

UNIVERSIDADE ESTADUAL DE CAMPINAS

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**METABOLISMO DO NITROGÊNIO NO SISTEMA
RADICULAR DE LEGUMINOSAS EM CONDIÇÃO DE
HIPOXIA**

Este exemplar corresponde à redação final
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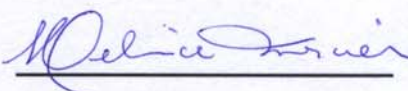
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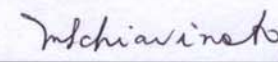
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ABREVIATURAS

ADH - álcool desidrogenase

ADP - Adenosina difosfato

Ala - alanina

AlaAT - alanina aminotransferase

AlaAT - alanine aminotransferase

Asp - ácido aspártico

AspAT - aspartato aminotransferase

ATP - adenosina trifosfato

cDNA - ácido desoxirribonucléico complementar

CoA - coenzima A

DNA - ácido desoxirribonucléico

FAD - flavina adenosina dinucleotídeo forma oxidada

FADH₂ - flavina adenosina dinucleotídeo forma reduzida

GABA - ácido gamma-aminobutírico

GABA-T - transaminase do ácido gamma-aminobutírico

GAD - descarboxilase do glutamato

GC-TOF-MS - gas chromatography - time-of-flight - mass spectrometry

Glu - ácido glutâmico

GOGAT - glutamato sintetase

GS - glutamina sintase

IDH - isocitrato desidrogenase

Lb - leghemoglobina

LbRNAi – mutante criado por RNA interferente para leghemoglobina

MAL - malato

MDH - malato desidrogenase

ME - enzima malica

NAD(P)⁺ - β -nicotinamida adenina dinucleotídeo, forma oxidada

NAD(P)H - β -nicotinamida adenina dinucleotídeo fosfato, forma reduzida

NifH - gene que codifica uma das subunidade do complexo nitrogenase

OAA - ácido oxaloacetato

OG (α -KG) - oxoglutarato (α -cetoglutarato)

OGDH - oxoglutarate dehydrogenase

ox - estado oxidado

PCR - reação em cadeia da polimerase

PDC - Piruvato descarboxilase

PEP - Fosfoenolpiruvato

PEPC - carboxilase do fosfoenolpiruvato

RNA - ácido ribonucléico

RNAi – RNA interferente

SDH - succinato dehidrogenase

TCA - ciclo do ácido cítrico

INTRODUÇÃO GERAL

Para as plantas, o principal efeito imposto pela inundação é o impedimento das trocas gasosas entre o sistema radicular e o espaço poroso do solo, visto que a resistência de difusão de muitos gases é aproximadamente 1000 vezes maior na água que no ar (Armstrong et al., 1994). Após 24 horas de inundação, com temperaturas ambiente superiores a 20°C, pode haver esgotamento total do O₂ do solo devido ao consumo por microorganismos, fauna e raízes (Armstrong et al., 1994; Drew et al., 2000; Liesack et al., 2000). Há hipoxia quando a pressão parcial de O₂ limita a produção de ATP pela mitocôndria (Drew, 1997), sendo o metabolismo hipóxico caracterizado pela atividade simultânea da respiração limitada (metabolismo aeróbico) e algum grau de fermentação (metabolismo anaeróbico). Sob condições hipóxicas, o metabolismo de um órgão vegetal pode ser heterogêneo, visto que as camadas externas recebem mais O₂ que as camadas internas (Ricard et al., 1994). A anoxia ocorre quando a produção de ATP pela fosforilação oxidativa é desprezível em relação ao gerado pela glicólise e fermentação (Drew, 1997). Condições de hipoxia são mais comuns na natureza que as de anoxia e podem promover efeitos adversos no crescimento (Drew, 1997).

Quando o sistema radicular é sujeito a encharcamento, a sobrevivência das raízes depende do metabolismo anaeróbico. Na respiração anaeróbica, pela falta de O₂ como acceptor final de elétrons, ocorre o acúmulo de intermediários no ciclo de Krebs, diminuição da disponibilidade de NAD(P)⁺ e acúmulo de piruvato. Nessas condições, a síntese de muitas proteínas da célula é suprimida enquanto que a

síntese de um grupo específico de proteínas aumenta (Sachs et al., 1980). Incluídas neste grupo são as enzimas álcool desidrogenase, lactato desidrogenase, piruvato descarboxilase, e várias enzimas da glicólise (Lazlo & St. Lawrence, 1983; Kelley & Freeling, 1984a; 1984b; Hoffman et al., 1986). Em consequência, acumulam vários produtos da glicólise, incluindo etanol, lactato, alguns ácidos orgânicos e aminoácidos (Ricard et al., 1994). Além de etanol e ácido láctico, o aminoácido alanina frequentemente se destaca como produto do metabolismo anaeróbico (Ricard et al., 1994).

Desta forma, três principais vias são ativadas durante o alagamento: a do etanol, a do ácido láctico e uma que é específica de plantas, que produz alanina a partir do glutamato e piruvato, envolvendo a AlaAT (alanina aminotransferase) (Ricard et al., 1994; Dennis et al., 2000; Sousa & Sodek, 2002). Em plantas sob condições normais de oxigenação, essas vias estão ausentes ou com atividade bastante reduzida, mas são rapidamente induzidas em baixa concentração de O_2 , o que sugere um papel no mecanismo de sobrevivência das plantas, embora não se conheça qual a extensão da contribuição e como elas se relacionam em baixo teor de O_2 (Dennis et al., 2000).

Embora a atividade conjunta da glicólise e fermentação permitam apenas a síntese limitada de ATP, pela fosforilação ao nível de substrato, regenera-se NAD^+ e remove-se o excesso de prótons (Dennis et al., 1992; Armstrong et al., 1994, Ricard et al., 1994; Morard & Silvestre, 1996, Drew, 1997; Sousa & Sodek, 2002). Neste caso, a respiração mitocondrial é limitada pela falta de O_2 comoceptor final de elétrons. A fermentação corresponde a um período excepcional e transitório no

metabolismo das raízes, no qual a produção de ATP cai de 36 para 2 moles por mol de glicose metabolizada (Dennis et al., 1992; Morard & Silvestre, 1996; Sousa & Sodek, 2002).

A fermentação alcoólica ramifica-se fora da via glicolítica a partir do piruvato. Num primeiro passo, piruvato é o substrato da piruvato descarboxilase, formando CO_2 e acetaldeído. O acetaldeído é tóxico para as plantas, e a sua redução para etanol, pela álcool desidrogenase, regenera NAD^+ , que é utilizado no metabolismo glicolítico para manter o fluxo de carbono em condições anaeróbias. Esta troca de caminho para produção de energia, da glicólise para a fermentação é uma adaptação das plantas para sobreviverem em condições de baixa disponibilidade de oxigênio (Huq et al., 1999).

Outras ramificações do piruvato incluem a sua transformação em OAA (via PEPCase) e MAL (via enzima málica), dois componentes do ciclo de Krebs. Atividade parcial do ciclo de Krebs pode ser uma estratégia importante em condições de hipoxia (Fox et al., 1994), mas o fenômeno foi pouco explorado. Esta atividade parcial do ciclo de Krebs envolve duas partes do ciclo funcionando em sentidos contrários. Um caminho segue a partir do OAA até o succinato, passando pelo MAL, no sentido oposto do habitual. Assim sendo, gera NAD^+ no lugar de NADH , favorecendo a fermentação. O outro caminho também segue a partir do OAA porém no sentido normal do ciclo, parando em αKG . Do ponto de vista do metabolismo de N estas ramificações do metabolismo do piruvato e o funcionamento parcial do ciclo gera ácidos orgânicos precursores dos aminoácidos Asp e Glu (a partir de OAA e αKG , respectivamente). Os aminoácidos Asp e Glu

podem ser precursores importantes para a formação de Ala (via AspAT e AlaAT), aminoácido este que se acumula em grandes quantidades nas raízes de soja sujeitas à hipoxia (Sousa & Sodek, 2003). A função deste processo, entretanto, é desconhecida, visto que a síntese de alanine a partir do piruvato não regenera NAD^+ (Sousa & Sodek, 2002), embora contribua para a regulação do pH celular (Reggiani et al., 1988).

Além das raízes, o sistema radicular de leguminosas é também constituído de nódulos, estruturas responsáveis pela fixação simbiótica de N_2 . Pouco se sabe sobre o comportamento do nódulo durante o alagamento do sistema radicular. Mesmo em condições normais há uma limitação no fornecimento de oxigênio para a fixação de nitrogênio, em função de uma barreira variável à difusão de oxigênio presente nos tecidos externos do nódulo que circundam a região central infectada (Hunt et al., 1989; Layzell et al., 1990). Portanto, o nódulo é naturalmente hipóxico (embora levemente) e por este motivo extremamente sensível ao alagamento em função da baixa disponibilidade de oxigênio (Lima & Sodek, 2003; Amarante & Sodek, 2006).

O processo de fixação de N_2 é bastante prejudicado pelo alagamento, quase que imediatamente (Amarante & Sodek, 2006), enquanto que as raízes entram em hipoxia apenas algumas horas depois do alagamento (Sousa & Sodek, 2003). Apesar da limitação pelo O_2 mesmo em condições normais (Hunt et al., 1989), o nódulo possui um eficiente mecanismo de transporte de O_2 para suprir a respiração do bacterióide e assim fornecer energia para a fixação de N_2 . No entanto, nenhuma tentativa foi feita para entender como outros aspectos do metabolismo de nitrogênio

no nódulo são afetados pelos baixos níveis de O_2 livre. A falta de O_2 no nódulo é uma situação completamente diferente da de uma raiz inundada, principalmente devido ao mecanismo que envolve leghemoglobina, que permite ao nódulo funcionar sob baixas concentrações de O_2 . Uma comparação de respostas para baixa concentração de O_2 livre deverá revelar aspectos interessantes do nódulo e da fisiologia da raiz, contribuindo para a compreensão dos processos envolvidos.

OBJETIVO GERAL

O objetivo geral deste trabalho foi determinar a relação entre metabolismo de nitrogênio em baixas concentrações de oxigênio no sistema radicular e o seu significado na tolerância da planta a este estresse, bem como relacionar alterações no metabolismo de aminoácidos ligados a piruvato e ácidos orgânicos do ciclo de Krebs com a hipoxia e elucidar o papel do oxigênio na regulação da fixação de nitrogênio pelos mecanismos de sensoriamento do nível energético.

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CHAPTER 01: Integration of carbon and nitrogen metabolism during hypoxia induced by waterlogging is crucial for survival of *Lotus japonicus*

1. RESUMO

O papel do metabolismo do nitrogênio na sobrevivência de plantas noduladas de *Lotus japonicus* foi investigado durante períodos de alagamento. O acúmulo de alanina revelou ser um dos pontos críticos durante a hipoxia, uma vez que esse foi o único aminoácido cuja biossíntese não foi inibida pela deficiência de nitrogênio observada em plantas mutantes (leghemoglobina-RNAi). As alterações metabólicas induzidas durante o período de alagamento podem ser mais bem explicadas pela ativação do metabolismo da alanina em combinação com uma atividade parcial do ciclo de Krebs. O resultado final deste cenário metabólico resulta no acúmulo de alanina e succinato bem como na produção extra de ATP sob hipoxia. A importância do metabolismo da alanina também estaria relacionada com a capacidade de regular o nível de piruvato. Esta e todas as outras alterações são discutidas no contexto de modelos atuais sobre a regulação do metabolismo da planta.

2. ABSTRACT

The role of nitrogen metabolism in the survival of prolonged periods of waterlogging was investigated in highly flood-tolerant, nodulated *Lotus japonicus* plants. Alanine production revealed to be a critical hypoxic pathway being the only amino acid whose biosynthesis is not inhibited by N-deficiency resulting from RNAi

silencing of nodular leghemoglobin. The metabolic changes which were induced following waterlogging can be best explained by the activation of alanine metabolism in combination with the modular operation of a split tricarboxylic acid (TCA) pathway. The sum result of this metabolic scenario is the accumulation of alanine and succinate and the production of extra ATP under hypoxia. The importance of alanine metabolism is discussed with respect to its ability to regulate the level of pyruvate, and this and all other changes are discussed in the context of current models concerning the regulation of plant metabolism.

3. INTRODUCTION

Plants initiate several responses to alleviate the consequences of oxygen deprivation during flooding or waterlogging of the soil. The combination of these adaptive responses is known as the "low oxygen escape syndrome" (Bailey-Serres and Voesenek, 2008). Among those adaptations, various anatomical and morphological changes such as aerenchyma formation (Justin and Armstrong, 1987; Kennedy et al., 1992), stem elongation (Voesenek and Blom, 1989; Armstrong et al., 1994), the presence of gas films around flooded leaves (Pederson et al., 2009), and increasing shoot biomass can be observed. The latter are supposed to help providing oxygen via photosynthesis (Mommer and Visser, 2005; Pederson et al., 2009).

A further class of hypoxic responses includes the down-regulation of a suite of energy, and therefore, oxygen-consuming metabolic pathways (Geigenberger,

2003). Examples of such metabolic adaptations to hypoxia include the down-regulation of storage metabolism (Geigenberger et al., 2000), the shift from invertase to sucrose synthase routes of sucrose hydrolysis (Bologa et al., 2003; Huang et al., 2008) and the inhibition of mitochondrial respiration (Gupta et al., 2009; Zabalza et al., 2009). These responses are already initiated before oxygen becomes limiting as a substrate for respiration. It is, therefore, suggested that these metabolic changes are important components of the survival strategy as they considerably extend the period of hypoxia which a plant can withstand.

When the oxygen availability decreases below the level at which oxygen becomes limiting for oxidative phosphorylation, plant cells will depend on alternative metabolic pathways to produce ATP. Under such anoxic circumstances, the major source for energy is the glycolytic pathway, which produces two ATP and two pyruvate molecules per unit of hexose whilst concomitantly reducing NAD^+ to NADH. In order to maintain glycolysis under anoxic conditions, NAD^+ must be continuously regenerated from NADH via fermentative reactions. Using pyruvate as substrate, fermentative metabolism either produces lactate, via lactate dehydrogenase or ethanol via two subsequent reactions catalyzed by pyruvate decarboxylase and alcohol dehydrogenase (Tadege et al., 1999). A drawback of these two pathways is that the end-products are toxic for the cell.

In addition to lactate and ethanol fermentation, many plant species accumulate alanine under anoxic conditions (Sousa and Sodek, 2003; Miyashita et al., 2007). Alanine can accumulate to high concentrations without harmful consequences. It is even suggested that alanine production would help to regulate

the pH balance within an anoxic cell (Reggiani et al., 1988). Nevertheless, it remains unclear how exactly the production of alanine supports anoxic metabolism, as no NADH oxidation occurs during the production of alanine (Sousa and Sodek, 2002). Accumulation of alanine without any alternative NADH oxidation would therefore lead to a decrease of the glycolytic flux due to NAD⁺ limitation.

Several metabolic pathways have been proposed to explain the accumulation of alanine upon anoxia. A rapid induction of the expression of AlaAT, a gene encoding for the enzyme alanine amino transferase, as well as an increase in the activity of the enzyme during flooding have previously been documented (Good and Crosby, 1989; Good and Muench, 1992; Muench and Good, 1994). These inductions could potentially explain the increased production of alanine. However, alanine accumulation was also observed in T-DNA knockout plants in which AlaAT activity was reduced to almost zero (Miyashita et al., 2007). Apparently, alanine production does not depend solely on the activity of AlaAT. An alternative reaction that is able to produce alanine is catalyzed by gamma-aminobutyric acid transaminase (GABA-T) using pyruvate as co-substrate. Similar to the AlaAT knockout plants, GABA-T null-mutants accumulated only slightly less alanine upon hypoxia as compared to wild type plants (Miyashita and Good, 2008). These results suggest at least partial redundancy of AlaAT and GABA-T under hypoxic conditions.

To better understand the role of alanine accumulation during hypoxia, metabolic changes were analyzed following root waterlogging of plants suffering from nitrogen limitation. Nodulated *Lotus japonicus* was grown on nitrogen-free substrate and exposed to several days of waterlogging. Changes in the levels of

metabolites of primary carbon and nitrogen metabolism were compared to changes that were observed in plants not able to fix nitrogen via symbiotic interaction with rhizobia. For this purpose, transgenic *L. japonicus* were used in which the genes encoding for Leghemoglobin were silenced via an RNAi approach (Ott et al., 2005; 2009). We preferred this transgenic approach to change the intrinsic nitrogen status of the plants to feeding experiments with various amounts of nitrate since it has been demonstrated that under anoxic conditions nitrate is converted via nitrate reductase into nitrite and subsequently to nitric oxide (Rockel et al., 2002; Planchet et al., 2005). Nitric oxide is known to be a versatile signaling molecule that could induce multiple pleiotropic effects (Besson-Bard et al., 2008).

Here we show that *L. japonicus* roots are very tolerant to waterlogging-induced hypoxia. However, their susceptibility to hypoxia increased upon nitrogen deprivation. Upon waterlogging, pronounced accumulation of succinate and alanine was observed that was independent of the N-status of the plant, whereas the metabolic behavior during flooding of all other amino acids was strongly influenced by the N-status of the plant. Based on our observations, a metabolic model is proposed which explains the role of alanine accumulation during waterlogging via reorganization of the tricarboxylic acid (TCA)-cycle in a modular manner where ATP is produced from an oxidizing pathway leading from oxoglutarate to succinate, whereas a parallel reducing branch oxidizes NADH during hypoxia. According to this scheme, alanine metabolism would also prevent pyruvate accumulation thereby facilitating the continued operation of glycolysis during waterlogging.

4. MATERIALS AND METHODS

4.1 Plant material and growth conditions

Wild type *Lotus japonicus* ecotype GIFU (B-129) and transgenic plants (LbRNAi) in which the expression of all 3 homologous genes encoding leghemoglobin in nodules were reduced to less than 3% of wild type via an RNAi approach (Ott et al., 2005) were grown from seeds in a controlled greenhouse environment (16 h day, 60% relative humidity, and 21/17°C day/night temperature regime). Seeds were sterilized in 2% sodium hypochlorite for 10 min, rinsed five times with sterile water, and germinated on moist, sterile filter paper for 3 days. Germinated seedlings were transferred to pots filled with 2-4 mm diameter hydroponic culture substrate granules (Lecaton Original, Fibo Exclay Deutschland GmbH, Lamstedt, Germany). Plants were watered daily, and twice per week, a nitrogen-free nutrient solution was supplied (0.5 mM CaCl₂; 0.5 mM KCl; 0.25 mM KH₂PO₄; 0.25 mM K₂HPO₄; 1.0 mM MgSO₄; 0.05 mM FeEDTA; Trace Elements – 9.1 µM MnCl₂; 0.046 mM H₃BO₃; 0.765 µM ZnCl₂; 0.56 µM NaMoO₄; 0.32 µM CuCl₂). Seven days after transferring seedlings to the pots, plants were inoculated with *Mesorhizobium loti* strain R7A.

Waterlogging of the plants was performed by placing the pots inside a second pot of similar size which was filled with N-free nutrient solution at one-third of normal strength sufficient to bring its level just above the substrate. Subsequently, nitrogen gas was bubbled through the solution until the concentration of dissolved oxygen decreased to 55 µM, which is the equivalent of 20% of air saturation. The oxygen

concentration of the solution was controlled throughout the course of the experiment, and was in the range 15 and 50 μM .

Plant organs (root, nodule and leaves) were harvested from 12-week-old plants directly into liquid nitrogen, and stored at -80°C . Six biological replicates were taken for each sample.

Plant survival during waterlogging was determined by counting the number of dead plants each day during a 30-day time period. The survival rate of wild type and LbRNAi plants was compared between non-flowering plants of the same age (4-5 weeks, wild type shoots were approximately 6-8 cm, and LbRNAi shoots were 2.5-3.0 cm long), as well as between plants of the same size (wild type plants were 2-3 weeks old, and LbRNAi plants were 4-5 weeks old).

4.2 Metabolic analyses

Nodulated roots and leaves were sampled during the course of the experiment at the time points as indicated in the text and immediately frozen in liquid nitrogen. Nodules were subsequently separated from the roots. Aliquots of 50 - 100 mg tissue fresh weight were ground in 2 ml Eppendorf tubes each containing a clean stainless steel metal ball (5 mm diameter) using a mixer-mill grinder (MM200; Retsch GmbH, Haan, Germany) for 2 min with a frequency of 30 Hz. Grinding components of the mill were cooled with liquid nitrogen to keep the samples frozen at all times. Metabolites were extracted as described by Lisec et al. (2006): by thoroughly shaking for 15 min at 70°C in a mixture of 1400 μl methanol supplemented with 60 μl 1.3 μM ribitol in water. Water-soluble metabolites were

separated by adding 750 μ l chloroform and 400 μ l water to the extract. Subsequently, the aqueous phase was transferred into clean Eppendorf tubes. Samples were dried at room temperature by vacuum centrifugation (Concentrator 5301, Eppendorf, Hamburg, Germany).

Derivatisation of the metabolites was performed by a methoxyamination reaction prior to GC-TOF-MS analysis. After baseline correction (ChromaTOF software version 1.00, Pegasus driver 1.61, LECO, St Joseph, MI, USA), peak heights of the mass (m/z) fragments were normalized to the internal standard (ribitol) and fresh weight of the samples. Annotation of the mass fragments was manually supervised using TagFinder, (Luedemann et al., 2008). Statistical analysis by two-way ANOVA of the data was done using the MeV (Multi Experiment Viewer) software (Saeed et al., 2003).

The amount of total amino acids was determined from methanol extracts using the ninhydrin method of Yemm and Cocking (1955) using a concentration series of leucine for quantification. Total soluble sugars were determined with the anthrone method of Graham and Smydzuk (1965) using glucose for the calibration curve. Starch was determined from the insoluble pellet as in Hendriks et al. (2003) using the Starch UV-method Kit (Roche) and total protein was measured as described by Bradford (1976) after solubilization of the insoluble pellet with 0.1 M NaOH. Absorbancies were read in a Spectra Max Plus microplate and cuvette spectrophotometer (Molecular Devices, Sunnyvale, USA).

4.3 Enzyme analysis

Enzyme extracts were prepared as described previously (Gibon et al., 2004), except Triton X100 was used at a concentration of 1% and glycerol at 20%. The maximum activity of 2-oxoglutarate dehydrogenase (EC 1.2.4.2) was determined exactly as described by Araújo et al. (2008). Aconitase (EC 4.2.1.3) activity was determined as detailed in Carrari et al. (2003), pyruvate dehydrogenase (EC 1.2.4.1) was assayed as described in Randall and Miernyk (1990), NAD-dependent isocitrate dehydrogenase (EC 1.1.1.41) as in Jenner et al. (2001), NAD-dependent malate dehydrogenase (EC 1.1.1.37) as described by Jenner et al. (2001), NAD-Malic enzyme (EC 1.1.1.39) as detailed in Kulichikhin et al. (2009), alanine aminotransferase (EC 2.6.1.2) as described by Good and Muench (1992), aspartate aminotransferase (EC 2.6.1.1) as described by Griffith and Vance (1989) and NADH-dependent glutamate synthase (EC 1.4.1.14) as described by Groat and Vance (1995).

4.4 Analysis of gene expression

Total RNA was extracted from nodulated roots using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNase treatment was done with the TURBO DNA-free kit (Ambion, Austin, USA). cDNA was prepared from 5 µg RNA using the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, USA). Real-time PCR amplification was carried out with the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster city, USA), using power sybr-green master mix (Applied Biosystems) and the primers described in

Supplementary Table II. Transcript levels of most genes were normalized against *ubiquitin* mRNA as described previously by Colebatch et al. (2004) except for the levels of bacterial *nifH* transcript that was normalized against the constitutively expressed gene *sigA* as described by Ott et al. (2005). Relative quantification of gene expression was performed using the comparative threshold cycle method, as described in the ABI PRISM 7900 Sequence Detection System User Bulletin number 2 (Applied Biosystems).

5. RESULTS

5.1 *Lotus japonicus* is highly tolerant to waterlogging

The effect of waterlogging on wild type *L. japonicus* plants was determined by visual observation. Even after 4 weeks, none of the plants of our test population showed any sign of reduced vitality though several morphological changes could be observed in the waterlogged plants (Fig. 1). These changes included the presence of etiolated stems, slightly yellowish leaves, delayed flowering and seed set and stunted root growth in comparison to non-waterlogged plants. Apparently, *L. japonicus* wild type plants are well able to adapt to prolonged periods of waterlogging, even though this occurs at the cost of seed production.

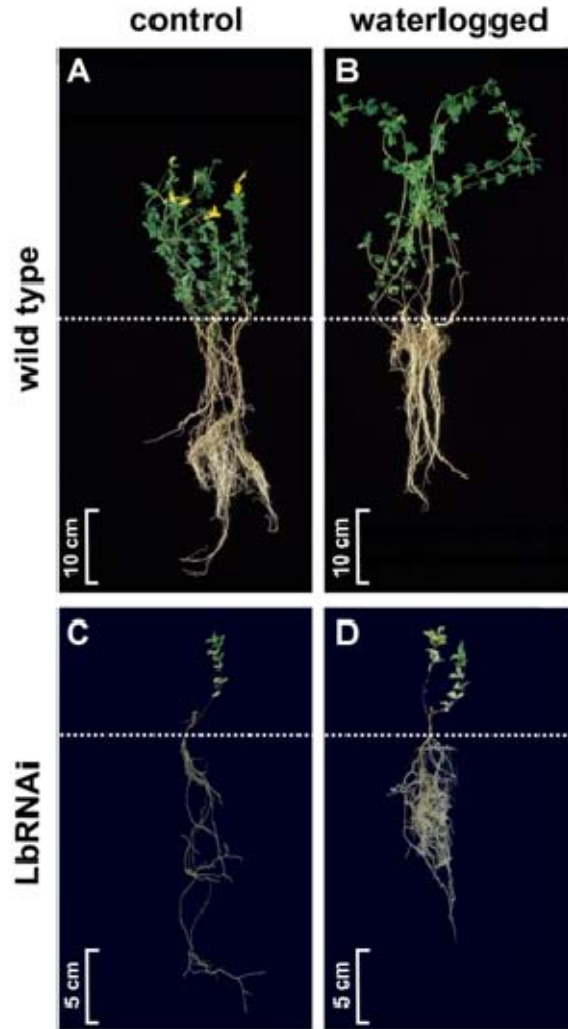


Figure 1. Phenotype of *L. japonicus* wild type and LbRNAi plants before and during waterlogging. Ten-weeks-old wild type (A, B) and LbRNAi (C, D) plants were photographed after a 4 weeks period of continuous waterlogging (B, D), or a similar growth period under control conditions (A, C). The photographs show representative images of the entire population. The dotted line indicates the transition from root to shoot.

Tolerance to waterlogging was investigated in more detail by determining the amount of plants surviving the course of the experiment (Fig. 2). Notably even after 4 weeks no dead wild type plants were observed. In contrast, plants in which the

genes encoding for nodular leghemoglobin (LegHb) were silenced by an RNAi approach already showed first signs of reduced vitality within the first week of waterlogging. These transgenic plants have been described in detail before (Ott et al., 2005; 2009), and various independent lines were shown to be identically affected by the RNAi silencing approach. Upon waterlogging, LbRNAi plants displayed a reduced but denser root system, and shoots produced more side branches (Fig. 1). After 4 weeks, about 25% of these plants were dead (Fig. 2). These data thus illustrate that the ability to fix nitrogen is required for maximum flooding resistance.

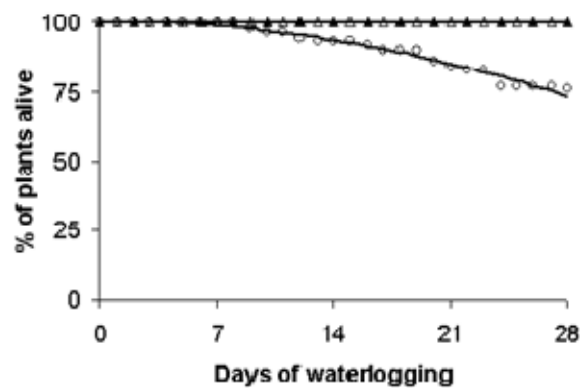


Figure 2. Survival rate of *L. japonicus* wild type and LbRNAi plants during an extended water-logging period. The amount of healthy plants was determined throughout a period of 30 days for LbRNAi plants (○), as well as for wild type plants of the same age (▲) or the same size (Δ). The error bars in the graph indicate the standard deviation.

5.2 Waterlogging induced changes in the expression of genes encoding enzymes for fermentation and nitrogen fixation

Waterlogging is generally assumed to reduce the availability of oxygen to the roots. The induction of the plant's response to hypoxia was verified by measuring the expression of alcohol dehydrogenase (*Adh*) and pyruvate decarboxylase (*Pdc*) (Fig. 3). These genes are all well known to be expressed upon hypoxia (Bologa et al., 2003; Ismond et al., 2003). As expected, the expression levels of *Adh* and *Pdc* increased during waterlogging in both wild type and the LbRNAi plants and during recovery the expression levels returned to the same levels as detected prior to the waterlogging treatment.

