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Genotypic variation in sulfur assimilation and metabolism of onion (*Allium cepa* L.) III. Characterization of sulfite reductase

Michael T. McManus [a,*], Srishti Joshi [a], Bruce Searle [c], Meeghan Pither-Joyce [b], Martin Shaw [b], Susanna Leung [a], Nick Albert [a], Masayoshi Shigyo [d], Jernej Jakse [e,f], Michael J. Havey [e], John McCallum [b]

[a] *Institute of Molecular BioSciences, Massey University, Private Bag 11222, Palmerston North, New Zealand*
[b] *New Zealand Institute for Plant and Food Research Limited, Private Bag 4704, Christchurch, New Zealand*
[c] *New Zealand Institute for Plant and Food Research Limited, Box 85, Hastings, New Zealand*
[d] *Department of Biological and Environmental Science, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan*
[e] *Department of Horticulture, 1575 Linden Drive, University of Wisconsin, Madison, WI 53706, USA*
[f] *Agronomy Department, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia*

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ABSTRACT

Genomic and cDNA sequences corresponding to a ferredoxin-sulfite reductase (SiR) have been cloned from bulb onion (*Allium cepa* L.) and the expression of the gene and activity of the enzyme characterized with respect to sulfur (S) supply. Cloning, mapping and expression studies revealed that onion has a single functional *SiR* gene and also expresses an unprocessed pseudogene (φ-SiR). Northern and qPCR analysis revealed differences in expression pattern between the *SiR* gene and the pseudogene. Western analysis using antibodies raised to a recombinant SiR revealed that the enzyme is present in chloroplasts and phylogenetic analysis has shown that the onion protein groups with lower eudicots. In hydroponically-grown plants, levels of SiR transcripts were significantly higher in the roots of S-sufficient when compared with S-deficient plants of the pungent cultivar 'W202A' but not the less pungent cultivar 'Texas Grano'. In these same treatments, a higher level of enzyme activity was observed in the S-sufficient treatment in leaves of both cultivars before and after bulbing. In a factorial field trial with and without sulfur fertilization, a statistically significant increase in SiR activity was observed in the leaves of the pungent cultivar 'Kojak' in response to added S but not in the less pungent cultivar 'Encore'.

1. Introduction

Onion and other *Allium* vegetables have been valued since antiquity for their characteristic flavors arising from organosulfur compounds, as well as the health-giving properties of these compounds (Griffiths et al., 2002). The flavor precursors of onion are comprised of a series of alk(en)yl cysteine sulfoxides (ACSOs) and their gamma-glutamyl peptides (Randle and Lancaster, 2002). Disruption of cells permits the action of alliinases on free ACSOs producing reactive sulfenic acids, while the distinctive tearing of onion arises from the action of a specific lachrymatory factor synthase (Imai et al., 2002) on 1-propenyl sulfenic acid, to yield the lachrymatory factor (LF) and a spectrum of downstream products (Block et al., 1996, 1997; Calvey et al., 1997). The levels of sulfur bioactives vary widely in onion, due to genetic and environmental effects, most notably sulfate availability (Randle and Lancaster, 2002), and genetic differences in uptake and partitioning of sulfate and organic sulfur compounds have also been reported (Randle et al., 1999; McCallum et al., 2002, 2011). Further, genetic linkage of two S-assimilation genes with a bulb pungency QTL suggests that regulation of S-assimilation may underpin genetic and environmental variation in bioactive S levels (McCallum et al., 2006b).

There have been fewer studies to date concerning regulation of S-assimilation enzymes in onion, but it is known that S-supply negatively influences APS reductase (APR; EC 1.8.99.2) activity at bulbing, while ATP sulfurylase (ATPS; EC 2.7.7.4) is positively influenced, again at bulbing (Thomas et al., 2011). This suggests that a feed-forward mechanism for S-assimilation may operate at the S-demanding bulbing stage, a concept supported by evidence, *in vitro*, of the formation of an ATPS–APR complex that may, *in vivo*, preferentially direct the flux of sulfate towards cysteine biosynthesis (Cumming et al., 2007). Further, a serine acetyltransferase (SAT; E.C. 2.3.1.30) gene from *Allium cepa* is not feed-back regulated by L-cysteine (McManus et al., 2005), in contrast to *Allium tuberosum* where Urano et al. (2000) characterized a feed-back regulated SAT.

