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Ectopic phytocystatin expression increases nodule numbers and influences the responses of soybean (*Glycine max*) to nitrogen deficiency

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This paper forms part of a special issue of *Phytochemistry* dedicated to the memory and legacy of Professor (Godfrey) Paul Bolwell, MA DSc (Oxon). (1946–2012), internationally-recognised plant biochemist and Regional Editor of *Phytochemistry* (2004–2012). He is much missed by his friends.

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ABSTRACT

Cysteine proteases and cystatins have many functions that remain poorly characterised, particularly in crop plants. We therefore investigated the responses of these proteins to nitrogen deficiency in wild-type soybeans and in two independent transgenic soybean lines (OCI-1 and OCI-2) that express the rice cystatin, oryzacystatin-I (OCI). Plants were grown for four weeks under either a high (5 mM) nitrate (HN) regime or in the absence of added nitrate (LN) in the absence or presence of symbiotic rhizobial bacteria. Under the LN regime all lines showed similar classic symptoms of nitrogen deficiency including lower shoot biomass and leaf chlorophyll. However, the LN-induced decreases in leaf protein and increases in root protein tended to be smaller in the OCI-1 and OCI-2 lines than in the wild type. When LN-plants were grown with rhizobia, OCI-1 and OCI-2 roots had significantly more crown nodules than wild-type plants. The growth nitrogen regime had a significant effect on the abundance of transcripts encoding vacuolar processing enzymes (VPEs), LN-dependent increases in VPE2 and VPE3 transcripts in all lines. However, the LN-dependent increases of VPE2 and VPE3 transcripts were significantly lower in the leaves of OCI-1 and OCI-2 plants than in the wild type. These results show that nitrogen availability regulates the leaf and root cysteine protease, VPE and cystatin transcript profiles in a manner that is in some cases influenced by ectopic OCI expression. Moreover, the OCI-dependent inhibition of papain-like cysteine proteases favours increased nodulation and enhanced tolerance to nitrogen limitation, as shown by the smaller LN-dependent decreases in leaf protein observed in the OCI-1 and OCI-2 plants relative to the wild type.

1. Introduction

The global population continues to rise by about eighty million people per year (Park et al., 2011). There is therefore an urgent need for improved varieties of crops, such as soybean that can sustain high yields even on poor soils (Tilman et al., 2011; Park et al., 2011). The production of improved crops with improved nitrogen use efficiencies so that they are better able to withstand low soil nitrogen availability is an essential requirement for future agriculture worldwide (Kant et al., 2010).

Grain and forage legumes account for nearly thirty percent of the worlds' primary crop production and are important contributors to the dietary protein intake of humans and animals (Graham and Vance, 2003). For example, soybean seeds provide more than 35% of the world's processed vegetable oil, which is used in bread and margarine production as well as industrial products (Van Heerden et al., 2003). Despite the high economic value of soybean, increases in yields have fallen behind those of other staple crops, such as cereals (Jeuffroy and Ney, 1997). This is particularly true in developing countries, where unfavourable environmental conditions can limit legumes productivity (Graham and Vance, 2000). Like other legumes, soybean is able

Abbreviations: CP, cysteine protease; CYS, cystatin; OCI, oryzacystatin-I; VPE, vacuolar processing enzyme.

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to form a mutually-beneficial symbiotic relationship with nitrogen-fixing bacteria (Oldroyd et al., 2011). Symbiotic nitrogen fixation occurring in soybean root nodules that house the bacteria supports high crop productivity even in nitrogen-poor soils (Moulin et al., 2001).

Nitrogen has long been recognised as one of the most important macronutrients required to sustain plant growth and a paradigm of plant responses to nitrogen limitation has been established that includes decreased photosynthesis, increased tissue carbon/nitrogen ratios, decreased shoot/root ratios and leaf carbohydrate accumulation, as well as extensive reprogramming of gene expression (Scheible et al., 2004; Bi et al., 2007). The remobilisation of nitrogen stored in photosynthetic proteins is of particular importance in supporting plant growth and development in situations of nitrogen limitation. Hence large increases in the abundance of transcripts encoding proteins involved in protein turnover and leaf senescence are characteristic of the nitrogen deficiency response (Crafts-Bradner et al., 1998; Horstensteiner and Feller, 2002).

Proteolysis, catalysed by the proteasome and a raft of different proteases, underpins leaf nitrogen remobilisation (Grudkowska and Zagdanska, 2004). Proteases fulfil many important functions in plants including the maturation and removal of abnormal or damaged proteins (Forsthoefel et al., 1998; Solomon et al., 1999; Grudkowska and Zagdanska, 2004). They are also crucial for the restructuring of the composition of the cellular protein network in response to developmental triggers and environmental stimuli. Cysteine proteases are involved in protein remobilisation and nitrogen recycling occurring during leaf senescence and programmed cell death for seed development (Beers et al., 2000; Kato et al., 2003; Esteban-Garcia et al., 2010). For example, the vacuolar processing enzyme (VPE) family of cysteine proteases, which are also known as legumains, play a crucial role in organ senescence and cell death. Loss of VPE function prevents cell death during the hypersensitive response to pathogens (Hara-Nishimura et al., 2005; Hatsugai et al., 2006). VPEs resemble mammalian caspases and they are localised in the vacuole. Their functions have been well-characterized in maturing seeds, where they play a key role in the processing of storage proteins (Shimada et al., 2003). They are able to activate pre-proteases by post-translational modification (Roberts et al., 2012).

