Leaf proteome of rapeseed subjected to sulphur restriction reveals numerous changes in proteins related to sulphur, carbon metabolisms and oxidative stress.

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Abstract

To identify the different metabolic processes involved in the tolerance of rapeseed to S restriction, oilseed rape was subjected to a sulphate restriction (Low S) for 35 days. Young leaves of S restricted plants shown morphological deficiency symptoms such as a higher anthocyanins content and a lower photosynthetic activity compared to the control. The analysis of young leaf proteome at Day 35 indicated that S and C metabolisms were affected by S limitation. This disruption leads to an increase in intercellular CO_2 concentration, associated with the induction of a β-carbonic anhydrase, which may facilitate its solubilization. Moreover, if S depletion occurred, those metabolic changes may lead to an oxidative stress, correlated by the induction of stress tolerance proteins such as BnD22, a Water Soluble Chlorophyll binding Protein which presents a dual function of protection of chlorophyll against ROS and a protease inhibitor activity.

Introduction

Sulphate (SO_4^{2-}) is the main form of sulphur (S) absorbed by plants and is assimilated into many compounds, such as cysteine, methionine, glutathione and secondary metabolites with various functions in plant metabolism (Leustek & Saito, 1999). The decline of industrial SO_2 rejections leads to a SO_4^{2-} depletion in soil, which impacts on both grain yield and oil quality of rapeseed (Brassica napus L.; Dubousset et al., 2010). Recent transcriptomic and metabolomic approaches have shown that alterations in expression levels of numerous genes associated with metabolic and physiological changes allow Arabidopsis thaliana to respond to S deficiency ((Hirai & Saito, 2004; Nikiforova et al., 2005). S limitation first involves a decrease in cysteine and an increase of its precursor, O-acetylserine (OAS). OAS accumulation is then assumed to regulate numerous genes expression such as genes implied in S uptake, assimilation and redistribution which are induced, improving S acquisition and utilization for plant growth. Rapeseed is also able to enhance S remobilization to sustain the S demand for growth through the induction of some sulphate transporters (Parmar et al., 2007) (Dubousset et al., 2009, 2010). Nevertheless, extended S limitation leads to an accumulation of amino acids, which seems to down-regulate nitrogen uptake and assimilation. In the same time, processes that increase the turnover of organic S and stress defence responses are induced. When it goes on, S limitation is assumed to repress growth and reduce the shoot:root ratio (for review see Hawkesford & Kok, 2006).

The decrease in cysteine and methionine content by S restriction could have an impact on the expression of essential proteins. This may lead to differences between transcriptomic and proteomic profiles as observed by Higashi et al. (2006). Therefore, the study of S limitation impacts on proteome should be a relevant approach to identify the metabolic pathways affected, since it integrates both transcriptional and post-transcriptional controls. In this context, our goal was to combine proteomic and physiological studies in order to provide new insights about the rapeseed responses and in particular the leaf metabolism modifications caused by a long-term S depletion occurring at the vegetative stage.

Materials and methods

After 55 d of growth under greenhouse conditions (16h/20°C-day and 8h/15°C-night, 400 µmoles photons $m^{-2}.s^{-1}$), Brassica napus L. (cv Capitol) plants were supplied with 500 µM (Control) or 8.7 µM (Low S) of $MgSO_4$ during 35 d. Control and Low S plants were sampled in quadruplicate at Day 0 and after 14, 21, 28 and 35 d of treatment. The relative chlorophylls and anthocyanins amounts were measured each week by the Multiplex ® system (Force A, Orsay, France). Photosynthetic activity and transpiration were measured from Day 28 with a LI-6400 portable system (LI-COR, Inc., Lincoln, NE, USA). Extraction of total proteins from leaf #16, 2-DE gels and protein identification by ESI-LC-MS/MS were performed according to protocols described by Desclos et al. (2008).

