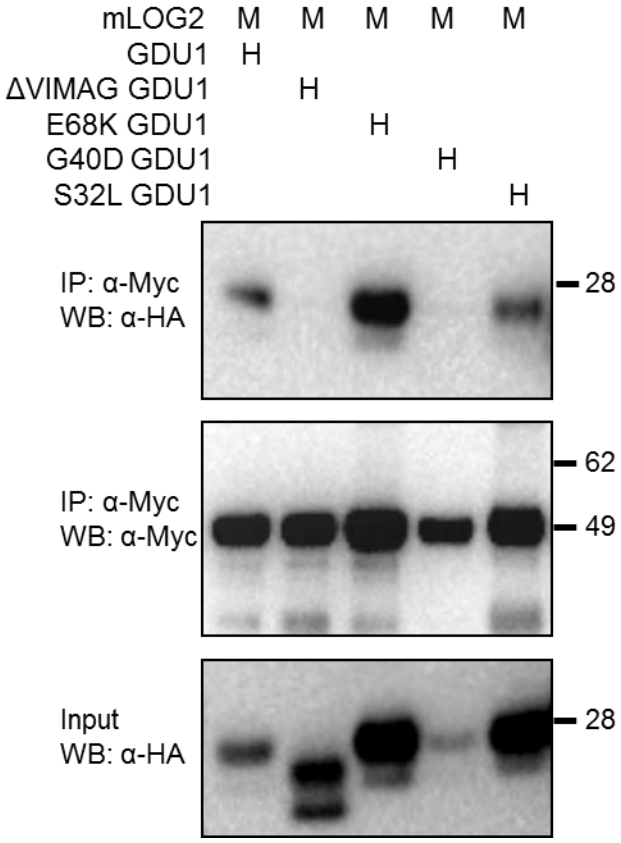

Figure 4-6. Co-immunoprecipitation of the GDU1 variants and mLOG2 expressed in *N. benthamiana*.
GDU1 variants were expressed as C-terminal HA fusion (H), mLOG2 (ubiquitination defective) as C-terminal Myc fusions (M) in N. bentamiana leaves. Proteins were extracted and immunoprecipitated (IP) with an anti-cMyc antibody, and detected by western blot (WB). Numbers on the right indicate molecular mass in kD. In this particular experiment, GDU1-HA did not accumulate at the same level as the other proteins.

Site directed mutagenesis to understand the S32A effect

Ser32 is highly conserved among GDU proteins, being sometimes replaced by Thr (in 13% of the ~100 analyzed sequences; data not shown), a residue with similar chemical properties. Running prediction algorithms such as PhosPhAT (Heazlewood et al., 2008; Durek et al., 2010) and PlantPhos (Lee et al., 2011) suggested that Ser32 can be phosphorylated (Fig. 4-7), despite the fact that this portion of the protein is considered to be extra-cellular. The PhosPhAT tool similarly predicted Ser28 can be phosphorylated. Ser32 was mutagenized to Thr, Asp, and Ala to test for the importance of the Ser *vs.* Asp and Ala, mimicking and suppressing phosphorylation respectively. Ser28 and Ser32 were mutagenized to Ala at the same time to ensure that lack of Ser32 phosphorylation could not be complemented by phosphorylation of Ser28. The mutagenized GDU1 proteins were stably expressed in Arabidopsis in fusion with the HA tag, and the size of the plants and protein accumulation were determined as above.

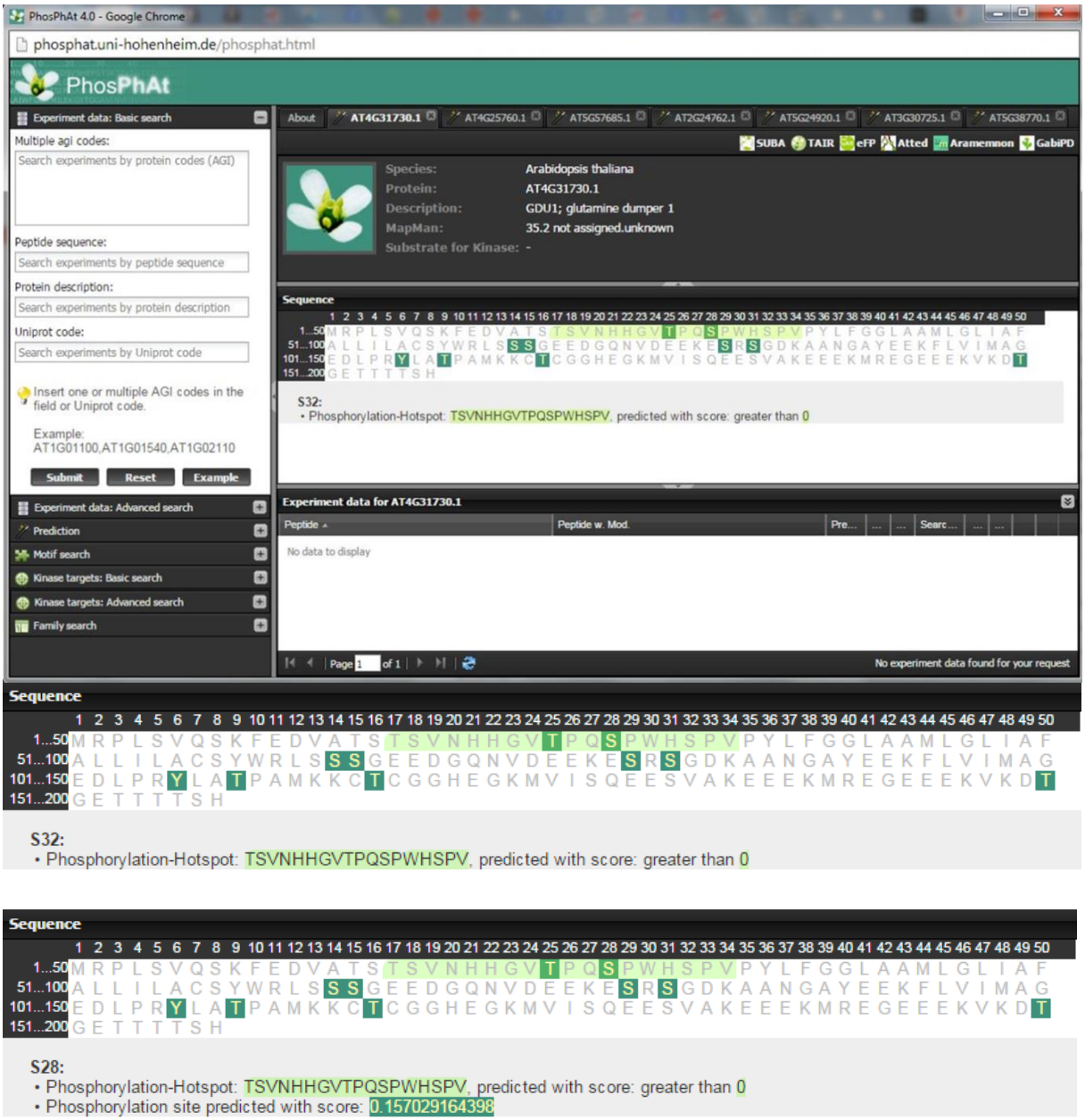

Figure 4-7. GDU1 phosphorylation site predicted by PhosPhAt 4.0.
Screenshot of GDU1 phosphorylation site predicted by PhosPhat 4.0. Top panel, screenshot showing all results. Middle panel, screenshot showing S32 phosphorylation score. Bottom panel, screenshot showing S28 phosphorylation score.

The size of the plants over-expressing the S32T mutant GDU1 was identical to the GDU1-HA over-expressors (Fig. 4-8A), suggesting that S32T GDU1 is fully functional. Over-expression of the S32D, S32A and S28+32A mutant proteins led

to plants with reduced size (80 to 100% of the wild type), but never as much as the GDU1 or S32T GDU1 over-expressors (60% of the wild type). Western blotting confirmed that all lines expressed the GDU1 protein (Fig. 4-8B).

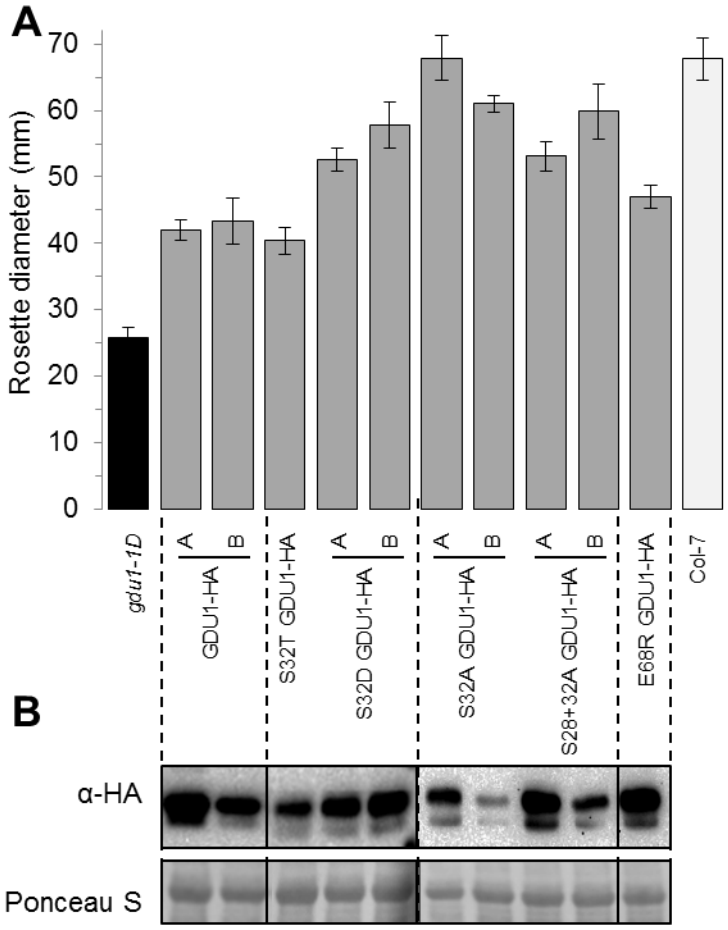

Figure 4-8. Analysis of plants over-expressing site-directed mutagenized GDU1 variants. Analysis of plants over-expressing the GDU1 variants. (A) Rosette diameter of four week-old Arabidopsis. Error bars are SEM (8 plants). Plants were grown at the same time as plants in Fig. 1. (B) Accumulation of the HA tagged GDU proteins in each line was estimated by western blot with an anti-HA antibody. Col-7 and *gdu1-1D* do not express any tagged protein and were not tested.

Gln transport analyses showed that the over-expression of the S32D, S32A and S32T variants led to plants with uptake and efflux similar to the plant over-expressing the wild type GDU1 (Fig. 4-5C). It is worth noting plants over-expressing the S32T variant displayed a higher efflux than the wild type GDU1 (Fig. 4-5B), suggesting that this protein is more active than GDU1. Interestingly, when the GDU1 protein bearing the double mutation S28+32A was over-expressed, Gln uptake and efflux were very similar to the S32L mutation, *i.e.* the *log1-4* mutation. No difference between the effect of the S32A and S32D mutations was detected by this assay.

Site directed mutagenesis to understand E68K effect

We tested whether the suppressing effect of the E68K mutation came from the change in charge (negative to positive) or from the addition of a Lys residue. For this purpose, Glu68 was mutagenized to Arg, and the variant protein expressed in Arabidopsis. This protein accumulated at the same level as the wild type protein (Fig. 4-8B), but the plants had a size similar to one over-expressing the wild type GDU1 (Fig. 4-8A). Transport assay showed that the plants over-expressing E68R behaved essentially similar to a GDU1 over-expressor (Fig. 4-5C), suggesting that the Arg at this position does not affect protein function at all. Since Arg is positive-charged, similar to Lys, and could not suppress GDU1 protein function, the

presence of an additional Lys residue in the C-terminal region of GDU1 is impacting its structure or its post-translational modification.

