Journal of Cereal Science 68 (2016) 122–131

Contents lists available at ScienceDirect

Journal of Cereal Science

journal homepage: www.elsevier.com/locate/jcs

# Food safety: Structure and expression of the asparagine synthetase gene family of wheat

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ARTICLE INFO

*Article history:*
Received 12 August 2015
Received in revised form
15 January 2016
Accepted 21 January 2016
Available online 26 January 2016

*Keywords:*
Nitrogen metabolism
Nutritional regulation of gene expression
Acrylamide
Crop composition and food safety

ABSTRACT

Asparagine is an important nitrogen storage and transport molecule, but its accumulation as a free amino acid in crops has implications for food safety because free asparagine is a precursor for acrylamide formation during cooking and processing. Asparagine synthesis occurs by the amidation of aspartate, catalysed by asparagine synthetase, and this study concerned the expression of asparagine synthetase (*TaASN*) genes in wheat. The expression of three genes, *TaASN1-3*, was studied in different tissues and in response to nitrogen and sulphur supply. The expression of *TaASN2* in the embryo and endosperm during mid to late grain development was the highest of any of the genes in any tissue. Both *TaASN1* and *TaASN2* increased in expression through grain development, and in the grain of field-grown plants during mid-development in response to sulphur deprivation. However, only *TaASN1* was affected by nitrogen or sulphur supply in pot-based experiments, showing complex tissue-specific and developmentally-changing responses. A putative N-motif or GCN4-like regulatory motif was found in the promoter of *TaASN1* genes from several cereal species. As the study was completed, a fourth gene, *TaASN4*, was identified from recently available genome data. Phylogenetic analysis showed that other cereal species have similar asparagine synthetase gene families to wheat.



## 1. Introduction

Asparagine plays a central role in nitrogen storage and transport in many plant species due to its relatively high ratio of nitrogen to carbon and its relative chemical inertia (Lea et al., 2007). It accumulates in its free (non-protein) form not only during normal physiological processes such as seed germination but also in response to a range of abiotic and biotic stresses (Lea et al., 2007), and understanding the mechanisms that are involved is important for improving crop yield and stress resistance. However, more pressingly, it also has implications for food safety because free asparagine is a precursor for acrylamide formation during high-temperature cooking and processing (reviewed by Curtis et al., 2014a; Halford et al., 2012). This has re-invigorated interest in the enzymes involved in asparagine synthesis and breakdown, and other metabolic pathways that could impact on free asparagine concentrations.

Mineral availability has a major influence on free asparagine accumulation in cereal grain, and highest concentrations occur when the plant has a plentiful supply of nitrogen but is unable to maintain a normal level of protein synthesis because of deficiencies in other nutrients. Unsurprisingly, therefore, nitrogen application correlates positively with free asparagine concentration (Martinek et al., 2009; Postles et al., 2013; Winkler and Schön, 1980), as do deficiencies in potassium, sulphur, phosphorus and magnesium (Lea et al., 2007). In wheat, sulphur deficiency in particular can result in dramatic increases in both the amount of free asparagine in the grain (Curtis et al., 2009, 2014b; Granvogl et al., 2007; Muttucumaru et al., 2006) and its distribution, with most asparagine accumulating in the embryo when sulphur supply is adequate but with high concentrations occurring in the endosperm under

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http://dx.doi.org/10.1016/j.jcs.2016.01.010

sulphur deficiency (Shewry et al., 2009). This means that whole-grain products are normally more prone to acrylamide formation than white flour products, but that white flour products are disproportionately affected by sulphur deficiency.

Asparagine synthesis occurs by the amidation of aspartate, catalysed by the enzyme asparagine synthetase. Early studies on pea (*Pisum sativum*) identified two, differentially-regulated asparagine synthetase genes (Tsai and Coruzzi, 1990) and two asparagine synthetase genes have already been cloned from wheat (*Triticum aestivum*) and called *TaASN1* and *TaASN2* (Wang et al., 2005). *TaASN1* expression in seedlings was shown to be up-regulated by salt and osmotic stress, and by treatment with abscisic acid (ABA) (Wang et al., 2005). Subsequently, its expression in leaves was shown to be induced by sulphur deficiency, but to be greatly reduced when a general control non-derepressible-2-type protein kinase, TaGCN2, was over-expressed (Byrne et al., 2012).

The study reported here concerned the differential expression of *TaASN1* and *TaASN2*, and a third, hitherto unidentified asparagine synthetase gene, *TaASN3*, in different organs of wheat and under varying levels of nitrogen and sulphur availability. As the study was completed, a fourth gene, *TaASN4*, was identified from recently available genome data.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Plant tissues were isolated from wheat (*T. aestivum*) cv. Cadenza. Seeds were surface sterilized in 70% ethanol for 1 min and 10% (v/v) sodium hypochlorite for 20 min with shaking, and then washed thoroughly with distilled water. The sterilized seeds were soaked in distilled water for 6–8 h and then placed in Petri dishes (90 mm) with two layers of moist filter paper at 4 °C for 2 days. The dishes were then transferred to room temperature to allow the seedlings to grow. After 1 week, the seedlings were transplanted to 20 cm diameter pots containing one third sand, one third perlite (typically 70–75% $SiO_2$, 12–15% $Al_2O_3$, 3–4% $Na_2O$, 3–5% $K_2O$, with traces of $Fe_2O_3$, MgO and CaO) and one third nutrient-free compost in a glasshouse with a 16 h day (supplemental lighting was used as necessary) and a minimum temperature of 16 °C. There were five plants per pot.

