Impact of methionine nutrition on the leaf proteome of *Lupinus angustifolius* L. and *Vicia faba* L.

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Abstract

Grain legumes possess a generally favourable seed protein amino acid composition but low contents of sulfur amino acids decrease their nutrient value. To enhance the methionine content of local grain legumes by plant breeding, phenotypical attributes for high methionine contents are required for the selection process. The efforts are of particular importance in organic agriculture as the use of genetically engineered plants, as well as the addition of synthetic feed additives, is prohibited and conventional breeding methods need to be improved. Previous studies revealed a partial correlation between methionine and leaf chlorophyll contents. In the present work the impact of applied methionine (1mM) on the plant protein and gene expression was investigated to gain further knowledge of possible regulatory functions of methionine. A down-regulation of high abundant proteins could be observed for *L. angustifolius* as well as *V. faba* due to methionine nutrition. In addition, in *V. faba* the expression of the methionine related enzyme *S*-adenosylmethionine:magnesium protoporphyrin IX *O*-methyltransferase was as well down-regulated due to methionine nutrition. These results contradict the expected beneficial effect of methionine nutrition on the plant proteome. Further experiments are necessary to verify the methionine-specificity of this effect and confirm these preliminary findings.

Introduction

Grain legumes have various agronomical benefits such as lower fertilizer usage, improvements of soil structure and lower pathogen abundance when part of crop rotations (Nemecek et al. 2008). Also they are particularly useful for organic farming as a protein source and part of farm-own feeding stuffs (Zollitsch et al. 2004).

The methionine (Met) and cysteine deficiency of grain legumes is a long-known obstacle to a wider use of the agronomical important legume species such as *L. angustifolius* and *V. Faba.* Several attempts to generate transgenic plants have been ventured in order to enhance the seed Met content (Amir, 2010). These were mainly directed at introducing genes of Met rich protein (e.g., zein from *Zea mays* L.) into

the legume genome. Other attempts include the manipulation of enzymes with regulatory functions in the biosynthesis pathway of Met (e.g., cystathione-γ-synthethase). A slight increase of sulfur-amino acid contents could be generated by both attempts, but no major improvement has been achieved. In organic farming the application of these methods is not allowed, meaning conventional breeding efforts are necessary. Today plant breeding approaches concentrate on the improvement of other traits such as yield and pathogen resistance. One reason for the negotiation of protein quality in conventional breeding programs might be the lack of applicable phenotypes or molecular markers (e.g., QTL) for high sulfur amino acid contents. In order to generate sustainable Met enriched cultivars, applicable selection criteria are required.

The present work investigates the impact of induced elevated Met (1mM) concentrations on the leaf protein expression. Hereby possible regulatory effects of soluble Met on protein expression are examined. The intention of the work is to reveal the cause for previously observed changes of the plant phenotype due to Met nutrition (Imsande, 2001 and Schumacher et al. 2009). Whether these altered phenotypes are a consequence of methionine-own regulatory functions or simply of enhanced plant nutrition is the main interest here.

Material and Methods

Experiments were performed with established cultivars of each plant species (Boruta (*L. angustifolius*) and Espresso (*V. faba*)). Plants were grown under constant conditions (23°C, 12/12h light, 120 µmol/m²) und supplied with Hoagland nutrient solution. For induced increased Met-concentration 1mM Met was added to the nutrient solution. Youngest full developed leaves were harvested and ground with liquid nitrogen. Total soluble protein was extracted with 50 mM Tris-Buffer (pH 7.5). Protein concentration was quantified according to Lowry (1951) after removal of the extraction buffer through TCA (10%) precipitation. First dimension separation was performed by isoelectric focusing with IPG-Strips from Biorad (ReadyStrip, 11cm; pH4-7) in a Biorad Protean Cell. The Second dimension separation was done with Protean II xi Cell and 12% Tris-/Tricine Gels.

In order to reduce Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase) content, it was precipitated according to Krishnan and Natarajan (2009). Gels were stained with Coomassie G250 solution and scanned, or pictures were taken by Geldoc imaging system.