Discussion

Suppressed mutants show only partially abolished Gln uptake

The characterization of the *log1-1* and three additional mutants (*log1-2*, *log1-3* and *log1-4*), all carrying mutations that suppressed the Gdu1D phenotype, led to unexpected observations. While the size of all these mutants is similar to the wild type and the early senescence observed in the leaves of *gdu1-1D* and *gdu1-5D* is absent, analysis of Gln uptake showed that the mutations do not suppress the Gdu1D phenotype completely (Fig. 4-2), with uptake and efflux of the mutants being intermediate between the wild type and the parental line *gdu1-5D*. Interestingly, the *Gdu1D* suppressor mutation *log2-1*, which affects the ubiquitin ligase LOG2, interactor of GDU1 (Pratelli et al., 2012), similarly does not completely suppress all characteristics of the Gdu1D phenotype: the wild type size is restored, the amino acid susceptibility is abolished, the plants do not secrete Gln anymore, but amino acid export is lowered to an intermediate level between the wild type and the parent (Pratelli and Pilot, unpublished data).

These mutations were obtained from a visual screening which led to the isolation of non-secreting plants of wild type size, possibly introducing a bias towards finding mutations that not necessarily affect transport, but rather than ones

that changes the plant size. The fact that both *log1* and *log2* mutations abolish the Gdu1D phenotype partially the same way suggest that they suppress the same component of the phenotype, which likely involves LOG2, leaving intact another component. This implies that the over-expression of GDU1 leads to several independent effects, some characterized by decreased size, induction of early senescence and amino acid tolerance, and other characterized by increased amino acid export.

The exact role of GDU1 is not known, and two proteins it interacts with have been identified, LOG2 and its homolog LUL1, two membrane-associated ubiquitin ligases (Pratelli et al., 2012). It is proposed that GDU1 and LOG2/LUL1 form a stable complex, stable enough to be co-immunoprecipitated, and involved in ubiquitination of yet unknown target(s) with a role in the regulation of amino acid transport (Pratelli et al., 2012). Loss of interaction with, or loss of expression of LOG2 is a simple explanation for the suppression of the Gdu1D phenotype by the *log1-1*and *log1-3* mutations, deletion of the VIMAG domain (Pratelli and Pilot, 2006) or the *log2* mutants (Pratelli et al., 2012). The fact that the loss of this interaction leads to a similar phenotype as the *log1-2* and *log1-4* mutations is striking, since the corresponding mutations in GDU1 (S32L and E68K) do not affect the interaction with LOG2 (Fig. 4-6). It has to be postulated that they affect other function of GDU1, which remains to be determined.

The G40D mutation might affect GDU1 protein stability

The G40D suppressor mutation in GDU1 has two obvious effects on protein's properties: it does not accumulate at the same level as the other proteins when expressed in *N. benthamiana* leaves (Fig. 4-3), and it does not co-immunoprecipitate with LOG2 (Fig. 4-6). This mutation is located in the transmembrane domain, and affects one of the conserved residues of this domain. In about 100 GDU proteins from higher plants, Gly40 is sometimes replaced by Ala (8%), Ser (4), Leu (1%) or Val (1%), but never by Asp. Gly are a very common residue in membrane helices, involved in glycine zipper in helix packing, enabling helix-helix interactions (Javadpour et al., 1999; Kim et al., 2005). Mutations of such important Gly residues led to abolition of transport (G114A DctA from *Sinorhizobium meliloti*; Trainer et al., 2007) or loss of dimer interaction in EmrE from *E. coli* (Elbaz et al., 2008), supposedly by disruption of the helix structure or ability of helices to interact. The sequence conservation of the GDU helix across different species is astonishing for a membrane protein, and one explanation could be that it is involved in interaction with membrane helices from other proteins. In this context, the G40D mutation likely affects such interaction, and may be the reason for the suppressor effect on the Gdu1D phenotype. Alternatively, the presence of a charge residue (Asp) residue inside the hydrophobic membrane helix destabilizes the protein, making it more difficult to

be integrated in the bilayer. This could explain the lower accumulation of the protein: membrane proteins that have problem to fold are typically degraded by the cellular quality control system (Nagy and Sanders, 2004; Houck and Cyr, 2012). While the localization of the G40D GDU1 protein was not dramatically changed in *N. benthamiana* leaves, this does not preclude any change in addressing to membrane subdomains. Such hypothesis is supported by the fact that G40D GDU1 does not interact with LOG2: these proteins would not interact if G40D GDU1 is targeted to membrane subdomains different than LOG2. Subcellular distribution change has indeed been observed for mutation affecting transmembrane Gly residues (Rosnoblet et al., 2013).

The Ser32 could be an external phosphorylation site

The other suppressor mutation *log2-4* affected Ser32, predicted to be phosphorylated, together with Ser28 (Fig. 4-7). It is not clear if these residues can be phosphorylated *in vivo*, because they are expected to lie in the extracellular region of the GDU1 protein. ATP has been found in the extracellular medium, and is supposed to be involved in phosphorylation of extracellular proteins, as well as in intercellular signaling or during pathogen recognition (Ndimba et al., 2003; Chivasa et al., 2005). The fact that the mutation of the conserved Ser32 to Leu suppresses the Gdu1D phenotype led to the hypothesis that phosphorylation is important in GDU1 function. Ser32 was mutagenized to Asp and Ala (to mimic or

suppress phosphorylation respectively) or Thr (another phosphorylation site), and over-expressed in plants. None of these mutations affected the functional properties of the GDU1 proteins related its effect in amino acid transport, since the over-expressors displayed similar Gln transport as GDU1 over-expressors (Fig. 4-5). On the contrary, while S32T over-expressors were small, S32A and S32D over-expressor had near wild type size (Fig. 4-8), suggesting that the S32T mutation has no effect on GDU1 functional properties, but that the S32A and S32D managed to dissociate the size and the transport components of the phenotype.

The double S28+32A mutations created a protein with functional properties very similar to the suppressor S32L GDU1, because the corresponding over-expressors had a similar phenotype both in term of size and transport (Figs. 4-5 and 4-8). Ser28 and Ser32 thus appear to have redundant function. In the hypothesis that they are extra-cellular phosphorylation sites, the S32L mutation would prevent Ser28 to be phosphorylated, possibly by affecting binding to a protein kinase.

Addition of Lys at position 68 could induce protein ubiquitination

The effect of the suppressor mutation E68K has been investigated to test the hypothesis that it locally changes the charge of the protein from negative to positive. Based on this hypothesis, the E68R mutation was expected to lead to a non-functional GDU1, which, when expressed in plants, would lead to the same effect at the E68K GDU1 in *log1-2*. Plants over-expressing E68R GDU1 were

smaller, and Gln transport was similar to the GDU1 over-expressors, suggesting that this variant GDU1 protein retained full GDU1 functionality. It is not explained why the E68K GDU1-HA over-expressors do not behave as the *log1-2* plants, and need further investigation. The presence of a Lys at this location appear to be important, while the C-terminal domain of GDU1, downstream from the transmembrane domain, contains already eight Lys (Fig. 4-1). Numerous hypotheses can be made to explain the effect of the mutation to Lys rather than to Arg in GDU1: while bearing the same charge, these two residues create very different hydrogen bonds with neighboring atoms, which could modify protein folding or interaction.

It has been found that mutation to a Lys residue in LOG2 at position 12 (R12K; the *log2-1* mutation) led to a suppression of the Gdu1D phenotype, while ubiquitin ligase activity and co-immunoprecipitation with GDU1 were not affected (Pratelli et al., 2012). This mutation affected the first Arg of a stretch of five Arg. A change in charge is probably not the reason for the suppressor effect of this mutation. One important post-translational modification occurring on Lys and not on Arg is linking to an ubiquitin molecule. This fact is of importance, since GDU1 interacts with the ubiquitin ligase LOG2, is ubiquitinated by LOG2 *in vitro*, and LOG2 is able to ubiquitinate itself *in vitro* (Pratelli et al., 2012). Addition of ubiquitin on R12K LOG2 or E68K GDU1 might not necessarily lead to protein degradation

(indeed, no decrease in E68K GDU1 stability has been observed compared to GDU1; Fig. 4-3), but to change in protein activity or ability to interact with partners. Indeed, ubiquitin has been found altering protein activity and protein-protein interactions (Schnell and Hicke, 2003).

Materials and methods

Plant material and growth

Arabidopsis thaliana (ecotype Col-7) lines were grown under 120 μE/m^2/s, 22 ℃, 16 h light /8 h dark on soil (Mix of Sunshine Mix 1 and Pro-mix HP at a 1:1 ratio) and were watered from below with 300 mg/l Miracle-Gro Fertilizer (Scotts, Marysville, OH, USA). Rosette diameters of about 8 plants from each line was measured with a ruler at about four weeks after sowing. *Arabidopsis thaliana* were transformed by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* GV3101 (pMP90). For transient expression of proteins in *N. benthamiana*, young leaves of five-week old plants were infiltrated with a suspension of *Agrobacterium tumefaciens* carrying the constructs of interest according to (Batoko et al., 2000), with the following modifications. The bacteria were grown overnight in LB supplemented with appropriate antibiotics, washed twice in 10 mM $MgCl_2$, 100 μM acetosyringone, and diluted to final OD_{600} of 0.05 before infiltration in *N. benthamiana* leaves.

Cloning and constructs

The Kunkel method was used for site directed mutagenesis (Kunkel et al., 1991), from sequences cloned by gateway cloning in the pDONR Zeo gateway vector, containing the f1 replication origin (D. Loqué personal communication). Mutagenized inserts were sequenced, and transferred by Gateway cloning (Life Technologies) to the binary vector pPWHTkan, derivative of pJHA212K ((Yoo et al., 2005), R. Pratelli and G. Pilot, unpublished data), and carrying in this order the CaMV 35S promoter, the Gateway cassette, a double HA tag sequence and the terminator of the small subunit of the Rubisco from pea (Pisum sativum; accession no. X00806).

Protein extraction, western blotting and co-immunoprecipitation

Protein extraction and western blotting were performed according to (Yu and Pilot, 2014) with following modifications. Leaves from each line (selected on kanamycin for seven days and transferred to soil and grown for three more weeks) were collected for protein extraction and western blot. Five hundred mg of leaves was ground with 1 ml of extraction buffer (50mM Tris-HCl, pH 7.3, 150mM NaCl, 10mM $MgCl_2$, 10mM DTT, 0.5% Nonidet P-40, and 1X Complete Protease Inhibitors (Roche)) on ice. Homogenate was centrifuged at 14,000 g and 4 ℃ for 15 min. Protein concentration of the supernatant was quantified by Bradford reagent. Twenty µg of total proteins were analyzed by SDS-PAGE (4%–12% polyacrylamide MES gel; Life Technologies) and western blotting. Proteins were

transferred on a nitrocellulose membrane (GE Healthcare) and detected using anti-HA (clone 3F10; Roche Diagnostics; 1:5,000) primary antibodies, anti-rat (Thermo Scientific) secondary antibodies, and the ECL-Prime western-blotting detection system (GE Healthcare). Co-immunoprecipitations were performed from *N. benthamiana* infiltrated leaves as described (Pratelli et al., 2012).

Amino acid uptake

Amino acid uptakes in plants were performed as described (Pratelli et al., 2010), from segregating T2 seeds selected on kanamycin before growing in liquid medium and uptake.

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Chapter V

Study of inducible GDU1 in Arabidopsis reveals connections between amino acid homeostasis and plant stress responses

Introduction

Amino acids participate in many important physiological processes in plants in addition to being the building blocks of proteins. Importantly, many amino acids are precursors of plant secondary metabolites, such as flavonoids (Falcone Ferreyra et al., 2012), alkaloids (Ziegler and Facchini, 2008), and glucosinolates (Kraker and Gershenzon, 2011), which are important for plant interactions with their environment. Amino acids are also the predominant form of transported organic nitrogen in plants. Amino acids are synthesized in either roots or mature leaves (source organs), depending on factors such as plant species and supplied nitrogen form, and transported to sink organs, such as root tips, flowers, and seeds, through the xylem and phloem (Tegeder, 2014). At the cellular level, amino acid transport across a biological membrane requires specialized proteins. Amino acid importers and exporters mediate transport of amino acids in opposite directions, either towards or from the cytosol, thus from or to the apoplasm, vacuoles or intracellular vesicles, respectively.