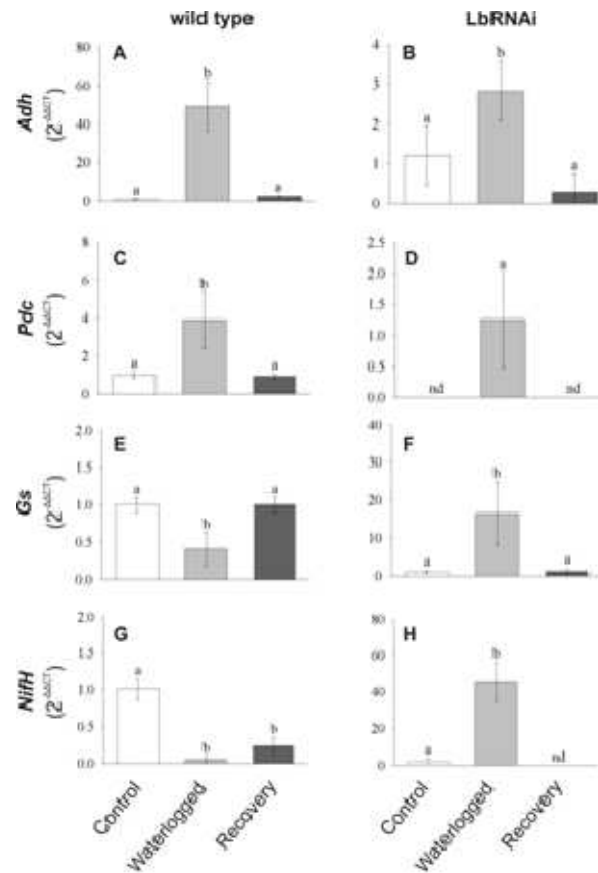


Figure 3. Changes in the transcript levels of hypoxia responsive genes Changes in the transcript levels of selected genes known to be induced by hypoxia. Samples from wild type (A, C, E, G) and LbRNAi (B, D, F, H) plants were analysed at normoxia, after 5 days of water-logging and after a 5 days recovery period. Values represent means \pm standard deviations. Bars that are marked with the same letter were judged by a one way ANOVA to be identical to the control ($P < 0.05$). Adh - alcohol dehydrogenase; Pdc - pyruvate decarboxylase; Gs - glutamine synthase; NifH – nitrogenase subunit H.

In addition to the expression of these hypoxia-induced genes, transcript levels of two additional genes encoding for glutamine synthase (*Gs*) and a subunit of the nitrogenase complex (*NifH*) were analyzed. These genes are known to be only expressed in nodules in which the oxygen concentration is so low that nitrogen fixation is possible. As expected, the expression level of both genes appeared to be very low in nodules of LbRNAi plants (Fig. 3). However, both genes were strongly induced during waterlogging of LbRNAi plants, again indicating that the oxygen concentration in these nodules had dropped. Expression levels of *Gs* and *NifH* in wild type nodules changed in a manner opposite to that observed in LbRNAi plants, being initially high, reduced during waterlogging and increased again during the recovery phase.

5.3 Quantitative changes of total amino acids, sugars, protein and starch induced by waterlogging differ between roots and shoot

Changes in the amount of total amino acids, sugars, protein and starch were determined before the start of the waterlogging treatment, as well as following 5 days of waterlogging and after 5 days of subsequent recovery from the treatment. In leaves of LbRNAi plants, the levels of total sugars and protein appeared to be slightly lower than in WT, whereas the amount of starch was about 45% higher, when assessed on a per gram fresh weight basis (Fig. 4). The amount of free amino acids was similar between the genotypes. Waterlogging had hardly any effect on the quantity of the above-mentioned metabolites in leaves, with only the level of protein

decreasing slightly in wild type leaves, and moderately increasing in LbRNAi plants. During the recovery phase, these changes were not reversed.

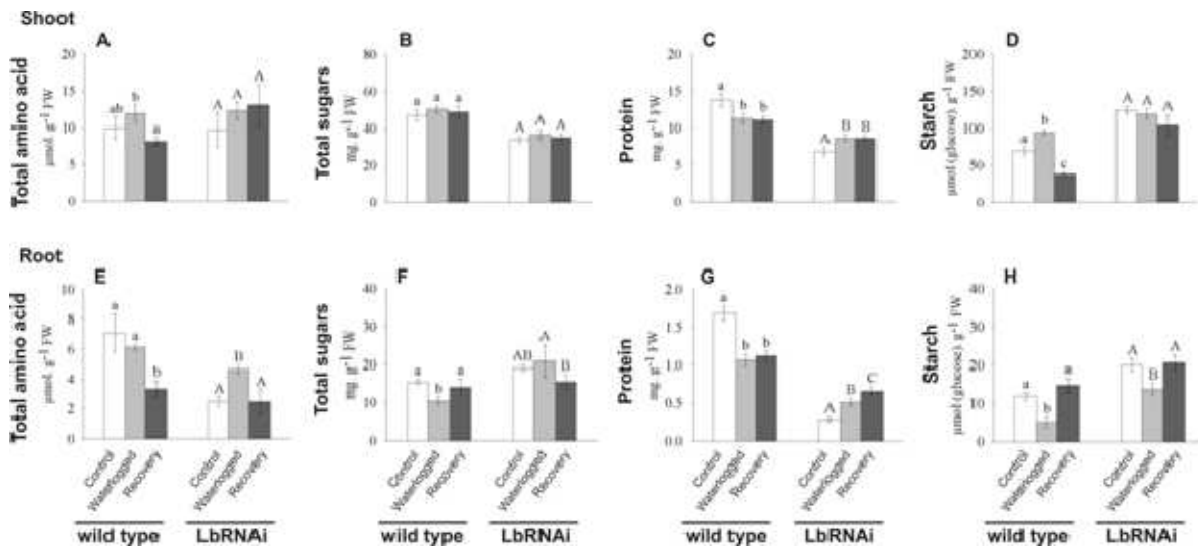


Figure 4. Determination of changes in total amino acids, protein, total soluble sugar and starch in *L. japonicus* wild type and LbRNAi plants. Quantification of total soluble amino acids (A,E), total soluble sugars (B,F), total protein (C,G) and starch (E,H) measured in shoot (A, B, C, D) and roots (E, F, G, H) of wild type and LbRNAi plants. Samples were taken prior to any treatment (control), after 5 days of waterlogging, and after 5 days recovery. Values are presented as means \pm standard deviation. Bars that are marked with a dissimilar letters are judged to be significantly different from each other (one-way ANOVA, $p < 0.05$). FW, fresh weight.

In roots, waterlogging-induced differences in the quantity of sugars, starch, amino acids and protein were pronounced. Even prior to waterlogging, the levels of free amino acids and total protein were significantly lower in the LbRNAi plants than in the wild type. In wild type plants, waterlogging induced a decrease in the amount of amino acids and total protein, which was not recoverable within five days after cessation of the treatment. In contrast, the amount of amino acids in the LbRNAi plants increased during waterlogging and recovered again after waterlogging was ceased. However, the amount of total protein increased during waterlogging, as well as during the subsequent recovery period.

In contrast to the N-containing compounds, the level of sugars and starch were significantly higher in the LbRNAi plant than in the wild type. Upon waterlogging, the levels of starch in both wild type and LbRNAi roots decreased, whereas the levels returned again to their initial levels during the recovery period. The amount of sugars decreased during waterlogging in wild type roots but not in those of LbRNAi plants.

In general, it is shown here that the hypoxic stress imposed on the plants by waterlogging induces strongest metabolic changes in roots, whereas the well-aerated parts of the plant remained rather unaffected. Furthermore, whereas N-assimilation is reduced by waterlogging in wild type plants, the opposite is observed in LbRNAi plants.

5.4 GCMS profiling

A comprehensive profiling of primary metabolism was obtained by gas-chromatography mass-spectrometry (Fernie et al., 2004; Lisec et al., 2006), on samples taken daily during the five-day period of waterlogging and the five subsequent days of the recovery phase. Over 40 metabolites were reproducibly identified in both genotypes across the three treatments, but not all metabolites were detected in each instance. An overview of the level of all metabolites determined across the experiment is provided in Supplemental Table I.

In order to investigate correlations between changes in the levels of metabolic intermediates of primary carbon and nitrogen metabolism, the levels of selected metabolites were plotted within the context of the metabolic pathways to which they belong. Visualizations of the data obtained for roots and nodules are shown in Fig. 5 and 6 respectively. In roots, changes in the level of almost all nitrogen-containing metabolites were detected in wild type plants, whereas only moderate changes were observed in the LbRNAi plants. In general, amino acids that are derived from precursors from the glycolytic pathway increased during waterlogging, whereas the levels declined for most amino acids that are linked to the TCA cycle. However, alanine, GABA and glutamate behaved differently, as these amino acids increased during waterlogging.

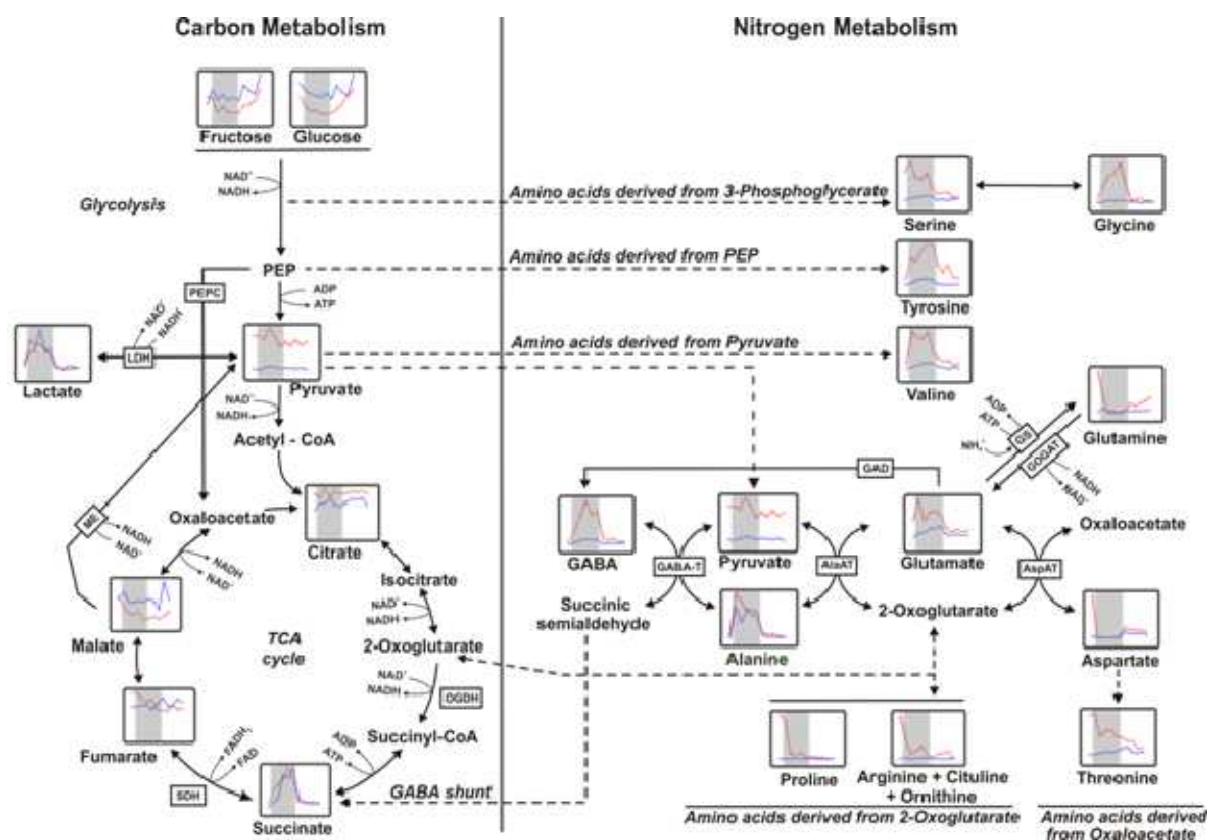


Figure 5. Visualization of changes in selected metabolites of primary carbon and nitrogen metabolism in roots of *L. japonicus* wild type and LbRNAi plants. Relative changes in the levels of metabolites as detected by GC-TOF-MS. The red line represents data obtained from wild type plants, and the blue line shows values measured in LbRNAi plants. The grey panel within each graph indicates the 5 day period during which the plants were waterlogged. Solid arrows indicate enzymatic reactions, and the dashed arrow link the same metabolite when this is present in two linked pathways. A full overview of the levels of all metabolites that were detected can be found in supplementary table I. The following enzyme names are abbreviated in the figure: AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; GABA-T, gamma-aminobutyric acid transaminase; GAD, Glutamate decarboxylase; GOGAT, glutamate synthase; GS, glutamine synthetase; ME, malic enzyme;

OGDH, oxoglutarate dehydrogenase; PEPC, posphoenolpyruvate carboxylase; SDH, succinate dehydrogenase.

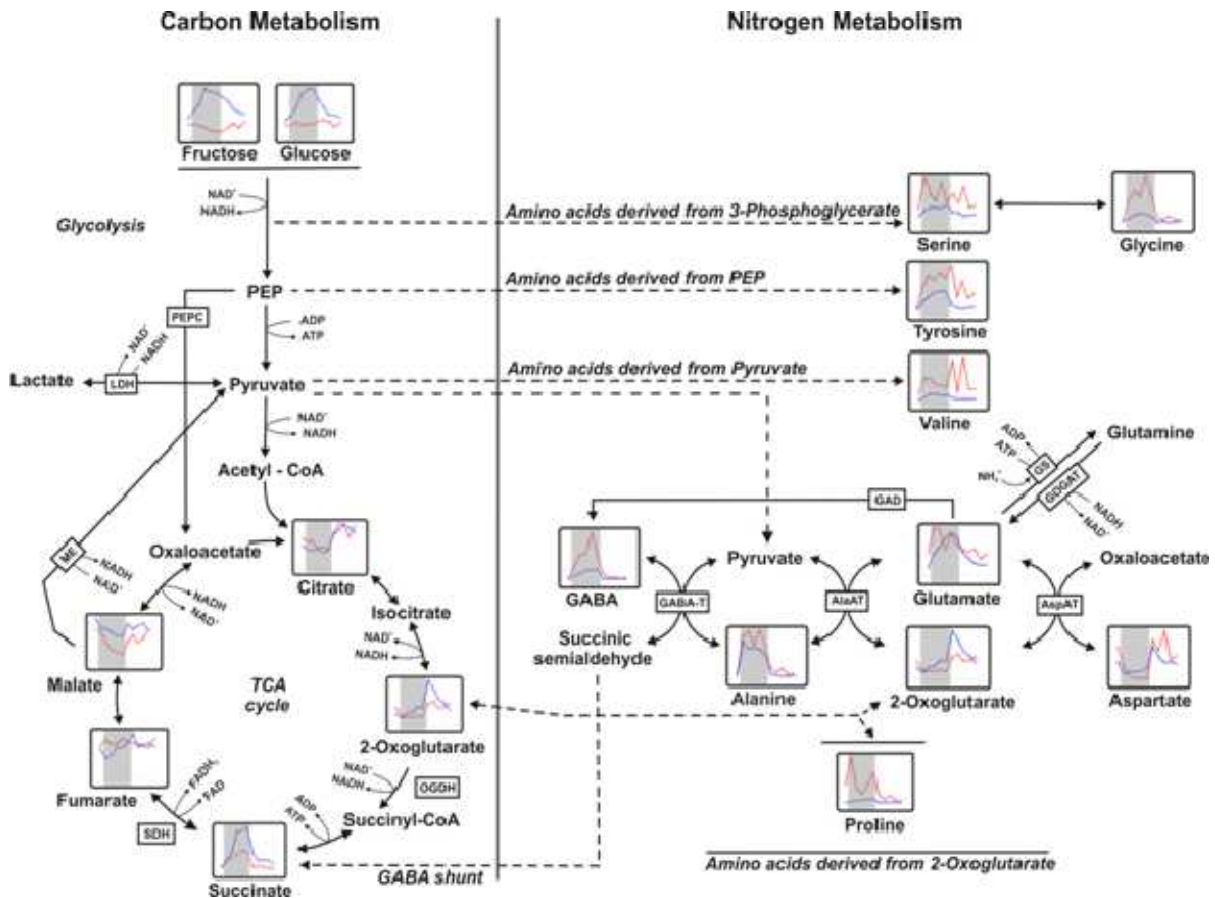


Figure 6. Visualization of changes in selected metabolites of primary carbon and nitrogen metabolism in nodules of *L. japonicus* wild type and LbRNAi plants. For an explanation of the experiment and the symbols and abbreviations used in the figure, it is referred to the caption of Fig. 5.

In contrast to the levels of amino acids, intermediates of glycolysis and the TCA cycle behaved rather similarly in both genotypes. A most pronounced increase was observed for the level of succinate. In nodules, the levels of amino acids

behaved similarly in both genotypes, but marked differences were observed in the metabolic behavior of fructose and glucose (a strong increase was observed during waterlogging in the LbRNAi plants, but no obvious change occurred in wild type) as well as succinate which increased in both genotypes, though in LbRNAi plants the increase was twice as strong.

5.5 TCA-cycle enzyme activities

The effect of flooding on the activities of pyruvate dehydrogenase (PDH; EC 1.2.4.1), aconitase (EC 4.2.1.3), NAD⁺-dependent isocitrate dehydrogenase (IDH; EC 1.1.1.41), 2-oxoglutarate dehydrogenase (OGDH; EC 1.2.4.2), NAD⁺-dependent malate dehydrogenase (MDH; EC 1.1.1.37), NAD⁺-dependent malic enzyme (ME; EC 1.1.1.39), alanine aminotransferase (AlaAT; EC 2.6.1.2), aspartate aminotransferase (AspAT; EC 2.6.1.1) and NADH-dependent glutamate synthase (GOGAT; EC 1.4.1.14) was determined in desalted enzyme extracts from roots of wild type plants (Fig. 7) for the enzyme activities as measured under normoxic control conditions were: PHD: 0.74 nmol min⁻¹ g⁻¹ FW; aconitase: 28.18 nmol min⁻¹ g⁻¹ FW; IDH: 13.78 nmol min⁻¹ g⁻¹ FW; OGDH: 0.36 nmol min⁻¹ g⁻¹ FW; MDH: 1.62 μmol min⁻¹ g⁻¹ FW; ME: 11.18 nmol min⁻¹ g⁻¹ FW; AlaAT: 3.39 μmol min⁻¹ g⁻¹ FW; AspAT 1.62 μmol min⁻¹ g⁻¹ FW; GOGAT 116.29 nmol min⁻¹ g⁻¹ FW. After 5 days of waterlogging, the activity of PDH did not change significantly, whereas aconitase, IDH, MDH and ME were significantly reduced to 12.99 nmol min⁻¹ g⁻¹ FW, 4.97 nmol min⁻¹ g⁻¹ FW, 0.46 μmol min⁻¹ g⁻¹ FW and 2.84 nmol min⁻¹ g⁻¹ FW, respectively. In contrast, the activity of OGDH, AlaAT, AspAT and GOGAT increased to 0.60 nmol

$\text{min}^{-1} \text{g}^{-1} \text{FW}$, $4.96 \mu\text{mol min}^{-1} \text{g}^{-1} \text{FW}$, $2.88 \mu\text{mol min}^{-1} \text{g}^{-1} \text{FW}$, $238.25 \text{ nmol min}^{-1} \text{g}^{-1} \text{FW}$ respectively. Of course, in vivo these enzymes are not necessarily active at maximum activity, but these data show unequivocally that various enzymes of the TCA-cycle are differentially regulated upon flooding.

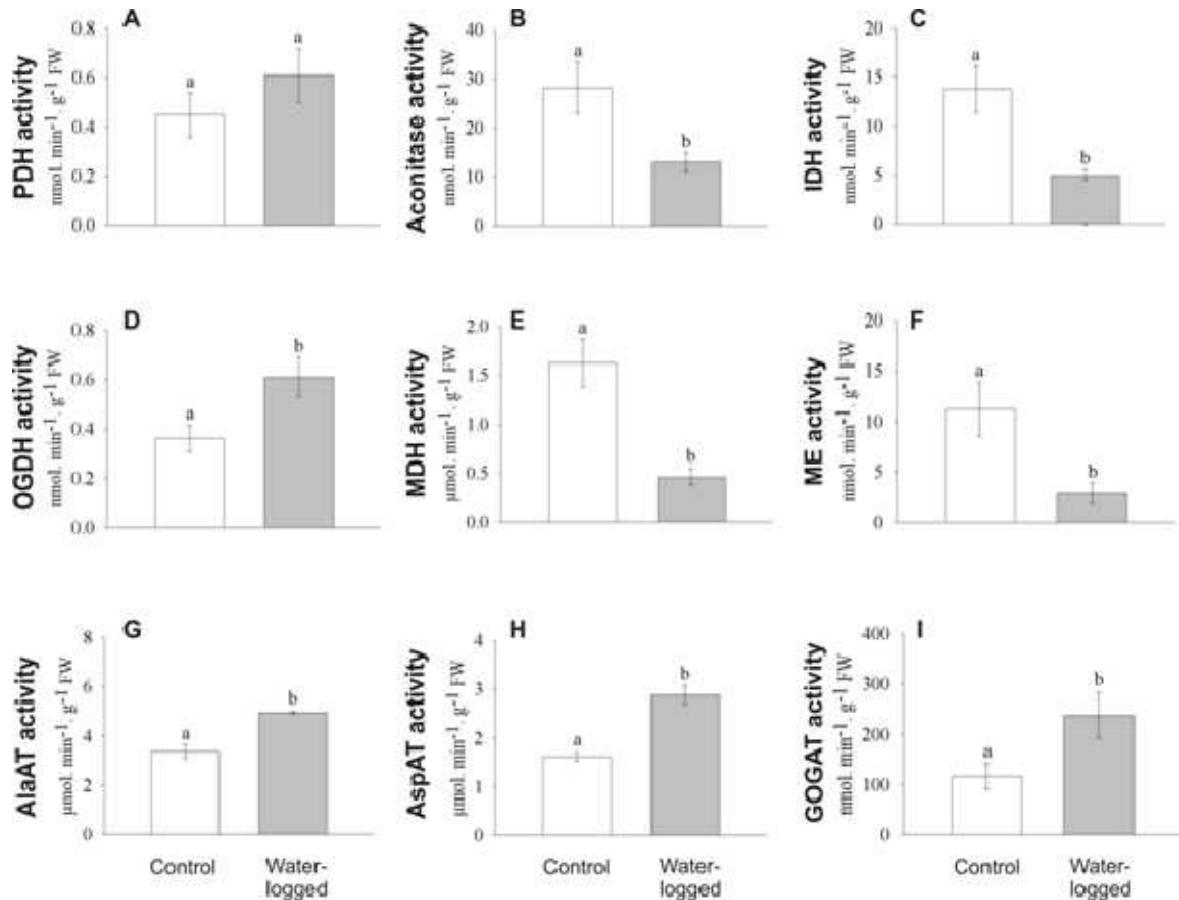


Figure 7. Changes of maximum enzyme activities from roots as induced by waterlogging of wild-type *L. japonicus* plants. The maximum activity of pyruvate dehydrogenase (A), aconitase (B), isocitrate dehydrogenase (C), oxoglutarate dehydrogenase (D), malate dehydrogenase (E), malic enzyme (F), alanine aminotransferase (G), aspartate aminotransferase (H) and glutamate synthase (I) were determined in root material that was collected from wild type plants grown under normoxic control conditions or after 5 days of

waterlogging. The bars indicate mean values \pm standard deviation of three independent biological replicate experiments. Means that differ significantly according to a one-way ANOVA ($P < 0.05$) are marked with different letters.

6. DISCUSSION

6.1 Tolerance to waterlogging is dependent on cellular N-status

Plants are able to initiate several adaptive responses in order to alleviate the consequences of oxygen deprivation during flooding or waterlogging of the soil. In this study, nodulated *L. japonicus* was shown to be very tolerant to prolonged waterlogging (Fig. 2). Waterlogged plants were characterized by reduced root growth and extensive stem elongation (Fig. 1), as well as the induction of genes encoding for the fermentative enzymes ADH, PDC, and AlaAT (Fig. 3). These adaptations are described to be part of the so-called low oxygen escape syndrome (Bailey-Serres and Voesenek, 2008) that delineates a characteristic response to hypoxia of flooding-tolerant plant species.

In addition to the morphological changes that were observed during waterlogging, both nitrogen and carbon metabolism are clearly affected in roots of wild type plants during this treatment. At the end of the five-day period of waterlogging, the amounts of protein and starch were reduced (Fig. 4). Similar observations were made in other studies in which various plant species including *Arabidopsis*, barley, wheat, soybean and potato were exposed to hypoxia (Thorne et al., 1982; Geigenberger et al., 2000; Gibon et al., 2002; Rolletschek et al., 2003; van

Dongen et al., 2004). These changes were best explained as part of the adaptive response of plants to hypoxia: by down-regulating the synthesis of starch and protein, plants limit their energy demand and therefore respiratory oxygen consumption can be reduced (Geigenberger, 2003; Gupta et al., 2009).

The depletion in protein and amino acids during hypoxia proceeds even beyond the waterlogging treatment, whereas the amounts of sugar and starch retained their original levels during the recovery period (Fig. 4). Apparently, amino acid metabolism is not only inhibited as an adaptive response to hypoxia, but also by a reduction in the rate of N-fixation due to hypoxia-induced energy deprivation leading to a prolonged depletion of cellular nitrogen levels. This hypothesis is further supported by the observation that the genes *NifH* and *GS*, which encode enzymes known to play essential roles in nodule nitrogen metabolism are strongly down-regulated during waterlogging (Fig. 3). Also in soybean, it was shown that N-fixation dropped severely during the first days of flooding (Thomas et al., 2005). Only after acclimation over a period of 10 days, during which aerenchyma developed, partial recovery of the nitrogen fixation capacity was observed in that study.

The importance of nitrogen metabolism for hypoxia tolerance was further investigated using *L. japonicus* plants in which the expression of nodular leghemoglobin (*Lb*) was silenced via an RNAi approach. The function of *Lb* in these transgenic plants has been elaborately characterized in various independent lines before (Ott et al., 2005; 2009), and we performed our extensive biochemical investigations here on the strongest stable LbRNAi line. LbRNAi plants are unable to decrease the oxygen concentration inside nodules to the level which is required for

nitrogen fixation by nitrogenase. Indeed, the levels of amino acids and protein were lower in LbRNAi plants as compared to the wild type, this difference being particularly prominent in roots. Moreover, LbRNAi plants were less resistant against waterlogging than wild type plants (Fig. 2). However, during waterlogging of LbRNAi plants, the level of amino acids and protein in both root and shoot increased surprisingly (Fig. 4). Probably, this increase is explained by the activation of nitrogenase due to the decrease in the concentration of oxygen within the nodules during waterlogging. Indeed, when LbRNAi plants were waterlogged, not only the genes encoding fermentative enzymes were induced but also the expression levels of *NifH* and *GS* increased (Fig. 3). However, it should be noted that even during waterlogging the levels of amino acids and protein documented in the transgenics still remained below the values measured in wild type indicating a low level of nitrogen fixation only.

6.2 Alanine metabolism appears indispensable for Lotus during hypoxia

The observations described above indicate a correlation between the nitrogen status of *L. japonicus* roots and the ability of this species to survive prolonged periods of waterlogging. We, therefore, performed a detailed analysis of changes in primary carbon and nitrogen metabolism following waterlogging. For this purpose we carried out GC-TOF-MS-based metabolite profiling on samples taken daily during five days of waterlogging and five subsequent days of the recovery phase (Supplemental Table I). The levels of various metabolites detected in roots (Fig. 5)

and nodules (Fig. 6) were plotted, within their metabolic context, onto a pathway map describing glycolysis, TCA-cycle and amino acid biosynthesis.

In roots of wild type plants, the majority of amino acids which are derived from intermediates of the TCA cycle decrease dramatically during waterlogging. In LbRNAi plants, the amount of these amino acids is already very low initially, and therefore no further decrease can be observed. Three amino acids behave differently from this general trend: in roots, alanine increases to a similar extent in both wild type and LbRNAi plants, whereas the levels of glutamate and GABA, both direct co-substrates for alanine synthesis, increase strongly during flooding in wild type but only moderately in roots of the LbRNAi line. The situation in nodules is slightly different. Especially striking is that the amount of glutamate in LbRNAi plants increases during water logging to the same extend as in wild type, which is probably explained by the slight activation of nitrogen fixation in the LbRNAi due to the decreased cellular oxygen concentration during flooding.

