Stress-induced changes in protease composition are determined by nitrogen supply in non-nodulating white clover

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Abstract

An inbreeding line of white clover has been identified which remains non-nodulated under appropriate physiological conditions and so the nitrogen concentration of the plant can be manipulated by altering the nitrate supply to the roots. Non-nodulating plants were used to test the hypothesis that acclimation to nitrogen limitation in white clover involves changes in protease activity and composition. These results indicate that acclimation to nitrogen limitation involves the realignment of constituent proteases without necessarily incurring significant changes in total protease activity. Plants grown at 2.5, 5.0, 7.5, and 10 mM nitrate showed a positive correlation between nitrate supply and foliar protein concentration. Protein profiles, revealed by Coomassie-stained SDS-PAGE, were unchanged between treatments for a given amount of protein. Serine, aspartate/metalloprotease, and two cysteine proteases were identified in the leaves. Although total protease activity per gram fresh weight was unchanged between treatments, the relative contributions of these four proteases was determined by nitrate supply. When plants were stressed further by withholding nitrate there was an increase in cysteine protease activity, but a senescence-related aspartate/metalloprotease was not visible. Hence, while protease expression in white clover leaves responded to the current and past nitrogen status of the plant, the proteases involved in remobilization during nutrient limitation were distinct from those involved during the main senescence period. It is suggested that nitrogen limitation induced an early, reversible stage of senescence in which perturbations in protease activity facilitated the degradation of non-essential proteins in order to increase the chances of plant survival or seed set.

Key words: Nitrate limitation, protease, proteolysis, white clover.

Introduction

Nutrient limitation, especially insufficient available nitrogen, can have major consequences for plant growth and productivity (Devienne-Barret et al., 2000; Andrews et al., 2001; Jenkins and Mahmood, 2003). In non-leguminous plants, sufficient soil nitrogen results in high foliar protein and this is often reflected by the extent of accumulation of Rubisco in the leaves of C3 plants (Delgado et al., 1994; Geiger et al., 1999) and PEPc in C4 plants (Sugiharto and Sugiyama, 1992). Extra nitrogen reserves can also be accumulated as storage proteins in leaves or seeds (Quick et al., 1992; Krishnan et al., 2000; Jain et al., 2001; Warren et al., 2003). Soil nitrogen availability also determines the abundance and activity of enzymes of nitrogen metabolism in leaves, for instance, asparagine synthetase is expressed in response to ammonium (Kawachi et al., 2002). Alternatively, an inadequate nitrogen (nitrate) supply to the roots results in decreased foliar protein (Delgado et al., 1994) and nitrate reductase activity (Li and Oaks, 1993), and less than 1 mM soil nitrate has been shown to affect directly the relative rates of synthesis and degradation of nitrate reductase in shoots and roots (Li and Oaks, 1993). Prolonged nitrogen limitation can result in accelerated leaf senescence and extensive loss of leaf protein (Crafts-Brandner et al., 1998; Hortensteiner and Feller, 2002), accompanied by increased activities of the enzymes of nitrate remobilization.
Legumes are not normally considered to be nitrogen limited because of the presence of nitrogen-fixing bacteria in the root nodules. However, nitrogen limitation can occur if the nodule population is partially or totally ineffective in terms of nitrogen fixation. For example, defoliation and drought can lead to premature nodule senescence which will limit the potential assimilation of molecular nitrogen (Gordon et al., 1990). In addition, recent evidence that the presence of heavy metals in soil are toxic to the root nodules (Karina et al., 2003) suggests that nitrogen limitation may be a consideration for legumes growing in contaminated soils.