While a number of amino acid importers have been identified involving source to sink partitioning of amino acids (Tegeder, 2014), very little is known about

amino acid exporters. So far, only CAT8 (Yang et al., 2010), BAT1 (Dundar and Bush, 2009), and SiAR1 (Ladwig et al., 2012) have been shown to mediate amino acid export from plant cells. The identification of *gdu1-1D* mutant showed that the Gdu1D phenotype is caused by overexpression of *GDU1* (Pilot et al., 2004). Previous reports indicate that GDU1 and other members of the same family probably regulate amino acid export and homeostasis, shown by boosted amino acid export, tolerance to toxic concentration of exogenous amino acids, and increased amino acid content in the GDU-overexpressors (Pratelli and Pilot, 2006; Pratelli and Pilot, 2007; Pratelli et al., 2010). Except for *GDU4* and *GDU7*, overexpression of *GDUs* reduced the size of the plants (Pratelli et al., 2010), suggesting plant growth is affected, which may due to perturbation in amino acid homeostasis.

Many plant defense compounds are derived from amino acid precursors, implying that the homeostasis of amino acids is crucial for plant immunity (Bednarek et al., 2009; Zeier, 2013). Interestingly, recent reports suggest that amino acid imbalance induces plant stress responses. Knock out of an amino acid transporter, LHT1, increased plant disease resistance in a SA-dependent manner, which is likely due to glutamine deficiency (Liu et al., 2010). Overexpression of the basic amino acid transporter CAT1 enhanced lysine uptake and increased plant resistance to *Pseudomonas syringae* by activating the SA pathway (Yang et al.,

2014). *gdu1-1D* exhibited enhanced ROS accumulation, activated defense response and strong disease resistance (Liu et al., 2010). Overexpression of *GDU3* also affected virus infection via activating the SA pathway (Chen et al., 2010). Strikingly, in all cases mentioned above, increased plant defense response was exerted by activating the SA pathway. Though some amino acids are precursors of certain plant hormones (Wang et al., 2002; Mano and Nemoto, 2012), such as ethylene and auxin, typical plant stress hormones, such as SA, JA, and ABA,are not derived from amino acids. So far, no direct causative link between amino acid imbalance and plant stress responses has been established.

Although the study of *gdu1-1D* provided valuable information about its role, it also hinders further characterization: the study of constitutive GDU1 over-expressors did not enable dissociating the primary effects of *GDU1* overexpression from plant adaptation to this overexpression. To elucidate the biochemical function of GDU1 and the pathways leading to the different aspects of the complex Gdu1D phenotype, we utilized a chemically inducible gene expression system. One of the obvious advantages using an inducible system is that it bypasses the situation that expression of a transgene compromises plant viability or fertility (Moore et al., 2006). However, in our case, we benefited from this system by identifying the primary effects of *GDU1* expression, dissecting the signaling pathways involved in the Gdu1D phenotype, and following the changes in amino acid content over time.

We proved that increase in amino acid export activity, caused by overexpression of *GDU1*, precedes stress-related phenotypes which involve ABA and SA.

Results

Study of GDU1 induction resolves the sequence of events caused by *GDU1* overexpression

The inducible GDU1 construct consisted in the pOp6/LhGR system (Craft et al., 2005) and introduced into *Arabidopsis thaliana* line 4c-S7 (provided by Dr. Ian Moore, University of Oxford, UK). 4c-S7 expresses a chimaeric transcription factor 'LhGR' under the control of the 35S promoter. This transcription factor is excluded from nucleus in the absence of glucocorticoids (Craft et al., 2005). GDU1 expression is controlled by the pOp6 promoter, which consists of six ideal lac operators positioned upstream of a minimal CaMV 35S promoter, and is silent in the absence of the artificial transcription factor LhGR. LhGR binding to glucocorticoids, for instance dexamethasone (DEX), confers conformational change and allows LhGR into nucleus, stringently activating GDU1 expression. Three independent inducible GDU1 lines, along with 4c-S7 (control), were grown on soil and tested for induction with DEX. As expected, *GDU1* expression was dramatically and quickly increased after induction: as soon as three hours after induction, transcript levels were at least three times the level in the original *gdu1-1D* mutant. *GDU1* expression peaked at 6-12h after induction, which is in

accordance with reports using the same system (Moore et al., 2006), with 5,000- to 10,000-fold increase, then slowly decreased to around 2,000-fold (Fig. 5-1). The peak expression for Line 1 was slightly delayed compared to Lines 2 and 3, which might reflect experimental variability since the latter two were treated in a different experiment. Nevertheless, *GDU1* expression kinetics was similar across the three inducible lines.

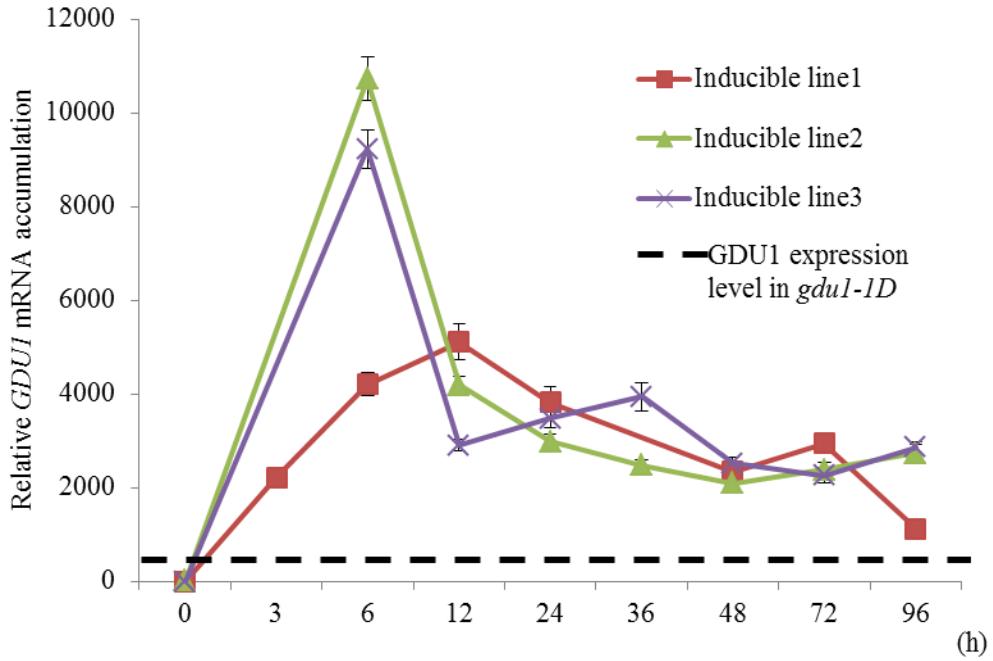

Figure 5-1. GDU1 expression after induction by DEX.
Three individual GDU1 inducible lines were induced by spraying 100 μM DEX at time 0. Plants were grown on soil for three week before induction. Error bars=SEM (n=3).

The induced lines displayed the main characteristic of the Gdu1D phenotype, the secretion of glutamine at the hydathodes (Fig. 5-2; (Pilot et al., 2004)). Interestingly, two to three days after induction, the induced lines developed lesions on leaves, similar to *gdu1-1D* (Fig. 5-3). Induction of *GDU1* triggered cell death

events, which aggravated over time (Fig. 5-4). Accumulation of reactive oxygen species (ROS) preceded cell death events, starting two days after induction (Fig. 5-5). Plant growth was delayed by *GDU1* induction, with reduced rosette size one week after induction, compared to the control (data not shown). Over-expression of *GDU1* thus triggered stress responses as soon as two days after induction and the stress level increased over time.

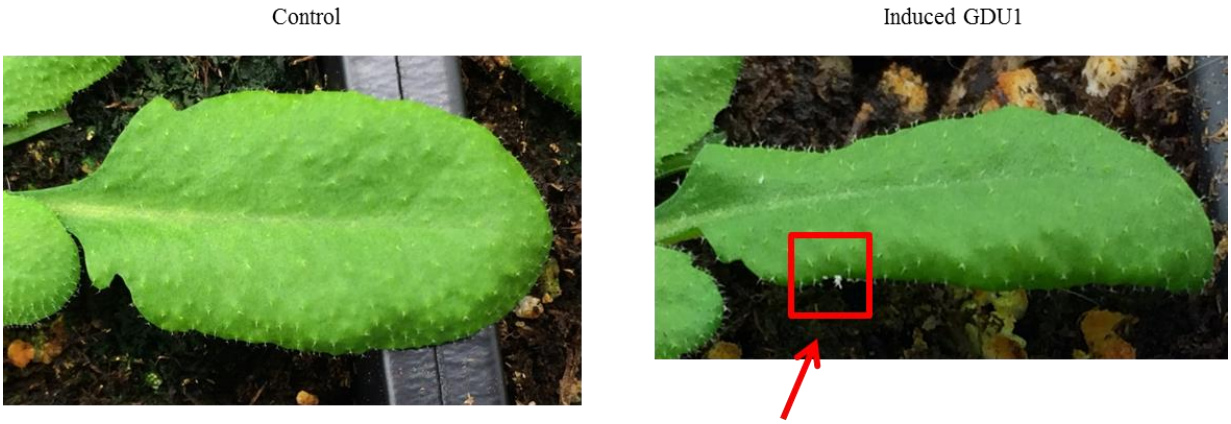

Figure 5-2. Glutamine secretion at the hydathodes in induced lines.
Two to three days after induction, secreted glutamine crystals were observed in the induced lines at the hydathodes (arrow).