The Mass spectrometric analysis of protein spots was carried out with LC-ESI Q-ToF analyses with an Easy-nLC system (Proxeon) coupled to a micrOTOF Q II MS (Bruker Daltonics).

In-gel digestion was carried out as described in Klodmann et al. 2010. Software from Bruker Daltonics was used for data processing and data analysis. The database search was carried out with ProteinScape (Bruker Daltonics) and the MASCOT search engine in the NCBInr database.

Total RNA was extracted and cDNA was synthesized with Nucleospin SMARTer PCR cDNA Synthesis Kit from Clontech. Polymerase chain reaction (PCR) was performed with Primers derived from the *Pisum sativum* (L.) *S*-Adenosylmethionine: magnesium protoporphyrin IX *O*-methyltransferase (MgProto) sequence published by Vasileuskaya et al. (2005).

Results

Second dimension separation of total soluble protein revealed a different expression of protein species between the treatments of *V. faba* (s. Fig. 1). Down regulation of several proteins could be observed due to Met nutrition. The protein pattern was very similar.

Total protein of *L. angustifolius* showed a similar effect of Met treatment on the protein expression (s. Fig. 2). A general decrease in spot intensity is visible, with few exceptions. Several prominently different

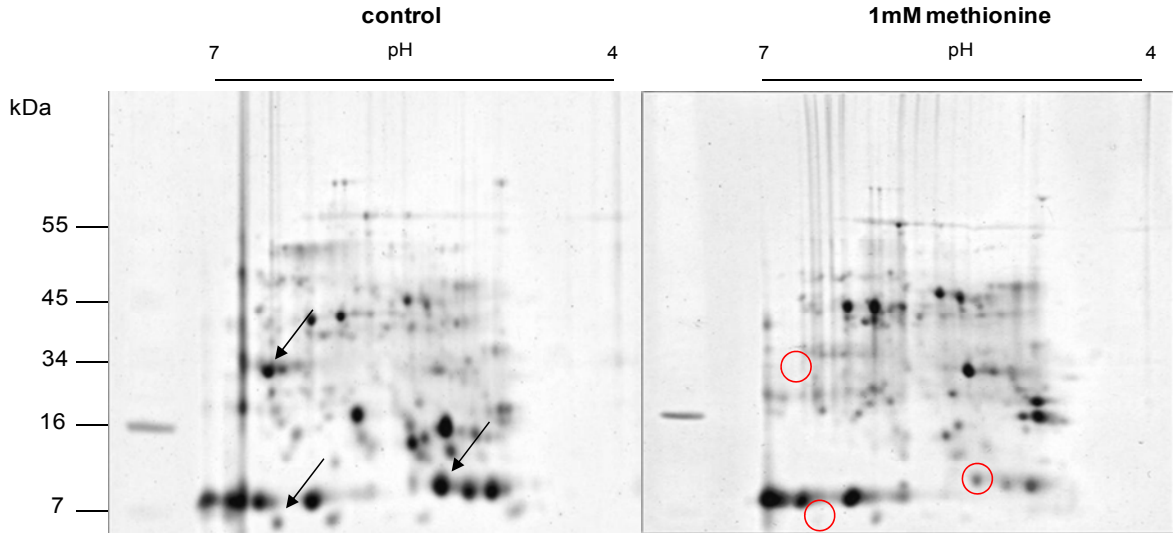

Figure 1: Soluble protein fraction (200µg) of *V. faba* control plants (left) and Met treated plants (right). Black arrows and red circles resp. indicate exemplary down-regulated proteins.