It has been suggested that, in wheat, low exogenous nitrogen supply causes the onset of senescence because a decrease in the cytoplasmic amino acid concentration acts as a signal for increased proteolysis, mediated by aspartic proteases (Barneix and Causin, 1996). In this study, non-nodulating clover plants were grown at four different nitrate supplies to test the hypothesis that perturbations in protease activity occur in legumes during acclimation to periods of nitrogen limitation. By exploiting the self-fertile allele of the self-incompatibility system, inbred lines of white clover have been developed (Abberton et al., 1998; Michaelson-Yeates et al., 1998). These are important tools because the resulting plants can exhibit characteristics governed by recessive genes. One such trait is the non-nodulating phenotype of some clover lines. Under physiological conditions of an acid rooting medium and an external nitrogen supply these plants remain non-nodulated and are entirely dependent on nitrogen supply from the soil to support plant growth and development (Abberton et al., 1998). Non-nodulating soybean has been used previously to demonstrate that the nitrogen supply at the roots determines the extent of nitrogen accumulation in the seed during grain-filling in legumes (Ohtake et al., 1999; Lhuillier-Soundele et al., 1999).

**Materials and methods**

**Plant material**

Inbred non-nodulating white clover plants with a range of protein concentrations were grown by varying mineral N application to rhizobia-free plants. White clover plants were grown in a controlled environment glasshouse at IGER. Supplementary lighting was provided by a bank of 18 Phillips SONT Agro 400 W bulbs which were computer-controlled to supply a minimum photosynthetic photon flux density (PPFD) of 200 µmol m⁻² s⁻¹ for a 16 h photoperiod starting at dawn. Temperature was regulated to 20/15 °C during the light and dark periods, respectively.

Seeds of non-nodulating white clover (Abberton et al., 1998; Michaelson-Yeates et al., 1998) were produced at IGER and sown in Fison’s Levingtons compost at a density of 50 seeds to a 15 cm pot and watered daily. After 3 weeks, 20 selected seedlings were transferred to 15 cm pots of Agsorb (8–16 mm diameter baked clay granules, pH 5.2) and watered. The plants were maintained free of rhizobia and, after 4 d, selected pots were fed on one of four nitrogen-containing nutrient solutions (2.5, 5.0, 7.5, and 10.0 mM NO₃⁻) prepared by adding KNO₃ to a nitrogen-free nutrient stock solution (Ryle et al., 1978). These concentrations were selected because earlier trials had confirmed that these treatments gave a range of herbage nitrogen concentrations from low to physiological levels. It was not possible to grow non-nodulating clover in the complete absence of KNO₃. Seedlings were grown for 6–8 weeks before experimental use. For characterization experiments, samples were taken from duplicate plants at each nitrate supply and the entire experiment was repeated on three occasions. Excised clover leaves were induced to senesce by placing them in the dark on dampened filter paper for 7 d at room temperature (Morris et al., 1996). In nitrogen-depletion experiments five plants were grown on each of 2.5, 5.0, 7.5, or 10 mM nitrate for 6 weeks, after which time they were watered with minus-N nutrient solution for a further 6 weeks.

**Biochemical measurements**

Protein was extracted from leaves and petioles which were ground to a fine powder in liquid nitrogen. Buffer (0.1 M TRIS/HCl pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% Triton X-100) was added at 5 ml g⁻¹ fresh weight (FW). Aliquots of the homogenate were removed for chlorophyll determination and the remainder centrifuged for 10 min at 10 000 g at 4 °C. The supernatant was removed and used in subsequent determinations of protein concentration and protease activity. Chlorophyll was extracted from leaf homogenates in acetone and determined according to Arnon (1949). Protein was determined by the dye-binding assay of Bradford (1976). Nitrogen and nitrate were determined according to the methods in Jackson et al. (1986). Protease activity was determined by either a clearing assay in which gelatin/agarose gels (pH 5.6) were incubated at 30 °C as described in Zaveleta-Mancera et al. (1999), in which case 1 unit of activity was assumed to be equivalent to a 1 mm diameter cleared region, or by digestion of azocasein as described by Morris et al. (1996), in which case 1 unit of activity was equivalent to 1 absorbance unit at 405 nm. Soluble sugars were extracted from leaves and petioles in 80% ethanol, and sugar concentration was quantified by HPLC (Cairns et al., 2002). Starch was extracted from the residue and analysed as described in Gallagher et al. (1997).