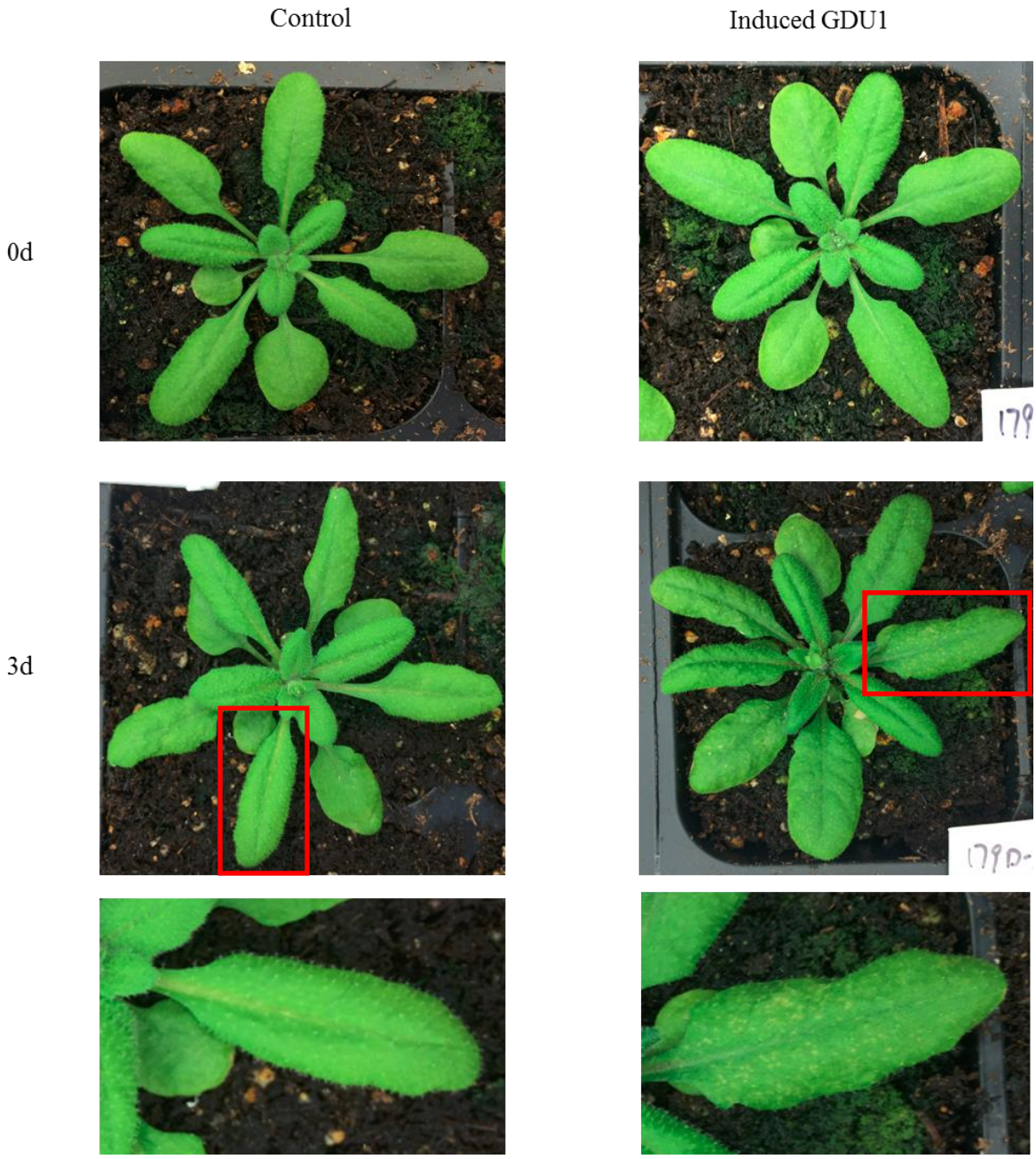

Figure 5-3. Lesions on leaves after GDU1 induction.
Left and right panels show presentative pictures of the control and induced GDU1 lines, respectively. Top panels, before induction (0 d). Middle panels, three days after induction. Bottom panels, magnified views of leaves three days after induction (highlighted by red rectangle in middle panels), showing healthy leaves (left) and leaves with lesions (right).

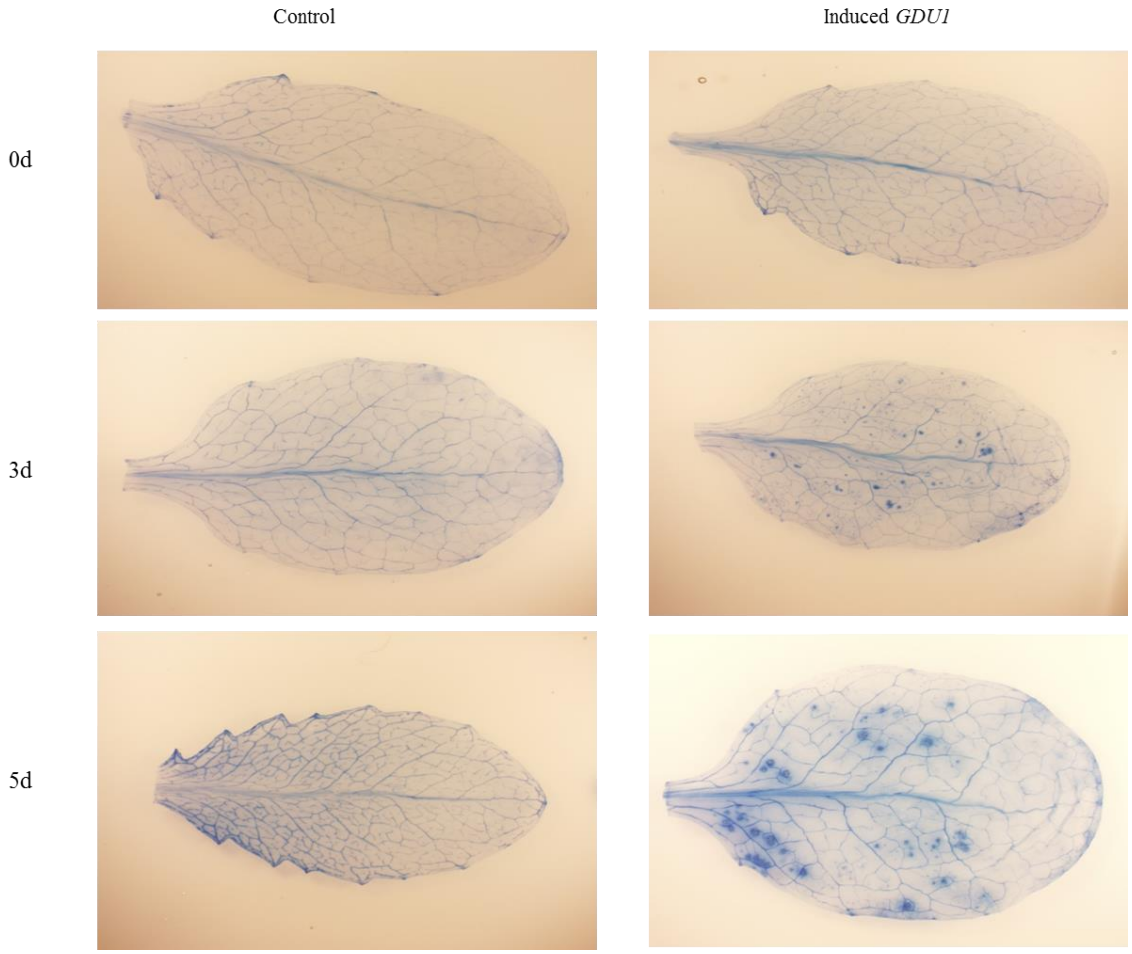

Figure 5-4. Trypan Blue staining showing cell death induced by GDU1 expression.
Left and right panels show representative leaves of the control and induced GDU1 lines, respectively. Top panels, before induction (0 d). Middle panels, three days after induction. Bottom panels, five days after induction. Dark blue regions indicate dead cells in leaves.

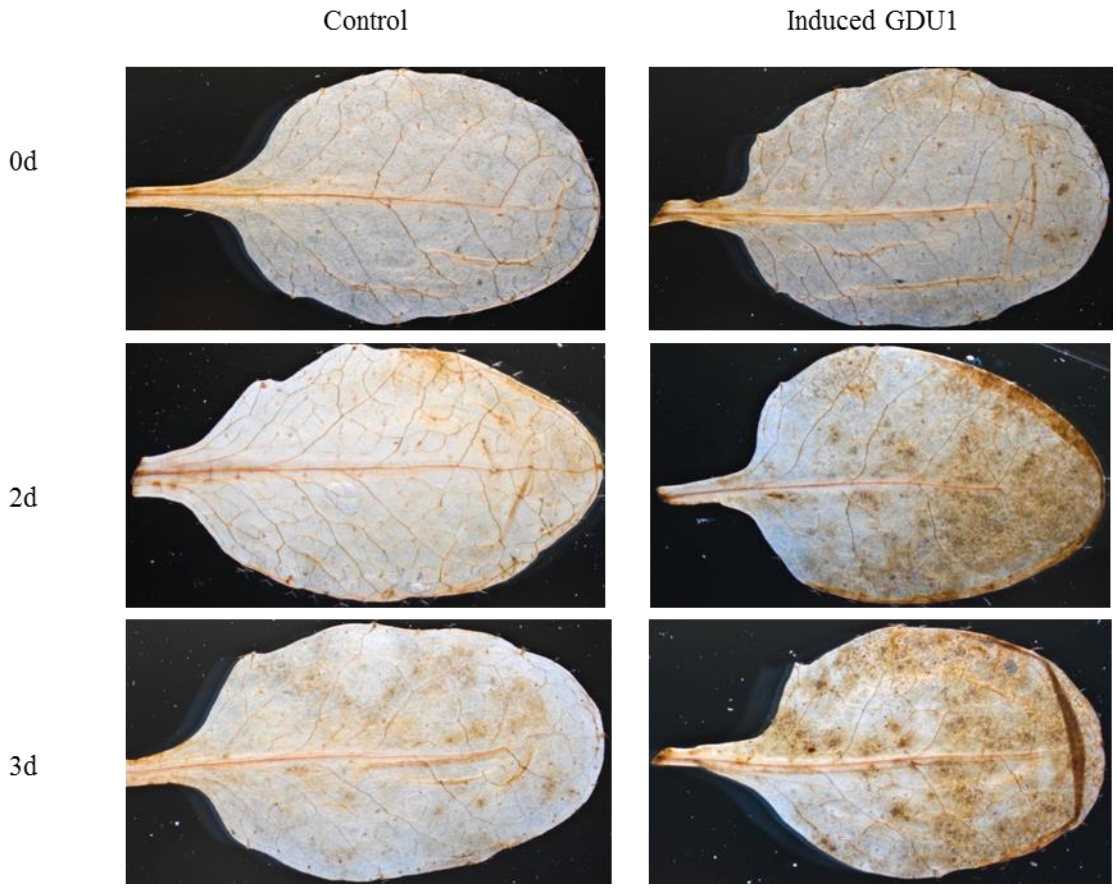

Figure 5-5. ROS accumulation increases after GDU1 induction.
Diaminobenzidine (DAB) staining showing ROS accumulation (brown dots and patches) after induction of GDU1. Left and right panels show representative leaves of the control and induced GDU1 lines, respectively. Top panels, before induction (0 d). Middle panels, two days after induction. Bottom panels, three days after induction.

Amino acid export activity is increased in *gdu1-1D* plants (Pratelli et al., 2010). In agreement with the Gdu1D phenotype, amino acid efflux was quickly increased after GDU1 induction. Within four hours after induction, glutamine efflux in the induced line almost doubled, compared to the control (Fig. 5-6). Even three hours after induction, amino acid efflux in induced GDU1 plants was similar as that in *gdu1-1D* (Fig. 5-7). While the efflux of glutamine, proline and leucine

was increased in the induced lines and *gdu1-1D*, the efflux of alanine remained inchanged, similar to the *gdu1-1D* mutant.

As previously shown for *gdu1-1D* plants (Pilot et al., 2004; Pratelli et al., 2010), amino acid content in leaves also increased after induction of GDU1, with the content of most of amino acids peaking at one day after induction, and then slowly decreasing (Fig. 5-8). Even five days after induction, the content of these amino acids still was two- to four-fold higher than the control. In contrast, the content of proline, an amino acid related to stress (Verslues and Sharma, 2010), was steadily increased from 12 hours to three days after induction, and then slightly decreased, which mimicked the progression of the observed stress response. On the other hand, the content of most amino acids did not change during the first 12 hours after induction, with the exception of glycine which transiently accumulated at six hours after induction.

Unlike the stress responses, which initiated about two days after GDU1 induction, the increases in amino acid efflux and content appeared much sooner, *i.e.* three hours and less than one day after induction, respectively, suggesting the primary biochemical function of GDU1 is related to regulation of amino acid export, rather than triggering plant stress responses.

