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Genotypic variation in sulphur assimilation and metabolism of onion (*Allium cepa* L.). II: Characterisation of ATP sulphurylase activity

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ABSTRACT

To investigate the regulation of sulphur (S)-assimilation in onion further at the biochemical level, the pungent cultivar W202A and the milder cultivar Texas Grano 438 PVP (TG) have been grown in S-sufficient (S^+; 4 meq S^{-1}) or S-deficient (S^-; 0.1 meq S^{-1}) growth conditions, and tissues excised at the seedling stage (pre-bulbing; *ca.* 10-weeks-old) and at the mature stage (bulbing; *ca.* 16-weeks-old). S-supply negatively influenced adenosine-5′-phosphosulphate (APS) reductase (APR) enzyme activity in both cultivars at bulbing only, and a higher abundance of APR was observed in both cultivars at bulbing in response to low S-supply. In contrast, S-supply significantly influenced ATP sulphurylase (ATPS) activity in leaf tissues of W202A only, and only at bulbing, while an increase in abundance in response to high S-supply was observed for both cultivars at bulbing. To investigate the regulation of the ATPS enzyme activity and accumulation further, activity was shown to decrease significantly in roots at bulbing in the S-deficient treatment in both cultivars, a difference that was only supported by western analyses in W202A. Phylogenetic analysis revealed that AcATPS1 groups in a broad monocot clade with the closest sequences identified in *Sorghum bicolour*, *Zea mays* and *Oryza sativa*, but with some support for a divergence of AcATPS1. Detection of ATPS in leaf extracts after two dimensional gel electrophoresis (2-DE) revealed that the protein may undergo post-translational modification with a differential pattern of ATPS accumulation detected in both cultivars over the developmental progression from the seedling to the bulbing stage. Treatment of leaf extracts of W202A to dephosphorylate proteins resulted in the loss of immuno-recognised ATPS spots after 2-DE separation, although enzyme activity was not influenced. These results are discussed in terms of the tiers of control that operate at the biochemical level in the reductive S-assimilation pathway in a S-accumulating species particularly during the high-S-demanding bulbing stage.

1. Introduction

Acquisition of the macro-element sulphur (S) is a critical process for the growth and development of higher plants and while the sulphur assimilation pathway in many higher plant species is now well described, the control of the S assimilation pathway with respect to the reductive biosynthesis of cysteine is still, comparatively, poorly understood (Kopriva and Rennenberg, 2004; Kopriva, 2006). In this respect, members of the genus *Allium*, including Chinese chive (*Allium tuberosum*), garlic (*Allium sativum*) and onion (*Allium cepa*) are of particular interest because they contain naturally high levels of further reduced organosulphur compounds, primarily sulfur-alk(en)yl cysteine sulfoxides (ACSOs) (Randle and Lancaster, 2002).

Pungency in onion is a heritable trait, and QTL mapping has shown that both ATPS and sulphite reductase (SiR) segregate in pungent onion lines (McCallum et al., 2006b). In a companion paper, significant differences in sulphate and cysteine pools in the mild and pungent genotypes, as well as differences in S assimilatory gene expression, have been shown (McCallum et al., 2011a). Thus as part of this wider study on the control of S-assimilation in onion (McCallum et al., 2002, 2005, 2006a, 2008, 2011a; McManus et al., 2005; Cumming et al., 2007), the regulation of APR, and ATPS in particular, in terms of enzyme activity and accumulation has been examined in response to S-supply. Most studies that use S-deficiency as a tool to dissect the molecular nature of pathway control, report responses after shorter periods of exposure (up to four days). In contrast, and of more relevance to field crops

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such as onion, are studies in which a S-deficiency is applied over more prolonged periods. Therefore, in this study, the biochemical regulation of the reductive S-assimilation pathway is investigated at both pre-bulbing and bulbing developmental stages. Further, the investigation also compares the pungent cultivar, 'W202A', with the less pungent cultivar, 'Texas Grano 438 PVP' (TG) to determine whether this cultivar difference in S metabolism reflected differences in enzyme activity or regulation measured.

2. Results and discussion

2.1. APR and ATPS enzyme activity and abundance in onion chloroplasts in response to S-supply

APR enzyme activity, measured in whole leaf extracts from plants grown in 18-L capacity tubs, was significantly higher in plants of both cultivars grown in low S-supply at the bulbing stage only (Fig. 1A). No significant differences in activity were observed between cultivars for both S-treatments and at both developmental stages, but a developmental effect was observed for both cultivars with significantly lower activity measured in extracts from plants at bulbing (Fig. 1A). For ATPS activity, measured in chloroplasts isolated from leaves of plants grown in 18-L capacity tubs, no significant differences were observed in both cultivars in response to S-supply at the pre-bulbing stage, although significantly higher activity with higher S-supply was observed at bulbing although only in W202A (Fig. 1A). Preliminary data reporting changes in APR and ATPS activity in onion has been shown previously (McCallum et al., 2006a), but the data presented here represents pooled analysis of two independent trials in 18-L capacity tubs conducted over two distinct seasons. These differences in activity are at variance with the APR and ATPS gene expression data in leaf tissue (McCallum et al., 2011a), where no significant differences were observed between S treatment at bulbing, and so may therefore represent the operation of some degree of either post-transcriptional or post-translational control. It is known that ATPS transcripts are regulated by a sulphur-starvation-inducible miR395 in *Arabidopsis* (Liang et al., 2010), and the observed higher activity in the S^+ treatment may arise through a decrease in the pool of ATPS transcripts in the S^- treatment leading to less translated enzyme. However, it is yet to be determined whether such transcripts are regulated by S-supply in onion and what is the effect on measurable enzyme activity.