**Electrophoresis**

Denaturing protein electrophoresis was performed according to Laemmli (1970). Semi-denaturing electrophoresis and staining of protease activity isoforms was performed as described previously (Kingston-Smith et al., 2003). Protease inhibition was achieved by pre-incubation of extracts with 10 µM E-64, 1 mM PMSF, 1 µM pepstatin A or 4 mM EDTA (final concentrations). These inhibitors were also included, as appropriate, in the development solution at the concentrations given above. Polypeptide and protease bands were quantified by densitometry (BioRad GS710 scanning densitometer equipped with Quantity One software; BioRad UK Ltd, Hemel Hempstead, UK).

**Statistical analysis**

Statistical analysis was performed by using the ANOVA function of the GENSTAT program (Baird et al., 2002).
Results

Characterization of non-nodulated white clover under different N supplies

Non-nodulated clover plants showed an obvious phenotypic effect when grown at different nitrogen concentrations. Above-ground dry matter (DM), total leaf nitrogen, and chlorophyll concentration increased significantly between treatments as nitrate supply increased from 2.5 mM to 10 mM (Table 1). There were no significant differences in total nitrogen of the petioles (data not shown). Leaves accumulated significantly more nitrate at 10 mM nitrate supply than at lower nutrient levels (Table 1).

The amount of extractable leaf protein increased progressively as nitrate supply increased, such that there was a doubling of protein concentration between plants grown at 2.5 mM and 10 mM nitrate (Table 1). Protein was extracted from leaves of plants grown at the various nitrate supplies and polypeptides were separated by denaturing gel electrophoresis. When loaded on an equal protein basis, the tracks were remarkably similar in composition and intensity of staining of particular bands (Fig. 1). Carbohydrate partitioning was affected by nitrate supply. Leaf starch showed a significant decrease with increasing nitrate supply, whereas there were no significant differences in water-soluble carbohydrate (WSC) between treatments (Table 1).

On a fresh weight basis, total foliar protease activity was similar between nitrate supply treatments, although a trend towards decreasing activity with increasing nitrate supply was detected when data were expressed on a protein basis (Fig. 2A). Further examination by semi-denaturing electrophoresis revealed three protease isoforms in mature leaves (identified here as a, c, and d) with Ms of 72, 42, and 37 kDa (Fig. 2B). Isoform d at 37 kDa was most abundant (47% of total protease by densitometry), in leaves of plants grown at 2.5 mM N compared with other N supplies (17% of total protease by densitometry), but was observed in all extracts made from senescent leaves. By contrast, isoform a at 72 kDa was most obvious in mature leaves of plants grown with 7.5 or 10 mM nitrate (>35% of total protease by densitometry compared with 6% in leaves of plants supplied with 2.5 mM nitrate). The 50 kDa isoform b was not detectable in mature leaves, although it increased with increasing nitrate supply in senescent leaves (Fig. 2B). Isoform c was more visible in senescent than mature clover leaves regardless of nitrate supply. In addition, isoform c was unaffected by nitrate supply in mature leaves, although there was a slight decrease in activity with nitrate supply in senescent leaves (Fig. 2B). By the inclusion of protease inhibitors during electrophoresis and staining, it was possible to deduce the protease activity class from the disappearance of specific bands in replica gels (Fig. 3). Isoform a was inhibited by PMSF and showed increased activity with EDTA, consistent with it being a serine protease (Fig. 3A, B). Isoform b was inhibited by both pepstatin and increasing concentrations of EDTA, suggesting either an aspartate or metalloprotease (Fig. 3C, D). Both isoforms c and d appeared to be cysteine proteases as they were inhibited by E-64 and activated by EDTA (Fig. 3E–H).