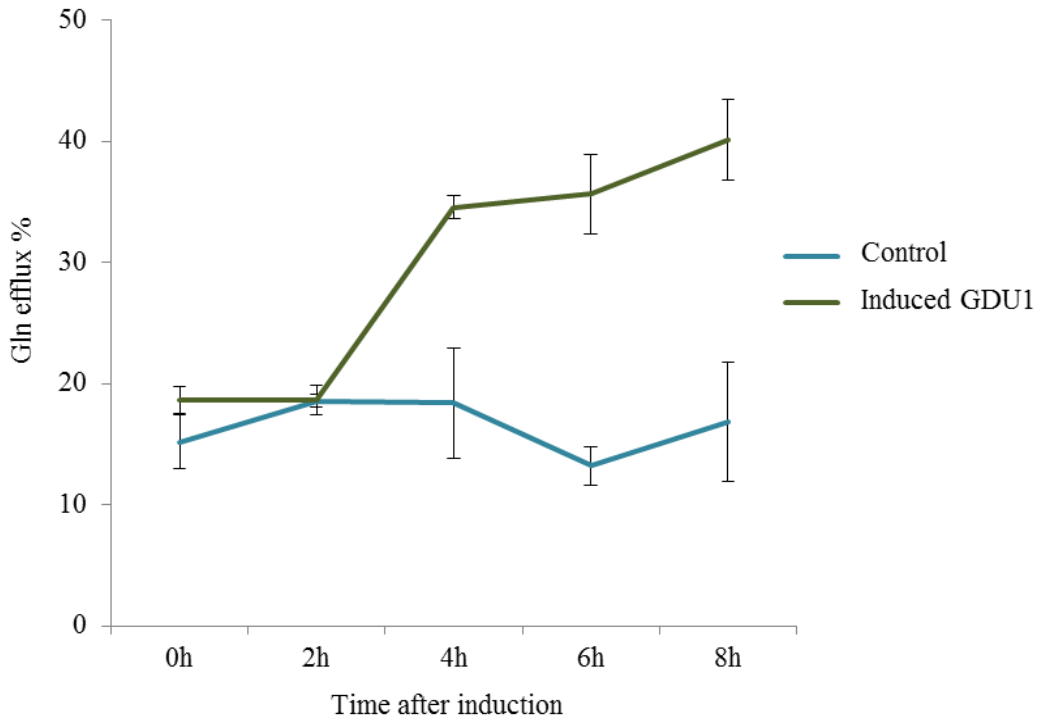

Figure 5-6. Gln efflux from the GDU1-induced line 1 and control.
Whole plantlets were grown in liquid medium for two weeks before the amino acid export experiment. Control and inducible GDU1 plants were treated with 30µM DEX. Error bars=SEM (n=3).

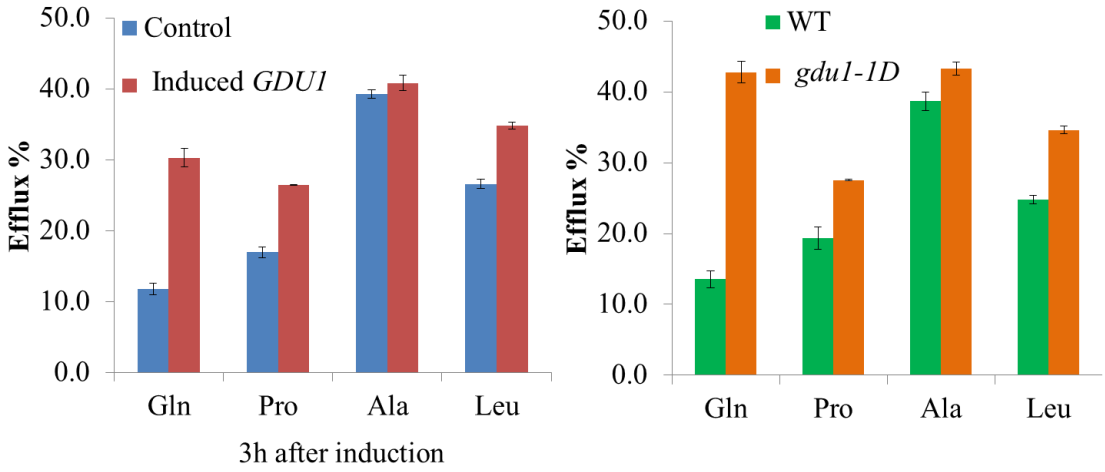

Figure 5-7. Amino acid efflux in induced GDU1 line and *gdu1-1D*.
Amino acid efflux of glutamine, proline, alanine and leucine in induced GDU1 line 1 (three hours after induction), compared to the control, and *gdu1-1D*, compared to the wild type (Col-7). Error bars=SEM (n=3).

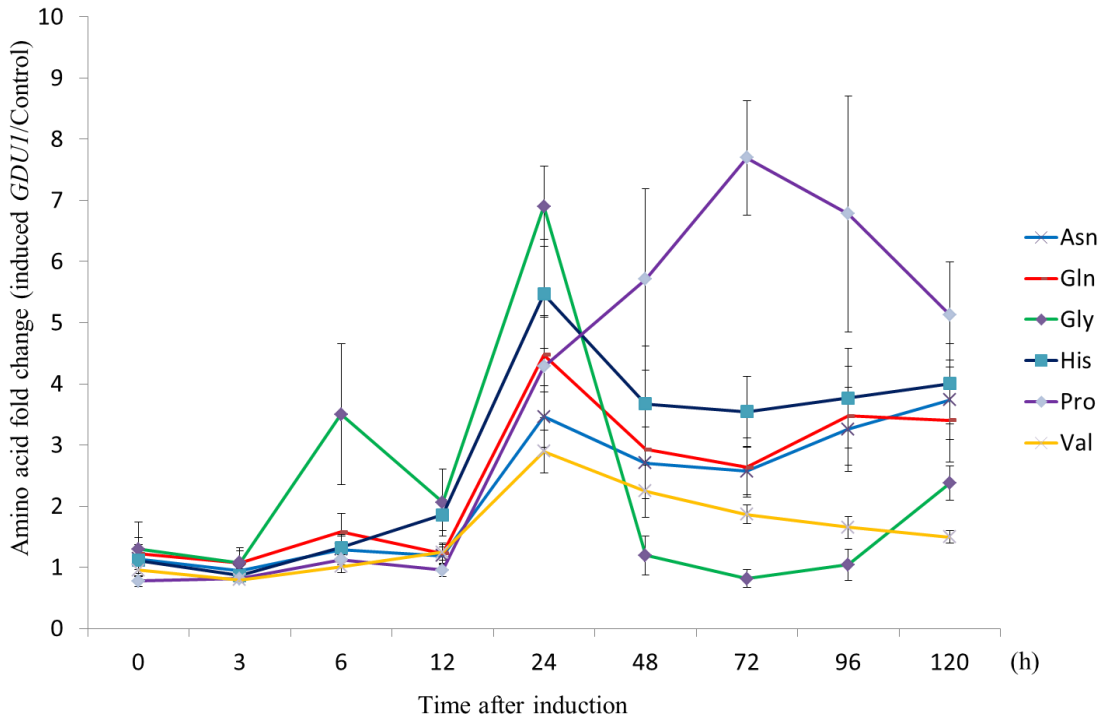

Figure 5-8. Free amino acid content in leaves in induced GDU1 line 1, compared to the control.
Amino acid content in leaves were quantified and normalized to the control, showing fold change. Error bars=SEM (n=3).

Induction of *GDU1* triggers ABA and SA responses

To pinpoint the pathways leading to the different phenotypes of the induced lines, the expression of marker genes for plant hormone signaling pathways, stresses, and metabolic enzymes were measured by qRT-PCR in two inducible GDU1 lines. Out of all the tested genes, marker genes from two signaling pathways stood out.

NCED3 encodes the major ABA biosynthetic enzyme, and *RD29A* is an ABA response gene (Yamaguchi-Shinozaki and Shinozaki, 1994; Barrero et al., 2006).

Interestingly, the expression of *NCED3* peaked at 12 hours after induction, and then quickly dropped after one day after induction, while the expression of *RD29A* peaked between 12 to 24 hours after induction (Fig. 5-9), suggesting that induction of *GDU1* triggered a transient outburst of ABA between 12 to 24 hours after induction.

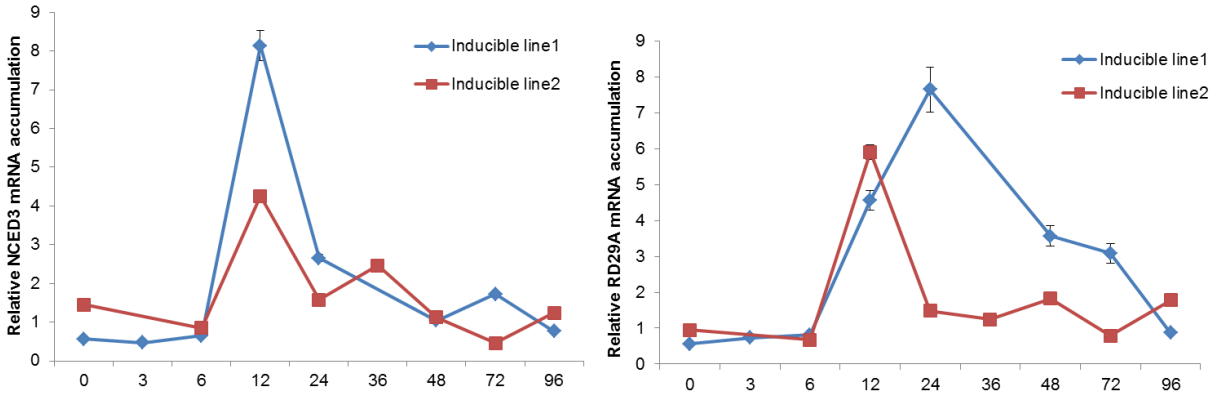

Figure 5-9. Expression of two marker genes in ABA signaling pathway after the induction of *GDU1*.
Relative gene expression of *NCED3* (left panel) and *RD29A* (right panel) after induction of *GDU1*. Relative gene expression is normalized to the control at the same time point. Error bars=SEM (n=3).

SID2 encodes the major SA biosynthetic enzyme and *PR1* responds strongly to SA signaling (Nawrath and Metraux, 1999). The expression of *SID2* peaked between one to two days after induction of *GDU1*, which was followed by *PR1* expression, peaking between two to three days after induction (Fig. 5-10). It is important to note that about two to three days after induction, the lesions and cell death were observed on leaves as well. Two signaling pathways are well characterized in triggering cell death in plants: one is caused by SA, and the other

one by jasmonic acid and ethylene (Pieterse et al., 2009). Since the expression of marker genes of jasmonic acid and ethylene did not chang during the first three days after induction (data not shown), it is more likely that SA accumulation led to the cell death observed after GDU1 induction.

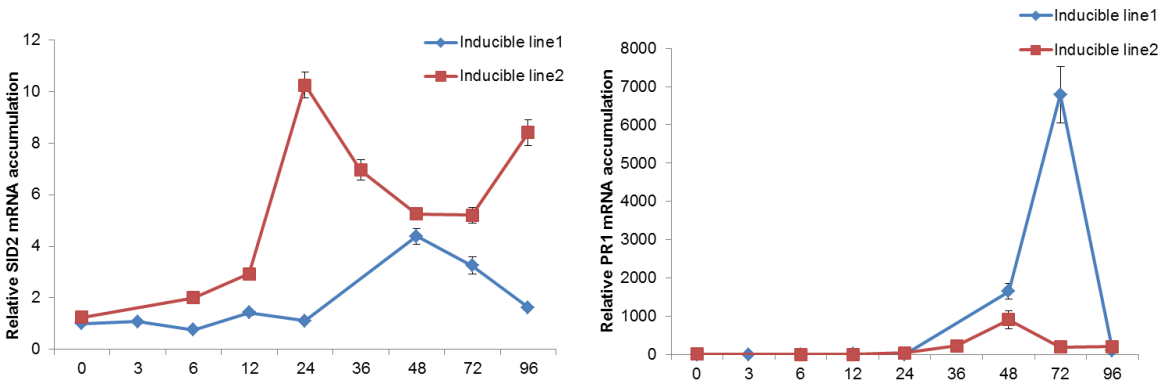

Figure 5-10. Expression of two marker genes of SA signaling pathway after induction of *GDU1*.
Relative gene expression of *SID2* (left panel) and *PR1* (right panel) after induction of *GDU1*. Relative gene expression is normalized to control at the same time point. Error bars=SEM (n=3).

When overlaying the expression of the marker genes and *GDU1* after induction, it is obvious ABA and SA signaling were happening sequentially (Fig. 5-11). The timing of ABA and SA signaling was shifted to earlier or later time points, depending on the strength and kinetics of *GDU1* induction. However, ABA signaling always followed *GDU1* induction, and always preceded SA signaling.