Results and Discussion

Growth and physiological modifications induced by S deprivation

Global shoot biomass is not significantly affected by Low S treatment (data not shown). This is consistent with studies conducted in B. oleracea (Koralewska et al., 2007, 2009) and oilseed rape (Dubousset et al., 2009). In these studies, the chlorophyll content of young leaves decreased in Low S plants, an observation not found in our experiment. Nevertheless, in young leaf (rank #16), a higher relative anthocyanins content and a lower photosynthetic activity, associated with a higher intercellular CO_2 concentration were measured after 35 d in Low S plants compared to control (Figs. 1A and 1B). These are signs of metabolic changes.

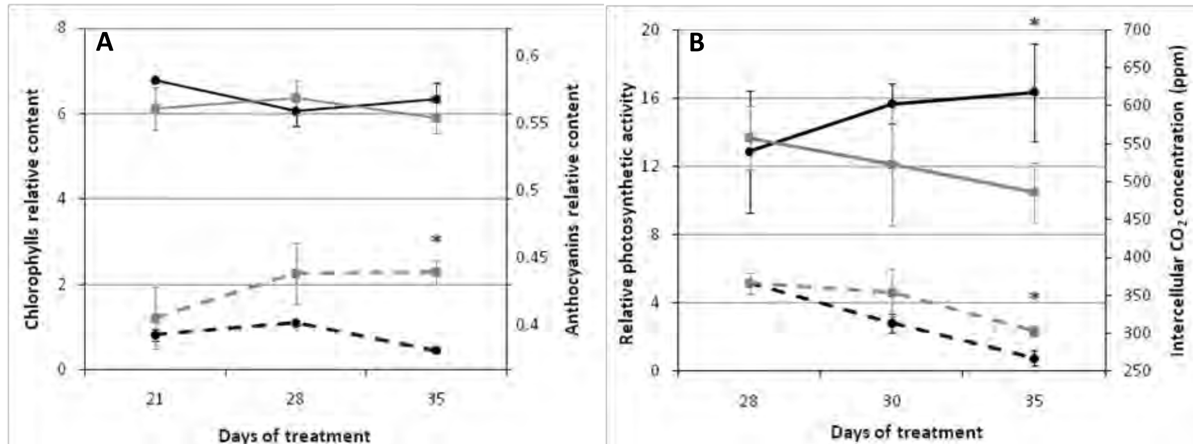

Figure 1: Chlorophylls (continuous lines) and anthocyanins (dotted lines) relative contents (**A**) and photosynthetic activity (continuous lines) and intercellular CO_2 concentration (dotted lines) (**B**) in the leaf rank #16 of control (Black) and S-restricted plants (Low S; Grey) after 21, 28, 30 or 35 d of treatment. Vertical bars indicate ± SE of the mean for n=3 and fit within the plot if not visible. *: Significant difference at $p \leq 0.05$.

Low S nutrition affects the proteome of young leaf (leaf rank #16)

At Day 35, in leaf #16, the total protein content was not significantly different between Control and Low S treatments (data not shown). Nevertheless, 2-DE analysis revealed 19 and 17 spots, respectively induced and repressed by Low S treatment, compared to the control. Using LC-MS / MS, 22 spots were clearly identified (Table I).

Proteomics changes associated to S metabolism

Induction of the putative Myrosinase-binding protein (spot no. 19) could provide S from glucosinolates degradation, suggesting that glucosinolates can be a S storage source in case of S deficiency. This finding is consistent with metabolome analysis performed in Arabidopsis thaliana that reported a decrease in glucosinolates accumulation for S restricted plants (Hirai & Saito, 2004). The slight induction observed for a vacuolar ATPase subunit (spot no. 22), could be implied in the induction of S remobilization through the induction of the sulphate efflux from the vacuole to sustain growth as previously described by Dubousset et al. (2009).