Table 1. Above-ground dry matter production (DM) and composition of leaves of non-nodulating clover grown at a range of nitrate supplies

Results from analysis of variance are presented from between one and four independent replications of an experimental block in which samples were destructively harvested from each of at least two plants grown at 2.5, 5.0, 7.5, or 10 mM nitrate. N, total nitrogen; WSC, water-soluble carbohydrate; LSD, least significant difference between means.

<table>
<thead>
<tr>
<th>Nitrate (mM)</th>
<th>DM (g)</th>
<th>N (mg g⁻¹ DM)</th>
<th>Protein (mg g⁻¹ DM)</th>
<th>Nitrate (mg g⁻¹ DM)</th>
<th>Chlorophyll (mg g⁻¹ DM)</th>
<th>Starch (mg g⁻¹ DM)</th>
<th>WSC (mg g⁻¹ DM)</th>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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for a particular inhibitor there was no detectable change in band density.

**Effect of nutrient stress on clover proteases**

In order to investigate the effect of nitrogen deprivation on the protein–protease relationship, plants were grown for 6 weeks with nitrate supplied as described above, after which time plants were given nutrient solution without nitrate (minus-N treatment) for 6 weeks. As the duration of the minus-N treatment progressed, leaf protein and chlorophyll values decreased towards those of the 2.5 mM nitrate pretreatment plants (Table 2). A significant time × nitrogen interaction was identified for chlorophyll and protein concentrations as a result of nitrogen deprivation, indicating that net protein concentration was a result of both prior nitrogen nutrition and duration of the minus-N treatment. Changes in leaf protein were accompanied by a gradual increase in protease activity regardless of the initial nitrate feeding regime (Table 2). This pattern began to reverse by the sixth week of treatment, possibly due to the very low levels of protein which were achieved prior to this sample time. Isoforms a, c, and d were present in leaves before the start of the minus-N treatment (Fig. 4). The gels were loaded on an equal fresh weight basis, so that intensity of staining reflects changes in net leaf protein between sample times (Table 2) as well as protease isoform abundance within samples. Hence, low staining of protease bands in plants initially grown at 10 mM nitrate reveals that protease proteins were a minor component of the protein pool, whereas they were relatively more abundant in leaves of plants grown at 2.5 mM nitrate before the start of the minus-N treatment. Isoform b was not observed to be a contributor to the protease activity during acclimation to lack of nitrate (Fig. 4). The band density of isoform c was relatively stable during minus-N treatment, and the relative abundance of isoform c was not affected by prior nitrate supply (mean percentage relative abundances were approximately 35%). The relative abundance of isoform d increased with time from 25% to 40%, regardless of the feeding regime before the onset of the minus-N treatment (Fig. 4). However, the time when isoform d was at maximum relative abundance depended on the previous nitrate supply; between weeks 3 and 4 for plants grown with 2.5 mM nitrate, but between weeks 4 and 5 for plants grown with 10 mM nitrate before the minus-N treatment (Fig. 4).

**Discussion**

Soil nitrogen supply is a key determinant of plant growth and productivity and it can directly influence the activity of enzymes of nitrogen metabolism in leaves of non-leguminous plants (Li and Oaks, 1993; Hortensteiner and Feller, 2002; Kawachi et al., 2002). By contrast with most legumes, the lack of nitrogen-fixing symbiotic bacteria in the root nodules of the white clover plants used here meant that these plants were entirely dependent on the uptake of nitrate by the roots to provide the nitrogen required for growth. As in non-leguminous plants, the non-nodulating clover showed a clear growth response to nitrate supplied at between 2.5 mM and 10 mM with an incremental rise in both total leaf nitrogen and extractable protein (Table 1). Murage et al. (1996) found that in Solanum nigrum, increasing the supply of nitrogen increased both protein and nitrate concentration of the leaves. However, in this study, a slight increase in the nitrate concentration of white clover leaves was observed only at the highest level of nitrate nutrition (Table 1).