It is most intriguing that the production of alanine under hypoxia appears to occur at the cost of nearly all other amino acids, especially when N-availability is limited. Even LbRNAi plants accumulate alanine to quantities that are indistinguishable from those of the wild type. Apparently, alanine biosynthesis upon hypoxia is of utmost importance for *L. japonicus* in the survival of waterlogging. This conclusion is sustained by the observation that AlaAT activity in increases significantly during hypoxia (Fig. 7).

The induction of AlaAT and the concomitant accumulation of alanine have been shown previously for many plant species; however, its significance has been

discussed (Sousa and Sodek, 2002). The benefit of alanine production as a fermentation pathway has also been questioned because no NAD^+ is regenerated during operation of the pathway, which would be required to drive glycolysis under hypoxia. In the current study, the significance of alanine accumulation is demonstrated since alanine accumulation does not decline even if plants are suffering from very low N-availability. It appears likely that alanine fermentation primarily functions to regulate the level of pyruvate. Pyruvate is not only a known activator of the alternative oxidase (Vanlerberghe et al., 1999), but has also recently been shown to interfere with the hypoxia-induced inhibition of respiration (Gupta et al., 2009; Zabalza et al., 2009). Therefore, in order to control the rate of respiratory oxygen consumption when the oxygen availability is low, it is important to prevent pyruvate accumulation. Alanine fermentation would be able to do just this, with the additional advantage of not producing toxic end-products such as lactate or ethanol.

6.3 Modular re-organization of the TCA cycle into oxidative and a reductive reactions

Amino acid metabolism cannot be regarded independently of primary carbon metabolism. Therefore we linked the changes in amino acid levels to the carbohydrates we determined from glycolysis and the TCA-cycle (Fig. 5 and 6). The increase in the amount of lactate in roots of both wild type and LbRNAi plants indicates the activation of fermentation during waterlogging. The decrease of both glucose and fructose in wild type roots confirms the observation described for total

sugars, and is also indicative for an increased glycolytic flux such as that which characterizes the fermentative Pasteur effect (Summers et al., 2000).

The levels of most intermediates of the TCA-cycle that we were able to determine did not change strongly during waterlogging, with the clear exception of succinate. In both roots and nodules of wild type and LbRNAi plants, the amount of succinate increased strongly during waterlogging. Succinate accumulation appears to be a general phenomenon in plants exposed to hypoxia, as similar observations were made for many other plant species (Menegus et al., 1989; Good and Muench, 1993; Narsai et al., 2009; van Dongen et al., 2009). It has recently been discussed that succinate accumulation upon various biotic and abiotic stresses could potentially be explained via activation of the GABA shunt (Fait et al., 2008). However, since all enzymes of this pathway exhibit pH optima between 8 and 10 (Satya Narayan and Nair, 1989; Shelp et al., 1995), it seems highly unlikely that a major role of the GABA-shunt would be to catalyze reactions under hypoxia. Therefore, we suggest that another explanation must exist for the simultaneous accumulation of succinate and GABA during water logging of *L. japonicus*.

The accumulation of succinate as observed in Fig. 5 and 6 is more likely to be explained by an inhibition of succinate dehydrogenase (SDH) under hypoxia. Since SDH participates in both the TCA cycle and the mitochondrial electron transport chain, the succinate oxidation activity of SDH will decrease when the ubiquinone pool is electron saturated. Concomitantly, the activity of aconitase, IDH, and MDH also decreased after 5 days of waterlogging to between 28 and 46% of the activities as determined in normoxic roots (Fig. 7B, 7C, 7E). In contrast, the capacity of OGDH

increased by approximately 33% (Fig. 7D). Probably, this might help to drive the production of ATP by succinyl CoA synthetase in the subsequent step of the TCA cycle and that would of course be most valuable under waterlogged conditions.

OGDH requires NAD^+ as co-substrate. Therefore, NADH must continuously be oxidized within the mitochondria to keep this reaction going, even when the mitochondrial electron transport chain is strongly inhibited due to the hypoxia. However, multiple evidence exist that regeneration of NAD^+ could occur via MDH by catalysing the reverse reaction leading to malate formation. First, metabolic flux analysis using ^{14}C -labeled carbohydrates in *Selenastrum minutum* revealed that under anoxia, the TCA-cycle can split in into two independent branches, both commencing from oxaloacetate. One branch proceeds in the common oxidative direction whereas the second branch follows the opposite reductive direction (Vanlerberghe et al., 1989; 1990). Second, flux balance analysis of a metabolic model of barley seeds subjected to various oxygen concentrations (Grafahrend-Belau et al., 2009), revealed that upon anoxia the TCA cycle breaks up in parts. It was predicted that the initial oxidizing reactions of the cycle are reversed to citrate production from oxoglutarate. Furthermore, it was suggested that the reaction catalyzed by MDH is reversed such that malate is produced and NADH is oxidized.

6.4 Integration of carbon and nitrogen metabolism under hypoxia

With modular activity of the TCA-cycle (see above) during periods of hypoxia in mind, accumulation of alanine becomes quite sensible. Figure 8 summarizes the

metabolic pathway that explains the changes in metabolite levels and enzyme activities we observed and discussed in the work described here.

Pyruvate reacts with glutamate to form alanine and oxoglutarate via AlaAT. This prevents pyruvate from accumulating and simultaneously oxoglutarate is produced which can react in the mitochondria to form succinate via OGDH and succinyl CoA synthetase to produce ATP. The NAD^+ that is required for this reaction is regenerated from NADH via MDH catalyzing the reaction from oxaloacetate (OAA) to malate. The OAA that is required as substrate for this reaction is produced by aspartate aminotransferase. Concomitantly, glutamate is produced, which is the co-substrate for alanine synthesis. To keep this cycle running, the pools of aspartate and glutamine deplete. Malate either reacts with NAD^+ via ME to form pyruvate which then can be used for alanine synthesis, or malate reacts via fumarate to succinate. Both pathways might function in parallel.

This pathway explains the role of alanine accumulation during hypoxia as well as the strong decline in most other TCA-cycle related amino acids. All other changes in metabolite levels (as indicated by the font size in Fig. 8) observed in roots and nodules of waterlogged Lotus are in agreement with the metabolic equilibriums that are expected to drive the metabolic flux from glycolysis, via alanine synthesis and oxoglutarate to succinate. Since all enzymes that are required for these reactions are known to have isoforms located within the mitochondria, the cycle can take place entirely within the mitochondrion. The amount of ATP gained via this hypoxic pathway is twice as much as that produced by glycolysis alone. The importance of alanine accumulation for surviving waterlogging of Lotus was shown here. The

relevance of this metabolic pathway in other plant species has now to be investigated.

Moreover, this reaction produces 2-oxoglutarate which can be used by oxoglutarate dehydrogenase (OGDH) and succinyl CoA synthetase to produce another ATP. Succinate will accumulate as the TCA-cycle will be further blocked due to the oxygen limitation at the reaction catalysed by succinate dehydrogenase. The mitochondrial NAD⁺ that is required to oxidize 2-oxoglutarate will be recycled via the enzyme malate dehydrogenase (MDH) which obtains its substrate either via PEP-carboxylase (PEPC) or via aspartate amino transferase (AspAT). The latter enzyme is known to be activated upon anoxia and provides glutamate as substrate for AlaAT. All reaction following on the production of pyruvate can be catalysed by enzymes that are located within the mitochondrion. The model explains why alanine accumulates upon anoxia in plants, and provides a pathway that improves the ATP production during anoxia.

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8. SUPPLEMENTARY TABLE

Supplementary table 1a. Overview of all metabolites determined in roots of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Analytical name	Biological Description				
	Metabolite Name	Metabolite ID	SumFormula	KEGG - ID	CAS - ID
A (amino acids)	A (amino acids)				
Alanine, DL- (2TMS)	Alanine	M000026	C3H7NO2	C00041	56-41-7
Glutamic acid, DL- (3TMS)	Glutamate	M000036	C5H9NO4	C00025	56-86-0
Glycine (3TMS)	Glycine	M000031	C2H5NO2	C00037	56-40-6
Phenylalanine, DL- (2TMS)	Phenylalanine	M000011	C9H11NO2	C00079	63-91-2
Proline, L- (2TMS)	Proline	M000029	C5H9NO2	C00148	147-85-3
Serine, DL- (3TMS)	Serine	M000015	C3H7NO3	C00065	56-45-1
Threonine, DL- (3TMS)	Threonine	M000016	C4H9NO3	C00188	72-19-5
Valine, DL- (2TMS)	Valine	M000030	C5H11NO2	C00183	72-18-4
Butyric acid, 4-amino- (3TMS)	GABA	M000114	C4H9NO2	C00334	56-12-2
Alanine, beta- (3TMS)	β-Alanine	M000027	C3H7NO2	C00099	107-95-9
Aspartic acid, L- (3TMS)	Aspartate	M000033	C4H7NO4	C00049	56-84-8
Glutamine, DL- (3TMS)	Glutamine	M000032	C5H10N2O3	C00064	56-85-9
Ornithine, DL- (4TMS)	Ornithine+Arginine+CitruLine	M000028	C5H12N2O2	C00077	70-26-8
Tyrosine, DL- (3TMS)	Tyrosine	M000035	C9H11NO3	C00082	60-18-4
B (organic acids)	B (organic acids)				
Malic acid, 2-methyl-, DL- (3TMS)	Citramalate	M000066	C5H6O4	C02226	498-23-7
Citric acid (4TMS)	Citrate	M000069	C6H8O7	C00158	77-92-9
Fumaric acid (2TMS)	Fumarate	M000067	C4H4O4	C00122	110-17-8
Lactic acid, DL- (2TMS)	Lactate	M000100	C3H6O3	C00186	79-33-4
Malic acid, DL- (3TMS)	Malate	M000065	C4H6O5	C00149	97-67-6
Malonic acid (2TMS)	Malonate	M000427	C3H4O4	C00383	141-82-2
Pyruvic acid (1MEOX) (1TMS)	Pyruvate	M000071	C3H4O3	C00022	127-17-3
Succinic acid (2TMS)	Succinate	M000074	C4H6O4	C00042	110-15-6
Galacturonic acid, D- (1MEOX) (5TMS)	Galacturonate	M000690	C6H10O7	C00333	685-73-4
C (aromatic acid)	C (aromatic acid)				
Benzoic acid (1TMS)	Benzoate	M000347	C7H6O2	C00180	65-85-0
D (N containing compounds)	D (N containing compounds)				

Putrescine (4TMS)	Putrescine	M000186	C4H12N2	C00134	110-60-1
E (sugars)	E (sugars)				
Fructose, D- (1MEOX) (5TMS)	Fructose	M000606	C6H12O6	C00095	57-48-7
Glucose, D- (1MEOX) (5TMS)	Glucose	M000040	C6H12O6	C00031	50-99-7
Maltose, D- (1MEOX) (8TMS)	Maltose	M000048	C12H22O11	C00897	69-79-4
Mannose, D- (1MEOX) (5TMS)	Mannose	M000633	C6H12O6	C00936	3458-28-4
Sucrose, D- (8TMS)	Sucrose	M000044	C12H22O11	C00089	57-50-1
Trehalose, alpha,alpha', D- (8TMS)	Trehalose	M000671	C12H22O11	C01083	99-20-7
Xylose, D- (1MEOX) (4TMS)	Xylose	M000579	C5H10O5	C00310	551-84-8
F (polyols)	F (polyols)				
Galactinol (9TMS)	Galactinol	M000673	C6H14O6	C01697	608-66-2
Glycerol (3TMS)	Glycerol	M000053	C3H8O3	C00116	56-81-5
Maltitol (9TMS)	Maltitol	M000058	C12H24O11		585-88-6
Mannitol, D- (6TMS)	Mannitol	M000687	C6H14O6	C00392	69-65-8
Inositol, myo- (6TMS)	Myo-Inositol	M000060	C6H12O6	C00137	87-89-8
Sorbitol, D- (6TMS)	Sorbitol	M000055	C6H14O6	C00794	50-70-4
G (phosphates)	G (phosphates)				
Fructose-6-phosphate (1MEOX) (6TMS)	Fructose-6P	M000510	C6H13O9P	C00085	643-13-0
Phosphoric acid (3TMS)	Phosphoric acid	M000075	H3O4P	C00009	7664-38-2

Supplementary table Ia. Overview of all metabolites determined in roots of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Analytical name	Analytical Description							
	CAS ID	Sum Formula	Analytical ID	Time expected	Time deviation	(m/z)	Cluster Size	Cluster match
A (amino acids)								
Alanine, DL- (2TMS)	2899-44-7	C3H7NO2	A138002-101	354060	-0.06	116	9	917
Glutamic acid, DL- (3TMS)	15985-07-6	C5H9NO4	A163001-101	507780	-0.09	246	5	908
Glycine (3TMS)	5630-82-0	C2H5NO2	A133001-101	325180	-0.03	248	12	974
Phenylalanine, DL- (2TMS)	2899-52-7	C9H11NO2	A164001-101	531145	-0.09	218	7	828
Proline, L- (2TMS)		C5H9NO2	A132003-101	338693	-0.01	142	6	995
Serine, DL- (3TMS)	7364-48-9	C3H7NO3	A138001-101	357523	-0.1	204	49	988
Threonine, DL- (3TMS)	64569-35-3	C4H9NO3	A140001-101	368365	-0.38	218	17	950
Valine, DL- (2TMS)	15984-93-7	C5H11NO2	A122001-101	271580	0	144	4	958
Butyric acid, 4-amino- (3TMS)	39508-23-1	C4H9NO2	A153003-101	342530	-0.13	174	61	909
Alanine, beta- (3TMS)	17891-86-0	C3H7NO2	A144001-101	394250	-0.05	248	7	892
Aspartic acid, L- (3TMS)	15985-05-4	C4H7NO4	A152002-101	457283	0.03	232	8	845
Glutamine, DL- (3TMS)	56145-13-2	C5H10N2O3	A178001-101	598300	0.01	227	0	795
Ornithine, DL- (4TMS)	55556-70-2	C5H12N2O2	A182002-101	570427	-0.04	174	2	928
Tyrosine, DL- (3TMS)	7415-19-2	C9H11NO3	A194002-101	658337	-0.07	218	18	962
B (organic acids)								
Malic acid, 2-methyl-, DL- (3TMS)		C5H6O4	A148001-101	425325	0.12	247	153	991
Citric acid (4TMS)	14330-97-3	C6H8O7	A182004-101	592883	-0.04	273	253	994
Fumaric acid (2TMS)	17962-03-7	C4H4O4	A137001-101	371255	-0.12	245	7	967
Lactic acid, DL- (2TMS)	17596-96-2	C3H6O3	A105001-101	190250	0.26	117	5	930
Malic acid, DL- (3TMS)	38166-11-9	C4H6O5	A149001-101	373395	-0.25	233	101	986
Malonic acid (2TMS)	18457-04-0	C3H4O4	A122003-101	300730	0.18	233	0	990
Pyruvic acid (1MEOX) (1TMS)	55493-92-0	C3H4O3	A104002-101	222650	0.01	115	0	940
Succinic acid (2TMS)	40309-57-7	C4H6O4	A134001-101	365427	-0.1	247	25	992
Galacturonic acid, D- (1MEOX) (5TMS)		C6H10O7	A196003-101	632880	0.34	333	11	893
C (aromatic acid)								
Benzoic acid (1TMS)	2078-12-8	C7H6O2	A128003-101	348220	-0.02	179	12	975
D (N containing compounds)								
Putrescine (4TMS)	39772-63-9	C4H12N2	A175002-101	517180	0.04	174	0	884
E (sugars)								
Fructose, D- (1MEOX) (5TMS)	56196-14-6	C6H12O6	A187002-101	579380	-0.13	307	101	978
Glucose, D- (1MEOX) (5TMS)	34152-44-8	C6H12O6	A189002-101	590400	-0.08	160	1	963

Maltose, D- (1MEOX) (8TMS)		C12H22O11	A277002-101	870355	-0.03	204	0	898
Mannose, D- (1MEOX) (5TMS)		C6H12O6	A188002-101	581930	-0.13	160	0	902
Sucrose, D- (8TMS)	19159-25-2	C12H22O11	A264001-101	840783	0.05	361	113	971
Trehalose, alpha,alpha'-, D- (8TMS)		C12H22O11	A274002-101	876240	-0.09	191	23	928
Xylose, D- (1MEOX) (4TMS)	56196-07-7	C5H10O5	A165001-101	493370	-0.15	307	0	976
F (polyols)								
Galactinol (9TMS)		C6H14O6	A299002-101	940280	-0.12	204	17	977
Glycerol (3TMS)	1785-22-0	C3H8O3	A129003-101	291783	-0.07	205	33	954
Maltitol (9TMS)		C12H24O11	A284001-101	889040	0.09	361	39	934
Mannitol, D- (6TMS)	14317-07-8	C6H14O6	A193002-101	583850	-0.46	319	18	897
Inositol, myo- (6TMS)	2582-79-8	C6H12O6	A209002-101	653910	0.03	305	126	985
Sorbitol, D- (6TMS)	14199-80-5	C6H14O6	A193001-101	588295	-0.11	319	0	833
G (phosphates)								
Fructose-6-phosphate (1MEOX) (6TMS)	55530-74-0	C6H13O9P	A232002-101	767060	0.04	315	0	675
Phosphoric acid (3TMS)	10497-05-9	H3O4P	A129001-101	333520	-0.14	314	78	983

Supplementary table 1a. Overview of all metabolites determined in roots of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Metabolite Name	Statistical		Wild Type Control	
	Induced by water-logging*	Between genotypes**	Day 0 mean	Dy 0 stdev
A (amino acids)				
Alanine	X		2.08E-05	1.47E-05
Glutamate	X	X	2.57E-03	1.46E-03
Glycine	X	X	2.63E-04	8.79E-05
Phenylalanine	X	X	2.27E-04	4.52E-05
Proline	X	X	4.34E-02	1.58E-02
Serine	X	X	1.98E-03	4.33E-04
Threonine	X	X	4.73E-04	9.42E-05
Valine	X	X	2.45E-04	1.23E-05
GABA	X	X	9.61E-04	1.73E-04
β-Alanine	X	X	1.42E-04	3.26E-05
Aspartate	X	X	5.18E-03	1.46E-03
Glutamine		X	4.82E-04	2.94E-04
Ornithine+Arginine+CitruLine	X	X	5.70E-04	2.85E-04
Tyrosine	X	X	1.18E-04	2.12E-05
B (organic acids)				
Citramalate			3.02E-03	3.97E-04
Citrate		X	3.24E-02	5.26E-03
Fumarate			1.63E-04	2.50E-05
Lactate	X		2.36E-04	4.58E-05
Malate	X	X	5.69E-03	3.63E-04
Malonate		X	1.20E-05	1.92E-06
Pyruvate		X	3.54E-04	3.48E-05
Succinate	X		3.03E-04	6.92E-05
Galacturonate		X	1.88E-05	3.31E-06
C (aromatic acid)				
Benzoate		X	3.26E-04	1.16E-05
D (N containing compounds)				
Putrescine	X	X	7.17E-05	1.31E-05
E (sugars)				
Fructose	X	X	5.27E-04	2.86E-04
Glucose	X	X	1.08E-03	2.54E-04

Maltose		X	2.72E-04	4.08E-05
Mannose		X	1.38E-05	9.94E-06
Sucrose		X	1.94E-02	2.70E-03
Trehalose	X	X	2.73E-04	7.53E-05
Xylose		X	1.26E-05	8.09E-06
F (polyols)				
Galactinol	X	X	1.35E-04	2.57E-05
Glycerol			2.64E-04	6.70E-05
Maltitol		X	4.41E-05	1.40E-05
Mannitol		X	3.55E-04	9.90E-05
Myo-Inositol		X	8.62E-04	1.61E-04
Sorbitol	X	X	5.94E-04	1.52E-04
G (phosphates)				
Fructose-6P			1.01E-05	4.19E-06
Phosphoric acid		X	1.75E-05	4.83E-06

*Statistical difference during the time point into genotype (two way anova significant at $P < 0.05$).

**Statistical difference between the genotype (two way anova significant at $P < 0.05$).

Supplementary table 1a. Overview of all metabolites determined in roots of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Metabolite Name	Wild Type Waterlogging									
	Day 1 mean	Day 1 stdev	Day 2 mean	Day 2 stdev	Day 3 mean	Day 3 stdev	Day 4 mean	Day 4 stdev	Day 5 mean	Day 5 stdev
A (amino acids)	0.000343	0.000094	0.000284	0.000037	0.000215	0.000091	0.000179	0.000045	0.000172	0.000088
Alanine	0.004840	0.003825	0.002543	0.000531	0.003522	0.000869	0.003555	0.000637	0.003081	0.000374
Glutamate	0.000647	0.000098	0.000971	0.000169	0.001039	0.000162	0.001352	0.000489	0.000762	0.000148
Glycine	0.000282	0.000063	0.000196	0.000027	0.000239	0.000054	0.000244	0.000093	0.000175	0.000031
Phenylalanine	0.037581	0.014508	0.004726	0.000765	0.003213	0.000747	0.007162	0.003806	0.001471	0.000696
Proline	0.002604	0.000741	0.001612	0.000209	0.001510	0.000339	0.001867	0.000406	0.001095	0.000432
Serine	0.000511	0.000161	0.000232	0.000042	0.000301	0.000041	0.000306	0.000083	0.000228	0.000085
Threonine	0.000976	0.000186	0.000817	0.000104	0.000785	0.000215	0.000942	0.000161	0.000494	0.000064
Valine	0.003156	0.000801	0.006244	0.001016	0.009534	0.001151	0.006600	0.003731	0.007283	0.001615
GABA	0.000367	0.000073	0.000338	0.000092	0.000474	0.000087	0.000359	0.000094	0.000325	0.000028
β-Alanine	0.000323	0.000081	0.000270	0.000045	0.000326	0.000069	0.000361	0.000051	0.000298	0.000059
Aspartate	0.000178	0.000055	0.000034	0.000005	0.000047	0.000023	0.000029	0.000011	0.000038	0.000001
Glutamine	0.000277	0.000186	0.000091	0.000005	0.000113	0.000044	0.000189	0.000060	0.000068	0.000031
Ornithine+Arginine+Citruine	0.000363	0.000081	0.000329	0.000034	0.000445	0.000069	0.000538	0.000214	0.000516	0.000060
Tyrosine										
B (organic acids)	0.003169	0.000495	0.003175	0.000769	0.002839	0.000550	0.003474	0.000606	0.003298	0.000595
Citramalate	0.028983	0.003749	0.036357	0.006658	0.029970	0.003912	0.034181	0.003585	0.032730	0.003514
Citrate	0.000164	0.000036	0.000141	0.000017	0.000130	0.000021	0.000137	0.000029	0.000099	0.000024
Fumarate	0.000908	0.000202	0.000786	0.000093	0.000788	0.000150	0.000662	0.000146	0.000524	0.000130
Lactate	0.003910	0.000428	0.002439	0.000441	0.002098	0.000676	0.002793	0.000675	0.000935	0.000228
Malate	0.000011	0.000003	0.000015	0.000005	0.000016	0.000007	0.000019	0.000010	0.000015	0.000004
Malonate	0.000345	0.000059	0.000322	0.000073	0.000427	0.000086	0.000351	0.000127	0.000248	0.000093
Pyruvate	0.000876	0.000218	0.001047	0.000164	0.001287	0.000275	0.001769	0.000329	0.001193	0.000246
Succinate	0.000012	0.000004	0.000017	0.000006	0.000017	0.000009	0.000020	0.000003	0.000018	0.000003
Galacturonate										
C (aromatic acid)	0.000416	0.000041	0.000386	0.000079	0.000480	0.000067	0.000403	0.000084	0.000447	0.000190
Benzoate										
D (N containing compounds)	0.000043	0.000016	0.000034	0.000010	0.000069	0.000015	0.000060	0.000015	0.000042	0.000010
Putrescine										
E (sugars)	0.000477	0.000122	0.000142	0.000048	0.000276	0.000211	0.000135	0.000069	0.000134	0.000044

Fructose	0.000611	0.000124	0.000365	0.000090	0.000397	0.000157	0.000240	0.000085	0.000228	0.000048
Glucose	0.000220	0.000023	0.000270	0.000050	0.000309	0.000010	0.000287	0.000009	0.000253	0.000024
Maltose	0.000052	0.000027	0.000039	0.000009	0.000044	0.000028	0.000049	0.000012	0.000047	0.000015
Mannose	0.019147	0.002640	0.023999	0.007518	0.016066	0.001532	0.022426	0.004524	0.020080	0.004071
Sucrose	0.000277	0.000133	0.000531	0.000078	0.000121	0.000029	0.000136	0.000032	0.000103	0.000028
Trehalose	0.000016	0.000011	0.000018	0.000004	0.000028	0.000017	0.000016	0.000005	0.000014	0.000007
Xylose										
F (polyols)	0.000063	0.000016	0.000051	0.000016	0.000047	0.000020	0.000024	0.000009	0.000026	0.000009
Galactinol	0.000378	0.000103	0.000367	0.000030	0.000302	0.000115	0.000292	0.000076	0.000340	0.000135
Glycerol	0.000034	0.000009	0.000050	0.000015	0.000056	0.000014	0.000057	0.000012	0.000044	0.000014
Maltitol	0.000548	0.000686	0.000329	0.000083	0.000255	0.000057	0.000220	0.000076	0.000271	0.000177
Mannitol	0.000755	0.000172	0.000759	0.000161	0.000980	0.000198	0.000985	0.000210	0.000779	0.000158
Myo-Inositol	0.000399	0.000119	0.000189	0.000047	0.000205	0.000088	0.000124	0.000051	0.000118	0.000023
Sorbitol										
G (phosphates)	0.000013	0.000007	0.000008	0.000000	0.000028	0.000004	0.000010	0.000002	0.000012	0.000007
Fructose-6P	0.000012	0.000005	0.000016	0.000004	0.000012	0.000006	0.000020	0.000007	0.000011	0.000002
Phosphoric acid	0.000343	0.000094	0.000284	0.000037	0.000215	0.000091	0.000179	0.000045	0.000172	0.000088

Supplementary table 1a. Overview of all metabolites determined in roots of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Metabolite Name	Wild Type Recovery									
	Day 6 mean	Day 6 stdev	Day 7 mean	Day 7 stdev	Day 8 mean	Day 8 stdev	Day 9 mean	Day 9 stdev	Day 10 mean	Day 10 stdev
A (amino acids)										
Alanine	4.31E-05	3.32E-06	3.57E-05	2.16E-05	3.64E-05	4.97E-06	2.33E-05	1.57E-05	1.39E-05	1.08E-05
Glutamate	1.76E-03	3.64E-04	1.35E-03	1.31E-04	1.61E-03	1.44E-04	1.39E-03	3.19E-04	1.49E-03	2.72E-04
Glycine	2.24E-04	7.38E-05	2.62E-04	6.78E-05	2.90E-04	4.74E-05	1.79E-04	5.90E-05	1.54E-04	3.69E-05
Phenylalanine	1.36E-04	2.08E-05	9.73E-05	9.63E-06	1.20E-04	1.72E-05	9.34E-05	1.42E-05	9.26E-05	2.26E-05
Proline	3.08E-03	8.14E-04	1.08E-03	2.41E-04	1.11E-03	1.92E-04	4.51E-04	1.30E-04	6.72E-04	1.17E-04
Serine	6.76E-04	1.32E-04	7.00E-04	1.20E-04	7.77E-04	1.15E-04	4.38E-04	7.88E-05	5.15E-04	1.00E-04
Threonine	2.28E-04	4.35E-05	1.89E-04	3.17E-05	1.87E-04	2.38E-05	1.05E-04	1.68E-05	1.15E-04	3.09E-05
Valine	3.53E-04	4.04E-05	2.95E-04	5.88E-05	3.06E-04	4.59E-05	2.04E-04	4.52E-05	2.45E-04	4.57E-05
GABA	1.73E-03	2.37E-04	1.19E-03	2.40E-04	1.45E-03	2.06E-04	9.16E-04	1.96E-04	9.16E-04	2.50E-04
β-Alanine	1.42E-04	3.61E-05	1.02E-04	1.83E-05	1.04E-04	2.05E-05	7.20E-05	1.36E-05	7.97E-05	1.18E-05
Aspartate	1.44E-03	2.93E-04	1.18E-03	1.17E-04	1.19E-03	1.75E-04	1.07E-03	3.07E-04	9.77E-04	2.09E-04
Glutamine	1.24E-04	6.98E-05	9.59E-05	5.16E-05	1.20E-04	3.87E-05	1.45E-04	7.27E-05	1.92E-04	1.06E-04
Ornithine+Arginine+Citruline	4.75E-05	2.19E-05	8.31E-05	1.37E-05	9.58E-05	1.33E-05	1.22E-04	5.27E-05	2.61E-05	7.57E-06
Tyrosine	2.92E-04	3.82E-05	2.01E-04	5.10E-05	2.90E-04	4.25E-05	1.34E-04	3.75E-05	1.28E-04	2.70E-05
B (organic acids)										
Citramalate	3.18E-03	4.48E-04	2.75E-03	3.13E-04	3.11E-03	4.33E-04	2.74E-03	5.65E-04	2.88E-03	4.91E-04
Citrate	3.26E-02	1.86E-03	3.41E-02	4.47E-03	3.36E-02	2.01E-03	3.49E-02	2.06E-03	3.29E-02	1.08E-03
Fumarate	9.72E-05	1.43E-05	9.30E-05	1.77E-05	1.18E-04	3.22E-05	1.02E-04	2.05E-05	1.04E-04	1.95E-05
Lactate	2.06E-04	3.98E-05	2.84E-04	3.44E-05	2.53E-04	5.00E-05	2.43E-04	3.91E-05	1.86E-04	7.02E-05
Malate	1.23E-03	1.72E-04	1.61E-03	5.30E-04	1.05E-03	1.02E-04	1.82E-03	4.25E-04	2.69E-03	1.60E-04
Malonate	1.52E-05	4.02E-06	1.79E-05	5.43E-06	1.54E-05	7.36E-06	2.75E-05	1.30E-05	1.13E-05	3.44E-06
Pyruvate	2.93E-04	3.24E-05	2.53E-04	3.37E-05	3.14E-04	3.45E-05	2.65E-04	4.05E-05	2.99E-04	6.63E-05
Succinate	3.47E-04	4.83E-05	2.87E-04	5.47E-05	3.09E-04	8.55E-05	2.25E-04	4.26E-05	2.18E-04	3.47E-05
Galacturonate	1.46E-05	1.41E-06	1.41E-05	5.15E-06	1.43E-05	5.66E-06	1.33E-05	1.76E-06	1.43E-05	5.79E-06
C (aromatic acid)										
Benzoate	3.81E-04	3.26E-05	3.82E-04	1.04E-04	5.83E-04	8.97E-05	4.80E-04	8.38E-05	4.29E-04	1.52E-05
D (N containing compounds)										
Putrescine	4.20E-05	1.96E-05	5.99E-05	1.75E-05	6.05E-05	5.67E-06	2.48E-05	9.01E-06	3.10E-05	5.80E-06
E (sugars)										