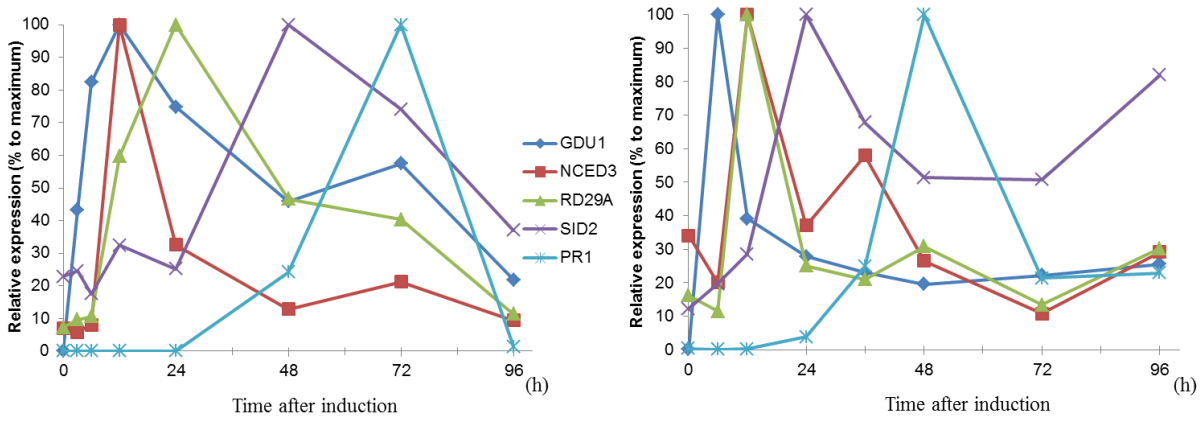

Figure 5-11. Expression of marker genes and *GDU1* after induction.
Expression of each gene was expressed relative to its maximum value and plotted over linear time scale. Left panel, expression in inducible line #1. Right panel, expression in inducible line #2.

A *GDU1* knock-out is less sensitive to drought

Since induction of *GDU1* triggered transient ABA signaling, and LOG2, a GDU1 interactor, has been characterized as a regulator in ABA-mediated drought stress responses (Kim and Kim, 2013), we hypothesized that *GDU1* is involved in ABA response. Under well-watered condition, the primary root growth of *gdu1-1D* and a *GDU1 knock-out* mutant (*gdu1-3*) were not different from the wild type (Fig. 5-12 A). Under severe drought condition, ABA inhibits primary root growth (Jiao et al., 2013), and the primary root growth of all lines was dramatically reduced. While the reduction in primary root elongation was similar for *gdu1-1D* and wild type plants, primary root elongation of *gdu1-3* was significantly greater than wild type under the drought condition (Fig. 5-12 B). This suggests that the over-expression of GDU1 does not affect the response, while suppression of *GDU1* expression makes the plants hyposensitive to ABA.

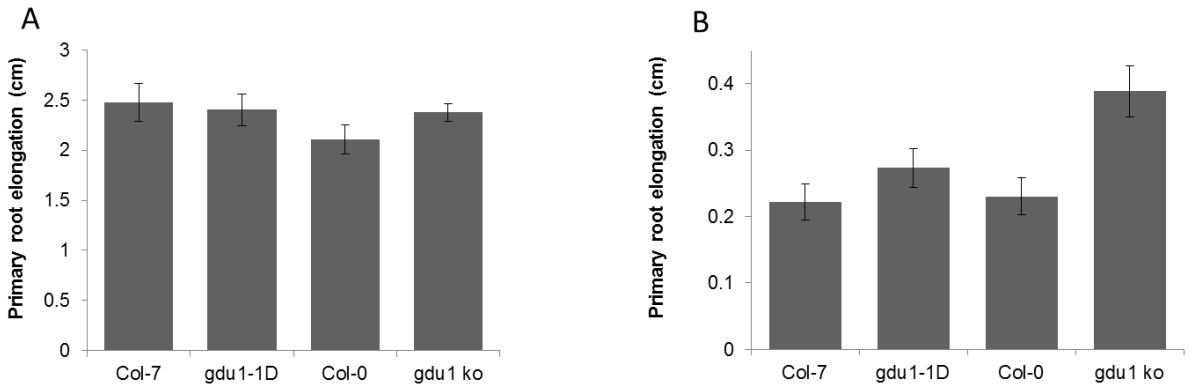

Figure 5-12. Primary root growth under well-watered and severe drought condition.
Col-7 and Col-0 are control of *gdu1-1D* and *gdu1-3* (*gdu1 knock-out*), respectively. Left panel, plants were grown *in vitro* under well-watered condition with water potential average of -0.04 MPa, while right panel represents plants grown under severe drought condition with water potential average of -0.88 MPa. Error bars=SEM.

SA leads to cell death triggered by induction of *GDU1*

To further characterize the role of SA in induced GDU1, *NahG*, which encodes a salicylate hydroxylase from *Pseudomonas putida* (You et al., 1991), which degrades SA, was introduced into *gdu1-1D*. In parallel, the *sid2-1* mutant (Nawrath and Metraux, 1999) was crossed with *gdu1-1D* to assess the role of *SID2* in the SA signaling triggered by induction of *GDU1*. Suppression of SA accumulation did not increase the size of *gdu1-1D* plants (Fig. 5-13). To verify the effectiveness of *NahG* and *sid2-1* in reducing SA signaling, expression of *PR1*, along with *GDU1*, was measured by qRT-PCR (Fig. 5-14). *GDU1* expression was similar in *gdu1-1D* and the *sid2-1 gdu1-1D* double mutant, and the *gdu1-1D* NahG plants. Expression of *NahG* strongly reduced *PR1* expression in *gdu1-1D*, compared to *gdu1-1D*, while presence of the *sid2-1* mutation reduced *PR1* expression to the level of the

wild type. Expression of *NahG* was thus more potent than *sid2-1* in reducing SA signaling in *gdu1-1D*. Interestingly, the *gdu1-1D sid2-1* double mutant still shows lesions on leaves, while introducing *NahG* into *gdu1-1D* completely suppressed the lesions (Fig. 5-15). This proved that cell death requires SA accumulation in *gdu1-1D* and likely in induced GDU1 lines. The fact that reducing SA response to a wild-type-comparable level did not suppress lesion development (*sid2-1* mutant) suggests that additional pathways are involved in the development of cell death. It is also possible that *GDU1* induction triggers a SA synthesis pathway independent of SID2, *e.g.* involving the phenylalanine ammonia lyase (Huang et al., 2010). It is also important to note that introducing *NahG* into *gdu1-1D* did not suppress glutamine secretion at the leaf margin and size reduction, suggesting that SA does not cause increased amino acid export.

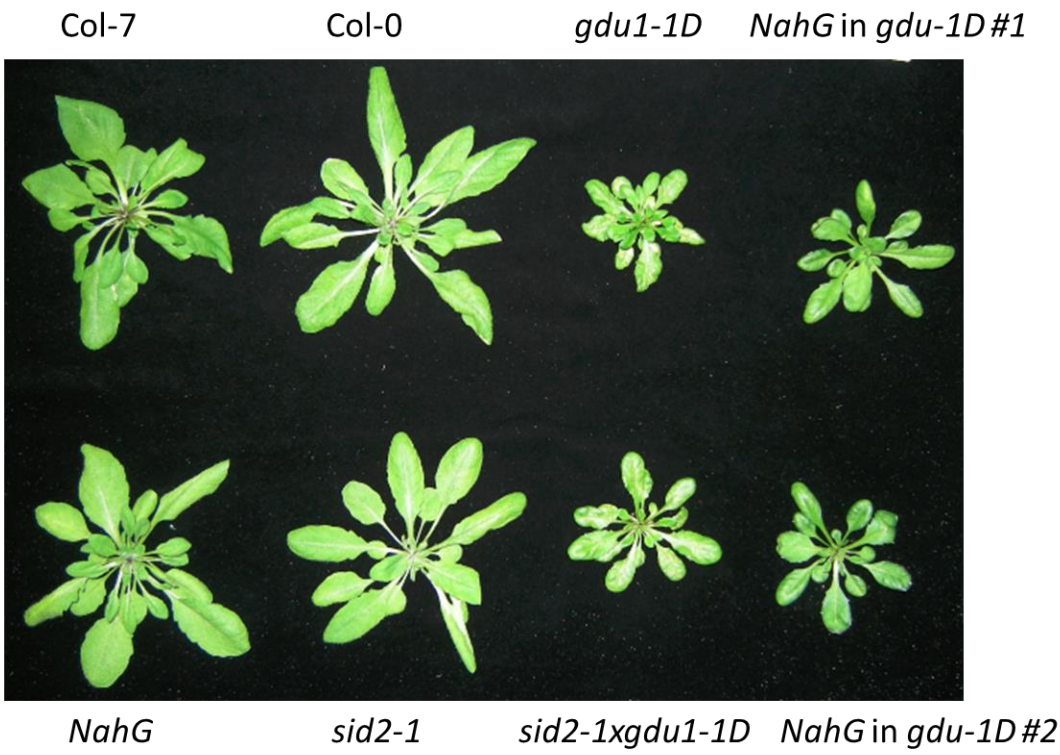

Figure 5-13. Reducing SA in *gdu1-1D* does not restore wild type plant size.
Five-week old plants, representative from each line, are shown.

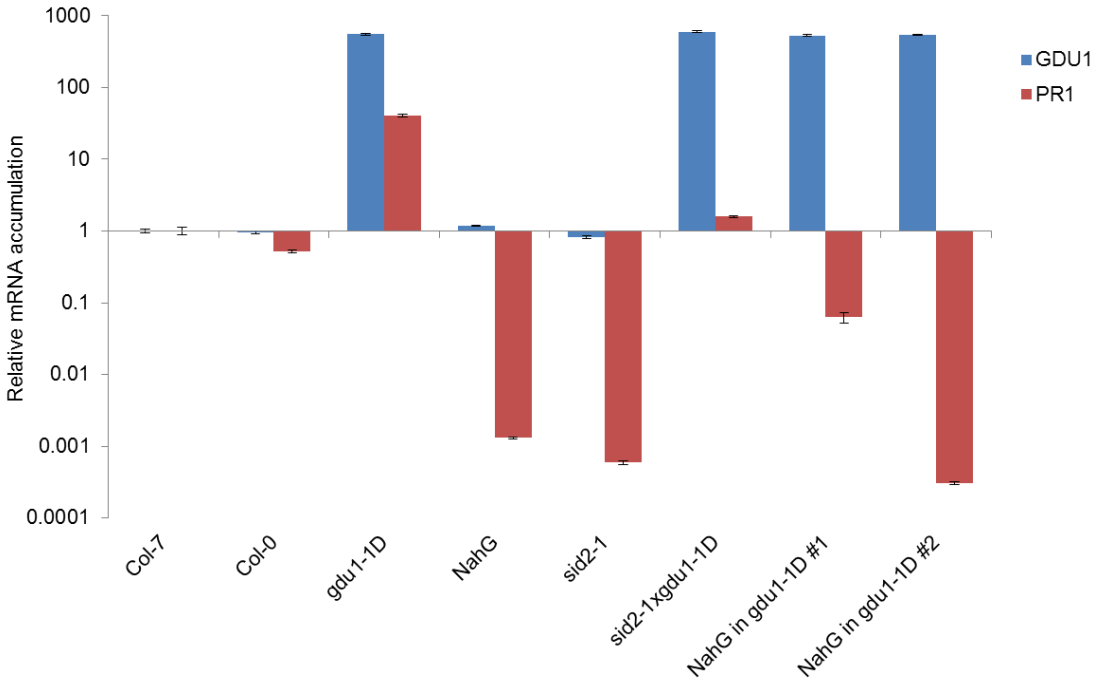

Figure 5-14. Relative gene expression of *GDU1* and *PR1*.
Relative gene expression was normalized to Col-7. Plants were grown on soil for five weeks before harvesting. Error bars=SEM (n=3).