The THI1 (spot no. 4) repression may lead to a preferential allocation of cysteine for protein and GSH synthesis since this protein is involved in the chloroplastic thiamine biosynthesis (Machado et al., 1996) from glyceraldehyde-3-phosphate and cysteine, two molecules whose levels are affected by S limitation (Nikiforova et al., 2005). The Glutathione S-transferase (spot no. 1) repression indicates that the xenobiotics detoxification capacity in leaf #16 seems to be affected at 35 d of S restriction. As a consequence, the young leaf could be sensitive to abiotic stress (Ryu et al., 2009).

Table I: LC-MS/MS identification of protein repressed (negative value of variation) and induced (positive value of variation) in leaf #16 after 35 d of S restriction (Low S) compared to Control plants. Significant ANOVA was followed by a Tukey test ($p \le 0.05$) carried out on the leaf-normalised spot volumes. Experimental and theoretical pI/Mr are also indicated. The assigned best-matched protein and its GenBank accession number are listed.

Spot No.	Factor of variation	Experimental pI / Mr	Theorical pI / Mr	Protein name / Species	NCBI Accession number
1	-1.42	6.5 / 24	8.5 / 59.1	Glutathione S-transferase / Brassica oleracea	gi|171921127
2	-1.31	5.6 / 21	5.8 / 26.3	Photosystem I light-harvesting chlorophyll a / b-binding protein / Nicotiana tabacum	gi|493723
3	-1.38	6.7 / 33	8.6 / 38.8	Contains similarity to ferredoxin-NADP+ reductase from Arabidopsis thaliana gb|AJ243705 and contains an oxidoreductase FAD / NAD-binding PF|00175 domain / Arabidopsis thaliana	gi|8778996
4	-1.8	5.5 / 31	5.8 / 36.6	THI1; protein homodimerization / Arabidopsis thaliana	gi|15239735
5	-1.3	5.5 / 65	5.3 / 55.2	Mitochondrial chaperonin (HSP60) / Arabidopsis thaliana	gi|2924773
6	-1.28	6.4 / 35	8.5 / 42.3	Malate dehydrogenase / Brassica rapa subsp. Pekinensis	gi|207667274
7	-1.51	5.8 / 19	6.8 / 21.8	Germin-like protein / Arabidopsis thaliana	gi|1755154
8	-1.43	6.2 / 19	6.8 / 21.8	Germin-like protein / Arabidopsis thaliana	gi|1755154
9	-1.35	6.2 / 20	6.8 / 21.8	Germin-like protein / Arabidopsis thaliana	gi|1755154
10	-1.45	6.8 / 30	6.2 / 27.5	Chain B, The Transient Complex Of Poplar Plastocyanin With Turnip Cytochrome F Determined With Paramagnetic Nmr / Brassica rapa	gi|67463833
11	-1.36	5.5 / 29	5.3 / 26.3	AT2G37660 / Arabidopsis thaliana	gi|227204455
12	-1.55	6.8 / 35	8.5 / 35.8	Mitochondrial malate dehydrogenase (NAD) / Arabidopsis thaliana	gi|18404382
13	+11.51	6.3 / 19	8.4 / 23.5	Heat stress-induced protein / Brassica oleracea var. capitata	gi|3319646
14	+3.08	5.3 / 20	5.1 / 23.3	Trypsin inhibitor propeptide / Brassica oleracea	gi|841208
15	+5.27	5.7 / 19	7.8 / 22.7	Water-soluble chlorophyll protein / Brassica oleracea var. acephala	gi|27530881
16	+2.92	5.4 / 59	5.1 / 55.3	ATP synthase CF1 alpha subunit / Brassica napus	gi|262400756
17	+7.86	6.6 / 29	6.5 / 28.8	BCA3 (ß CARBONIC ANHYDRASE 4); carbonate dehydratase / zinc ion binding / Arabidopsis thaliana	gi|15220853
18	+1.49	6.1 / 17	6.7 / 21.8	Cu-Zn Superoxide dismutase / Arabidopsis thaliana	gi|3273753
19	+2.29	5.2 / 49	5.4 / 20.6	Putative myrosinase-binding protein 3 / Brassica rapa subsp. Pekinensis	gi|33285912
20	+2.3	5.7 / 43	6.3 / 40.9	12-oxophytodienoate reductase / Arabidopsis thaliana	gi|2765083
21	+1.86	5.4 / 51	7.6 / 48.7	Aminotransferase class I and II family protein / Arabidopsis thaliana	gi|15217440
22	+1.32	5.2 / 57	5 / 54.7	Nucleotide-binding subunit of vacuolar ATPase / Arabidopsis thaliana	gi|166627