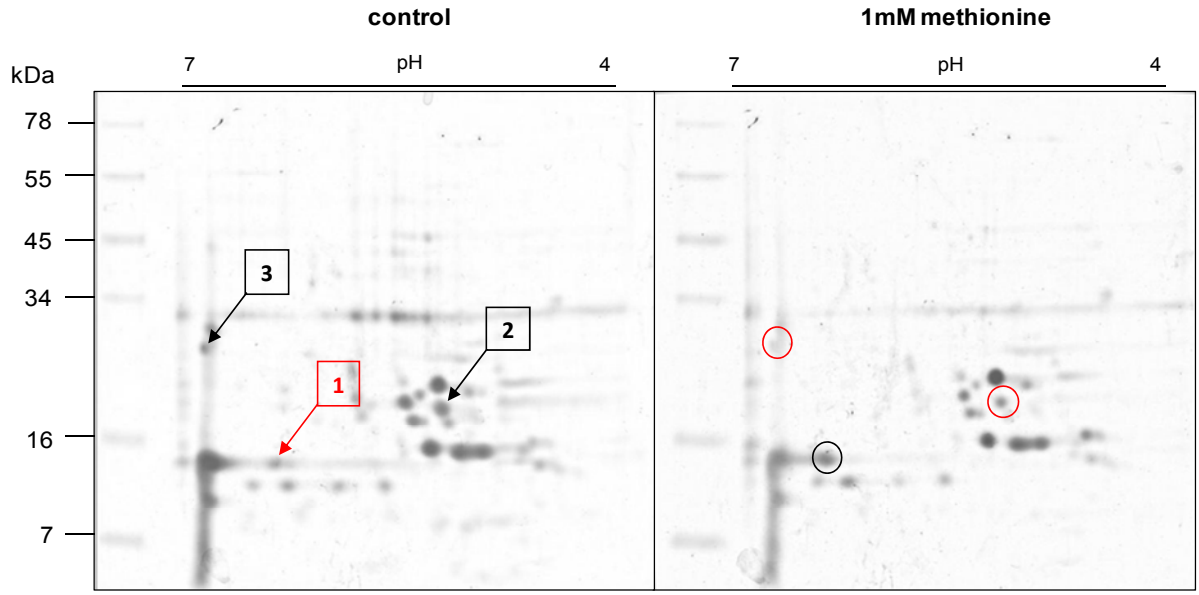

Figure 2: Soluble protein fraction (200µg) of *L. angustifolius* control plants (left) and Met treated plants (right). Selected, down- (red) or up-regulated (black) proteins marked by arrows resp. cycles.

spots were selected for MS-analysis (s. Tab. 1).

Table 1: Mass spectroscopic analysis of selected, differently expressed proteins within the total protein fraction of *L. angustifolius*.

No.	Accession	Name	Mascot Score	MW [kDa]	SC [%]
1	RBS2_PEA	Ribulose bisphosphate carboxylase small chain 3C	221	20,23	24
2	RBL_ALIPL	Ribulose bisphosphate carboxylase large chain (Fragment)	220	43,94	11
3	G3PB_PEA	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor	215	48,07	14

MW=molecular weight; SC=sequence coverage

Large chain of Rubisco and Glyceraldehyde-3-phosphate dehydrogenase could be identified as down-regulated proteins.

The high abundance of Rubisco proteins within the total protein extract made it difficult to identify further proteins. To analyse the Met impact on other, less abundant protein species, the Rubisco content in the sample was reduced by phytate precipitation.

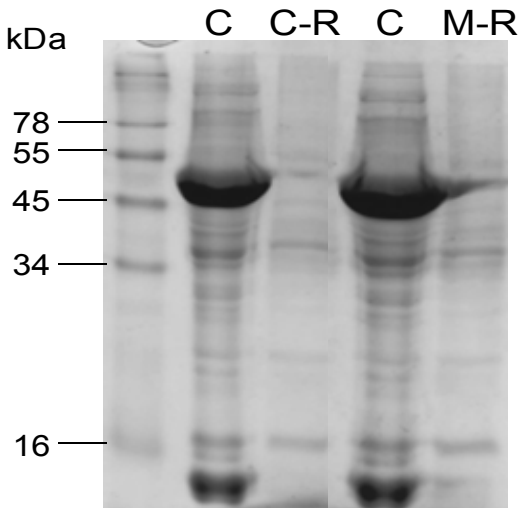

Figure 3: SDS-PAGE of total leaf protein (10µl; *L. angustifolius*) before and after precipitation of Rubisco compounds (C=control; M=Met treated plants; -R=after Rubisco precipitation)

In Figure 3 the result of the Rubisco precipitation experiment is shown. Total soluble protein was applied to lanes before and after precipitation. The concentration of the large chain of Rubisco is clearly depleted (band at 51kDa). Other protein concentrations were reduced by the treatment as well, but to a lesser degree.