Recently, the possibility of SiR activity as a significant control point in S-assimilation has been brought into focus with knock-down experiments in *Arabidopsis thaliana* which indicated that

* Corresponding author. Tel.: +64 6 356 9099; fax: +64 6 350 5688.
E-mail address: M.T.McManus@massey.ac.nz (M.T. McManus).

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down-regulation of the enzyme causes a 'bottleneck' in the flux of S through the reduced pathway (Khan et al., 2010). As SiR only occurs as a single gene in *A. thaliana*, it might be expected that down-regulation at this step will significantly influence the rate of S-flux, although for onion, the large demand for reduced S-containing precursors at bulbing may also support the possibility that SiR is a key enzyme in this Allium species. Thus it is important to determine whether a single copy also exists in onion and how its expression may be regulated in an S-accumulating species. In this regard, we previously reported the cloning and partial characterization of the *SiR* gene from onion (McCallum et al., 2002) and the close linkage of this gene with an ATP sulfurylase locus and a QTL for bulb pungency (McCallum et al., 2006b) suggesting that the enzyme may be an important regulatory point. In this study, therefore, we have characterized the genomic organization, expression and activity of SiR, including responses to S-supply, to better understand the role of the enzyme in the regulation of S-assimilation in onion.

2. Results

2.1. Genomic and cDNA cloning of SiR loci

We previously identified three cDNAs with weak similarity to plant SiRs (GB accessions BI095555, BI729456, BM192999) by randomly sequencing 477 clones from a differential cDNA library from onion root enriched for genes up-regulated by S deprivation (McCallum et al., 2002). The consensus nucleotide sequence of these three clones show weak homology ($e = 10^{-6}$) by BLASTX comparison with a 100 amino acid region corresponding to exons 5 and 6 of *A. thaliana* SiR (At5g04590). However they contain a premature stop codon and a downstream region with no significant similarity to plant SiRs, leading us to postulate that they are products of a transcribed pseudogene. We designate the putative pseudogene as φ-SiR. Primers designed to the homologous region were used to amplify downstream sequences by 3′-RACE with high homology to plant SiRs (McCallum et al., 2002). By aligning these sequences (AF403293, AF403293) with the partial φ-SiR cDNA sequences, nested primers were designed to selectively amplify four distinct φ-SiR cDNA clones (Genbank accessions AY750683–AY750686) by 3′-RACE from total leaf RNA of the heterozygous, open-pollinated onion variety 'Pukekohe Longkeeper'. These differed by several single-nucleotide polymorphisms and one apparent insertion-deletion. Initial attempts to clone 5′ regions of SiR by 5′-RACE were unsuccessful so TAIL-PCR was used to isolate 5′ CDS regions (Genbank AF403293, AF403294). Using the consensus sequence data, the full sulfite reductase coding region was amplified from cDNA of the doubled haploid line 'CUDH2107'.

PCR screening of a partial onion BAC library using a primer set designed to regions shared SiR and φ-SiR cDNA sequences was previously used to identify a single clone (Genbank DQ273270; Jakše et al., 2008) which showed homology to exons 4–6 of plant sulfite reductases (Bork et al., 1998). Gene prediction programmes did not predict gene models consistent with a functional sulfite reductase gene in this sequence. Alignment of the genomic sequence with φ-SiR cDNAs using gmap revealed three exons downstream of the SiR-homologous regions that could produce the observed cDNAs through canonical splice sites (Fig. 1) The 12 bp insertion/deletion polymorphism distinguishing the φ-SiR cDNA clones was consistent with use of alternative canonical AG splice acceptors at base positions 53,108:53,109 and 53,121:53,123.