Proteomics changes associated to C metabolism and protection against oxidative stress

The carbon metabolism appears to be affected by 35 d of S limitation, particularly the photosynthetic metabolism, that leads to a C fixation decline (Fig. 1B), and a higher intercellular CO_2 concentration. At the proteomic level, these disruptions are corroborated by the modulation of numerous proteins, such as the repression of a putative FNR (spot no. 3) and a chloroplast MDH (spot no. 6). The repression of the latter may indicate a relative reduction of malate export (Minárik et al., 2002). Nevertheless, this proteomic analysis also shows an induction of proteins implied in maintaining energy production in the young leaf subjected to Low S treatment, such as the ATP synthase α-subunit (spot no. 16) and BnD22 (Water-Soluble Chlorophyll binding Protein, spot no. 15), involved in chlorophyll protection against ROS and in maintenance of protein content (Desclos et al., 2008). Indeed, chlorophyll and protein contents are not affected by the S limitation applied in our study. The reduction of photosynthetic activity and the proteins modulations discussed above suggest an alteration of the coupling between light and dark photosynthesis reactions, which could lead to an accumulation of CO_2 at the cellular level. This

accumulation was measured at the intercellular level in our study. The β-Carbonic anhydrase (spot no. 17) which is directly involved in CO_2 metabolism, associated with the Calvin cycle (Jebanathirajah & Coleman, 1998), found strongly induced in our study, could then facilitate the CO_2 solubilisation and thus promote its fixation during the dark photosynthesis reactions.

This alteration of photosynthetic processes could also lead to the formation of ROS, such as $O_2^{\cdot-}$ by the Mehler reaction. The induction of the chloroplastic Cu-Zn SOD (spot no. 18) also suggests that S limitation causes an oxidative stress in the young leaf. This protein could then convert this toxic superoxide anion into H_2O_2. The induction of BnD22, and the repression of protein showing similarity with Germin-like proteins (spots no. 7, 8 and 9), which could have an oxalate oxydase activity generating H_2O_2 and CO_2, may also be involved in reducing oxidative stress. The higher anthocyanins content observed in the leaf #16 also suggests an induction of processes associated with oxidative stress protection after 35 d of S limitation.

In addition to its impact on S metabolism, THI1 repression could have a negative impact on C metabolism, whether on glycolysis/neoglucogenesis or on chlorophyll synthesis. Indeed, thiamine, a S containing molecule, is the precursor of thiamine pyrophosphate, a co-enzyme involved in C metabolism such as Pyruvate carboxylase, Pyruvate oxidase or Transketolase activities (Lindqvist & Schneider, 1993). This repression could cause chlorosis and mitochondrial DNA damage if S restriction is extended beyond 35 d, since this protein is also involved in mitochondrial DNA damage tolerance (Machado et al., 1996).

Proteomics changes associated with others metabolisms:

As for THI1, the repression of HSP60 (spot no. 5) is a sign of a mitochondrial stress caused by Low S. According to the Arabidopsis thaliana transcriptome response to S restriction (Hirai et al., 2003; Hirai & Saito, 2004), our proteomic study showed an induction of 12-oxophytodienoate reductase (spot no. 20), that catalyzes the last step of jasmonate biosynthesis. Jasmonate could have a positive effect on S metabolism in case of S limitation, through the induction of many genes, as suggested by the work of Jost et al. (2005), but could result, ultimately, to the death of cells expressing this phytohormone, particularly via the induction of an oxidative stress.

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