Fructose	1.26E-04	3.39E-05	2.99E-04	1.25E-04	3.31E-04	7.88E-05	4.44E-04	9.95E-05	7.35E-04	1.94E-04
Glucose	2.67E-04	4.69E-05	4.52E-04	1.28E-04	7.08E-04	2.40E-04	1.20E-03	5.22E-04	1.45E-03	2.38E-04
Maltose	2.38E-04	2.69E-05	2.19E-04	3.27E-05	2.67E-04	1.01E-05	2.13E-04	1.48E-05	2.40E-04	1.36E-05
Mannose	2.19E-05	8.68E-06	2.19E-05	7.63E-06	3.41E-05	9.19E-06	3.41E-05	7.22E-06	3.23E-05	1.37E-05
Sucrose	2.08E-02	3.37E-03	1.95E-02	4.07E-03	1.93E-02	2.39E-03	2.02E-02	2.66E-03	1.66E-02	1.37E-03
Trehalose	1.65E-04	5.79E-05	1.28E-04	2.75E-05	2.09E-04	6.96E-05	2.00E-04	5.59E-05	2.43E-04	4.66E-05
Xylose	9.29E-06	2.10E-06	1.26E-05	6.41E-06	1.35E-05	4.90E-06	2.11E-05	2.57E-06	4.05E-05	2.24E-05
F (polyols)										
Galactinol	1.27E-04	3.20E-05	2.00E-04	5.80E-05	2.53E-04	4.22E-05	3.14E-04	6.28E-05	2.55E-04	5.30E-05
Glycerol	3.41E-04	1.49E-04	3.40E-04	8.56E-05	3.43E-04	9.97E-05	3.66E-04	9.24E-05	3.39E-04	1.62E-04
Maltitol	4.44E-05	1.26E-05	4.67E-05	8.72E-06	5.11E-05	4.35E-06	4.12E-05	7.18E-06	5.09E-05	1.56E-05
Mannitol	3.24E-04	1.33E-04	2.63E-04	5.32E-05	2.58E-04	8.22E-05	3.42E-04	8.70E-05	3.31E-04	8.35E-05
Myo-Inositol	7.54E-04	1.54E-04	8.77E-04	1.62E-04	8.94E-04	1.66E-04	7.59E-04	1.06E-04	9.01E-04	1.44E-04
Sorbitol	1.19E-04	1.27E-05	2.96E-04	1.32E-04	4.19E-04	1.49E-04	6.64E-04	3.31E-04	1.49E-03	1.11E-03
G (phosphates)										
Fructose-6P	8.05E-06	4.27E-06	6.38E-06	2.92E-06	7.05E-06	2.31E-06	8.64E-06	5.08E-06	7.04E-06	3.87E-06
Phosphoric acid	1.01E-05	3.06E-06	1.78E-05	9.41E-06	8.88E-06	1.33E-06	9.70E-06	3.76E-06	1.27E-05	4.68E-06

Supplementary table 1a. Overview of all metabolites determined in roots of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Metabolite Name	Statistical		LbRNAi Control	
	Induced by water-logging *	Between genotypes **	Day 0 mean	Dy 0 stdev
A (amino acids)				
Alanine	X		1.0689E-05	3.01408E-06
Glutamate	X	X	0.000806035	3.82129E-05
Glycine		X	0.00016768	4.09025E-05
Phenylalanine	X	X	4.34213E-05	4.47536E-06
Proline	X	X	0.000417274	0.000256015
Serine	X	X	0.000286736	9.86786E-05
Threonine	X	X	4.12881E-05	4.30839E-06
Valine	X	X	0.000150142	4.60574E-05
GABA	X	X	0.000412066	6.69384E-05
β-Alanine	X	X	3.12107E-05	7.55817E-06
Aspartate	X	X	0.000466881	6.73156E-05
Glutamine		X	3.82548E-05	1.59783E-05
Ornithine+Arginine+Citru line		X	2.81322E-05	6.64969E-06
Tyrosine	X	X	5.33725E-05	1.50229E-05
B (organic acids)				
Citramalate			0.00329539	0.000535277
Citrate	X	X	0.01941046	0.001455402
Fumarate			0.0001104	1.63981E-05
Lactate	X		0.000334388	0.000111641
Malate	X	X	0.005314316	0.001234803
Malonate	X	X	1.74448E-05	5.49971E-06
Pyruvate		X	4.21798E-05	5.59012E-06
Succinate	X		0.000134529	2.16245E-05
Galacturonate		X	3.87821E-05	2.93639E-06
C (aromatic acid)				
Benzoate	X	X	0.000279725	5.71942E-05
D (N containing compounds)				
Putrescine		X	1.58846E-05	4.29988E-06
E (sugars)				

Fructose	X	X	0.000359326	7.97195E-05
Glucose		X	0.001779602	0.001338193
Maltose		X	0.000334646	6.25868E-05
Mannose	X	X	1.25086E-05	2.61716E-06
Sucrose		X	0.017372227	0.003923432
Trehalose	X	X	0.000112639	1.879E-05
Xylose		X	3.20938E-05	2.17506E-05
F (polyols)				
Galactinol		X	9.15867E-05	2.06553E-05
Glycerol			0.000435846	0.00012309
Maltitol		X	6.34738E-05	1.54118E-05
Mannitol		X	0.00019801	6.89383E-05
Myo-Inositol	X	X	0.001295718	0.000281698
Sorbitol		X	0.001077899	0.000842761
G (phosphates)				
Fructose-6P			8.95534E-06	4.20698E-06
Phosphoric acid		X	0.000290527	5.7275E-05

*Statistical difference during the time point into genotype (two way anova significant at $P < 0.05$).

**Statistical difference between the genotype (two way anova significant at $P < 0.05$).

Supplementary table 1a. Overview of all metabolites determined in roots of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Metabolite Name	LbRNAi Waterlogging									
	Day 1 mean	Day 1 stdev	Day 2 mean	Day 2 stdev	Day 3 mean	Day 3 stdev	Day 4 mean	Day 4 stdev	Day 5 mean	Day 5 stdev
A (amino acids)										
Alanine	0.00024	6.75E-05	0.00013	3.46E-05	0.00023	0.00016	0.00021	0.00011	0.00021	0.00004
Glutamate	0.00125	2.06E-04	0.00134	2.60E-04	0.00172	0.00038	0.00180	0.00018	0.00196	0.00047
Glycine	0.00018	7.00E-05	0.00024	6.29E-05	0.00022	0.00006	0.00024	0.00005	0.00022	0.00006
Phenylalanine	0.00005	1.41E-05	0.00008	3.69E-06	0.00007	0.00001	0.00007	0.00001	0.00006	0.00001
Proline	0.00041	1.33E-04	0.00053	7.57E-05	0.00061	0.00014	0.00060	0.00012	0.00059	0.00012
Serine	0.00040	6.96E-05	0.00043	8.15E-05	0.00050	0.00009	0.00053	0.00007	0.00054	0.00010
Threonine	0.00004	1.69E-05	0.00006	8.40E-06	0.00007	0.00001	0.00006	0.00001	0.00008	0.00001
Valine	0.00018	5.76E-05	0.00021	2.53E-05	0.00025	0.00007	0.00028	0.00005	0.00026	0.00007
GABA	0.00064	1.25E-04	0.00118	4.88E-04	0.00140	0.00031	0.00129	0.00041	0.00133	0.00032
β-Alanine	0.00007	1.75E-05	0.00013	1.36E-05	0.00017	0.00004	0.00015	0.00003	0.00015	0.00003
Aspartate	0.00027	1.14E-04	0.00028	8.15E-05	0.00028	0.00005	0.00033	0.00004	0.00045	0.00005
Glutamine	0.00008	7.92E-05	0.00003	3.51E-06	0.00002	0.00000	0.00002	0.00001	0.00004	0.00002
Ornithine+Arginine+CitruLine	0.00004	7.32E-06	0.00003	1.07E-05	0.00003	0.00001	0.00003	0.00001	0.00003	0.00000
Tyrosine	0.00007	1.02E-05	0.00010	7.90E-06	0.00012	0.00001	0.00012	0.00002	0.00011	0.00002
B (organic acids)										
Citramalate	0.00324	3.57E-04	0.00288	7.42E-04	0.00347	0.00036	0.00319	0.00039	0.00271	0.00042
Citrate	0.02448	4.76E-03	0.02435	3.88E-03	0.02805	0.00290	0.02266	0.00305	0.02220	0.00340
Fumarate	0.00011	3.07E-05	0.00012	1.40E-05	0.00012	0.00003	0.00010	0.00002	0.00012	0.00002
Lactate	0.00060	8.57E-05	0.00062	5.80E-05	0.00118	0.00053	0.00058	0.00005	0.00070	0.00026
Malate	0.00688	2.74E-03	0.00484	8.68E-04	0.00456	0.00075	0.00493	0.00068	0.00478	0.00059
Malonate	0.00002	4.29E-06	0.00004	1.04E-05	0.00004	0.00001	0.00003	0.00001	0.00003	0.00000
Pyruvate	0.00004	1.36E-05	0.00006	4.54E-06	0.00007	0.00001	0.00007	0.00001	0.00005	0.00000
Succinate	0.00035	9.13E-05	0.00049	1.48E-04	0.00146	0.00070	0.00132	0.00028	0.00175	0.00025
Galacturonate	0.00004	9.65E-06	0.00004	8.49E-06	0.00004	0.00001	0.00004	0.00000	0.00003	0.00002
C (aromatic acid)										
Benzoate	0.00026	5.97E-05	0.00027	6.89E-05	0.00044	0.00012	0.00026	0.00006	0.00032	0.00004
D (N containing compounds)										
Putrescine	0.00001	5.19E-06	0.00001	4.58E-06	0.00002	0.00000	0.00001	0.00000	0.00002	0.00000
E (sugars)										
Fructose	0.00070	1.52E-04	0.00045	1.76E-04	0.00057	0.00011	0.00043	0.00009	0.00051	0.00008
Glucose	0.00140	2.56E-04	0.00126	3.32E-04	0.00114	0.00027	0.00108	0.00032	0.00127	0.00022

Maltose	0.00028	9.93E-05	0.00037	4.41E-05	0.00040	0.00007	0.00037	0.00000	0.00040	0.00006
Mannose	0.00002	3.78E-06	0.00002	4.93E-06	0.00003	0.00001	0.00003	0.00001	0.00004	0.00001
Sucrose	0.02099	8.11E-03	0.01538	5.46E-03	0.01705	0.00240	0.01569	0.00216	0.01469	0.00381
Trehalose	0.00014	3.00E-05	0.00009	2.48E-05	0.00005	0.00002	0.00004	0.00001	0.00007	0.00002
Xylose	0.00004	1.13E-05	0.00003	9.33E-06	0.00005	0.00001	0.00004	0.00000	0.00003	0.00000
F (polyols)										
Galactinol	0.00003	1.13E-05	0.00003	7.05E-06	0.00003	0.00001	0.00002	0.00001	0.00003	0.00001
Glycerol	0.00035	9.05E-05	0.00023	5.16E-05	0.00036	0.00010	0.00029	0.00008	0.00032	0.00011
Maltitol	0.00006	2.44E-05	0.00008	2.21E-05	0.00009	0.00002	0.00009	0.00002	0.00008	0.00002
Mannitol	0.00035	1.33E-04	0.00022	5.57E-05	0.00013	0.00004	0.00011	0.00003	0.00020	0.00005
Myo-Inositol	0.00126	4.38E-04	0.00154	3.21E-04	0.00203	0.00039	0.00196	0.00043	0.00223	0.00053
Sorbitol	0.00077	1.64E-04	0.00084	2.14E-04	0.00066	0.00015	0.00057	0.00011	0.00066	0.00010
G (phosphates)										
Fructose-6P	0.00001	3.39E-06	0.00001	4.37E-06	0.00001	0.00001	0.00001	0.00000	0.00001	0.00000
Phosphoric acid	0.00023	1.37E-04	0.00028	7.52E-05	0.00062	0.00081	0.00060	0.00049	0.00020	0.00004

Supplementary table 1a. Overview of all metabolites determined in roots of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery.

Metabolite Name	LbRNAi Recovery									
	Day 6 mean	Day 6 stdev	Day 7 mean	Day 7 stdev	Day 8 mean	Day 8 stdev	Day 9 mean	Day 9 stdev	Day 10 mean	Day 10 stdev
A (amino acids)										
Alanine	0.00003	0.000011	0.00002	6.41E-06	1.82E-05	7.79E-06	0.00002	0.000010	1.96E-05	1.11E-05
Glutamate	0.00112	0.000302	0.00102	3.23E-04	1.11E-03	4.03E-05	0.00098	0.000164	1.02E-03	1.40E-04
Glycine	0.00019	0.000053	0.00019	4.75E-05	1.46E-04	1.49E-05	0.00020	0.000073	2.08E-04	3.93E-05
Phenylalanine	0.00005	0.000006	0.00005	1.22E-05	4.42E-05	6.56E-06	0.00005	0.000006	5.27E-05	9.77E-06
Proline	0.00029	0.000068	0.00021	8.79E-05	2.03E-04	4.14E-05	0.00020	0.000034	1.88E-04	3.11E-05
Serine	0.00030	0.000047	0.00033	8.15E-05	2.71E-04	4.27E-05	0.00032	0.000069	3.70E-04	5.47E-05
Threonine	0.00011	0.000013	0.00012	3.01E-05	6.08E-05	4.84E-06	0.00006	0.000009	6.70E-05	1.69E-05
Valine	0.00019	0.000050	0.00017	4.81E-05	1.38E-04	2.69E-05	0.00017	0.000039	1.48E-04	2.51E-05
GABA	0.00045	0.000082	0.00049	1.07E-04	5.04E-04	5.83E-05	0.00046	0.000073	5.00E-04	8.32E-05
β-Alanine	0.00006	0.000011	0.00006	1.56E-05	4.72E-05	8.18E-06	0.00005	0.000010	5.03E-05	6.56E-06
Aspartate	0.00095	0.000140	0.00077	1.87E-04	7.52E-04	1.17E-04	0.00078	0.000089	6.85E-04	7.10E-05
Glutamine	0.00004	0.000010	0.00004	2.14E-05	4.30E-05	1.89E-05	0.00004	0.000005	5.55E-05	4.39E-05
Ornithine+Arginine+CitruLine	0.00002	0.000008	0.00003	1.07E-05	2.21E-05	7.91E-06	0.00003	0.000011	3.12E-05	1.20E-05
Tyrosine	0.00005	0.000008	0.00005	8.87E-06	4.77E-05	4.48E-06	0.00006	0.000009	5.89E-05	1.21E-05
B (organic acids)										
Citramalate	0.00314	0.000560	0.00299	1.89E-04	2.90E-03	6.22E-04	0.00334	0.000452	2.95E-03	4.60E-04
Citrate	0.02485	0.001776	0.02815	2.85E-03	2.74E-02	3.92E-03	0.02861	0.003147	2.29E-02	3.46E-03
Fumarate	0.00015	0.000060	0.00011	3.12E-05	1.16E-04	2.18E-05	0.00014	0.000028	1.29E-04	2.12E-05
Lactate	0.00024	0.000068	0.00017	5.39E-05	1.63E-04	3.45E-05	0.00018	0.000034	1.94E-04	4.93E-05
Malate	0.00528	0.000730	0.00491	6.04E-04	3.19E-03	4.88E-04	0.00905	0.000462	5.14E-03	5.61E-04
Malonate	0.00004	0.000007	0.00003	5.65E-06	2.71E-05	8.53E-06	0.00003	0.000014	1.84E-05	6.04E-06
Pyruvate	0.00005	0.000012	0.00005	2.76E-06	4.18E-05	5.58E-06	0.00006	0.000006	3.88E-05	2.55E-06
Succinate	0.00047	0.000082	0.00035	8.41E-05	2.75E-04	5.55E-05	0.00035	0.000069	2.84E-04	7.68E-05
Galacturonate	0.00004	0.000003	0.00004	5.48E-06	3.28E-05	2.00E-05	0.00005	0.000006	4.15E-05	6.03E-06
C (aromatic acid)										
Benzoate	0.00034	0.000066	0.00029	7.82E-05	3.10E-04	5.35E-05	0.00038	0.000049	3.09E-04	6.20E-05
D (N containing compounds)										
Putrescine	0.00002	0.000002	0.00002	4.32E-06	1.89E-05	3.01E-06	0.00002	0.000001	1.46E-05	1.57E-06
E (sugars)										
Fructose	0.00043	0.000124	0.00082	2.57E-04	6.92E-04	1.49E-04	0.00060	0.000130	1.09E-03	2.96E-04
Glucose	0.00100	0.000238	0.00167	5.67E-04	1.33E-03	2.62E-04	0.00116	0.000219	2.23E-03	3.54E-04

Maltose	0.00037	0.000027	0.00033	2.94E-05	3.46E-04	1.62E-05	0.00041	0.000052	3.63E-04	8.01E-05
Mannose	0.00003	0.000003	0.00003	5.39E-06	2.06E-05	2.25E-06	0.00001	0.000006	2.32E-05	2.73E-06
Sucrose	0.02265	0.004321	0.01737	3.74E-03	1.73E-02	3.71E-03	0.01613	0.002406	1.54E-02	1.54E-03
Trehalose	0.00005	0.000017	0.00004	1.31E-05	1.23E-04	2.87E-05	0.00009	0.000016	1.59E-04	4.96E-05
Xylose	0.00003	0.000005	0.00003	7.67E-06	2.95E-05	3.41E-06	0.00003	0.000003	4.39E-05	1.01E-05
F (polyols)										
Galactinol	0.00029	0.000467	0.00008	2.33E-05	1.03E-04	2.41E-05	0.00009	0.000018	1.41E-04	3.03E-05
Glycerol	0.00029	0.000064	0.00031	1.05E-04	2.99E-04	8.93E-05	0.00025	0.000031	2.56E-04	5.97E-05
Maltitol	0.00009	0.000009	0.00008	9.44E-06	8.69E-05	7.43E-06	0.00010	0.000015	9.06E-05	2.24E-05
Mannitol	0.00016	0.000065	0.00009	2.90E-05	1.75E-04	4.02E-05	0.00011	0.000028	1.88E-04	5.30E-05
Myo-Inositol	0.00183	0.000268	0.00164	4.00E-04	2.08E-03	3.45E-04	0.00225	0.000341	2.07E-03	4.06E-04
Sorbitol	0.00055	0.000140	0.00093	3.39E-04	6.69E-04	2.08E-04	0.00066	0.000128	1.25E-03	2.32E-04
G (phosphates)										
Fructose-6P	0.00001	0.000002	0.00001	5.22E-06	7.09E-06	1.53E-06	0.00001	0.000002	7.65E-06	5.55E-07
Phosphoric acid	0.00390	0.004637	0.00041	8.10E-05	6.54E-05	1.03E-05	0.00128	0.000343	5.51E-05	2.12E-05

Supplementary table Ib. Overview of all metabolites determined in nodules of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Analytical name	Biological Description				
	Metabolite Name	Metabolite ID	SumFormula	KEGG - ID	CAS - ID
A (amino acids)	A (amino acids)				
Alanine, DL- (2TMS)	Alanine	M000026	C3H7NO2	C00041	56-41-7
Glutamic acid, DL- (3TMS)	Glutamate	M000036	C5H9NO4	C00025	56-86-0
Glycine (3TMS)	Glycine	M000031	C2H5NO2	C00037	56-40-6
Phenylalanine, DL- (2TMS)	Phenylalanine	M000011	C9H11NO2	C00079	63-91-2
Proline, L- (2TMS)	Proline	M000029	C5H9NO2	C00148	147-85-3
Serine, DL- (3TMS)	Serine	M000015	C3H7NO3	C00065	56-45-1
Valine, DL- (2TMS)	Valine	M000030	C5H11NO2	C00183	72-18-4
Butyric acid, 4-amino- (3TMS)	GABA	M000114	C4H9NO2	C00334	56-12-2
Alanine, beta- (3TMS)	β-Alanine	M000027	C3H7NO2	C00099	107-95-9
Aspartic acid, L- (3TMS)	Aspartate	M000033	C4H7NO4	C00049	56-84-8
Tyrosine, DL- (3TMS)	Tyrosine	M000035	C9H11NO3	C00082	60-18-4
Asparagine, DL- (4TMS)	Asparagine	M000013	C4H8N2O3	C00152	70-47-3
Tryptophan, DL- (3TMS)	Tryptophan	M000012	C11H12N2O2	C00078	73-22-3
B (organic acids)	B (organic acids)				
Malic acid, 2-methyl-, DL- (3TMS)	Citramalate	M000066	C5H6O4	C02226	498-23-7
Citric acid (4TMS)	Citrate	M000069	C6H8O7	C00158	77-92-9
Fumaric acid (2TMS)	Fumarate	M000067	C4H4O4	C00122	110-17-8
Malic acid, DL- (3TMS)	Malate	M000065	C4H6O5	C00149	97-67-6
Succinic acid (2TMS)	Succinate	M000074	C4H6O4	C00042	110-15-6
Glutaric acid, 2-oxo- (1MEOX) (2TMS)	2-Oxoglutarate	M000571	C5H6O5	C00026	328-50-7
Threonic acid (4TMS)	Threonate	M000078	C4H8O5	C01620	7306-96-9
C (aromatic acid)	C (aromatic acid)				
Benzoic acid (1TMS)	Benzoate	M000347	C7H6O2	C00180	65-85-0
D (N containing compounds)	D (N containing compounds)				
Putrescine (4TMS)	Putrescine	M000186	C4H12N2	C00134	110-60-1
Urea (2TMS)	Urea	M000364	CH4N2O	C00086	57-13-6
E (sugars)	E (sugars)				
Fructose, D- (1MEOX) (5TMS)	Fructose	M000606	C6H12O6	C00095	57-48-7
Glucose, D- (1MEOX) (5TMS)	Glucose	M000040	C6H12O6	C00031	50-99-7
Mannose, D- (1MEOX) (5TMS)	Mannose	M000633	C6H12O6	C00936	3458-28-4
Sucrose, D- (8TMS)	Sucrose	M000044	C12H22O11	C00089	57-50-1
Trehalose, alpha,alpha'-, D- (8TMS)	Trehalose	M000671	C12H22O11	C01083	99-20-7

F (polyols)	F (polyols)				
Glycerol (3TMS)	Glycerol	M000053	C3H8O3	C00116	56-81-5
Inositol, myo- (6TMS)	Myo-Inositol	M000060	C6H12O6	C00137	87-89-8
G (phosphates)	G (phosphates)				
Fructose-6-phosphate (1MEOX) (6TMS)	Fructose-6P	M000510	C6H13O9P	C00085	643-13-0
Phosphoric acid (3TMS)	Phosphoric acid	M000075	H3O4P	C00009	7664-38-2

Supplementary table Ib. Overview of all metabolites determined in nodules of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Analytical name	Analytical Description							
	CAS ID	Sum Formula	Analytical ID	Time expected	Time deviation	(m/z)	Cluster Size	Cluster match
A (amino acids)								
Alanine, DL- (2TMS)	2899-44-7	C3H7NO2	A138002-101	354060	-0.06	116	9	917
Glutamic acid, DL- (3TMS)	15985-07-6	C5H9NO4	A163001-101	507780	-0.09	246	5	908
Glycine (3TMS)	5630-82-0	C2H5NO2	A133001-101	325180	-0.03	248	12	974
Phenylalanine, DL- (2TMS)	2899-52-7	C9H11NO2	A164001-101	531145	-0.09	218	7	828
Proline, L- (2TMS)		C5H9NO2	A132003-101	338693	-0.01	142	6	995
Serine, DL- (3TMS)	7364-48-9	C3H7NO3	A138001-101	357523	-0.1	204	49	988
Valine, DL- (2TMS)	15984-93-7	C5H11NO2	A122001-101	271580	0	144	4	958
Butyric acid, 4-amino- (3TMS)	39508-23-1	C4H9NO2	A153003-101	342530	-0.13	174	61	909
Alanine, beta- (3TMS)	17891-86-0	C3H7NO2	A144001-101	394250	-0.05	248	7	892
Aspartic acid, L- (3TMS)	15985-05-4	C4H7NO4	A152002-101	457283	0.03	232	8	845
Tyrosine, DL- (3TMS)	7415-19-2	C9H11NO3	A194002-101	658337	-0.07	218	18	962
Asparagine, DL- (4TMS)		C4H8N2O3	A164007-101	479050	-0.05	188	6	740
Tryptophan, DL- (3TMS)	55429-28-2	C11H12N2O2	A223001-101	790560	-0.08	202	92	703
B (organic acids)								
Malic acid, 2-methyl-, DL- (3TMS)		C5H6O4	A148001-101	425325	0.12	247	153	991
Citric acid (4TMS)	14330-97-3	C6H8O7	A182004-101	592883	-0.04	273	253	994
Fumaric acid (2TMS)	17962-03-7	C4H4O4	A137001-101	371255	-0.12	245	7	967
Malic acid, DL- (3TMS)	38166-11-9	C4H6O5	A149001-101	373395	-0.25	233	101	986
Succinic acid (2TMS)	40309-57-7	C4H6O4	A134001-101	365427	-0.1	247	25	992
Glutaric acid, 2-oxo- (1MEOX) (2TMS)	60022-87-9	C5H6O5	A158004-101	523847	-0.09	198	9	896
Threonic acid (4TMS)	38191-88-7	C4H8O5	A156001-101	458330	-0.2	292	25	975
C (aromatic acid)								
Benzoic acid (1TMS)	2078-12-8	C7H6O2	A128003-101	348220	-0.02	179	12	975
D (N containing compounds)								
Putrescine (4TMS)	39772-63-9	C4H12N2	A175002-101	517180	0.04	174	0	884
Urea (2TMS)	18297-63-7	CH4N2O	A127002-101	340357	0.18	189	4	987
E (sugars)								
Fructose, D- (1MEOX) (5TMS)	56196-14-6	C6H12O6	A187002-101	579380	-0.13	307	101	978
Glucose, D- (1MEOX) (5TMS)	34152-44-8	C6H12O6	A189002-101	590400	-0.08	160	1	963

Mannose, D- (1MEOX) (5TMS)		C6H12O6	A188002-101	581930	-0.13	160	0	902
Sucrose, D- (8TMS)	19159-25-2	C12H22O11	A264001-101	840783	0.05	361	113	971
Trehalose, alpha,alpha'-, D- (8TMS)		C12H22O11	A274002-101	876240	-0.09	191	23	928
F (polyols)								
Glycerol (3TMS)	1785-22-0	C3H8O3	A129003-101	291783	-0.07	205	33	954
Inositol, myo- (6TMS)	2582-79-8	C6H12O6	A209002-101	653910	0.03	305	126	985
G (phosphates)								
Fructose-6-phosphate (1MEOX) (6TMS)	55530-74-0	C6H13O9P	A232002-101	767060	0.04	315	0	675
Phosphoric acid (3TMS)	10497-05-9	H3O4P	A129001-101	333520	-0.14	314	78	983

Supplementary table Ib. Overview of all metabolites determined in nodules of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Metabolite Name	Statistical		Wild Type Control	
	Induced by water-logging*	Between genotypes**	Day 0 mean	Dy 0 stdev
A (amino acids)				
Alanine	X		0.012873403	0.002738686
Glutamate	X	X	0.043069169	0.017028219
Glycine	X	X	0.003163347	0.000489475
Phenylalanine	X	X	0.003733796	0.001189989
Proline		X	0.047700616	0.015677112
Serine	X	X	0.005233044	0.00052084
Valine	X	X	0.007116301	0.001759492
GABA	X	X	0.003508077	0.000213954
β-Alanine	X	X	0.000800517	9.5523E-05
Aspartate	X	X	0.017906042	0.002704951
Tyrosine	X	X	0.001955688	0.000637431
Asparagine	X	X	0.054764222	0.008727639
Tryptophan	X	X	0.020649277	0.004670627
B (organic acids)				
Citramalate	X	X	0.097883694	0.037902148
Citrate	X		0.094225144	0.007574635
Fumarate		X	0.002036647	0.000253854
Malate	X	X	0.039137077	0.003342144
Succinate	X	X	0.001869818	0.000122171
2-Oxoglutarate	X	X	0.000775166	0.000164669
Threonate			0	0
C (aromatic acid)				
Benzoate			0.002732364	9.16308E-05
D (N containing compounds)				
Putrescine	X	X	0.003465858	0.000490841
Urea	X		0.000852853	0.000116902
E (sugars)				
Fructose	X	X	0.000541687	0.000221165
Glucose		X	0.000386826	3.63256E-05

Mannose		X	0.000968851	0.000224319
Sucrose		X	0.325424785	0.044654173
Trehalose	X	X	0.001013028	0.000289991
F (polyols)				
Glycerol		X	0.005433734	0.000714128
Myo-Inositol	X	X	0.009709146	0.001430719
G (phosphates)				
Fructose-6P			0	0
Phosphoric acid	X	X	0.003423642	0.000743751

*Statistical difference during the time point into genotype (two way anova significant at $P < 0.05$).