The enzyme data is also supported by protein abundance at bulbing (as determined by western analysis; Fig. 1B). For APR, higher accumulation was observed in leaves of plants growing in the S^- media, while higher accumulation of ATPS protein was observed in chloroplast extracts isolated from plants grown in the S^+ media.

The biochemical regulation of the S-assimilation pathway is perhaps best characterised in *Arabidopsis thaliana* (Koprivova et al., 2000; Kopriva, 2006). Using $^{35}SO_4^{2-}$, a flux control co-efficient value of 0.7 to 0.9 has been calculated for the reduction of APS to sulphite by APR (Vauclare et al., 2002), and under S-starvation, the activity of APR has been shown to increase in a series of studies (reviewed in Kopriva, 2006). In common with such studies, we have also measured a significant increase in APR activity and protein accumulation (as determined by western analyses), but only at the bulbing stage. This suggests that some primacy of control may exist at this developmental stage (bulbing) in onion when high S accumulation is occurring while, and unlike *Arabidopsis*, no control is operating at the pre-bulbing (seedling) stage. However, no cultivar differences were observed which does support the gene expression studies (McCallum et al., 2011a) and suggests that any genetic variation in pungency may not be at the level of APR.

For ATPS, the significantly higher activity in response to higher S-supply at the bulbing stage represents a departure from the control of the S-assimilation pathway more commonly observed in model species such as *Arabidopsis* (Vauclare et al., 2002) but also in other species that have been characterised including canola, *Zea mays* and *Brassica juncea* (Lappartient and Touraine, 1996; Clarkson et al., 1999; Lee and Kang, 2005). In these species, significantly higher activity is observed in tissues excised from plants subjected to S-deficient growth conditions. However, given the accumulation of reduced S-containing compounds that also occurs at bulbing (Randle and Lancaster, 2002), these observations suggest that the pathway in onion may operate more in a 'feed-forward' mode with ATPS representing the first point of this regulation (i.e. higher activity with higher S-supply), albeit in the more pungent W202A line. The relatively high cysteine content, particularly at bulbing, observed by McCallum et al. (2011a) may also support this. Further, as there is no significant difference in ATPS gene expression in leaf tissue between S-treatment in either cultivar (McCallum et al., 2011a), changes at the protein level may therefore act as a control point in the pathway. In further support, a complex between ATPS and APR has been postulated, the formation of which would support S flux preferentially through the reductive pathway (Cumming et al., 2007).

Together, this onion chloroplast data did suggest that in the W202A cultivar, at least, regulation of ATPS enzyme activity and abundance may represent a significant control point for the reductive S-assimilation pathway. Thus we sought to undertake further characterisation of the ATPS enzyme.

2.2. ATPS activity in root tissues in response to S-supply

As the first part of the more detailed examination on the regulation of the ATPS enzyme, activity was measured in crude extracts of roots excised from plants grown in 1-L capacity tubs (Fig. 2A). At the pre-bulbing stage, there were no differences in activity between the S-supply treatments in either cultivar and, likewise, no differences were observed between the two cultivars. At the bulbing stage, significantly higher enzyme activity was observed in the S-deficient treatment in both cultivars. A developmental effect was again observed with a significantly reduced level of activity measured in roots of plants of both cultivars maintained in the S^+ media at the bulbing stage in comparison with the pre-bulbing stage (Fig. 2A). The abundance of ATPS was higher in root extracts of both cultivars grown in S^+ media, an observation that was in contrast with the enzyme data (Fig. 2B). In bulbing plants, a higher abundance was observed in W202A in plants grown in low S-supply, in agreement with the enzyme activity measurements (Fig. 2B). However, no major differences in abundance were observed in root extracts of TG maintained in S-sufficient or S-deficient media, which is, again, in contrast with the activity data.

In contrast to shoot tissue, the higher activity in roots of S-deprived onion is consistent with activity measurements in canola and *B. juncea* (Lappartient and Touraine, 1996; Lappartient et al., 1999; Lee and Kang, 2005) but is in contrast to the onion chloroplast data. However, what is consistent is that any differences in activity that are observed occur at bulbing when significant accumulation of reduced S-containing ACSOs is occurring. The decrease in activity in roots in S-sufficient plants coincided with the observed increases in activity in S-sufficient plants in leaf tissue suggesting that some co-ordination of response may operate in onion such that during bulbing, ATPS activity in roots decreases in preference to an increase in the leaves for eventual assimilation into the bulb. This observed co-ordination of response through the