For the nitrogen feeding study, 24 pots were used, feeding was started one week after transplanting and each pot received 300 mL of liquid every day. Plants were supplied with either a medium containing a full nutrient complement of potassium, phosphate, calcium, magnesium, sodium, iron, nitrate (2 mM $Ca(NO_3)_2$ and 1.6 mM $Mg(NO_3)_2$) and sulphate ions (1.1 mM $MgSO_4$) (Curtis et al., 2009; Muttucumaru et al., 2006), or the same medium containing one-tenth the concentration of nitrate (0.2 mM $Ca(NO_3)_2$ and 0.16 mM $Mg(NO_3)_2$). The pots were arranged in a randomised block design with three blocks, giving three replicates of a two treatments by four time points (7, 14, 21 and 28 days post-anthesis) factorial structure. Grain and flag leaf were collected at the four time points (destructive samples from plants in pots), frozen in liquid nitrogen and stored at −70 °C. Embryo and endosperm from grain at 14 days post-anthesis were separated for tissue-specific analysis.

For the nitrogen and sulphur feeding study, seeds were sown and plants were potted up and provided with liquid feed in the same way. This time, 48 pots were used with five plants per pot. The plants were supplied with four different media, each containing a full complement of potassium, phosphate, calcium, magnesium, sodium and iron (Curtis et al., 2009; Muttucumaru et al., 2006) in addition to different concentrations of nitrate and sulphate. These were: 2 mM $Ca(NO_3)_2$, 1.6 mM $Mg(NO_3)_2$ and 1.1 mM $MgSO_4$ (the $N^+S^+$ treatment); 0.2 mM $Ca(NO_3)_2$, 0.16 mM $Mg(NO_3)_2$ and 1.1 mM $MgSO_4$ (the $N{-}S^+$ treatment); 2 mM $Ca(NO_3)_2$, 1.6 mM $Mg(NO_3)_2$ and 0.11 mM $MgSO_4$ (the $N^+S^-$ treatment); 0.2 mM $Ca(NO_3)_2$, 0.16 mM $Mg(NO_3)_2$ and 0.11 mM $MgSO_4$ (the $N{-}S^-$ treatment). After four weeks, the concentration of sulphate was reduced to zero in the two sulphur-deficient media. The pots were arranged in a randomised block design with three blocks, giving three replicates of a two nitrogen ($N^+$, $N^-$) by two sulphur ($S^+$, $S^-$) by four time points (14, 21, 28, 35 days post-anthesis) factorial structure. Flag leaf, stem, root and grain were collected at 14, 21, 28 and 35 days post-anthesis, and the embryo and endosperm tissues were removed from the grain and separated (samples were not taken at 7 days because the embryo and endosperm are not differentiated at that stage). The material was frozen in liquid nitrogen and kept at −70 °C.

### 2.2. Expression analyses by real-time quantitative polymerase chain reaction (qPCR)

RNA was extracted from powdered tissue using the hot phenol method (Verwoerd et al., 1989), with some modification. Approximately 0.5 mL of frozen, powered tissue was suspended in 1 mL of hot (80 °C) phenol and extraction buffer (1:1, v/v). Chloroform:isoamyl alcohol (24:1, v/v) (0.5 mL) was added and mixed well before separation of the phases by centrifugation for 5 min at 4 °C. The aqueous phase was transferred to another tube and the extraction with chloroform:isoamyl alcohol was repeated. Total RNA was then precipitated from the solution by adding lithium chloride to a concentration of 4 M and keeping the solution at 4 °C overnight. After collection of the total RNA by centrifugation and a 70% ethanol wash, any DNA contamination was removed by DNaseI treatment. After further purification by extraction with phenol:-chloroform:isoamyl alcohol (25:24:1, v/v/v) and chloroform:isoamyl alcohol, the RNA was precipitated with ethanol and dissolved in diethylpyrocarbonate-treated water. The RNA concentration was measured using a Nanodrop-1000 Spectrophotometer (Thermo Fisher, Wilmington, DE, USA) and its quality checked by electrophoresis through a 1% agarose gel.

RNA samples that had been prepared in the same way were also available from winter wheat, cv. Spark, that had been grown in a field trial on the Rothamsted Research farm at Woburn, Bedfordshire, UK, in 2007–2008. The soil at this site is a sandy loam with very poor nutrient retention and is severely sulphur deficient (extractable soil sulphate concentrations range from 0.5–1.8 mg sulphur per kg; Riley et al., 2002). The plants had been supplied with either 0 ($S^-$) or 40 kg sulphur ($S^+$) per hectare, in a split plot design, the sulphur being applied as gypsum (calcium sulphate dihydrate) at the tillering stage in March 2008. All of the plants received nitrogen at 200 kg per hectare as ammonium nitrate. There were three replicate samples for each treatment ($S^+$, $S^-$) at each of four time points (7, 14, 21 and 28 days post-anthesis).

First-strand cDNA synthesis was performed using SuperscriptIII (Invitrogen, Life Technologies Ltd, Paisley, UK) and 2 μg DNase-treated RNA, according to the manufacturer's protocol. The qPCR reaction mix consisted of 10 μL 2 × SYBR Green JumpStart Taq ReadyMix (Sigma, Poole, UK), 2.5 μL cDNA, 1 μL of a 10 μM stock solution of each primer and 0.2 μL ROX reference dye (to normalise the fluorescent reporter signal) in a final volume of 20 μL. The reaction was performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, California, USA) and the amplification conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and then at 62 °C for 1 min. Data were collected during the 62 °C step and the melting curves were calculated after cycle 45. The primers for qPCR were designed by Primer3 (Untergasser et al., 2012) and are shown in Supplementary