**Statistical difference between the genotype (two way anova significant at $P < 0.05$).

Supplementary table Ib. Overview of all metabolites determined in nodules of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Metabolite Name	Wild Type Waterlogging									
	Day 1 mean	Day 1 stdev	Day 2 mean	Day 2 stdev	Day 3 mean	Day 3 stdev	Day 4 mean	Day 4 stdev	Day 5 mean	Day 5 stdev
A (amino acids)										
Alanine	0.1146	0.0161	0.12860	0.02049	0.0915	0.0037	0.1314	0.0159	0.0909	0.0079
Glutamate	0.0873	0.0165	0.08458	0.00334	0.0495	0.0034	0.0747	0.0040	0.0570	0.0095
Glycine	0.0199	0.0072	0.02709	0.00463	0.0222	0.0024	0.0403	0.0062	0.0234	0.0013
Phenylalanine	0.0095	0.0028	0.01087	0.00060	0.0073	0.0012	0.0062	0.0009	0.0069	0.0004
Proline	0.1137	0.1339	0.03788	0.00825	0.0240	0.0079	0.0483	0.0270	0.0744	0.0948
Serine	0.0152	0.0022	0.00971	0.00094	0.0076	0.0010	0.0136	0.0021	0.0073	0.0002
Valine	0.0184	0.0032	0.01813	0.00134	0.0134	0.0023	0.0132	0.0033	0.0122	0.0021
GABA	0.0332	0.0054	0.06295	0.00705	0.0656	0.0044	0.1172	0.0228	0.0602	0.0062
β-Alanine	0.0027	0.0006	0.00317	0.00011	0.0034	0.0004	0.0050	0.0004	0.0031	0.0003
Aspartate	0.0064	0.0010	0.00193	0.00040	0.0007	0.0001	0.0009	0.0000	0.0005	0.0004
Tyrosine	0.0077	0.0010	0.01129	0.00293	0.0097	0.0011	0.0122	0.0035	0.0102	0.0006
Asparagine	0.0419	0.0209	0.00406	0.00049	0.0016	0.0003	0.0027	0.0006	0.0039	0.0027
Tryptophan	0.0331	0.0071	0.02808	0.00191	0.0307	0.0036	0.0205	0.0035	0.0273	0.0029
B (organic acids)										
Citramalate	0.1018	0.0348	0.06691	0.00496	0.1065	0.0149	0.0947	0.0077	0.0824	0.0089
Citrate	0.0810	0.0097	0.08646	0.00902	0.0607	0.0156	0.0497	0.0073	0.0533	0.0185
Fumarate	0.0024	0.0003	0.00217	0.00043	0.0021	0.0003	0.0025	0.0003	0.0023	0.0002
Malate	0.0354	0.0082	0.02251	0.00085	0.0145	0.0025	0.0129	0.0028	0.0099	0.0021
Succinate	0.0067	0.0023	0.00790	0.00024	0.0092	0.0014	0.0114	0.0002	0.0114	0.0025
2-Oxoglutarate	0.0004	0.0001	0.00037	0.00003	0.0004	0.0001	0.0004	0.0001	0.0004	0.0001
Threonate	0.0000	0.0000	0.00000	0.00000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
C (aromatic acid)										
Benzoate	0.0027	0.0005	0.00283	0.00034	0.0027	0.0003	0.0025	0.0007	0.0026	0.0002
D (N containing compounds)										
Putrescine	0.0053	0.0004	0.00775	0.00022	0.0073	0.0005	0.0104	0.0033	0.0088	0.0027
Urea	0.0009	0.0002	0.00060	0.00005	0.0006	0.0001	0.0006	0.0001	0.0008	0.0001
E (sugars)										
Fructose	0.0006	0.0002	0.00050	0.00017	0.0004	0.0002	0.0002	0.0001	0.0002	0.0000
Glucose	0.0003	0.0001	0.00048	0.00006	0.0004	0.0001	0.0004	0.0001	0.0004	0.0002

Mannose	0.0008	0.0001	0.00099	0.00004	0.0010	0.0002	0.0010	0.0001	0.0011	0.0003
Sucrose	0.3448	0.0585	0.33934	0.02806	0.3206	0.0358	0.3140	0.0109	0.2970	0.0431
Trehalose	0.0015	0.0001	0.00085	0.00019	0.0006	0.0001	0.0006	0.0001	0.0004	0.0002
F (polyols)										
Glycerol	0.0075	0.0018	0.00811	0.00125	0.0067	0.0008	0.0074	0.0008	0.0095	0.0028
Myo-Inositol	0.0066	0.0010	0.01034	0.00104	0.0081	0.0006	0.0099	0.0009	0.0101	0.0018
G (phosphates)										
Fructose-6P	0.0000	0.0000	0.00012	0.00004	0.0002	0.0001	0.0002	0.0001	0.0002	0.0000
Phosphoric acid	0.0059	0.0007	0.00681	0.00027	0.0057	0.0002	0.0083	0.0014	0.0071	0.0016

Supplementary table Ib. Overview of all metabolites determined in nodules of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Metabolite Name	Wild Type Recovery									
	Day 6 mean	Day 6 stdev	Day 7 mean	Day 7 stdev	Day 8 mean	Day 8 stdev	Day 9 mean	Day 9 stdev	Day 10 mean	Day 10 stdev
A (amino acids)										
Alanine	0.0275	0.0062	0.0171	0.0015	0.0279	0.0048	0.0121	0.0027	0.0122	0.0006
Glutamate	0.0500	0.0056	0.0451	0.0073	0.0507	0.0169	0.0331	0.0048	0.0424	0.0017
Glycine	0.0055	0.0013	0.0035	0.0002	0.0067	0.0022	0.0039	0.0010	0.0037	0.0003
Phenylalanine	0.0078	0.0011	0.0030	0.0002	0.0077	0.0028	0.0040	0.0005	0.0059	0.0011
Proline	0.0224	0.0099	0.0091	0.0055	0.0195	0.0101	0.0066	0.0018	0.0078	0.0026
Serine	0.0098	0.0012	0.0071	0.0011	0.0122	0.0038	0.0057	0.0014	0.0061	0.0004
Valine	0.0316	0.0036	0.0086	0.0018	0.0351	0.0061	0.0107	0.0018	0.0107	0.0012
GABA	0.0187	0.0043	0.0051	0.0009	0.0080	0.0020	0.0046	0.0004	0.0059	0.0006
β-Alanine	0.0020	0.0002	0.0008	0.0001	0.0010	0.0001	0.0006	0.0001	0.0007	0.0001
Aspartate	0.0219	0.0009	0.0156	0.0019	0.0270	0.0011	0.0107	0.0010	0.0130	0.0006
Tyrosine	0.0144	0.0004	0.0044	0.0010	0.0094	0.0018	0.0043	0.0015	0.0055	0.0009
Asparagine	0.0096	0.0017	0.0201	0.0034	0.0150	0.0033	0.0152	0.0025	0.0213	0.0038
Tryptophan	0.0310	0.0084	0.0227	0.0063	0.0333	0.0017	0.0262	0.0040	0.0381	0.0063
B (organic acids)										
Citramalate	0.0680	0.0208	0.0726	0.0028	0.1286	0.0229	0.0534	0.0028	0.0835	0.0067
Citrate	0.1097	0.0076	0.1387	0.0062	0.1386	0.0148	0.0953	0.0049	0.1335	0.0108
Fumarate	0.0022	0.0004	0.0021	0.0004	0.0021	0.0002	0.0020	0.0003	0.0025	0.0002
Malate	0.0252	0.0019	0.0326	0.0026	0.0241	0.0024	0.0284	0.0052	0.0393	0.0024
Succinate	0.0024	0.0001	0.0026	0.0003	0.0021	0.0001	0.0017	0.0002	0.0020	0.0001
2-Oxoglutarate	0.0009	0.0001	0.0011	0.0003	0.0008	0.0001	0.0008	0.0001	0.0009	0.0001
Threonate	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
C (aromatic acid)										
Benzoate	0.0028	0.0001	0.0025	0.0004	0.0029	0.0004	0.0025	0.0002	0.0029	0.0003
D (N containing compounds)										
Putrescine	0.0133	0.0022	0.0104	0.0027	0.0136	0.0010	0.0073	0.0007	0.0127	0.0003
Urea	0.0009	0.0002	0.0009	0.0000	0.0011	0.0002	0.0010	0.0003	0.0005	0.0001
E (sugars)										
Fructose	0.0002	0.0001	0.0004	0.0001	0.0006	0.0001	0.0004	0.0001	0.0007	0.0001
Glucose	0.0005	0.0001	0.0005	0.0001	0.0005	0.0002	0.0003	0.0001	0.0005	0.0001

Mannose	0.0010	0.0003	0.0009	0.0001	0.0010	0.0003	0.0009	0.0002	0.0010	0.0000
Sucrose	0.3357	0.0218	0.3390	0.0069	0.3680	0.0085	0.3126	0.0205	0.3418	0.0396
Trehalose	0.0004	0.0002	0.0004	0.0001	0.0007	0.0001	0.0005	0.0001	0.0005	0.0001
F (polyols)										
Glycerol	0.0093	0.0022	0.0065	0.0010	0.0075	0.0010	0.0073	0.0019	0.0065	0.0011
Myo-Inositol	0.0069	0.0002	0.0083	0.0020	0.0107	0.0008	0.0060	0.0013	0.0093	0.0004
G (phosphates)										
Fructose-6P	0.0001	0.0000	0.0001	0.0000	0.0001	0.0000	0.0001	0.0000	0.0001	0.0000
Phosphoric acid	0.0042	0.0008	0.0037	0.0004	0.0037	0.0002	0.0035	0.0002	0.0030	0.0009

Supplementary table Ib. Overview of all metabolites determined in nodules of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Metabolite Name	Statistical		LbRNAi Control	
	Induced by water-logging *	Between genotypes **	Day 0 mean	Dy 0 stdev
A (amino acids)				
Alanine	X		0.008772308	0.001683332
Glutamate	X	X	0.016756073	0.000467754
Glycine	X	X	0.002336018	0.000474729
Phenylalanine	X	X	0.000615822	9.01652E-05
Proline		X	0.002275672	9.04247E-05
Serine	X	X	0.002656369	0.000407855
Valine	X	X	0.00236575	0.000310106
GABA	X	X	0.003063438	8.09832E-05
β-Alanine	X	X	0.00041914	5.41247E-05
Aspartate	X	X	0.009675324	0.000627692
Tyrosine	X	X	0.001283478	0.000228152
Asparagine	X	X	0.002893519	0.000278767
Tryptophan	X	X	0.002938256	0.000201894
B (organic acids)				
Citramalate	X	X	0.177600513	0.018134903
Citrate	X		0.070423284	0.005172322
Fumarate		X	0.001866579	0.00023128
Malate	X	X	0.053735771	0.003118947
Succinate	X	X	0.002433078	6.82173E-05
2-Oxoglutarate	X	X	0.000504827	6.25182E-05
Threonate			0.006811759	0.000317428
C (aromatic acid)				
Benzoate			0.00255193	0.000212744
D (N containing compounds)				
Putrescine	X	X	0.000732426	6.7801E-05
Urea	X		0.00120028	0.000394119
E (sugars)				
Fructose	X	X	0.000843274	0.000209209
Glucose	X	X	0.000513506	6.04469E-05

Mannose	X	X	0.000545045	2.64204E-05
Sucrose		X	0.427079906	0.020756363
Trehalose	X	X	0.000372935	4.04919E-05
F (polyols)				
Glycerol		X	0.006582361	0.000418079
Myo-Inositol	X	X	0.017659835	0.001784824
G (phosphates)				
Fructose-6P			0.000100039	2.44999E-05
Phosphoric acid	X	X	0.046315204	0.007519802

*Statistical difference during the time point into genotype (two way anova significant at $P < 0.05$).

**Statistical difference between the genotype (two way anova significant at $P < 0.05$).

Supplementary table Ib. Overview of all metabolites determined in nodules of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery. (continue....)

Metabolite Name	LbRNAi Waterlogging									
	Day 1 mean	Day 1 stdev	Day 2 mean	Day 2 stdev	Day 3 mean	Day 3 stdev	Day 4 mean	Day 4 stdev	Day 5 mean	Day 5 stdev
A (amino acids)										
Alanine	0.1033	0.0171	0.0805	0.0156	0.0858	0.0021	0.0795	0.0046	0.0644	0.0094
Glutamate	0.0411	0.0049	0.0550	0.0022	0.0704	0.0095	0.0795	0.0107	0.0709	0.0043
Glycine	0.0049	0.0002	0.0073	0.0010	0.0094	0.0005	0.0083	0.0009	0.0068	0.0008
Phenylalanine	0.0012	0.0001	0.0016	0.0001	0.0020	0.0003	0.0018	0.0002	0.0020	0.0001
Proline	0.0056	0.0010	0.0071	0.0017	0.0093	0.0020	0.0102	0.0002	0.0124	0.0014
Serine	0.0048	0.0007	0.0054	0.0007	0.0054	0.0007	0.0052	0.0001	0.0065	0.0013
Valine	0.0046	0.0006	0.0066	0.0016	0.0071	0.0001	0.0058	0.0008	0.0060	0.0019
GABA	0.0107	0.0023	0.0164	0.0014	0.0253	0.0021	0.0262	0.0049	0.0283	0.0008
β-Alanine	0.0011	0.0002	0.0018	0.0001	0.0025	0.0003	0.0026	0.0002	0.0030	0.0006
Aspartate	0.0032	0.0008	0.0044	0.0009	0.0043	0.0005	0.0049	0.0004	0.0064	0.0004
Tyrosine	0.0032	0.0008	0.0044	0.0006	0.0060	0.0005	0.0063	0.0002	0.0065	0.0001
Asparagine	0.0006	0.0001	0.0005	0.0002	0.0004	0.0001	0.0003	4.80E-05	0.0005	0.0002
Tryptophan	0.0033	0.0008	0.0040	0.0005	0.0047	0.0007	0.0041	0.0006	0.0045	0.0008
B (organic acids)										
Citramalate	0.1825	0.0161	0.1447	0.0085	0.1726	0.0215	0.1430	0.0283	0.1707	0.0031
Citrate	0.0576	0.0207	0.0547	0.0074	0.0624	0.0039	0.0660	0.0037	0.0569	0.0037
Fumarate	0.0013	0.0002	0.0014	0.0002	0.0019	0.0003	0.0019	0.0003	0.0022	0.0002
Malate	0.0404	0.0076	0.0406	0.0053	0.0353	0.0063	0.0319	0.0062	0.0310	0.0018
Succinate	0.0059	0.0009	0.0107	0.0016	0.0221	0.0028	0.0230	0.0018	0.0247	0.0040
2-Oxoglutarate	0.0003	0.0002	0.0005	0.0003	0.0006	0.0000	0.0008	5.19E-05	0.0008	4.39E-05
Threonate	0.0057	0.0006	0.0066	0.0014	0.0067	0.0009	0.0063	0.0007	0.0061	0.0008
C (aromatic acid)										
Benzoate	0.0023	0.0003	0.0024	0.0006	0.0025	0.0004	0.0020	0.0004	0.0022	0.0002
D (N containing compounds)										
Putrescine	0.0009	0.0002	0.0010	0.0002	0.0015	0.0001	0.0013	0.0001	0.0012	0.0001
Urea	0.0009	0.0002	0.0006	0.0002	0.0005	0.0002	0.0004	3.32E-05	0.0006	0.0000
E (sugars)										
Fructose	0.0012	0.0003	0.0015	0.0003	0.0023	0.0003	0.0021	0.0002	0.0020	0.0003
Glucose	0.0007	0.0002	0.0009	0.0002	0.0014	0.0003	0.0016	0.0001	0.0016	0.0001

Mannose	0.0009	0.0001	0.0011	1.96E-05	0.0014	0.0002	0.0016	0.0002	0.0019	0.0002
Sucrose	0.4249	0.0465	0.4555	0.0350	0.4238	0.0585	0.4252	0.0363	0.4061	0.0560
Trehalose	0.0004	0.0001	0.0003	5.48E-05	0.0002	0.0000	0.0002	4.35E-05	0.0004	0.0001
F (polyols)										
Glycerol	0.0057	0.0016	0.0070	0.0005	0.0058	0.0008	0.0055	0.0008	0.0062	0.0009
Myo-Inositol	0.0211	0.0053	0.0285	0.0067	0.0405	0.0063	0.0432	0.0009	0.0522	0.0048
G (phosphates)										
Fructose-6P	0.0003	4.85E-05	0.0005	0.0002	0.0006	0.0001	0.0006	8.88E-05	0.0005	0.0001
Phosphoric acid	0.0285	0.0115	0.0383	0.0106	0.0233	0.0051	0.0304	0.0081	0.0210	0.0056

Supplementary table Ib. Overview of all metabolites determined in nodules of *Lotus japonicus* wild type and LbRNAi plants during a 5 days period of waterlogging and 5 days of subsequent recovery.

Metabolite Name	LbRNAi Recovery									
	Day 6 mean	Day 6 stdev	Day 7 mean	Day 7 stdev	Day 8 mean	Day 8 stdev	Day 9 mean	Day 9 stdev	Day 10 mean	Day 10 stdev
A (amino acids)										
Alanine	0.0118	0.0022	0.0094	0.00309	0.0063	0.0010	0.0070	0.00045	0.0075	0.00120
Glutamate	0.0460	0.0011	0.0345	0.00952	0.0248	0.0033	0.0230	0.00305	0.0219	0.00357
Glycine	0.0021	0.0004	0.0023	0.00093	0.0020	0.0002	0.0021	0.00028	0.0027	0.00006
Phenylalanine	0.0009	0.0000	0.0008	0.00016	0.0007	0.0000	0.0007	0.00004	0.0008	0.00004
Proline	0.0030	0.0004	0.0032	0.00050	0.0030	0.0002	0.0028	0.00021	0.0029	0.00022
Serine	0.0041	0.0002	0.0039	0.00099	0.0029	0.0002	0.0032	0.00044	0.0034	0.00023
Valine	0.0041	0.0010	0.0034	0.00125	0.0031	0.0001	0.0033	0.00056	0.0035	0.00040
GABA	0.0028	0.0022	0.0038	0.00062	0.0037	0.0002	0.0035	0.00031	0.0037	0.00055
β-Alanine	0.0009	0.0002	0.0008	0.00017	0.0007	0.0001	0.0006	0.00002	0.0008	0.00009
Aspartate	0.0180	0.0014	0.0133	0.00131	0.0092	0.0016	0.0097	0.00160	0.0087	0.00092
Tyrosine	0.0026	0.0002	0.0019	0.00034	0.0016	0.0000	0.0018	0.00007	0.0020	0.00017
Asparagine	0.0026	0.0000	0.0025	0.00060	0.0028	0.0002	0.0026	0.00061	0.0023	0.00051
Tryptophan	0.0031	0.0004	0.0027	0.00009	0.0032	0.0007	0.0035	0.00058	0.0037	0.00014
B (organic acids)										
Citramalate	0.1903	0.0128	0.1895	0.03669	0.2104	0.0268	0.2122	0.02312	0.2362	0.02941
Citrate	0.1052	0.0085	0.1198	0.00130	0.1359	0.0179	0.1262	0.00468	0.1103	0.01511
Fumarate	0.0027	0.0007	0.0020	0.00035	0.0021	0.0003	0.0022	0.00035	0.0020	0.00037
Malate	0.0461	0.0059	0.0392	0.00268	0.0431	0.0063	0.0467	0.00201	0.0383	0.00285
Succinate	0.0100	0.0008	0.0060	0.00028	0.0063	0.0018	0.0061	0.00027	0.0040	0.00015
2-Oxoglutarate	0.0019	0.0003	0.0016	0.00026	0.0012	0.0005	0.0010	0.00006	0.0007	0.00020
Threonate	0.0000	0	0.0000	0.00000	0.0000	0.0000	0.0000	0.00000	0.0000	0.00000
C (aromatic acid)										
Benzoate	0.0026	0.0006	0.0023	0.00066	0.0029	0.0005	0.0032	0.00016	0.0029	0.00042
D (N containing compounds)										
Putrescine	0.0009	5.03E-05	0.0011	0.00010	0.0010	0.0001	0.0010	0.00012	0.0009	0.00031
Urea	0.0008	7.66E-05	0.0008	0.00004	0.0008	0.0002	0.0011	0.00004	0.0013	0.00033
E (sugars)										
Fructose	0.0019	4.05E-04	0.0017	0.00010	0.0013	0.0001	0.0011	0.00006	0.0011	0.00032
Glucose	0.0012	1.76E-04	0.0010	0.00022	0.0007	0.0000	0.0007	0.00002	0.0006	0.00012

Mannose	0.0011	1.43E-04	0.0010	0.00013	0.0008	0.0001	0.0009	0.00007	0.0009	0.00004
Sucrose	0.4271	2.13E-02	0.4141	0.06044	0.4371	0.0010	0.4017	0.07550	0.3882	0.01850
Trehalose	0.0003	2.39E-05	0.0002	0.00006	0.0004	0.0001	0.0003	0.00007	0.0006	0.00003
F (polyols)										
Glycerol	0.0065	5.19E-04	0.0070	0.00076	0.0064	0.0008	0.0059	0.00149	0.0061	0.00073
Myo-Inositol	0.0433	3.97E-03	0.0403	0.00764	0.0303	0.0016	0.0284	0.00194	0.0263	0.00668
G (phosphates)										
Fructose-6P	0.0002	7.00E-05	0.0002	0.00006	0.0001	0.0000	0.0001	0.00001	0.0002	0.00001
Phosphoric acid	0.0473	4.72E-03	0.0422	0.00242	0.0286	0.0016	0.0388	0.01040	0.0140	0.00551

Supplementary table II. Overview of the primers used for quantitative RT-PCR.

Transcript Identifier	Gene name	Sequence forward primer	Sequence reverse primer	Reference
Lja.3082	Alcohol dehydrogenase (ADH)	TCTTTGGACTTGGAGCTGTTGGC	TGATGCACCAGAAACCCTTGCC	this publication
Lja.3240	Glutamine synthetase 1 (GS1)	GGGAATTCCAAGTTGGCCCTTCAG	AACGAGCAACCCACACCTCATC	this publication
Lja.14091	Elongation factor 1a	GGATCTCAAGCGTGGTTTCGTG	TGACTTGGGAGGTGAAGTTTGC	this publication
Lja.14113	Actin	TTGGAGCTGAGAGGTTCCGTTG	ACCCGCAGCTTCCATTCCAATC	this publication
Lja.8201	Tubulin	ACACGTGCTCTTGTTCCCTCCTC	TGCTACTGCTGAGGATGATGGG	this publication
PDC 1	Pyruvate decarboxylase 1 (PDC1)	GCCTGCGAATTGTGGTTATGA	TAGCGCCTACTGACCAACCAA	Andreadeli <i>et al.</i> 2009
Nitrogenase	nifH	TCCAAGCTCATCCACTTCGTG	AGTCCGGCGCATACTGGATTA	Ott <i>et al.</i> 2005
Ubiquitin	TC3806	TTCACCTTGTGCTCCGTCTTC	AACAACAGCACACACAGACAATCC	Ott <i>et al.</i> 2005
SigA	sigA	GCCCTCTGCTCGACCTTTCC	AGCATCGCCATCGTGTCTCTC	Ott <i>et al.</i> 2005
LjUbi1	ubiquitin10	TTCACCTTGTGCTCCGTCTTC	AACAACAGCACACACAGCCAATCC	Ott <i>et al.</i> 2009

Andreadeli A, Flemetakis E, Axarli I, Dimou M, Udvardi MK, Katinakis P, Labrou NE (2009) Cloning and characterization of *Lotus japonicus* formate dehydrogenase: A possible correlation with hypoxia. *Biochimica et Biophysica Acta* **1794**: 976–984

Ott T, van Dongen J T, Günther C, Krusell L, Desbrosses G, Vigeolas H, Bock V, Czechowski T, Geigenberger P, Udvardi M K (2005) Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Current Biology*. **15**: 531–535.

Ott T, Sullivan J, James E K, Flemetakis E, Günther C, Gibon Y, Ronson C, Udvardi M K (2009) Absence of symbiotic leghemoglobins alters bacteroid and plant cell differentiation during development of *Lotus japonicus* root nodules. *Molecular Plant-Microbe Interactions* **22**: 800-808.

CHAPTER 02: Regulation of expression and activity of alanine aminotransferase in various organs of soybean (*Glycine max*) in relation to the nitrogen source during hypoxic stress

1. RESUMO

O papel da alanina aminotransferase (AlaAT) foi investigado em relação ao expressão gênica, atividade enzimática e conteúdo de alanina produzido em tecidos vegetais. Três cópias do gene alanina aminotransferase foram encontradas no genoma de soja. Através de primers genéricos expressão dos genes AlaAT foi encontrada em todos os órgãos testados (raiz, nódulo, folhas e vagens), apesar de mostrar considerável variação. Também foi detectada atividade da AlaAT em todos os órgãos testados. O isogene que mais respondeu à hipoxia foi o GmAlaAT1. A atividade da AlaAT permaneceu alta durante o período de reoxigenação, enquanto a expressão gênica e o conteúdo de alanina foram praticamente recuperados ao nível normal, o que indicaria que o papel da AlaAT poderia estar relacionado de alguma forma também com a conversão de alanina em piruvato e glutamato durante o período de pós-hipoxia. Os genes *GmAlaAT* também responderam ao conteúdo de nitrogênio presente na solução de alagamento. Quando amônio foi utilizado como fonte de nitrogênio tanto a expressão gênica como a atividade enzimática da AlaAT apresentaram valores maiores que em presença de nitrato. Os resultados presentes nesse trabalho sugerem que AlaAT não somente é importante durante o período de hipoxia, mais também durante a fase de recuperação.

2. ABSTRACT

To investigate the function of alanine aminotransferase (AlaAT) in soybean during hypoxic stress, regulation at the transcript level was analyzed, together with the enzyme activity and alanine content of the tissue. Analysis of public EST databases revealed three alanine aminotransferase isoenzymes encoded by the soybean genome. Using generic primers expression of AlaAT was found in all organs tested (root, nodule, leaves and pods), although the level of expression showed considerable variation. Activity of AlaAT was also detected in all these organs. The isoform that responded most strongly to hypoxia was GmAlaAT1. AlaAT activity remained high during re-oxygenation but the transcript level and the alanine content decreased, indicating a role for AlaAT in the catabolism of alanine after a period of low oxygen availability. GmAlaAT also responded to nitrogen availability in the solution during waterlogging. Ammonium as nitrogen source induced both the expression and activity of *AlaAT* more than when nitrate was supplied in the nutrient solution. The results presented here suggest that AlaAT might not only be important during hypoxia, but also during the recovery phase.