The Rubisco-depleted total protein solution was used for 2D-analysis. Figure 4 displays Gels with protein samples of *L. angustifolius*. The protein pattern between the treatments is very similar, while protein expression is generally reduced in Met treated plants. Most visible protein spots of the control sample are more intense than in the sample of Met treated plants. Accordant results were gained for samples of *V. faba* (cp. Fig. 5). Here the protein pattern is as well similar between the treatments. No novel expressed proteins could be detected due to Met nutrition. Exclusive, comparatively higher expressed proteins in the Met treated plants were selected for sequence analysis. Results are shown in Table 2. Through the MS-analysis each spot was identified as Polyphenol oxidase A1, probably representing intermediates of the same protein.

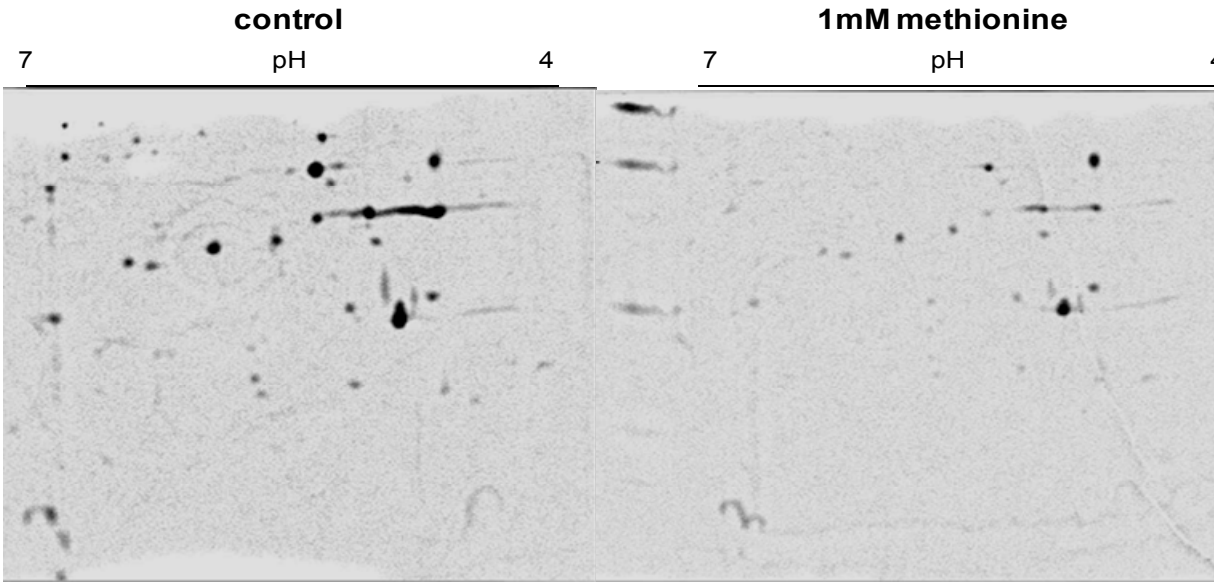

Figure 4: Soluble protein fraction (150µg) of *L. angustifolius* control plants and Met treated plants after removal of Rubisco and Rubisco precursors. Pictures were recorded with Geldoc camera.

Experiments with plant RNA-transcripts were performed for the Met chlorophyll branch point enzyme MgProto.