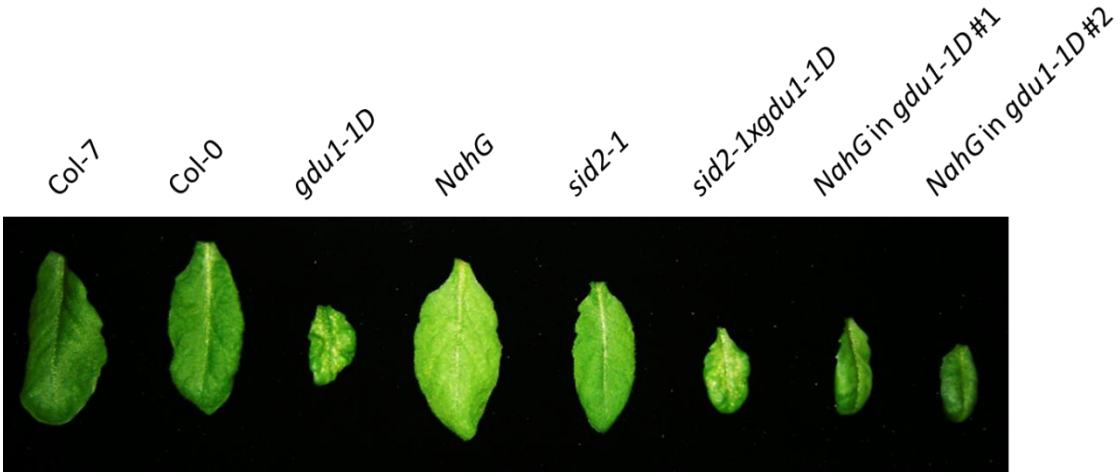

Figure 5-15. Eliminating SA in *gdu1-1D* suppresses lesion formation in *gdu1-1D*.
Plants were grown on soil for five weeks, and leaves were cut and photographed the same time.

gdu1-1D is more resistant to Hyaloperonospora arabidopsidis, but also more susceptible to Pseudomonas syringae.

SA is considered as one of the most important plant hormones in plant defense responses to plant pathogens (Pieterse et al., 2009). It can be speculated that increased accumulation of SA by *GDU1* over-expression render the plants more resistant to pathogens. However, enhancement of free amino acid content in *gdu1-1D* leaves and increased amino acid export could improve pathogen feeding on *gdu1-1D* plants. The susceptibility of *gdu1-1D* to plant pathogens is thus difficult to predict because of these two contradicting factors. We tested the susceptibility of *GDU1* over-expressors to an obligate biotrophic oomycete pathogen, *Hyaloperonospora arabidopsidis* (*Hpa*), and a hemibiotrophic bacteria, *Pseudomonas syringae pv. tomato* DC3000 *(Pto* DC3000).

Hpa depends on nutrients extracted from hosts by the feeding structures called haustoria. Additionally, *Hpa* is sensitive to plant SA level, since SA is an essential component in both ETI and basal resistance (McDowell et al., 2000). GDU1 over-expressors challenged with *Hpa* were more resistant (Fig. 5-16), suggesting elevated SA outweighed the effects by increased amino acids to *Hpa*.

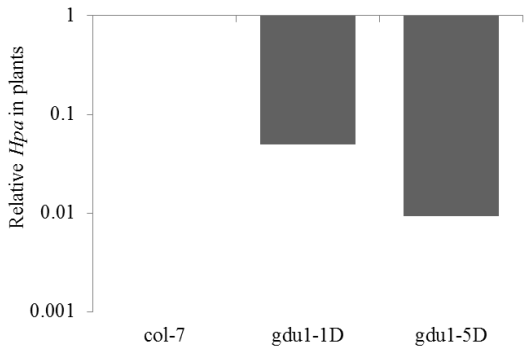

Figure 5-16. Relative *Hpa* multiplication in plants.
Normalized *Hpa* growth in two different GDU1 over-expressors, compared to control, Col-7, 3 days after inoculation.

When challenged with *Pto* DC3000 ΔCEL (CEL effectors removed to weaken ability to grow in plants, Alfano et al., 2000), *GDU1* overexpressors were more susceptible to this pathogen (Fig. 5-17), suggesting amino acid availability is a limiting factor for its growth in plants. To elucidate the effects of SA and amino acid availability to *Pseudomonas syringae*, *gdu1-1D*, *gdu1-1D sid2-1* double mutant and *NahG*-expressing *gdu1-1D* were challenged with *Pto* DC3000 ΔCEL. Interestingly, decreasing SA levels by *NahG* expression or by the *sid2-1* mutation in wild type plants led to similar increased susceptibility as *gdu1-1D*. Over-

expressing *GDU1* and decreasing SA levels had additive effects on the susceptibility (Fig. 5-18). While amino acid availability and SA levels are both limiting factors for *Pseudomonas syringae* multiplication in plants, the effect of increase in amino acid content or export takes over the elevated plant defense caused by SA accumulation in *gdu1-1D.*

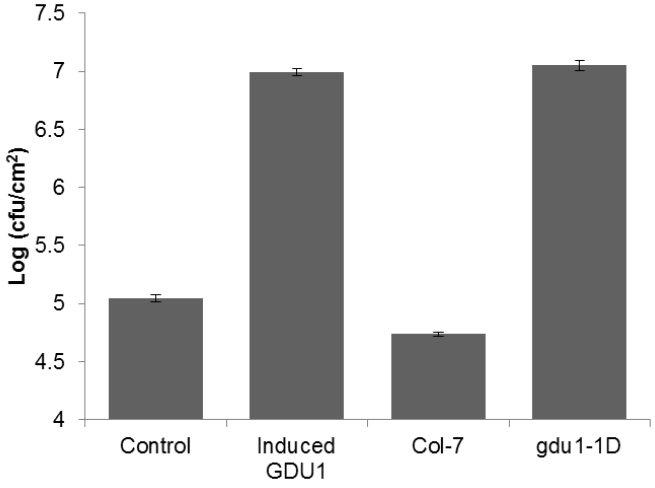

Figure 5-17. Plants overexpressing *GDU1* are more susceptible to *Pseudomonas syringae.*
Pseudomonas syringae growth in plants 3 days post inoculation. Error bars=SEM (n=3). Cfu, colony forming unit.

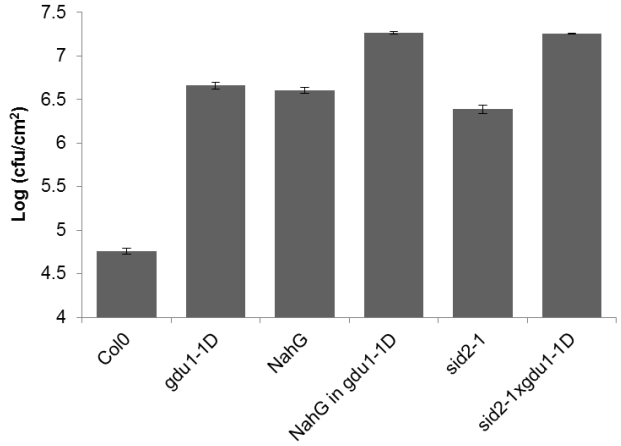

Figure 5-18. *Pseudomonas syringae* growth in plants 3 days post inoculation.
Error bars=SEM (n=3). Cfu, colony forming unit.

Discussion

The primary biochemical function of GDU1 is related to regulation of amino acid export

Though the characterization of the *gdu1-1D* mutant with constitutive expression provided valuable information about GDU1 biochemical function, study of the chemically inducible gene expression system brought more details about GDU1 role. For the first time, it allowed us to dissociate the effects caused by *GDU1* overexpression, rather than the pleiotropic effects found in the constitutive GDU1 over-expressor. Expression of *GDU1* was dramatically and quickly increased after induction (Fig. 5-1), which made it an ideal system to monitor transcriptional reprograming along the time course to find the timing of important pathways contributing to the different characteristics of the Gdu1D phenotype. Induction of *GDU1* first augmented amino acid export (Fig. 5-6), which was followed by increased amino acid content (Fig. 5-8), while ROS accumulation (Fig. 5-5) and cell death (Fig. 5-3 and 5-4) were only observed after 2-3 days. At three hours after induction, neither stress responses nor changes in amino acid content could be detected, but amino acid export was already enhanced. This confirmed our speculation that the primary biochemical function of GDU1 is related to regulation of amino acid export (Pilot et al., 2004; Pratelli et al., 2010).

When *NahG,* which encodes a salicylate hydroxylase, was introduced into *gdu1-1D*, glutamine was still secreted, although the plants showed no SA signaling and lesion on the leaves. This indicates that while SA accumulation is involved in the lesion formation after *GDU1* induction, it is not involved in the pathways leading to increased amino acid content and export in *gdu1-1D*.

Although the mechanism of increased amino acid export by overexpression of *GDU1* is still elusive, the inducible GDU1 system provides a promising tool. At three hours after induction, overexpression of *GDU1* actively augmented amino acid export, but no notable secondary effects had been triggered. Even at 7 h, RNASeq analysis found that no gene involved in amino acid transport or metabolism displayed strong changes in expression that could explain the enhanced export (data not shown). This suggests that GDU1 regulates amino acid export at the protein level, possibly controlling membrane protein trafficking and/or stability. It would be interesting to compare the protein profile of induced GDU1 lines with the control at this time point to find candidates in this process: according to our hypothesis, the accumulation of the candidate proteins is expected to increase. Since GDU1 is localized at the plasma membrane and endosomes (Pratelli et al., 2012), it would be more interesting to purify proteins from these membranes.

Could imbalance in amino acid homeostasis trigger defense responses in *gdu1-1D*?

Though the overexpression of *GDU1* disrupts amino acid homeostasis, it is unclear if the imbalance of amino acid homeostasis in *gdu1-1D* is the reason for the activated defense responses. *Liu et al.* proposed that glutamine deficiency may be a signal triggering defense response by activating SA pathway in plants (Liu et al., 2010). Characterization of mutants in *ASPARTATE KINASE2 (AK2)* and *DIHYDRODIPICOLINATE SYNTHASE2 (DHDPS2)* revealed that threonine accumulation helped plant defend to *Hpa* infection (Stuttmann et al., 2011). Pipecolic acid, a lysine catabolite, has shown to be a critical regulator of plant systemic acquired resistance (Návarová et al., 2012). Though a few reports support the hypothesis that imbalance in amino acid homeostasis leads to plant defense responses, whether this is the case in *gdu1-1D* remain to be determined. In addition, the critical amino acids for this process are unknown.