3. INTRODUCTION

Alanine aminotransferase (AlaAT, EC 2.6.1.2) is a pyridoxal phosphate-dependent enzyme usually found in the all plant parts. Thus, it may be found not only in leaves and roots, but also in other tissues like the endosperm (Kikuchi et al., 1999) and flowers (Igarashi et al., 2003). This broad expression profile of AlaAT indicates that the enzyme is involved in an essential biochemical reaction during the whole life cycle of the plant. Indeed, the enzyme catalyses the reversible reaction between pyruvate and glutamate into alanine and oxoglutarate, thereby linking primary carbon metabolism with the synthesis of various amino acids.

During hypoxic conditions, AlaAT is suggested to play a special role, since it has been shown for various plant species that the activity of AlaAT as well as the accumulation of alanine increase when the oxygen availability decreases (Good and Muench, 1993; Muench and Good, 1994; Rocha et al, Chapter 1). Other metabolic changes that occur simultaneously during hypoxia are a decrease of the adenylate energy charge, acidification of the cytosol due to lactate accumulation and the loss of carbon due to an increased glycolytic flux and ethanol fermentation. Whereas the production of lactate and ethanol have detrimental side effects, alanine accumulation does not cause damage to the cell and indeed helps keeping the glycolytic flux and avoids the loss of carbon.

The role of AlaAT during the recovery phase after hypoxic stress is probably as important for the understanding of flooding tolerance (Fan et al, 1988) as its biochemical behavior during hypoxia. Sousa and Sodek (2003) demonstrated that

AlaAT activity increased during hypoxia more so after the accumulation of alanine had reached its maximum. Therefore, they suggested that the major role of AlaAT might be during the recovery phase, since the level of alanine returned to pre-hypoxic levels within 24h of return to normoxia. Miyashita et al. (2007) confirmed this idea by showing that the *Arabidopsis* *AlaAT1* knock out mutant (*alaat1-1*) was able to accumulate alanine during hypoxia like wild type plants, whereas the decrease of the levels of alanine during the re-oxygenation phase was delayed. It has been suggested that during the hypoxic phase, the plant prepares itself for a rapid recovery once oxygen becomes available again. This hypothesis is mainly based on the observation that many genes that encode proteins with an important function during the recovery phase are already expressed during the hypoxic stress (Drew, 1997). Furthermore, most metabolites recover rapidly to their normal level once hypoxia is over (Barret-Lennard et al., 1988; Fan et al., 1988; Albrecht et al., 1993; Sousa and Sodek, 2003). AlaAT has exactly this kind of regulation pattern as it is up-regulated at the transcriptional level during hypoxia and during the re-oxygenation phase the high levels of AlaAT can ensure the rapid conversion of accumulated alanine back into glutamate (Miyashita et al., 2007).

The *AlaAT* gene family of *Arabidopsis* was shown to be comprised of four homologues. Two of them encode for enzymes that not only function as AlaAT, but can also act as glutamate:glyoxylate aminotransferase (GGAT1 and GGAT2; Igarashi et al., 2003; Liepman and Olsen, 2003). Subcellular fractionation analysis has shown that the activity of GGAT was principally located in peroxisomes of *Arabidopsis* (Igarashi et al. 2003; Liepman and Olsen, 2003). It was suggested that these enzymes play an important role in photorespiration and amino acid metabolism

(Igarashi et al. 2003 ; Liepman and Olsen, 2003). The other two genes, *AlaAT1* and *AlaAT2*, were not characterized in detail for *Arabidopsis*, but *in silico* prediction of their localisation suggested that *AlaAT2* is a mitochondrial enzyme and *AlaAT1* is located in the cytosol (Liepman and Olsen, 2003).

Due to its important role in nitrogen and carbon metabolism in plants, AlaAT has been intensively studied by several groups (Sousa and Sodek, 2003; Ricoult et al., 2006; Good et al., 2007; Miyashita et al., 2007; Beatty et al., 2009). However, no research has been performed on soybean at the molecular level. Therefore we set out to characterize the *AlaAT* multigene family in soybean plants and investigate the regulation of AlaAT expression and activity in various plant organs, as well as under different conditions, such as different nitrogen sources and changing oxygen availability

4. MATERIALS AND METHODS

4.1 Plant material and growth conditions

Soybean plants (*Glycine max* L. Merrill cv. IAC- 17), inoculated with *Bradyrhizobium elkanii* strain SEMIA 5019, were grown in a greenhouse under natural light and temperature conditions. Three plants were grown together in one plastic pot with a volume of 3 L containing vermiculite as substrate and supplied with 200 mL N-free nutrient solution twice per week (Hoagland and Arnon, 1950) (CaCl₂ 0.5 mM; KCl 0.5 mM; KH₂PO₄ 0.25 mM; K₂HPO₄ 0.25 mM; MgSO₄ 1.0 mM; FeEDTA 0.05 mM; Trace Elements – MnCl₂ 9.1 µM; H₃BO₃ 0.046 mM; ZnCl₂ 0.765 µM; NaMoO₄ 0.56 µM; CuCl₂ 0.32 µM). Shortly before flowering, the pots were transferred

to containers and the root system flooded with N-free nutrient solution at 1/3 strength. When nitrogen sources were tested, KNO_3 or $(\text{NH}_4)_2\text{SO}_4$ was added to the N-free nutrient solution to a final concentration of 5 mM of nitrogen. Waterlogging was maintained during 3 days. Non-waterlogged control plants were set up simultaneously, and supplied with 200 mL full strength N-free nutrient solution (vermiculite capacity of 600 mL). Plants that were cultivated without inoculation were supplied with a nutrient solution supplemented with 15mM KNO_3 . At harvest, samples of pods, leaf, root and nodules were frozen in liquid nitrogen and subsequently lyophilized. The lyophilized material was stored at -20°C in a desiccator containing silica gel.

4.2 Identification of AlaAT sequences

ESTs from soybean AlaAT were selected from the NCBI database (national center for biotechnology information, <www.ncbi.nlm.nih.gov>) using the keywords “alanine aminotransferase and Glycine max”. Alignment of the ESTs to build contigs was done with the BioEdit Sequence Alignment Editor (<www.mbio.ncsu.edu/BioEdit/bioedit.html>).

4.3 Classification and sub-cellular localization of AlaAT

Phylogenetic analysis of alanine aminotransferase protein sequences that were identified in public databases (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>) from both monocot and dicot plant species was carried out in order to define possible subclasses that might share functional homologies. An unrooted tree was created

applying the neighbour-joining method with MEGA4 (Tamura et al., 2007) (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Protein sequences used included: *Glycine max* GmAlaAT1 (ABW17196.1), GmAlaAT2 (ABW17197.1) and GmAlaAT3 (ABW17198.1); *Arabidopsis thaliana* AtAlaAT1 (AAF82782.1), AtAlaAT2 (NP565040.2), AtAOAT1 (NP564192.2) and AtAOAT2 (NP177215.1); *Medicago truncatula* MtmAlaAT and MtcAlaAT (Ricoult et al., 2006); *Populus trichocarpa* PtAlaAT1 (XP002315675.1), PtAlaAT2 (XP002312679.1), PtAlaAT3 (XP002331223.1) and PtAlaAT4 (XP002304255.1); *Chlamydomonas reinhardtii* CrAlaAT1 (XP001695350.1) and CrAlaAT2 (XP001698518.1); *Oryza sativa* cv Japonica Os07g42600 (EEE67593.1), Os10g25130 (NP001064504.1), Os03g08530 (ABF94336.1), Os10g25140 (ABB47495.2), Os09g26380 (NP001063248.1) and Os07g01760 (NP001058716.1); *Physcomitrella patens* subsp. *patens* PpAlaAT1 (XP001769989.1), PpAlaAT2 (XP001753102.1), PpGGT1 (XP001777071.1), and PpGGT2 (XP001782822.1).

Three algorithms were used to predict the sub-cellular localization of AlaAT: PSORT [<http://psort.nibb.ac.jp/form.html>] (Nakai and Horton, 1999); BaCelLo (<http://gpcr.biocomp.unibo.it/bacello>) (Pierleoni et al., 2006); TargetP (<http://www.cbs.dtu.dk/services/TargetP>) (Emanuelsson et al., 2000) and Pence Proteome Analyst Specialized Subcellular Localization of Proteins (Lu et al., 2004).

4.4 Southern blot

In order to determine the abundance of *AlaAT* homologues encoded by the soybean genome, southern blotting was performed. Genomic DNA was extracted from fresh leaf tissues of soybean plants using a DNeasy Plant Maxi Kit (24)

(Quiagen, Hilden, Germany). 15-20 mg of DNA was digested with BamHI, MfeI, and AluI, restriction enzymes that have a cleavage site outside of the probe region. The restriction fragments were separated by gel electrophoresis on a 0.8% (w/v) agarose gel and transferred to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, UK) fixed using UV-light.

Generic primers were designed to obtain PCR products of 499 bp which were used as templates for the production of the radioactive labeled probe. The sequence of these primers was as follows:

Sense: 5' – CGGGGCAGGTTCTTGGTGAGGC – 3'

Antisense: 5' – CAGGCGAATTTGGGGGAACAGG – 3'

Radioactive labeling of the DNA probe with [α -³²P]dCTP was performed using Klenow fragment DNA polymerase and the Megaprime DNA Labelling System (GE Healthcare, Fairfield, US) according to the manufacturer's instructions. Hybridization was performed overnight at 65°C. After thoroughly washing the membranes with SSC, the filters were wrapped in plastic foil and radioactive signals detected using a Typhoon Trio + phospho imager (Amersham, Piscataway, US).

4.5 Amino acid analysis

To determine changes in the alanine content of plants exposed to the various treatments, total amino acids were extracted with 10 ml of methanol:chloroform:water (12/5/3 v/v) per gram plant material (Bieleski and Turner, 1966). The aqueous phase was recovered after phase separation and individual amino acids were analysed as

their OPA derivatives by reverse-phase HPLC, as described previously (Puiatti and Sodek, 1999).

4.6 Aminotransferase assays

AlaAT enzyme activity was determined in various tissues of soybean plants after the treatments indicated in the text. Plant material was ground with a mortar and pestle in 5 volumes of 50 mM Tris/HCl pH 7.5 containing 1 mM DDT. All operations were carried out at 4°C. The homogenate was centrifuged at 10 000 x g for 20 min and an aliquot of the supernatant was desalted using a PD10 column (GE Healthcare, Buckinghamshire, UK). The eluted protein fraction was assayed for AlaAT (EC 2.6.1.2) essentially as described by Good and Muench (1992). The AlaAT assay contained, in a final volume of 3 ml, 10 mM L-alanine, 5 mM oxoglutarate, 0.1 mM NADH, 50 mM Tris-HCl (pH 7.5) and 5 units of lactate dehydrogenase (Sigma L5132). After adding extract to the reaction buffer, light absorbtion was determined at 30 sec intervals at 340 nm using a spectrophotometer Ultrospec 1000 (Pharmacia-Biotec, Cambridge, UK) with a temperature-controlled cuvetteholder at 30°C.

Total protein content of the enzyme extract was measured as described by Bradford (1976)

4.7 Semi-quantitative RT-PCR

In order to determine expression changes of *AlaAT* genes, plant material of soybean was ground in liquid nitrogen using a pestle and mortar. For RNA extraction 0.9 g of root or 0.6 g of nodule, leaf or pod was mixed with 3 mL of TRIzol Reagent

(Invitrogen, Carlsbad, USA) following the manufacturer's recommendations. The RNA samples were stored at -80°C until further processing. For the synthesis of cDNA, 5.0 µg of total RNA was first treated with Turbo DNA-free (Ambion, Austin, USA) to remove DNA contamination. Subsequently, cDNA was synthesized using oligo(dt) 12-18 primers and reverse transcriptase (Superscript II - Invitrogen, Carlsbad, USA). Subsequently, the cDNA was used as template for a polymerase chain reaction (PCR) using gene-specific primers for the various *AlaAT* homologues. Previous experiments were done to identify the proper cycle number at which non-saturated signal intensities were obtained for all ethidium bromide stained PCR products after agarose gel electrophoresis. The following program was used in the thermal cycler: an initial step of 3 min at 96°C, followed by 1 min at 94°C, 1 min at 60°C and 1 min at 72°C (30 cycles) with 5 min final extension at 72°C. The verification of amplification of the fragments was performed by separation in agarose gel 1% in TAE buffer. Transcript levels were normalized against actin mRNA.

The synthesis of the first strand of cDNAs was done without dilution for Generic, *GmAlaAt1*, *GmAlaAt2* and *Actin* primers, as well as concentrated 5 x (first PCR) and a second PCR carried out with the product of the first for *GmAlaAT3* primers.

For primer design, the option *PrimerSelect* of the program *Lasergene* (DNASTAR, Madison, USA) was used. For the construction of the generic and specific probes of AlaAT, primers were designed from the cDNA sequences from soybean, which are deposited in a public database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>). To ensure the specificity of the probes, the primers were designed

at the C-terminal side, where the largest differences between the homologues were observed (Supplementary Figure 1a). The primers used were:

Generic: Sense: 5' – CGGGGCAGGTTCTTGGTGAGGC – 3'

Antisense: 5' – CAGGCGAATTTGGGGGAACAGG – 3'

GmAlaAT1: Sense: 5' – CTTCTGCGCCACCGTCACCA – 3'

Antisense: 5' – CAGGGCTTGCACCATCAGTCAT – 3'

GmAlaAT2: Sense: 5' – TGGCCACAATTGAAGGACGAG – 3'

Antisense: 5' – GCATCAGCAGGGAATAGCAGT – 3'

GmAlaAT3: Sense: 5' – CTTGCAAACCTGGTTGGTGTTC – 3'

Antisense: 5' – GCATCAGCAGGGAATAGCAGT – 3'

Actin: Sense: 5' – GCTCCTAGGGCTGTCTTTCC – 3'

Antisense: 5' – CTCAGCAGAGGTGGTGAACA – 3'

5. RESULTS

5.1 *In silico* identification and characterisation of *AlaAT* genes in soybean

From the NCBI database 195 soybean ESTs were selected that showed homology with known *AlaAT* sequences from other plant species. By alignment of these 195 ESTs we identified 11 contigs, three of which were represented by 35 to 89 ESTs each and appeared to encode full coding sequences for *AlaAT* homologues. Southern blot hybridization confirmed this number of *AlaAT* homologues in soybean, since a maximum of three clear bands appeared after hybridization with a generic

probe for *AlaAT* (Fig 1). A very weak, fourth band could be observed in some of the genome digestions, but since its intensity was so much weaker than that of the other bands, it is likely that the sequence similarity between the genomic fragment and the probe was not very high for this band.

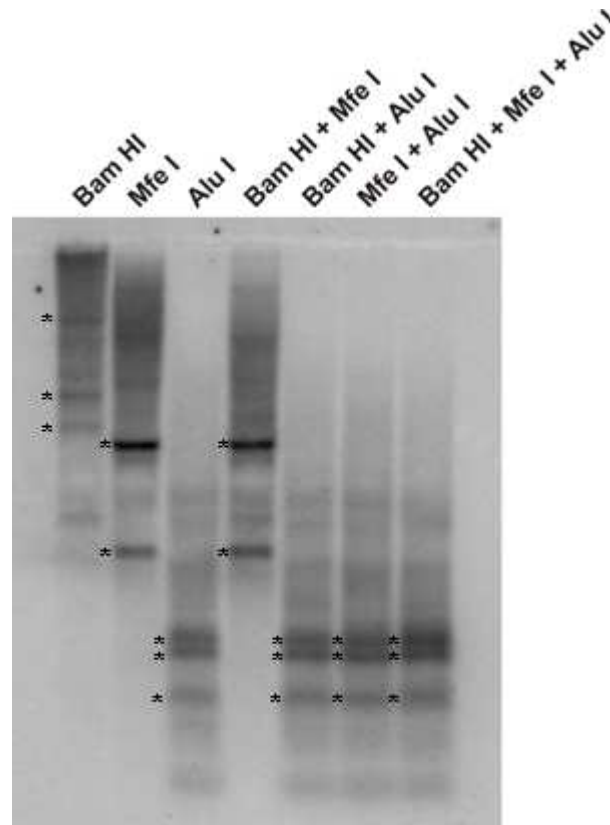


Fig 1. Southern blot analysis of Soybean plants. Genomic DNA was digested with BamHI, MfeI and / or AluI and hybridized with a 499 bp probe against *AlaAT*. BamHI, and MfeI did not cleave inside the coding sequence of the three *AlaAT* homologs that were identified for Soybean, whereas AluI did cleave inside the coding sequence of all three homologs, but outside the region for which the probe was designed. (*) indicate main bands observed after specific probe hybridization.

The three genes that were identified were named *GmAlaAT1*, *GmAlaAT2* and *GmAlaAT3*, and an alignment of the protein sequences is shown in Figure 2. The cDNA sequences (Supplementary Figure 1b, 1c and 1d) are deposited in the NCBI database with the following gene identification numbers: *GmAlaAT1* (EU165371), *GmAlaAT2* (EU165372), *GmAlaAT3* (EU165373).

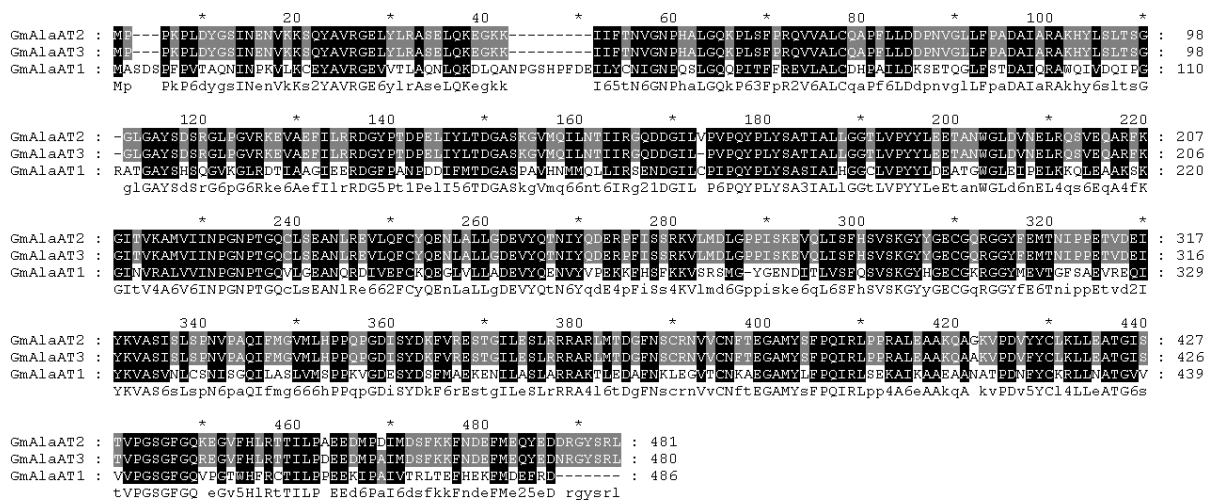


Fig 2. Isogene alignment of AlaAT from soybean adult plants. Multiple sequence alignment of Glycine max AlaAT isoforms run by GeneDoc (<http://www.psc.edu/biomed/genedoc>; Nicholas et al., 1997). Shaded boxes represent different levels of conservation among the AlaAT proteins in soybean (black=100%, grey=66%). A consensus amino acid sequence is also shown in the fourth row, where upper case letters indicate residues conserved among the three isoforms and lower case letters the residues conserved in two isoforms only.

Classification of the AlaAT homologues was done by a sequence analysis comparing all full length protein sequences currently present in the the databases for

AlaAT (Supplementary Figure II). The neighbour-joining phylogenetic tree that resulted from this analysis produced a clear separation into two sub-families (Fig 3).

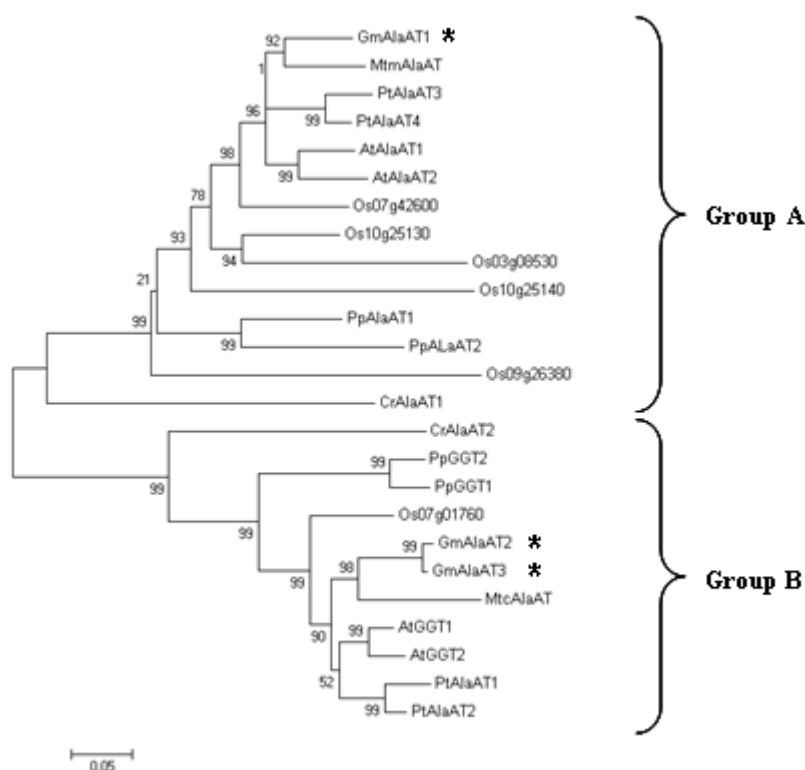


Fig 3. Phylogenetic tree of all known AlaAT proteins from various plant species.

Included in the phylogenetic tree are *Glycine max* GmAlaAT1 (ABW17196.1), GmAlaAT2 (ABW17197.1) and GmAlaAT3 (ABW17198.1); *Arabidopsis thaliana* AtAlaAT1 (AAF82782.1), AtAlaAT2 (NP_565040.2), AtAOAT1 (NP_564192.2) and AtAOAT2 (NP_177215.1); *Medicago truncatula* MtmAlaAT and MtcAlaAT (Ricoult et al., 2006); *Populus trichocarpa* PtAlaAT1 (XP_002315675.1), PtAlaAT2 (XP_002312679.1), PtAlaAT3 (XP_002331223.1) and PtAlaAT4 (XP_002304255.1); *Chlamydomonas reinhardtii* CrAlaAT1(XP_001695350.1) and CrAlaAT2 (XP_001698518.1); *Oryza sativa* Japonica Group Os07g42600 (EEE67593.1), Os10g25130 (NP_001064504.1), Os03g08530 (ABF94336.1), Os10g25140 (ABB47495.2), Os09g26380 (NP_001063248.1) and

Os07g01760 (NP_001058716.1); *Physcomitrella patens* subsp. *patens* PpAlaAT1 (XP_001769989.1), PpAlaAT2 (XP_001753102.1), PpGGT1 (XP_001777071.1) and PpGGT2 (XP_001782822.1). Phylogenetic analyses based on protein sequence were performed via MEGA4 (Kumar et al., 2008; Tamura et al., 2007) and CLUSTALW (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

Prediction of subcellular compartmentalization was done with four different algorithms (Bacello, TargetP, Ponce and PSORT) for *Glycine max* and all species cited in 4.3. Unfortunately the result was not clear, since for some of these proteins the 4 algorithms returned 4 different predictions (data not shown). Therefore, no conclusive prediction of the subcellular localization of the soybean homologues could be obtained.

5.2 Analysis of the expression of AlaAT in different organs of soybean

In order to check the general distribution of AlaAT in adult soybean plants we analysed the activity of AlaAT, determined the alanine content and measured the expression of the three soybean *AlaAT* homologues in roots, nodules, leaves and pods of both nodulated and non-nodulated plants. The amount of alanine varied strongly between the various organs, as well as between nodulated and non-nodulated plants (Fig 4 A). In non-nodulated plants, leaves contained the highest levels of alanine on a dry weight basis whereas in nodulated plants the pods had the highest alanine content.

The activity of AlaAT was similar in all tissues except for the roots where the activity was about half that of the other organs (Fig 4 B). Nodulation had little influence on the activity of AlaAT for any of the organs tested.

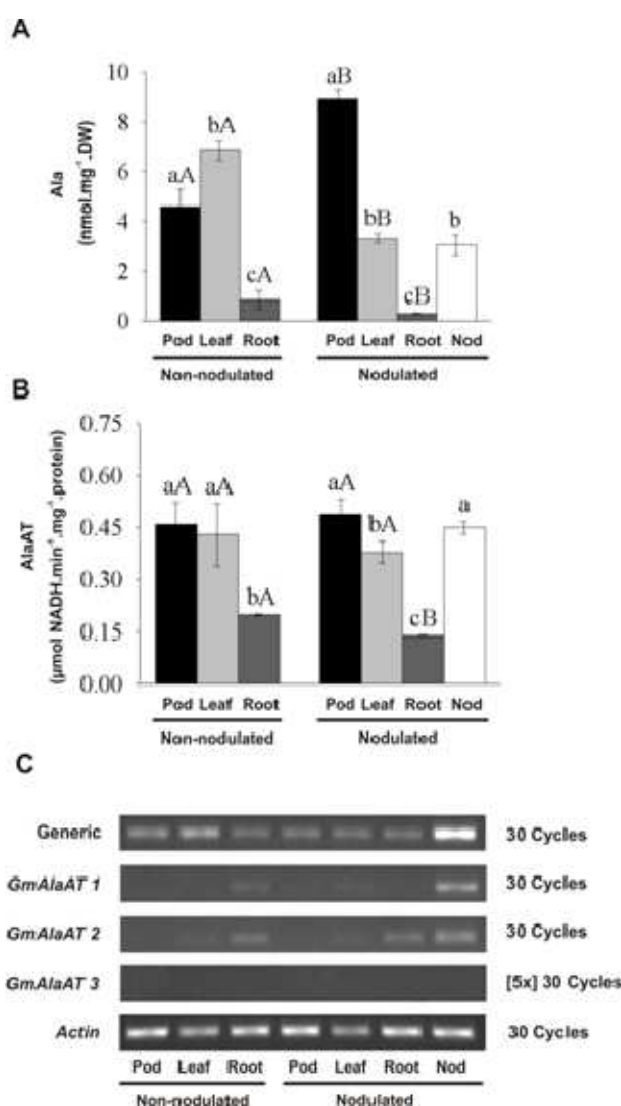


Fig 4. General distribution of AlaAT in soybean plants. Nodulated plants were cultivated in the absence of nitrate (totally dependent on symbiotic nitrogen fixation) and non-nodulated plants were fed with 15 mM KNO₃. A) Alanine content in different organs of soybean plants. B) AlaAT activity from extracts of different organs of soybean plants. C) Semiquantative SQ-RT-PCR analysis of *GmAlaAT1*, *GmAlaAT2* and *GmAlaAT3* expression in different tissues of

soybean plants. Both generic primers and homologue-specific primers were used to detect *AlaAT* mRNA. *Actin* was used as constitutive gene for normalization of the samples. Data represent the mean \pm standard deviation, $n = 4$. Means that differ significantly according to a one-way ANOVA ($P < 0.05$) followed by the Tukey test are marked with different letters. Capital letters indicate difference between cultivation condition for the same organ and small letters the difference between organs.

Tissue specific expression of the *AlaAT* homologues was determined by semi-quantitative reverse transcription PCR (SQ-RT-PCR). Four pairs of PCR primers were designed; three of them were specific for one homologue only, whereas the fourth pair was generic and could hybridize with all homologs. The analysis with the generic primers showed that *AlaAT* genes were expressed in all organs of the adult plant, independent of the nodulation status of the plant (Fig 4 C). Strongest expression was observed in the nodules. Analysis using the gene specific primers indicated that this expression signal is mainly due to *AlaAT1* and *AlaAT2* expression, whereas expression of *AlaAT3* was very hard to detect. Furthermore, *GmAlaAT1* and *GmAlaAT2* were both shown to have considerable expression levels in roots of both nodulated and non-nodulated plants, whereas in leaves and pods both gene homologues were only slightly expressed. *AlaAT3* was only very weakly expressed in all tissues investigated.

5.3 Effect of hypoxia on AlaAT in soybean

It has been shown previously (Sousa and Sodek, 2003) that hypoxia induces an increase in both the alanine content and the AlaAT activity in roots of non-nodulated soybean plants during hypoxia. To analyse which homologue is responsible for this hypoxic response, we characterized the gene expression of each during hypoxia for both non-nodulated and nodulated plants.

Prior to the waterlogging treatment, the content of alanine in root tissue was the same in both nodulated and non-nodulated plants, but during the 3-day period of hypoxia alanine increased 45-times in the roots of non-nodulated plants and only 4.6 times in root tissue of nodulated plants (Fig. 5). Independent of the nodulation status, the alanine values recovered completely over the 3-day period of re-oxygenation to the values from before the hypoxic treatment. The amount of Ala in the nodules itself did not change during or after the waterlogging treatment.