The expression of the Met related MgProto Gene in *V. faba* was investigated by semi-quantitative PCR (s. Figure 6). Calculated products a for housekeeping gene (actin; 421bp) and MgProto (284bp) were obtained. Met treated plants possessed a lower expression of MgProto than control plants. In this experiment the effect of cysteine nutrition (1mM) was additionally investigated. Cysteine treated plants show

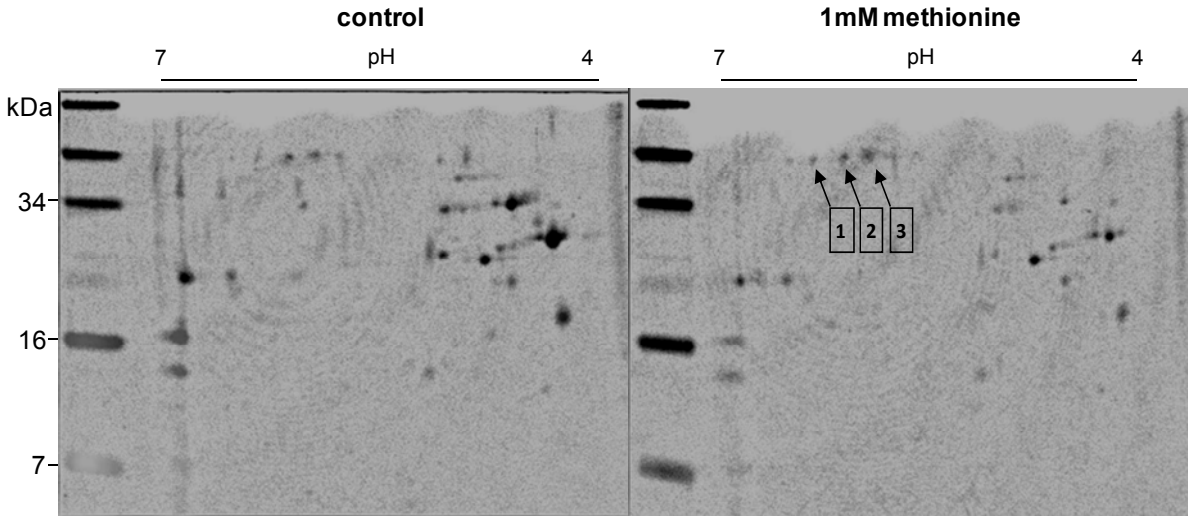

Figure 5: Soluble protein fraction (150µg) of *V. faba* control plants and Met treated plants after removal of Rubisco and Rubisco precursors. Pictures were recorded with Geldoc camera. Arrows indicate selected spots for MS-analysis.

a higher expression of MgProto than con-

Table 2: Mass spectroscopic analysis of selected proteins within the total protein fraction of *V. faba* after Rubisco precipitation.

Number	Accession	Name	Mascot Score	MW [kDa]	SC [%]
1	PPO_VICFA	Polyphenol oxidase A1	179	68,5	7.59 %
2	PPO_VICFA	Polyphenol oxidase A1	352	43,94	9.74 %
3	PPO_VICFA	Polyphenol oxidase A1	184	68,5	6.27 %

MW=molecular weight; SC=sequence coverage

trol and Met treated plants. No gene product could be obtained with the applied Primer for *L. angustifolius* cDNA.