The activity of cysteine proteases is regulated *in situ* by interactions with tight-binding inhibitors called cystatins. Cystatins inhibit cysteine proteases of the papain C1A family in a reversible manner (Martinez and Diaz, 2008; Martinez et al., 2012). The rice endosperm cystatin, called oryzacystatin I (OCI), comprises 102 amino acids and has a tertiary structure consisting of a central α-helix and a five-stranded anti-parallel β-sheets with no disulphide bonds (Benchabane et al., 2010). OCI, which is perhaps the best-characterised plant cystatin to date, has a highly conserved QXVXG motif that is required for cysteine protease inhibition (Arai et al., 1991; Jenko et al., 2003). Phytocystatins regulate cysteine protease-mediated protein turnover during growth and development. The coordinated expression of transcripts encoding cysteine protease and cystatin interacting partners has been reported in senescent spinach leaves (Tajima et al., 2011). Ectopic OCI expression not only altered the growth and development of tobacco (Van der Vyver et al., 2003; Prins et al., 2008), soybean and *Arabidopsis thaliana* plants (Quain et al., 2014) but also enhanced tolerance to abiotic stresses, such as low temperatures and drought (Prins et al., 2008; Quain et al., 2014).

Soybeans can suffer from nitrogen deficiency under field conditions, particularly at flowering when the nodules start to senesce or when seeds are either planted without inoculation of soils with appropriate symbiotic bacteria, particularly in areas where soybean has not been grown previously, or on acid soils that prevent successful nodulation (Mengel and Ruiz-Diaz, 2012). Abiotic stresses, such as defoliation, drought and exposure to heavy metals, can cause premature nodule senescence resulting in impaired symbiotic nitrogen fixation (Gordon et al., 1990; Karina et al., 2003).

The recent release of the complete soybean genome (Schmutz et al., 2010) and the RNAseq atlas of genes expressed in fourteen different soybean tissues (Severin et al., 2010) allows the accurate identification and characterisation of soybean cystatins, cysteine proteases and VPEs. The Phytozome database (www.phytozome.net) currently contains over 300 cystatin-like sequences from the Viridiplantae kingdom, 706 C1 cysteine protease sequences as well as 362 C13 cysteine protease (VPE-type) sequences. However, the exact functions of most of these proteins remain largely uncharacterised (Severin et al., 2010). In this study, we selected a small number of model cysteine proteases, VPEs and cystatins that were reported to be expressed in leaves and roots in the Phytozome database. Our aim was to explore whether the levels of transcripts encoding these proteins was influenced by ectopic OCI expression in soybean plants that had been grown for four weeks with either a high (5 mM) nitrate (HN) supply or in the absence of added nitrate (LN). We selected two independent transgenic OCI expressing soybean lines (OCI-1 and OCI-2) that had different levels of OCI transcripts in the leaves (Quain et al., 2014). The variation in the levels of expression between different independent transgenic lines is consistent with the known features of 35S-driven transgene expression in *A. thaliana*, in which a bimodal expression pattern has been reported consisting of 20% high-level expressers and 80% low-level expressers (De Bolle et al., 2003). We compared the effects of ectopic OCI expression in the responses of soybeans to LN using these transgenic lines (Quain et al., 2014). Our earlier studies had indicated that OCI-1 line had about half the levels of the OCI protein in their leaves than the OCI-2 line (Quain et al., 2014). The data presented here show that growth under LN conditions induced changes in the levels of transcripts encoding cysteine proteases, VPEs and cystatins, and that the LN-dependent responses of the soybean plants are modified in the OCI-1 and OCI-2 lines.

2. Experimental

2.1. Plant growth

Soybean (*Glycine max* cultivar Williams) transformation was performed as described previously (Quain et al., 2014). Two independent transformed soybean lines (OCI-1 and OCI-2) that had been produced by selfing the primary transformants twice were used in this study. The lines were selected on the basis of high OCI transcript abundance and OCI protein in leaves and other tissues (Quain et al., 2014). Seeds of wild-type soybeans and OCI-1 and OCI-2 lines were sown in pots containing vermiculite. A minimum of twenty plants per line were analysed in each experiment. Plants were grown in a controlled environment chamber at day/night temperatures of 28 °C/20 °C, respectively, and an irradiance of 400 $mol^{-2}s^{-1}$with a 12 h day/12 h night cycle. Plants were supplied daily with distilled water. 100 ml full-strength Hoagland's solution per plant was added twice per week to plants grown under HN (high nitrogen) conditions while those grown under LN (low nitrogen) conditions were provided twice per week with nitrogen-free Hoagland's solution. Hoagland's solution consisted of KNO_3 (5.0 mM), $MgSO_4{\cdot}7H_2O$ (2 mM), KH_2PO_4 (1 mM), FeDTA (0.1 mM), $CaCl_2{\cdot}2H_2O$ (5 mM) and KCl (0.05 mM). Macronutrient salts were added to the solution [H_3BO_3 (46 μM), $MnSO_4{\cdot}4H_2O$ (3.9 μM), $ZnSO_4{\cdot}7H_2O$ (3.9 μM), $CuSO_4{\cdot}5H_2O$ (1 μM), $Na_2MoO_4{\cdot}2H_2O$ (0.1 μM)], deionised water was added to attain the required volume and the pH was adjusted to 6.8 with 1 M NaOH. The LN