The changes in AlaAT activity as induced by waterlogging and the subsequent recovery period were similar to those changes observed for gene expression (Fig 5 B). In root tissue of non-nodulated plants hypoxic conditions induced AlaAT activity by about 2 times, and a three-fold increase was observed in roots from nodulated plants. During the recovery phase, the activity of AlaAT decreased slightly again, but even three days after the waterlogging treatment the activity of AlaAT remained about 1.5 to 1.9 times higher than the activity that was measured before the start of the experiment. In nodules, the activity of AlaAT did not change, neither during nor after waterlogging.

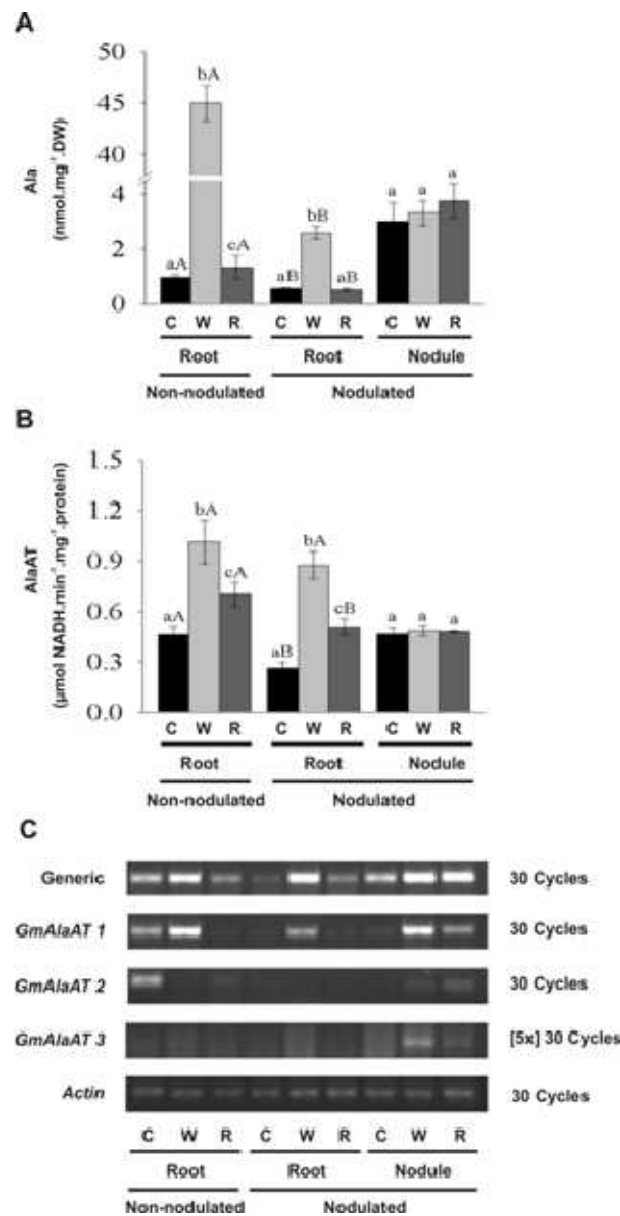


Fig 5. Hypoxic response in soybean plants. A) Alanine content in roots and nodule of soybean plants. C, control treatment; W, after 3 days of waterlogging; R, after 3 days of recovery from hypoxia. B) AlaAT activity in root extracts of soybean plants C) Analysis by SQ-RT-PCR of *GmAlaAT1*, *GmAlaAT2* and *GmAlaAT3* expression in roots of soybean plants. Both generic primers and homologue-specific primers were used to detect AlaAT mRNA. *Actin* was used as constitutive gene for normalization of the samples. All values represent the

means \pm standard deviation, $n = 4$. Means that differ significantly according to a one-way ANOVA ($P < 0.05$) followed by the Tukey test are marked with different letters. Capital letters indicate difference between cultivation condition for the same organ and small letters the difference between organs.

Expression analysis of the three AlaAT homologues revealed that in roots only the *GmAlaAT1* transcript increased during waterlogging and it declined to low levels during the recovery period (Figure 5 C). In nodules, the expression of *GmAlaAT1* increased strongly during waterlogging and the transcript did not decrease completely during the 3-day recovery period. In contrast to *GmAlaAT1*, the *GmAlaAT2* transcript decreased in roots during waterlogging, but in nodules, it increased slightly. No transcript of *GmAlaAT3* was detected in roots under any condition, but expression increased during waterlogging in nodules.

5.4 Effect of NO_3^- or NH_4^+ fertilisation on AlaAT in roots of non-nodulated soybean plants after waterlogging

To investigate the impact of nitrogen availability on the regulation of non-nodulated soybean root AlaAT during hypoxia, we characterized the expression pattern of *AlaAT* genes under hypoxic stress in presence or absence of either nitrate or ammonium. In roots of non-waterlogged plants, the content of alanine was relatively low and did not depend on the nitrogen availability of the plants (Fig. 6 A). However, during waterlogging the amount of Ala increased strongly from 0.74 before waterlogging to 23.97 nmol mg^{-1} DW during waterlogging in the absence of any N supply. In the presence of nitrate the Ala content increased from 1.21 to 78.05 nmol

mg⁻¹ DW whereas with ammonium the increase in Ala was even stronger, rising from 1.68 to 232.26 nmol mg⁻¹ DW (Fig 6 A). A similar tendency could be observed for the activity of AlaAT (Fig 6 B). No difference in AlaAT activity was observed between control plants (before waterlogging) that were treated with various N supplies. During waterlogging, the activity of AlaAT increased by a factor of 2 during hypoxia in absence of N, and it increased 2.5 times in the presence of NO₃⁻ and 2.6 times in the presence of NH₄⁺. These data indicate that the external source of nitrogen strongly affects alanine accumulation during waterlogging with a similar but relatively much smaller effect on AlaAT activity.

Generic primers for *AlaAT* genes reveal strong expression during hypoxia in all the nitrogen treatments, but there is little variation induced by the absence of N or NO₃⁻ fertilization (Fig 6 C). In presence of NH₄⁺ the expression of *AlaAT* is higher than with NO₃⁻. *GmAlaAT1* followed the same expression pattern as found for generic *AlaAT*. The expression of *GmAlaAT2* which was especially weak in the absence of NO₃⁻ increased slightly during waterlogging of plants in the absence of N supply, whereas in the presence of NO₃⁻ or NH₄⁺ expression of *GmAlaAT2* decreased during waterlogging. No expression of *GmAlaAT3* could be detected in any of the treatments tested.

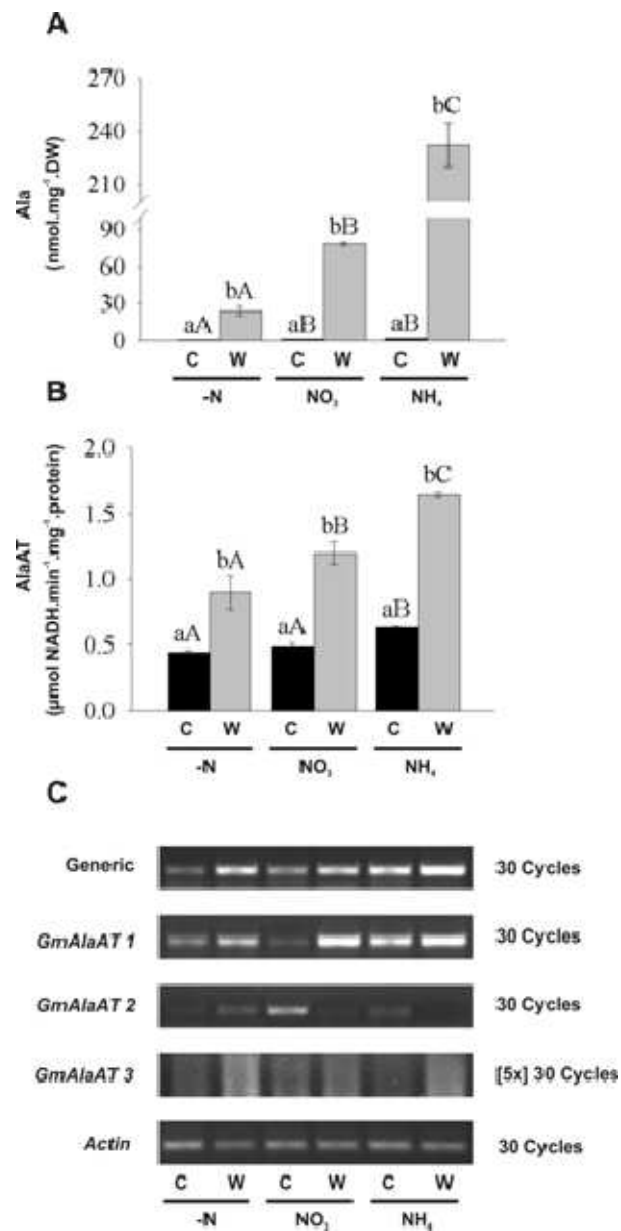


Fig 6. Hypoxic response in relation to different nitrogen fertilization conditions of non-nodulated soybean plants. A) Alanine content of roots of soybean plants B) AlaAT activity in root extracts of soybean plants. C) Analysis by SQ-RT-PCR of *GmAlaAT1*, *GmAlaAT2* and *GmAlaAT3* expression in roots of soybean plants. Both generic primers and homologue-specific primers were used to detect AlaAT mRNA. *Actin* was used as constitutive gene for normalization of the samples. Plants were given different nutrient solutions that contained

either nitrate (NO_3^-), ammonia (NH_4^+) or no nitrogen (-N). C, control samples taken after three days of feeding plants with different sources of nitrogen under normoxia conditions; W, samples taken after 3 days of feeding plants with different sources of nitrogen while being waterlogged. Values represent means \pm standard deviation, $n = 4$. Means that differ significantly according to a one-way ANOVA ($P < 0.05$) followed by the Tukey test are marked with different letters. Small letters indicate difference between normoxia and hypoxia condition within the same nitrogen treatment. Capital letters indicate difference between nitrogen treatment within the same condition (normoxia or hypoxia).

5.5 Effect of NO_3^- or NH_4^+ supply on AlaAT in roots of nodulated soybean plants during waterlogging

A similar experiment to that described above was carried out on nodulated plants, grown in the absence of mineral N. Again, Ala accumulated in roots during waterlogging (Fig 7 A), but levels were not as high as those determined for non-nodulated plants. In the absence of any external nitrogen supply, the amount of alanine increased during waterlogging by a factor of 11, rising from 0.60 to 6.85 nmol mg^{-1} DW. In the presence of NO_3^- during waterlogging, Ala accumulation was some 24 times greater, rising from 0.74 to 17.57 nmol mg^{-1} DW, and 22.3 times greater when roots were supplemented with NH_4^+ (2.32 to 51.65 nmol mg^{-1} DW). In nodules no significant change in the amount of alanine was found for plants without nitrogen supply, as well as in plants supplemented with NO_3^- . However, nodules from plants fed with ammonium showed a 17-times increase in Ala concentration during waterlogging. These data can explain a similar relationship between N source and

Ala production found by Thomas and Sodek (2006) with respect to xylem sap Ala transport in soybean plants during waterlogging.

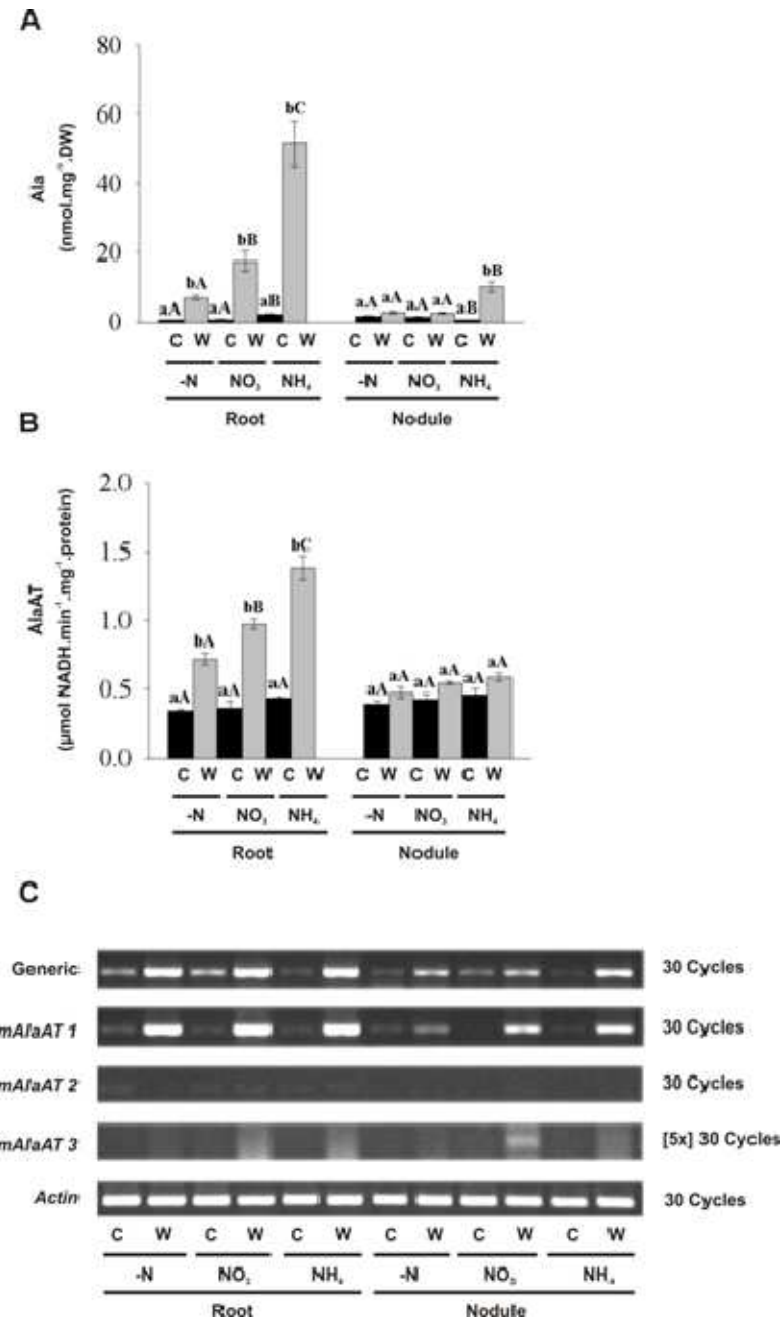


Fig 7. Hypoxic response in relation to different nitrogen fertilization conditions of nodulated soybean plants. A) Alanine content of the root system of soybean plants cultivated in vermiculite without mineral N (nodulated plants). The root systems were flooded (W) with

nutrient solutions containing different sources of nitrogen: nitrate (NO_3^-), ammonia (NH_4^+) and no nitrogen (-N). B) AlaAT activity in root extracts of soybean. C) Analysis by SQ-RT-PCR of *GmAlaAT1*, *GmAlaAT2* and *GmAlaAT3* expression in roots of soybean plants. Both generic primers and homologue-specific primers were used to detect AlaAT mRNA. *Actin* was used as constitutive gene for normalization of the samples. C, samples taken before the waterlogging treatment; W, samples taken after 3 days of waterlogging; -N, treatment without no nitrogen supply. Values represent means \pm standard deviation, $n = 4$. Means that differ significantly according to a one-way ANOVA ($P < 0.05$) followed by the Tukey test are marked with different letters. Small letters indicate difference between normoxia and hypoxia condition within the same nitrogen treatment. Capital letters indicate difference between nitrogen treatment within the same condition (normoxia or hypoxia).

The change in activity of AlaAT as induced by waterlogging was very similar between nodulated and non-nodulated plants. Upon waterlogging, AlaAT activity in nodulated plants increased 2 times in the absence of any external nitrogen source, whereas in the presence of NO_3^- an increase of 2.7 times was observed, and increased 3.8 times in the presence of NH_4^+ (Fig 7 B).

Independent of the nitrogen treatment, the level of the *GmAlaAT1* transcript in both roots and nodules showed a strong increase during hypoxia (Fig 7 C). *GmAlaAT2* was expressed at a negligible level in all conditions tested. The expression level of *GmAlaAT3* was very low in all treatments with an exception for nodules of plants treated with NO_3^- during waterlogging.

6. DISCUSSION

The function of AlaAT in soybean was investigated. Analysis of the available EST database information revealed that at least three different homologues are encoded by the soybean genome. Sequence analysis with all available plant homologues known to date revealed a subdivision of the gene family into two major subclasses. One of these subclasses contained one of the soybean homologues *GmAlaAT1*, whereas both *GmAlaAT2* and *GmAlaAT3* were included in the second subclass.

A detailed functional analysis of *Arabidopsis* homologues revealed that various AlaAT isoenzymes have different substrate selectivity (Igarashi et al., 2003; Liepman and Olsen, 2003). In *Arabidopsis*, four *AlaAT* isogenes have been described. Two of them, *AtAlaAT1* and *AtAlaAT2* encode true alanine aminotransferases, and two others, *AtGGAT1* and *AtGGAT2*, were shown to have glutamate: glyoxylate aminotransferase (GGAT) activity also (Igarashi et al., 2003; Liepman and Olsen, 2003). It would be interesting to learn whether this functional division can be extended to the other members of the gene subclasses but this would require the determination of the substrate specificity of each of the cited enzymes.

Using generic primers to investigate *AlaAT* expression it was found that the expression of this gene was very similar for all organs analyzed except for nodules which showed a much higher expression level. Notably, changes in the activity of the enzyme did not correlate with the level of transcript, as revealed by the generic primer analysis. Apparently, posttranscriptional regulation of AlaAT is important for the

proper regulation of the activity of the enzyme. This is in agreement with earlier studies on *Arabidopsis* showing that gene expression and activity of most enzymes of primary metabolism do not correlate (Blaesing et al. 2005).

It is well established that root AlaAT is highly activated upon hypoxia (Sousa and Sodek, 2003; Ricoult et al., 2006; Good et al., 2007; Miyashita et al., 2007; Beatty et al., 2009; Rocha et al., Chapter 1). In this study the hypoxic response of AlaAT was shown to be limited to the isoform *GmAlaAT1*. Three days after the return from waterlogging, no transcript of *AlaAT* could be detected. In addition, the level of alanine declined rapidly to its value before waterlogging. Furthermore, AlaAT enzyme activity decreased. However, this activity remained at a level that was significantly above that found before waterlogging. Probably, during the recovery period, AlaAT catalyses the reverse reaction, thus helping to decrease the amount of alanine that was stored during waterlogging. Indeed, using ¹⁵N-isotope labeling of glutamate and alanine, Ricoult et al. (2006) showed for *Medicago truncatula* that the activity of AlaAT during hypoxia acts in the direction to form alanine, whereas the reaction was reversed during normoxia. This would help making the carbon and nitrogen that was stored as alanine during hypoxia rapidly available for the plant after the hypoxic stress was over (Sousa and Sodek, 2003; Miyashita et al., 2007).

An alternative explanation for the role of AlaAT during hypoxia was raised by Drew (1997) who suggested that accumulation of alanine could improve tolerance to hypoxia via the activation of the glycolytic flux which increases the amount of ATP produced by the glycolytic pathway. A similar mechanism is described by the so-called Pasteur Effect when ethanol or lactate fermentation is activated. However, a bottleneck of this reaction is the depletion of NAD⁺. This reaction would therefore only

be helpful for the production of ATP, as long as NAD^+ remains available. However, Rocha et al. (Chapter 1) described an extended reaction pathway which suggested that the production of alanine could be directly linked to the TCA cycle via ketoglutarate, which is produced concomitantly with Ala by AlaAT. This would allow the plant to save NAD^+ consumption in the TCA cycle by isocitrate dehydrogenase, but retains the production of ATP by oxoglutarate dehydrogenase.

Further, the importance of AlaAT activity during hypoxia was stressed by Gupta et al. (2009) and Zabalza et al (2009). They suggested that the production of Ala from pyruvate could be important for regulating the level of pyruvate, as it was shown that high concentrations of pyruvate can lead to the activation of respiratory oxygen consumption, e.g. via the activation of the alternative oxidase in the mitochondria. Especially during hypoxia, the consumption of oxygen should be reduced to a minimum. Therefore mechanisms to prevent pyruvate accumulation are required.

In this way alanine can be involved in a series of events that are advantageous to the plant during hypoxia. In contrast to the fermentative production of ethanol, the accumulation of alanine would reduce the loss of carbon during hypoxia, as Ala can be stored at high concentrations without detrimental side-effects. Furthermore, high amounts of alanine were found in the xylem of waterlogged plants (Sousa and Sodek, 2003) indicating not only that even higher amounts of Ala are produced in the hypoxic roots but that Ala probably acts as an agent to recycle carbon and nitrogen from the hypoxic root to the normoxic shoot. This would help to improve plant tolerance during hypoxia.

To investigate the effect of the nitrogen source on the activation of AlaAT during waterlogging, we investigated the expression and activity of AlaAT in both nodulated and non-nodulated plants that received various nitrogen supplements. In general, the amounts of Ala that were measured in non-nodulated plants was much higher than in nodulated plants. This is likely explained by the NO_3^- fertilization of these plants during growth resulting in the accumulation of NO_3^- in root tissue followed by its mobilization during hypoxia (Brandão and Sodek, 2009). However, in both nodulated and non-nodulated plants, the accumulation of Ala was lowest in plants that were given no further N supplement during waterlogging, whereas the highest levels of Ala were found in waterlogged plants that obtained ammonium as N-substrate. Apparently, the activity of AlaAT can be regulated post-transcriptionally. The high rate of Ala production during hypoxia in the presence of NH_4^+ has also been observed by Vanlerberghe and Turpin (1990). They suggested that the synthesis of Ala is part of an NH_4^+ detoxification mechanism without changing the energy or the redox potential of the cell. This could also explain our results. In either case, the correlation between AlaAT activity and Ala accumulation that we observed does indicate that AlaAT can be responsible for the accumulation of Ala during waterlogging.

It should be noted, however, that the role of AlaAT is not limited to hypoxia. By measuring the time-course of both AlaAT activity and Ala in the roots of soybean plants during waterlogging and on return to normoxia, Sousa and Sodek (2003) observed that much of the increase in AlaAT activity during waterlogging occurred after most of the increase in Ala had occurred, while the decrease in Ala on return to normoxia was very rapid, coinciding with the elevated AlaAT levels. They concluded

that, although AlaAT may well be responsible for Ala formation, the actual increase in activity during waterlogging had a limited role in Ala accumulation but a very important role in the utilization of Ala when plants recover from the stress induced by waterlogging. This conclusion was confirmed by Miyashita et al. (2007) who showed a retardation of the decrease in Ala after waterlogging of an *Arabidopsis* AlaAT1 null-mutant in which the overall AlaAT activity was drastically reduced. This indicates that Ala can accumulate during waterlogging even if the capacity of AlaAT is reduced, but that during the recovery phase a decrease in AlaAT capacity becomes detrimental.

Summarizing our data, evidence is provided here that Soybean has three AlaAT homologues which can be categorized in two different gene sub-families. The first subfamily contains homologues similar to GmAlaAT1, whereas GmAlaAT2 and 3 cluster together in the second subfamily. During hypoxia, only the expression of GmAlaAT1 is induced, but the activity of AlaAT under hypoxia varies depending on the nitrogen source. The activation of AlaAT during waterlogging and the concomitant accumulation of Ala indicate a role of AlaAT in hypoxic metabolism. Nevertheless, it should be considered that the enzyme catalyses an equilibrium reaction and thus AlaAT could also play a role in Ala breakdown during the recovery from waterlogging stress. Further research, including metabolic flux analysis, will be required in the future to understand the impact of the nitrogen source on Ala metabolism during waterlogging and recovery.

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8. SUPPLEMENTARY TABLE

Supplementary Figure 1a. Nucleotide sequence of cDNA clone of AlaAT isogenes from *Glycine max*. Start and Stop codons are highlighted in red. The 3' and 5' untranslated region are highlighted in blue. The primers utilized in this work are shaded in green (forward) and yellow (reverse).

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GmAlaAT3 -----
GmAlaAT2 CACACACTCTTCAATCAAATTGAAGCAGAAAGCCAAAGGCCAGAGAGACCCCTGTTATCT
GmAlaAT1 -----

GmAlaAT3 -----TCTATTATTTGCAAACTGGTGGCTT
GmAlaAT2 ACGGAGAGACCCCTTACACTCCAAAGCTTGGCAAAATTGAAGAGCAACATTATCCCTTT
GmAlaAT1 -----

GmAlaAT3 ATTTGTTAAGCCATATGTTATATTCCTTTAAGTATTCTGAGTCTACATATTTGATGAA
GmAlaAT2 GGATTAATAAAGCCATCTCAAATCTCAATTCCAATAATCCG--TTAGCACTTTTTTCAGT
GmAlaAT1 -----

GmAlaAT3 GGTTACTGATTAAGTATTCAGAGAAGTTC-CTAATTAAGGAGTCTAAGTCTACATATCAG
GmAlaAT2 GGCT-CTGGT---GCTGCCATTGCGTTCACCAACCGCCACTGCCACACATACACTTCAC
GmAlaAT1 -----

GmAlaAT3 TATTACTTCACTAGTTAGCTTGAATTCTGTTATGAAGAAATCTAAGTTCCTTAACAGA
GmAlaAT2 TACAGACAA-ATAAATT---TCACACTCTTTTGTCTGACACGTATAACATTCTGAACATA
GmAlaAT1 -----CACCTTTTCAACCGTT-CACTCATTTTGGGGGAGG
                *   *   *               *   *   *   *

GmAlaAT3 AAACACTTCACATGTAGTAGAGTAAATGAGCAACACTCTTCTTCAATCAAGCGAAGGTGG
GmAlaAT2 AACTACTTGGTTTTTTCCACACCCGAAT-----ACTCTTCTTCAATCAAGCGAAGGTGG
GmAlaAT1 AATCAACCTTCACCATCCCTTTCTTCT-----TCTTCTTCTCTTCCCCA---TTT
                **           *           *           *   *   *   *   *

GmAlaAT3 TCTC-----GATTGCAAGTGATCCACGATACACTCTACGCTTCACAAAACCTTCAA
GmAlaAT2 TCTCACTTGACTCGATTGCAAGTGATCCACGACACACTGTTTCGCTTCACAAAAGCTTCGA
GmAlaAT1 TCTC-----GCTCTCGTTTCTTCTCTCTACGCCGTTCTC---CATGGCTTCTGA
                ***           *   *   *   *   *   *   *   *   *   *   *   *

GmAlaAT3 ACCCAATTCCACAG-----AATTCTTCTGTTCACCATGCCACCAAGCCTT
GmAlaAT2 ATCCAATTCCCTCAGTAACATCTGCAAGAAGTCTTCTCCTTCACCATGCCACCAAGCCTT
GmAlaAT1 TTCCCTTTTCTTG-----TCACCGCTCAAAACATCAACCCCAAGGTTT
                **   **   *   *           **   *           *   *   *   *

GmAlaAT3 TAGACTATGGGTCAATAAATGAAAACGTGAAGAAGAGTCAATATGCTGTCAGAGGTG--A
GmAlaAT2 TAGACTATGGGTCAATAAATGAAAACGTGAAGAAGAGTCAATATGCTGTCAGAGGTG--A
GmAlaAT1 TGAAATGTGAGTATGCTGTTAGA-----GGAGAGGTTGTACACTTGCCAGAAATTTGCA
                *   *   *   *   *   *   *   *   *   *   *   *   *

GmAlaAT3 ATTATACCTTCGAGCTTCTGAACTTCAGAAAGAGGGCAAAAAGATTATCTTTACTAATGT
GmAlaAT2 ATTATACCTTCGAGCTTCTGAGCTTCAGAAAGAGGGCAAAAAGATTATATTACTAATGT
GmAlaAT1 AAAGGATTTACAGGCCAATCCAGGCTCTCACCATTGATGAGATACTTTACTGCAACAT
                *           *   *   *   *   *   *   *   *   *   *

GmAlaAT3 TGGCAACCCGCATGCATTGGGACAGAAACCACTGAGCTTCCCTCGCCAGGTTGTTGCTTT
GmAlaAT2 TGGCAACCCACATGCATTGGGACAGAAACCACTGAGCTTCCCTCGTCAGGTTGTTGCTTT
GmAlaAT1 TGGAAATCCTCAGTCTCTGGGCCAGCAGCCAATAACTTTTTTCCGAGAGGTTCTTGCAAT
                ***   **   *   *   *   *   *   *   *   *   *   *   *

GmAlaAT3 GTGCCAAGCTCCATTCTACTTGATGATCCTAATGTTGGACTGCTATTCCTGCTGATGC

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GmAlaAT3 GAAGAAGAGCAAGGCTAATGACTGATGGATTCAACAGTTGCAGAAATGTGGTTGTAATT
GmAlaAT2 GAAGAAGAGCAAGGCTAATGACTGATGGATTCAACAGTTGCAGAAATGTGGTTGTAATT
GmAlaAT1 CCAGGCGTGCAAAGACACTAGAAGATGCATTCAACAAATTAGAGGGTGAACATGCAACA
* *