Discussion

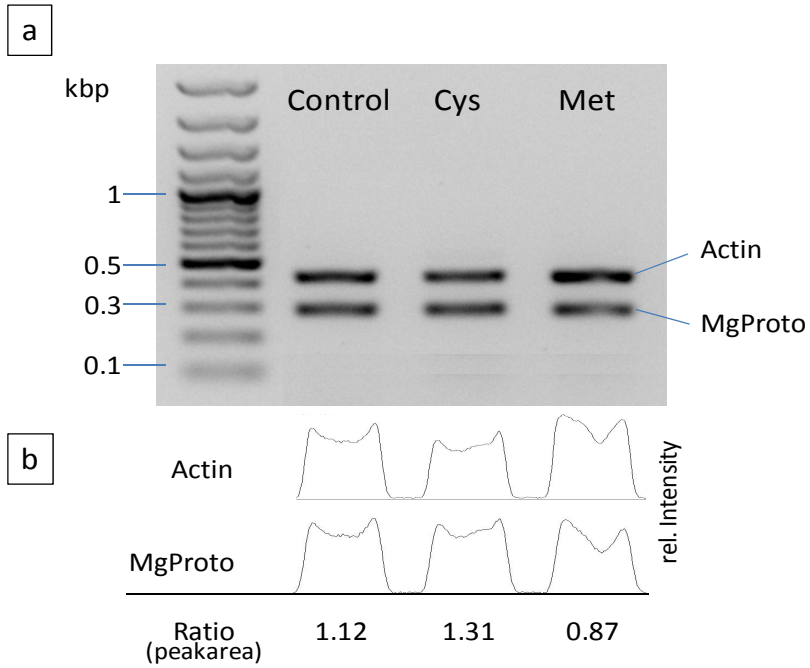

Figure 6: Reverse transcriptase-PCR with cDNA of leaf RNA from *V. faba* treated with Cystein (Cys), Met or untreated. a: 1µg of PCR product was applied per lane. b: Relative intensity of bands was determined with ImageJ software.

In the present work the impact of extracellular methinone on the plant protein biochemistry was investigated. The ability of many plant species to assimilate soluble amino acids (AA) (e.g., Met) from the soil has been observed previously (e.g., Soldal and Nissen, 1978). In a work by Imsande (2001) a phenotypical effect of supplied Met (0.1mM) on the leaf colour of *Glycine max* (L.) was observed. Similar results for *L. angustifolius* and *V. faba* were obtained in a previously published work (Schumacher et al. 2009).

In the present paper, this effect should be investigated further for *L. angustifolius* and *V. faba* in order to gain insight into the regulatory functions of soluble Met.

Presented results indicate a down regulation of housekeeping proteins due to Met nutrition for *L. angustifolius* and *V. faba* plants. Since nitrogen and sulfur nutrition generally lead to enhanced protein expression rates (Zorb et al., 2010), the effect is clearly not caused by an additional N and S supply through Met.

Furthermore Rubisco expression levels are in particular elevated by increased N supply (Imai et al., 2008). In the present work the down-regulation of the large subunit of Rubisco due to Met treatment for *L. angustifolius* could be observed. On the other hand, the small subunit of Rubisco was up-regulated in relation to other proteins. While the reason for the observed change of protein expression levels is unknown, regulatory functions of soluble Met have been claimed for protein synthesis (e.g., by Bagga et al., 2005). Bagga et al. found that the expression of transgenic β-zein was induced by high levels of soluble Met.

Furthermore the indirect regulatory function of Met as a precursor for S-adenosylmethionine (SAM) is known (Amir, 2010). SAM, the primary cellular methyl group donor, participates in all kinds of metabolic reactions, such as membrane and chlorophyll synthesis and regulates these processes as well (Amir, 2010).

In this regard the expression of MgProto, for which SAM functions as substrate, was investigated. The Met treatment caused a down-regulation of MgProto, possibly due to a feedback inhibition by synthesis products. The previously observed, the accumulation of chlorophylls due to Met treatment in *V. faba* and *L. angustifolius* might be related to this effect (cp. Schumacher et al. 2009).

Hitherto the reason for the reduction of protein expression levels due to the Met treatment has not been identified. Arshad et al. (1992) observed a slight reduction of root and shoot biomass in *Albizia lebbeck* (L.) after treatment with high Met (1g/kg soil) but not for lower concentrations. Amir (2010) mentions the possible toxic effect of high levels of Met, which might serve as an explanation for decreased plant growth. Further experiments are necessary to evaluate whether the observed down-regulation of proteins is indeed Met-specific. Plants supplied with cysteine instead of Met will be analyzed in order to judge the Met specificity of the observed effect and to compare the transcriptome level by Suppression Subtractive Hybridization (SSH). This will provide further insight into the regulatory functions of soluble Met on gene expression as well as protein and pigment synthesis.

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