How plants maintain amino acid homeostasis and sense its status is also unclear. Recent reports suggested plant glutamate-like receptors (GLRs) are candidates for amino acid sensors. Unlike their homologs in mammals, ionotropic glutamate receptors (iGluRs), plant GLR receptors show broad ligand specificities (Price et al., 2012; Forde and Roberts, 2014). At least 12 of the 20 proteogenic amino acids could act as plant GLR agonists, triggering membrane depolarization

and calcium influx (Qi et al., 2006; Vincill et al., 2012; Tapken et al., 2013). Some GLRs seemed to be involved in defense against pathogens: *Glr3.3* mutants are more susceptible to the hemibiotrophic bacteria, *Pseudomonas syringae*, and the biotrophic oomycete pathogen, *Hyaloperonospora arabidopsidis* (Li et al., 2013; Manzoor et al., 2013). Summarizing the current literature, Forde and Roberts suggested that plant GLRs may sense amino acids and regulate plant defense response (Forde and Roberts, 2014). It would be interesting to see the role of GLRs in *gdu-1D*, for instance, by introducing the inducible *GDU1* into *GLR* mutants and wild type, and testing for defense response.

gdu1-1D showed mixed resistance to plant pathogens

Since the SA pathway is activated in *gdu1-1D*, and SA is an essential component of plant innate immunity, we hypothesized that *gdu1-1D* is more resistant to plant pathogens. This hypothesis was correct in some cases. Multiplication of the biotropic pathogen *Hpa* was strongly reduced in *gdu1-1D* (Fig. 5-16). On the other hand, the increased amino acid content and export in *gdu1-1D* made the plant more susceptible to *Pseudomonas syringae*, suggesting that amino acid availability is a major limiting factor for bacterium multiplication. The fact that reducing SA content promoted *Pseudomonas syringae* multiplication in plants and the increased susceptibility of plants overexpressing *GDU1* to *Pseudomonas syringae* implies that elevated defense caused by SA accumulation

in *gdu1-1D* was vanquished by increased nutrient availability to the pathogen. The difference between *Hpa* and *P. syringae* growth might arise from different feeding strategies (use of haustorium, or feeding directly from the apoplasm), or sensitivity to the SA-mediated response of the plant. Indeed, *P. syringae* was shown to adjust its nutrient utilization pathways to fit nutrients abundant in the leaf apoplasm (Rico and Preston, 2008).

gdu1-3 is less sensitive to drought, probably due to reduced LOG2 activity

Induction of *GDU1* triggered a transient ABA response, before SA signaling. ABA plays an important role in many aspects of plant development and stress responses, such as seed germination, stomata opening, and root growth under dehydration (Cutler et al., 2010). Under the severe drought condition, primary root growth is inhibited through ABA signaling pathway (Jiao et al., 2013). While the primary root elongation of *gdu1-1D* under the severe drought condition did not show any difference with the control, the GDU1 knockout mutant, *gdu1-3*, primary root grew longer than the control, suggesting *gdu1-3* is hyposensitive to ABA. Previously, Kim and Kim found that suppression of *LOG2*, a GDU1 interactor, causes hyposensitive to ABA (Kim and Kim, 2013). Therefore, we suspect that suppression of *GDU1* may reduce LOG2 activity, conferring hyposensitivity to ABA. The dual roles of LOG2, in ABA response (Kim and Kim, 2013) and

regulation of amino acid export (Pratelli et al., 2012), may explain the diversity of responses in plant caused by GDU1 over-expression.

Materials and methods

Plant material and growth

Arabidopsis thaliana plants were grown under 120 μE/m^2/s, 22 ℃, 16 h light /8 h dark on soil (Mix of Sunshine Mix 1 and Pro-mix HP at a 1:1 ratio) and were watered from below with 300 mg/l Miracle-Gro Fertilizer (Scotts, Marysville, OH, USA). Arabidopsis thaliana were transformed by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* GV3101 (pMP90).

Cloning and constructs

GDU1 coding sequence and the terminator of the small subunit of the Rubisco from pea (Pisum sativum; accession no. X00806) were inserted into the multiple cloning sites in pH-TOP vector obtained from Ian Moore, University of Oxford, UK.

Induction of GDU1

Soil grown three-week old plants were sprayed with 100 μM dexamethasone, which was dissolved in DMSO before dilution, and 0.03% silwet-77 in the final solution. Leaves from three individual plants from each line were collected at each indicated time point for RNA extraction, amino acid analysis, Trypan blue

staining, and DAB staining. For plants grown in liquid media for uptake experiments, plantlets were induced by 30 μM dexamethasone in media.

RNA extraction and quantitative RT-PCR

RNA extraction and quantitative RT-PCR were performed as described (Yu and Pilot, 2014).

Amino acid uptake and quantitation

Amino acid uptake in plants and quantitation were performed as described (Pratelli et al., 2010).

Staining methods

Trypan blue staining and DAB staining were performed according to (Pogány et al., 2009), with the following modifications. For Trypan blue staining, leaves, after trypan blue staining, were destained with chloral hydrate.

Root elongation assay

Root elongation assay were performed according to (Weele et al., 2000), with following modifications. Surface sterilized seeds were sowed on plates containing half-strength MS medium with 0.5% (w/v) Suc, pH 5.7, 0.9% (w/v) agar. Plates were stored in 4^{o}C for two days before transferring to growth chamber. On day 0, seeds on plates were placed in a growth chamber under short day condition (20 ℃, 120 μE/m^2/s, 10 h light /14 h dark). Six days after plating, similar seedlings (roots

1.5-2.0 cm long, cotyledons expanded) were transferred to plates with lower water potential or control plates with the same water potential. For each experiment, three plates with six seedlings from each line were prepared. Plants were grown vertically in the previous conditions for seven days. Plates were scanned and root lengths were calculated using ImageJ (http://imagej.nih.gov/ij/). Water potentials were measured using WP4C dewpoint potentiometer (Decagon Devices) at the end of each experiment, and reported as the mean of the measured potentials.

Pseudomonas syringae inoculations and sampling

Pseudomonas syringae inoculations and sampling were performed according to (Boureau et al., 2002), with following modifications. Bacteria *Pto* DC3000 ΔCEL was cultivated in 28 ℃ on NYGA media plate for two days, and resuspended in 10 mM $MgCl_2$ and 0.02% silwet-77 solution and diluted to OD600 =0.2. And the plant was pre-moistured one day before inoculation by being sprayed with water and covered by plastic cover. Then the bacteria solution was sprayed on to plant leaf until both abaxial and adaxial sides were saturated. The inoculated plant was covered by plastic cover to keep moisture for two days then got uncovered and cultivated at normal growth condition for another one day. Leaf discs were randomly sampled from inoculated leaves at the third day. For induced plants, plants were induced by spraying 100 μM dexamethasone and 0.03% silwet-77 solution two days before inoculations.

Hyaloperonospora arabidopsidis inoculations and sampling

Hyaloperonospora arabidopsidis inoculations and sampling were performed as described (Anderson et al., 2012).

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Chapter VI

Summary and future directions

Overexpression of *GDU1* causes profound modifications on plant physiology and interactions with its environment. Because of the glutamine secretion, initial characterization on the *gdu1-1D* mutant focused on amino acid transport, especially export, and metabolism. In addition, *gdu1-1D* develops lesions on leaves without pathogen invasion. To better understand the biochemical function of GDU1, two approaches were undertaken: one was to study mutations that suppress the Gdu1D phenotype and the other was to characterize the Gdu1D phenotype using a new approach.

GDU1 is a small membrane protein with only one transmembrane domain, unlikely to be an amino acid transporter. Therefore, its function was expected to be exerted by interacting with other proteins. We anticipated that characterizing mutations in GDU1 and GDU1 interactors would enable to better understand its structure-function relationship, especially regarding its interaction with regulators of amino acid transport. LOG2, an E3 ubiquitin ligase, was isolated as a GDU1 interactor and the GDU1-LOG2 interaction was found necessary for the Gdu1D phenotype. While GDU1 can be ubiquitinated by LOG2 *in vitro*, it appeared that LOG2 regulates GDU1 functions in a non-proteasome-dependent manner. It was

thus hypothesized that LOG2 regulates GDU1 localization at the plasma membrane (Chapter II). Based on my later work on GDU1, I speculate that overexpression of *GDU1* stabilizes LOG2 localization at the plasma membrane, where LOG2 ubiquitinates and targets for degradation a negative regulator of an amino acid exporter. In this way, overexpression of *GDU1* is expected to cause increased amino acid export activity.

Characterization of intragenic mutations in GDU1 revealed that while the size of the *log1* mutants was similar to the wild type, the amino acid export activity of those mutants was intermediate between *gdu1-1D* and the wild type, which indicates that *log1* mutations do not completely suppress the Gdu1D phenotype (Chapter IV). The *log1-1* and *log1-3* mutations abolished GDU1 interaction with LOG2. However, it should be noted that GDU1 also interacts with LUL1, a LOG2 homolog (Pratelli et al., 2012). Thus, while those two mutations affected the interaction with LOG2, they may not affect the interaction with LUL1. If LUL1 is involved in the regulation of amino acid transport, this hypothesis may explain the observation of the intermediate increased amino acid export activity of the *log1* mutants. Study of the *log1-2* and *log1-4* mutations implied that protein phosphorylation and ubiquitination regulate GDU1 functions, which suggests a high complexity in the regulation on amino acid export.

The GDU family is composed of seven members in Arabidopsis. They share two conserved domains, the transmembrane domain and the VIMAG domain. The VIMAG domain is necessary for interaction with LOG2, thus essential for their functions. Overexpression of the *GDUs* leads to a similar phenotype, such as increased amino acid export and amino acid content in leaves, and reduced plant size (except GDU4 and GDU7), so they probably have similar roles in the regulation of amino acid export (Pratelli et al., 2010). The differences in expression profiles of *GDUs* suggest they fulfil different roles in certain developmental stages and organs. As part of a project that could not be added to this dissertation, RNAseq analyses of plants over-expressing various *GDUs* was carried out. The study indicated that while these mutants showed increased stress responses and decreased activities of metabolism and development, lines over-expressing different *GDUs* showed different stress responses: some displayed response to biotic stress, while others to abiotic stress. It thus seems that the GDUs have specific roles in different processes while sharing a role in regulating amino acid export.

Although early studies of constitutive GDU1 over-expressors provided some information about the biochemical function of the protein, we were not able to differentiate well the various effects caused by *GDU1* overexpression. To separate these effects, we utilized an inducible gene expression system (Chapter V). This

way, we showed that increase in amino acid export precedes stress-related phenotypes, which involved ABA and SA. My work proved that the lesion formation in *gdu1-1D* is dependent on the SA-pathway and I showed that overexpression of *GDU1* influences plant sensitivity to pathogens in two opposite ways. Increased amino acid availability increased pathogen susceptibility, while constitutive SA accumulation concomitantly elevated defense responses.

While no direct link between amino acid imbalance and plant stress responses has been established so far, the inducible *GDU1* expression system provided an excellent tool to study this question. Pipecolic acid, a lysine catabolite, has been shown to stimulate SA biosynthesis, and a priming signal for systemic acquired resistance (Návarová et al., 2012). It will be interesting to test whether pipecolic acid is involved in the elevated defense response seen in *GDU1* over-expressors. GLRs have been suggested to be amino acid sensors, regulating plant defense response (Forde and Roberts, 2014), and as such they would be candidates involved in plant defense response triggered by GDU1 overexpression. The fact that leucine is proposed to regulate many stress-response genes (Hannah et al., 2010) suggests that some amino acids are more important than others in this process. Which of them are responsible for triggering defense response? Are they equivalently potent?

The work reported in this dissertation sheds light on how amino acid imbalance interacts with various plant signaling pathways and stress responses and shows that the inducible expression system provides a promising tool for further detailed study on this topic.

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Appendix

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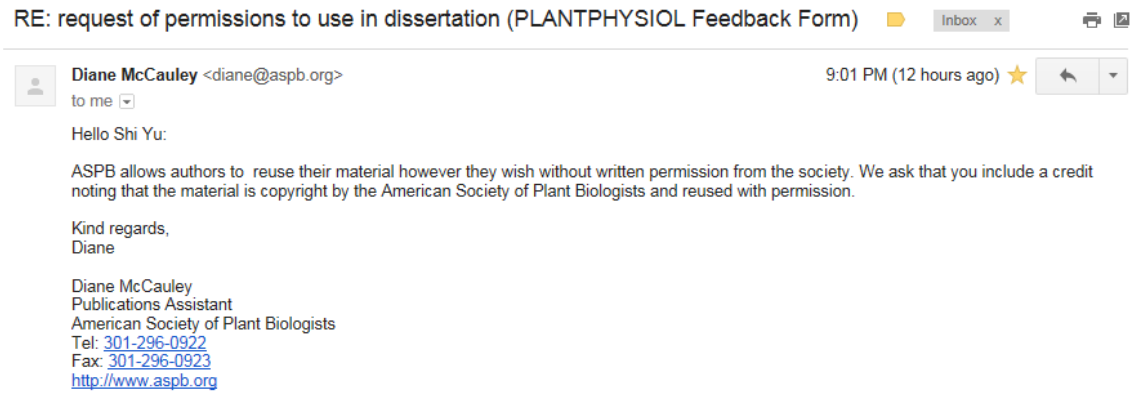

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