GmAlaAT3 TTACTGAAGGTGCCATGTATTTCCTCCTCAAATACGTTTGCCGCCAAGAGCTTTAGAGG
GmAlaAT2 TTACTGAAGGTGCCATGTATTTCCTCCTCAAATACGTTTGCCGCCAAGAGCTTTAGAGG
GmAlaAT1 AAGCAGAAGGGGCAATGTA**CCTGTTCCCCCAAATTCGCCTG**TCCGAAAAGGCTATCAAG
* *

GmAlaAT3 CTGCTAAACAGGCTGCAAAGGTTCCAGATGTTTCTACTGCCTCAAACCTTTG---GAAG
GmAlaAT2 CTGCTAAACAGGCTGCAAAGGTTCCAGATGTTTCTACTGCCTCAAACCTTTG---GAAG
GmAlaAT1 CTGC---AGAGGCTGCAAATGCAACACCTGATACTTCTATTGCAAACGCTTGCTTAATG
* *

GmAlaAT3 CCACTGGCATATCCACTGTTCTCTGGTTTCCAGATTGGACAGAGAGAAGGGGTCTTCCATT
GmAlaAT2 CCACTGGCATATCCACTGTTCTCTGGTTTCCAGATTGGTCAGAAAGAAGGGGTCTTCCATT
GmAlaAT1 CCACAGGAGTCGTTGTTGTTCTCTGGTTTCTGGTTTGGACAGGTTCTTGGCACATGGCATT
* *

GmAlaAT3 TGAGGACTACTATTTTACCAGACGAGGAAGACATGCCCGCTATTATGGATAGTTTCAAAA
GmAlaAT2 TGAGGACAACTATTTTACCAGCTGAGGAAGACATGCCCTGATATTATGGATAGTTTCAAAA
GmAlaAT1 TTAGGTGCACCATATTGCCTCCAGAAGAAAAGATTCCGGCCATCGTCACCCGCTTGACTG
* *

GmAlaAT3 AGTTCAATGATGAATTCATGGAGCAATACGAAGACAATAGAGGTTATTCAAGGTTGTAAG
GmAlaAT2 AGTTCAATGATGAATTCATGGAGCAATACGAAGACGATAGAGGTTATTCAAGGTTGTAAG
GmAlaAT1 AGTTCCATGAAAAATTCATGGATGAGTTCCGTGAC**TA-AGTCTACTTGAATTTATCCG**
* *

GmAlaAT3 AAAATTAT-----CGTAAAGATGCATTATACTCTTGTGCTTTTGTAAG-----
GmAlaAT2 AAAATCAT-----TGTAAGGCTGCATTATACTCTTGCCTTTTGGAAGTTCTTCATC
GmAlaAT1 TCAATTTTACGGGTACCCAATTGATTTTGTGAGCGATGGATTTTGATGTTAGAGAAAA
* *

GmAlaAT3 -----AA-AAACATAACCTCCCTTTCTACTATTTGTCCT---T
GmAlaAT2 CATCCATCCCTCGGAGAGAAAA-AAACATAACCTCCCTTTCTACTATTTGTCCTTCATT
GmAlaAT1 TAGTCTCTTTTGTTCAGGAATAAATTGTCAACTCCTTATATATTGTCATCTTGAA
* *

GmAlaAT3 GGAGATAATTCTTAAACCTTACACTTTCTTCTATATCATCTCTGAACCTTACAATA-----
GmAlaAT2 GGAGATTATTCTTAAACCTTACACTTTCTTCTATATCATCTCTGAACCTTACAATATTTCG
GmAlaAT1 TATGCAAGGCGTGAAAAGATATTGGTTTTCTCCATTCTATAAAAAAAAAAAAAA---A
* *

GmAlaAT3 -----AAA---AATTTCGGCTG-----CAAAAAAAAAAAAA-----
GmAlaAT2 GCTGTATATTTAATCTCTCTTGAACCTTTTATCAAAAAAAAAAAAAATGAAAAAATCAAAA
GmAlaAT1 AATATAAATCTCGTTGTAATCGAATAAAGCTTCGTATTAGAATAAAAAAAAAAAAA-----
* *

GmAlaAT3 -----
GmAlaAT2 AAAATCGTCTTGAATGTGATGGTTTTCGTCATGTGTTAAATGTCCTAAGAAAAAGATT
GmAlaAT1 -----

GmAlaAT3 -----
GmAlaAT2 TGACATAGATATATATAAGGTCTAGATATGTTTTTTGTCTTGGAAGTTAGTGTTTTTT
GmAlaAT1 -----

GmAlaAT3 -----
GmAlaAT2 TTATTCGTAA
GmAlaAT1 -----

Supplementary Figure Ib. Nucleotide sequence of cDNA clone GmAlaAT1 from *Glycine max*.

GmAlaAT1 (EU165371) (bankit1019367)

1	CACCTTTTCAACCGTTCACTCATTCTTCTGCGCCACCGTCACCATCACCATCCCTTTCCTTCTTCTTCTCTCTCCCC	80
	M A S D S P F P V T A Q N	
81	ATTTTCTCGCTCTCGTTTCTTCTCCTCTACGCCGTTCTCCATGGCTTCTGATTCCCTTTTCTGTACCGCTCAAAACA	160
	I N P K V L K C E Y A V R G E V V T L A Q N L Q K D L	
161	TCAACCCCAAGGTTCTGAAATGTAGTATGCTGTAGAGGAGAGGTTGTCACACTTGCCCAGAATTGCAAAAGGATTTA	240
	Q A N P G S H P F D E I L Y C N I G N P Q S L G Q Q P	
241	CAGGCCAATCCAGGCTCTCACCCATTGATGAGATACTTTACTGCAACATTGGAATCCTCAGTCTCTGGGCCAGCAGCC	320
	I T F F R E V L A L C D H P A I L D K S E T Q G L F	
321	AATAACTTTTTTCCGAGAGGTTCTTGCAATTATGTGACCATCCAGCTATATTAGACAAAAGTGAACACAGGGTTTGTTC	400
	S T D A I Q R A W Q I V D Q I P G R A T G A Y S H S Q	
401	GTACCGACGCAATACAGCGAGCATGGCAGATTGTGGATCAGATTCTGGGAGAGCAACTGGTGCCTATAGCCATAGTCAG	480
	G V K G L R D T I A A G I E E R D G F P A N P D D I F	
481	GGTGTCAAGGGCTTGGCTGATACAATAGCTGCTGGAATTGAAGAGCGTGATGGTTTTCTGCCAATCCTGATGACATTTT	560
	M T D G A S P A V H N M M Q L L I R S E N D G I L C	
561	CATGACTGATGGTGAAGCCCTGCTGTCCATAATATGATGCAATTACTCATTAGATCAGAAAATGATGGTATTCTGTGTC	640
	P I P Q Y P L Y S A S I A L H G G C L V P Y Y L D E A	
641	CCATTCCACAGTACCCCTCTGTACTCAGCCTCAATTGCCCTCCATGGTGGCTGCCTGGTACCTTATTATCTAGATGAAGCA	720
	T G W G L E I P E L K K Q L E A A K S K G I N V R A L	
721	ACAGGCTGGGGGTTGGAATACCTGAACCAAGAAGCAATTGGAGGCTGCCAAGTCTAAGGGCATCAATGTTAGGGCTTT	800
	V V I N P G N P T G Q V L G E A N Q R D I V E F C K	
801	AGTTGTTATAATCCTGGCAATCCAACGGGGCAGGTTCTTGGTGAGGCAAATCAGCGAGATATAGTAGAGTTTGAAGC	880
	Q E G L V L L A D E V Y Q E N V Y V P E K K F H S F K	
881	AAGAAGGTTTGGTTCTTTTAGCTGATGAGGTATATCAAGAAAACGTTTATGTTCTGAGAAGAAATTCACCTTTTCAAG	960
	K V S R S M G Y G E N D I T L V S F Q S V S K G Y H G	
961	AAGGTATCTCGGTCCATGGGATATGGTGAGATGATACACCTTAGTATCTTTCAATCAGTCTCCAAAGGCTATCACGG	1040
	E C G K R G G Y M E V T G F S A E V R E Q I Y K V A	
1041	GGAGTGTGGGAACGAGGAGGTTATATGGAGGTGACTGGGTTTTCTGCAGAAGTGAGGGAACAAATATATAAAGTGGCAT	1120
	S V N L C S N I S G Q I L A S L V M S P P K V G D E S	
1121	CTGTCAACCTTTGCTCTAATATCTCTGGTCAATTCTTGCAAGCTTGGTCATGAGTCCACCCAAGGTTGGAGATGAGTCC	1200
	Y D S F M A E K E N I L A S L A R R A K T L E D A F N	
1201	TATGACTCATTGCTGAGAGGAGAATATTTTGGCGTCCCTTGCCAGGCGTGCAAAGACACTAGAAGATGCATTCAA	1280
	K L E G V T C N K A E G A M Y L F P Q I R L S E K A	
1281	CAAATTAGAGGGTGTAACATGCAACAAAGCAGAAGGGCAATGTACCTGTTCGCCAAATTCGCCTGTCCGAAAAGGCTA	1360
	I K A A E A A N A T P D N F Y C K R L L N A T G V V V	
1361	TCAAAGCTGCAGAGGCTGCAATGCAACACCTGATAACTTCTATTGCAAACGCTTGCTTAATGCCACAGGAGTCGTTGTT	1440
	V P G S G F G Q V P G T W H F R C T I L P P E E K I P	
1441	GTTCTCGGTTCTGGTTTGGACAGGTTCTGGCACATGGCATTTTAGGTGCACCATATTGCCTCCAGAAGAAAAGATTCC	1520
	A I V T R L T E F H E K F M D E F R D *	
1521	GGCCATCGTCACCCGCTTGACTGAGTTCCATGAAAATTGATGGATGAGTTCCGTGACTAAGTCTACTTGAATTTTATC	1600
1601	CGTCAATTTTACGGGTACCCAATTGATATTTGATGAGCGATGGATTTTGTAGTTAGAGAAAATAGTCTCTTTTGTTC	1680
1681	AGGAATAAATTTGTCAACTCCTTATTATATTGCACTCTTGAATATGCAAGGCGTGAAAAGATATTGGTTTCTCCATT	1760
1761	TTATAAAAAAAAAAAAAAAAAAATAAATCTCGTTGTAATCGAATAAACTTCGTATTAGAATAAAAAAAAAAAAAA	1833

Supplementary Figure 1c. Nucleotide sequence of cDNA clone GmAlaAT2 from *Glycine max*.

GmAlaAT2(EU165372) (bankit1019379)

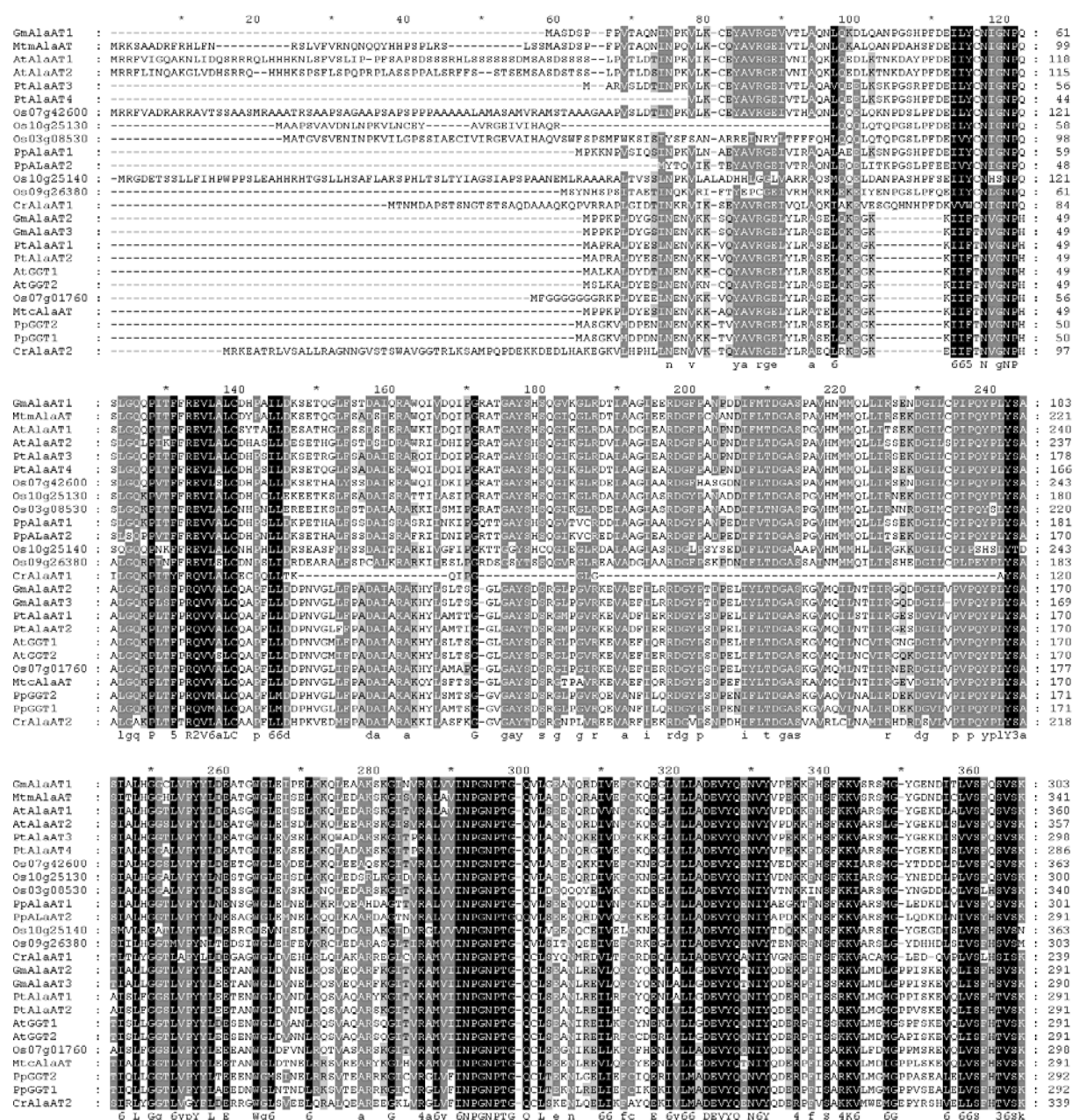
1	CACACACTCTTCAATCAAATTGAAGCAGAAAGCCAAAGGCCAGAGAGAGCCCTGTTATCTACGGAGAGACCCTTACACTC	80
81	CAAAGCTTGGCCACAATTGAAGGACGAGCATTATCCCTTTGGATTAAATAAGCCATCTCAAATCTCAATTCCAATAATCC	160
161	GTTAGCACTTTTTCAGTGGCTCTGGTGTGCCATTGCGTTCCACCAACCGCCACTGCCACACATACACTTCACTACAGA	240
241	CAAATAAATTTACACTCTTTTGTCTGACACGTATAACATTCTGAATAAACTACTTGGTTTTTCCCACACCGAATACT	320
321	CTTCTTCAATCAAGCGAAGGTGGTCTCACTTGACTCGATTGCAAGTGATCCACGACACACTGTTTCGCTTCACAAAAGCTT	400
M P P K P L D Y G S I		
401	CGAATCCAATTCTCTCAGTAACATCTGCAAGAAGTCTTCTCCTTACCATTGCCACCAAAGCCTTTAGACTATGGGTCAATA	480
N E N V K K S Q Y A V R G E L Y L R A S E L Q K E G K		
481	AATGAAAACGTGAAGAAGAGTCAATATGCTGTGAGAGGTGAATTATACCTTCGAGCTTCTGAGCTTCAGAAAGAGGGCAA	560
K I I F T N V G N P H A L G Q K P L S F P R Q V V A		
561	AAAGATTATATTACTAATGTTGGCAACCCACATGCATTGGGACAGAAACCACTGAGCTTCCTCGTCAGGTTGTTGCTT	640
L C Q A P F L L D D P N V G L L F P A D A I A R A K H		
641	TGTGCCAAGCTCCATTCTACTTGATGATCCTAATGTTGGACTGCTATTCCCTGCTGATGCAATTGCAAGAGCTAAACAC	720
Y L S L T S G G L G A Y S D S R G L P G V R K E V A E		
721	TATCTTTCATTGACCTCGGGCGGTTTAGGCGCTTATAGTGACTCCGCGGCCTTCCAGGAGTGAGGAAGGAAGTGGCTGA	800
F I L R R D G Y P T D P E L I Y L T D G A S K G V M		
801	GTTCACTACTCAGGCGTGATGGTTATCCAACCTGATCCAGAACTCATATATCTCACTGATGGTCCAGCAAGGGGGTGATGC	880
Q I L N T I I R G Q D D G I L V P V P Q Y P L Y S A T		
881	AGATATTAATACTATCATCAGAGGTCAAGACGATGGGATTTTGGTTCCAGTCCACAATACCCACTCTACTCAGCAACA	960
I A L L G G T L V P Y Y L E E T A N W G L D V N E L R		
961	ATTGCTCTGCTTGGTGGTACCCTTGTTCATACTACCTTGAAGAGACAGCAAATTGGGGTCTTGATGTTAATGAACCTCG	1040
Q S V E Q A R F K G I T V K A M V I I N P G N P T G		
1041	TCAATCAGTTGAGCAGGCTCGCTTTAAAGGAATAACTGTTAAAGCAATGGTGATCATAAATCCTGGAAACCTACTGGTC	1120
Q C L S E A N L R E V L Q F C Y Q E N L A L L G D E V		
1121	AATGCCTTAGTGAAGCTAATCTAAGAGAGGTTTGAATCTGTTATCAAGAAAATTTAGCCTTGCTTGGAGATGAGGTT	1200
Y Q T N I Y Q D E R P F I S S R K V L M D L G P P I S		
1201	TACCAGACAAATATATATCAGGATGAACGACCTTCATTAGTTCTAGAAAAGTTTTGATGGACTTGGGGCCACCTATAAG	1280
K E V Q L I S F H S V S K G Y Y G E C G Q R G G Y F		
1281	CAAGGAAGTCCAGCTTATTTCTTTTCACTCCGTGTCAAAGGTTATTATGGTGAATGTGGACAGCGAGGTGGATATTTTG	1360
E M T N I P P E T V D E I Y K V A S I S L S P N V P A		
1361	AAATGACCAACATTCTCCAGAGACAGTTGATGAGATCTACAAGGTTGCATCAATATCACTTAGTCCAAATGTTCCAGCA	1440
Q I F M G V M L H P P Q P G D I S Y D K F V R E S T G		
1441	CAAATATTTATGGGAGTTATGCTCCATCCACCTCAACCTGGAGATATTTCTTATGACAAATTTGTTAGGAGAGACACCGG	1520
I L E S L R R R A R L M T D G F N S C R N V V C N F		
1521	AATACTTGAATCACTGAGAAGAAGAGCAAGGCTAATGACTGATGGATTCAACAGTTGCAGAAATGTGGTTTGTAAATTTTA	1600
T E G A M Y S F P Q I R L P P R A L E A A K Q A G K V		
1601	CTGAAGGTGCCATGTATTCAATCCCTCAAATACGTTTGCCGCCAAGAGCTTTAGAGGCTGCTAAACAGGCTGGAAAGGTT	1680
P D V Y Y C L K L L E A T G I S T V P G S G F G Q K E		
1681	CCAGATGTTTACTACTGCCTCAAACCTTTTGAAGCCACTGGCATATCCACTGTTCTGTTTCAGGATTTGGTCAGAAAGA	1760
G V F H L R T T I L P A E E D M P D I M D S F K K F		
1761	AGGGGTGTTCCATTTGAGGACAACTATTTTACCAGCTGAGGAAGACATGCCTGATATTATGGATAGTTTCAAAAAGTTCA	1840
N D E F M E Q Y E D D R G Y S R L *		
1841	ATGATGAATTCATGGAGCAATACGAAGACGATAGAGGTTATTCAAGGTTGTAAGAAAAATCATTGTAAAAGCTGCATTATA	1920
1921	CTCTTGGCGCTTTTGGAGGTTCTTCATCCATCCATCCCTCGGAGAGAAAAAACATAACCTCCGTTTCTACTATTGTGCC	2000
2001	TTCAATGGAGATTATCTTAAACCTTACACTTTCTCTATATCATCTCTGAACCTTACAATATTTCGGCTGTATATTTAAT	2080
2081	CTCTCTGAACCTTTTATCAAAAAAATAAGTGAATAAATCAAAAAAATCGTCTTGAATGTGATGGTTTTCGCTCATG	2160
2161	TGTTTAAATGTCCTAAGAAAAAGATTGACATAGATATATATAAGGTCTAGATATGTTTTTGTCTTGGTAAGTTAGTG	2240
2241	TTTTTTTTTATCTGTAA	2258

Supplementary Figure Id. Nucleotide sequence of cDNA clone GmAlaAT3 from *Glycine max*.

GmAlaAT3 (EU165373) (bankit1019388)

1	TCTATTATCTTGCAAACCTGGTTGGTGTTCATTGTGTAAGCCATATGTTATATTCCTTTAAGTATTCTGAGTCTACATAT	80
81	TTGATGAAGGTTACTGATTAAGTATTACAGAGAAGTTCCTAATTAAGGAGTCTAAGTCTACATATCAGTATTTACTTCACT	160
161	AGTTAGCTTGAATTCGTATTGAAGAAATCTAAGTTCCTTAACAGAAAACACTTCACATGTAGTAGAGTAAATGAGCAA	240
241	CACCTCTTCTTCAATCAAGCGAAGGTGGTCTCGATTGCAAGTGATCCACGATACACTCTACGCTTCACAAAACCTTCAAAC	320
321	CCAATTCCACAGAATTCTTCTGCTTCACCATGCCACCAAAGCCTTTAGACTATGGGTCAATAAATGAAAACGTGAAGAAG	400
401	AGTCAATATGCTGTGAGAGGTGAATTATACCTTCGAGCTTCTGAACCTTCAGAAAGAGGGCAAAAAGATTATCTTTACTAA	480
481	TGTTGGCAACCCGCATGCATTGGGACAGAAACCACTGAGCTTCCCTCGCCAGGTTGTTGCTTTGTGCCAAGCTCCATTCC	560
561	TACTTGATGATCCTAATGTTGGACTGCTATTCCTGCTGATGCAATTGCAAGAGCTAAACACTATCTTTCATTGACCTCG	640
641	GGTGGTTTAGGTGCTTATAGTGACTCCCGTGGCCTTCCAGGAGTGAGGAAGGAAGTTGCTGAGTTCATACTCAGGCGTGA	720
721	TGTTATCCAACCTGATCCAGAGCTCATATATCTCACTGATGGTGCAAGCAAAGGAGTGATGCAGATATTAAATACTATCA	800
801	TCAGGGTCAAGACGATGGGATTTTGCCAGTCCCACAATACCCACTCTACTCAGCAACAATTGCTCTGCTTGGTGGTACC	880
881	CTTGTTCCATACCTACCTTGAAGAGACAGCAAATTTGGGGTCTTGATGTTAATGAACCTTCGTCATCAGTTGAGCAGGCTCG	960
961	CTTTAAAGGAATAACTGTTAAAGCAATGGTGATCATAAATCCTGGAAACCCCTACTGGTCAATGCCTTAGTGAGCTAATC	1040
1041	TAAGAGAGGTTTTCGAATTCTGTTATCAAGAAAATTTAGCCTTGCTTGAGAGATGAGGTTTACCAGACAAATATATATCAG	1120
1121	GATGAACGACCCCTTCATTAGTTCTAGAAAGGTTTGTATGGACTTGGGGCCACCTATAAGCAAGGAAGTCCAGCTTATTTTC	1200
1201	TTTTCACTCCGTGTCAAAAGGTTATTATGGTGAATGTGGACAGCGAGGTGGATATTTTGAAATGACCAACATTCTCTCCAG	1280
1281	AGACAGTTGATGATCTACAAGGTTGCATCAATATCACTTAGTCCAAATGTTCCAGCACAAATATTTATGGGAGTTATG	1360
1361	CTCCATCCACCTCAACCTGGAGATATTTCTTATGACAAATTTGTTAGGGAGAGCACCGGAATACTTGAATCACTGAGAAG	1440
1441	AAGAGCAAGGCTAATGACTGATGGATTCAACAGTTGCAGAAATGTGGTTTGTAATTTTACTGAAGGTGCCATGTATTTCAT	1520
1521	TCCCTCAAATACGTTTGCGCCAAGAGCTTTAGAGGCTGCTAAACAGGCTGCAAAGGTTCCAGATGTTTCTACTGCCTC	1600
1601	AAACTTTTGAAGCCACTGGCATATCACTGTTCCTGGTTCCAGGATTTGGACAGAGAGAAGGGGCTTCCATTGAGGAC	1680
1681	TACTATTTTACCAGACGAGGAAGACATGCCGCTATTATGGATAGTTTCAAAAAGTTCAATGATGAATTCATGGAGCAAT	1760
1761	Y E D N R G Y S R L *	
1841	ACGAAGACAATAGAGGTTATTCAAGGTTGTAAGAAAATTATCGTAAAAGATGCATTATACTCTTGCTGCTTTGTAAGAAA	1840
1921	ACAATAAAAAATTTTCGCGTGCACAAAAA	1954

Supplementary Figure II. Multiple sequence alignment with hierarchical clustering of *Glycine max* rum by GeneDoc (<http://www.psc.edu/biomed/genedoc>) (Nicholas et al., 1997). The other plants species are described in the phylogenetic tree (Fig. 3). The alignment analyses was based on protein sequence. Black shading indicates 100%, dark grey shading 80%, and light grey shading 60% identity. In the consensus amino acid sequence, capital letters indicate 100% identity and small letters indicate more than 75% identity.



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*          380          *          400          *          420          *          440          *          460          *          480
GmAlaAT1 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 425
MtmAlaAT : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 463
AtAlaAT1 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 482
AtAlaAT2 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 479
PtAlaAT3 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 420
PtAlaAT4 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 408
Os07g42600 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 485
Os10g25130 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 422
Os03g08530 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 462
PpAlaAT1 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 423
PpAlaAT2 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 413
Os10g25140 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 485
Os09g26380 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 425
CrAlaAT1 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 344
GmAlaAT2 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 413
GmAlaAT3 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 412
PtAlaAT1 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 413
PtAlaAT2 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 413
AtGGT1 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 413
AtGGT2 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 413
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MtcAlaAT : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 413
PpGGT2 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 414
PpGGT1 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 414
CrAlaAT2 : GNGGCCRRGGYNEVGFSPAEVRDQIKVASVNDGNTGGQILASIVSPFVYCHREYDSEMAKENTASTARATLEADAPKLEGVYCNKAGGMILFPQRIYGEKAIKAEAKNATD : 461
G GCGC RGGY Ect 26yK as6 N Q6 66 Ppk G #5 5 E 56 tA 6 N 6 CN EGaeY FF lp a aa pD

*          500          *          520          *          540          *          560
GmAlaAT1 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 486
MtmAlaAT : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 524
AtAlaAT1 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 543
AtAlaAT2 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 540
PtAlaAT3 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 481
PtAlaAT4 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 469
Os07g42600 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 546
Os10g25130 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 483
Os03g08530 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 537
PpAlaAT1 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 484
PpAlaAT2 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 461
Os10g25140 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 546
Os09g26380 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 486
CrAlaAT1 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 393
GmAlaAT2 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 481
GmAlaAT3 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 480
PtAlaAT1 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 481
PtAlaAT2 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 481
AtGGT1 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 481
AtGGT2 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 481
Os07g01760 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 485
MtcAlaAT : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 474
PpGGT2 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 480
PpGGT1 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 480
CrAlaAT2 : NNYCKLLNATGIVVPGSGFQVPTMHFR-CQLL-CDKTPALVTRLTEHEK-----FNDEFRD----- : 521
Y LL atG6 vPG8gqq g h r t lp e f

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CONCLUSÃO GERAL

A importância do acúmulo de alanina durante o estresse por hipoxia foi mostrada nesse trabalho através do uso de plantas mutantes de *Lotus japonicum* (LbRNAi). Essas plantas, apesar de deficientes em nitrogênio, mostraram ser capazes de acumular alanina em nível comparável aos de plantas selvagens. O acúmulo de alanina também mostrou ser parte de uma cadeia cíclica de eventos onde um rearranjo do ciclo tricarboxílico culminaria na produção extra de ATP.

Embora se tenha demonstrado que não somente a síntese de alanina seria induzida pela presença de fontes externas de nitrogênio mas também a expressão gênica e a atividade enzimática da AlaAT, ainda não está claro se realmente existe uma correlação entre conteúdo de alanina e tolerância ao alagamento. Desta forma podemos concluir que outros mecanismos também possam estar envolvidos com a tolerância ao alagamento e precisariam ser investigados em maiores detalhes.