2.2. Genomic organization of SiR loci

Southern hybridization using the partial SiR cDNA clone (AF403293) obtained by 3′-RACE revealed simple banding in onion and asparagus (but not garlic) consistent with a single locus

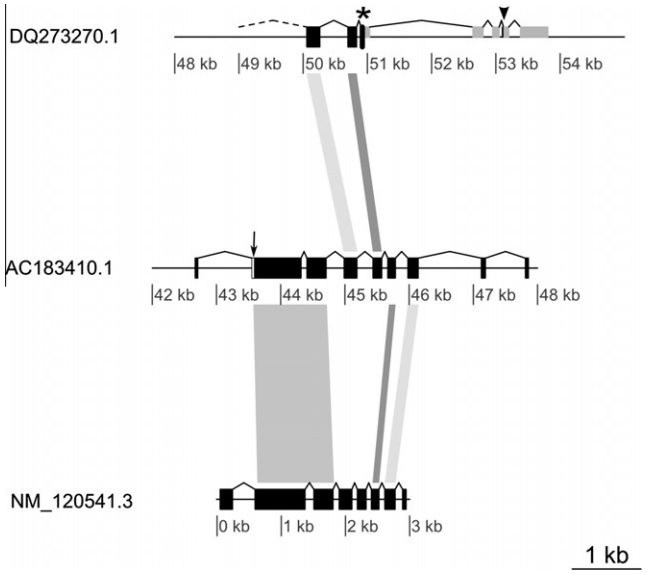

Fig. 1. Comparison of intron–exon structures of sulfite reductases of *Arabidopsis* and asparagus with the SiR pseudogene of onion. A premature stop in the onion pseudogene is denoted by an asterisk and the site of an alternative splicing observed in cDNAs is denoted by an arrowhead. The sequence location of the conserved N-terminal peptide motif reported in *A. tubersosum* SiR by Takahashi et al. (1996b) is denoted by a black line.

(Fig. 2A). This clone only has 100 bp with significant similarity to the pseudogene. However use of a partial cDNA (Genbank accession CF434863) with 330 bp overlap revealed banding consistent with more than one locus (Fig. 2B). Hybridization of a partial φ-SiR cDNA (GB accession BI095555) to genomic DNA revealed a very high copy number (Fig. 2C) suggesting that the downstream region in this clone lacking homology to SiR may represent the insertion of a repetitive element.

We previously reported the mapping of SiR to chromosome 3 in two onion populations (Martin et al., 2005; McCallum et al., 2006b) based on RFLP, SNP and SSCP polymorphisms and Ohara et al. (2005) mapped SiR to chromosome 3 in *Allium fistulosum*. SSCP gels used to map SiR in the 'W202A × Texas Grano 438' F_2 population (McCallum et al., 2006b) exhibited additional segregating bands that could not be readily scored for mapping. Because efforts to design polymorphic markers to the φ-SiR locus were unsuccessful, primer sets designed to simple sequence repeats (SSRs) identified on the BAC clone were evaluated. The primer set ACM304, designed to flank a $(CA)_7$ SSR, revealed a polymorphic single locus amplicon which was located to chromosome 5 by amplification in a panel of monosomic alien addition lines (Fig. 3). This assignment was further verified by mapping in the interspecific *Allium* cross, *A. cepa* × *Allium roylei*, which revealed linkage to the chromosome 5 group containing isozyme marker PGI-1 (van Heusden et al., 2000; McCallum et al., 2012).

2.3. Characterization and comparison of the amino acid sequence of SiR

Comparison of a deduced 630 amino acid residue sequence of onion SiR from the doubled haploid line 'CUDH2107', revealed high sequence identity with SiR sequences from other species (Fig. 4). Using the subcellular localization tool, TargetP, a plastid localization is predicted with the deduced sequence containing a putative 52 amino acid transit peptide. Takahashi et al. (1996b) determined an N-terminal sequence of EVKRSKVEI for a purified SiR protein from *A. tuberosum*, and this exact motif can be identified in the monocot sequences compared in Fig. 4 (rice, maize and Asparagus), but not in Arabidopsis (or in sequences from soybean, grape,