An exogenous nitrate supply has been shown to be essential for the accumulation of the storage protein β-conglycinin by non-nodulating soybean and pea (Ohtake et al., 1996; Krishnan et al., 2000). It was possible that, in non-nodulating white clover, the observed increase in leaf protein at higher nitrate supplies was due to the accumulation of either storage Rubisco (Dalling, 1985) or a vegetative storage protein. However, the results from protein measurement and gel electrophoresis indicate that the increase in leaf protein observed here was due to an overall increase in protein synthesis and was not because of an increase in specific proteins. The proportion of the protein
pool accounted for by Rubisco was remarkably consistent between nitrate treatments (Fig. 1). Likewise, if the observed 2-fold increase in leaf protein between the highest and the lowest nitrate treatments was due to a storage protein, the corresponding peptide band would have been highly visible, but no suitable candidates were observed.

Maximum nitrogen use efficiency with limited nitrogen supply can be achieved by the remobilization of nitrogen stored as proteins, which is then utilized in reproductive or vegetative organs. Remobilization of stored nitrogen is a key part of grain-fill. Hence, seed yield can be severely restricted if leaf protein concentrations are low as a result of non-nodulation of legumes or low soil nitrogen supply to non-legumes (Tsai et al., 1991; Lhuillier-Soundele et al., 1999; Miceli et al., 2000). It has been shown that protease activities in the roots of a perennial grass change according to the requirements for nitrogen remobilization (Thornton and Bausenwein, 2000). It has also been suggested that the decreased abundance of Rubisco polypeptides during growth of maize at low soil nitrogen was a result of enhanced proteolysis (Tsai et al., 1991). In the current study, non-nodulating clover plants were used to identify changes in leaf protease activity in relation to growth under limited nitrogen supply. Given that a 2-fold increase in leaf protein was observed between 2.5 mM and 10 mM nitrate supply in the mature leaves of non-nodulating clover, the lack of a corresponding decrease in total protease activity was surprising (Fig. 2A). However, it is possible that fine control of protease activity in white clover leaves was achieved by endogenous inhibitors such as serpins (Roberts et al., 2003), rather than by turnover of the protease proteins. Yamauchi et al. (2002) have previously found that nitrogen-starved cucumber cotyledons also had lower protease activities than the controls and this could be explained by the specific loss of one protease isoform. Although total protease activity in non-nodulating white clover was little altered by nitrate supply, the protease isoform composition of the leaves was different between 2.5 mM and 10 mM nitrate treatments. By contrast with the results of Yamauchi et al. (2002), one protease activity (isoform d) was present at higher relative abundance in plants grown at 2.5 mM nitrate than in the other treatments. According to its response to incubation with E-64 and EDTA this is a cysteine protease. Asp et al. (2004) have

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Fig. 3. Inhibition of protease isoforms by 1 mM PMSF, 10 μM E-64, 1 μM pepstatin and 0, 4, or 10 mM EDTA. Effect of (A) PMSF and (B) EDTA on protease a; (C) pepstatin and (D) EDTA on protease b; (E) E-64 and (F) EDTA on protease c; (G) E64 and (H) EDTA on protease d. Representative images are presented where inhibition of activity was detected by comparative densitometry of images in negative: solid lines, untreated controls; dashed lines, inhibitor treated.
recently characterized a 32 kDa cysteine protease from white clover and it is possible that this is the same protease as isoform d.

One explanation for the presence of high protease activity at low leaf protein could involve signalling via cytoplasmic amino acid concentration (Barneix and Causin, 1996). Barneix and Causin (1996) suggest that exogenous nitrate (and to greater effect, ammonium) supply determines the cytoplasmic free amino acid concentration in leaves. Hence, under high nitrogen supply the maintenance of proteolysis at a low level permits the generation of sufficient amino acids which can be exported to nitrogen-sinks. Under low nitrogen supply the low leaf protein coupled with low rates of proteolysis means a decrease in cytoplasmatic amino acid concentration, which results in the increased activity of aspartic proteases and premature senescence (Barneix and Causin, 1996). This elegant hypothesis may need further refinement to take into account the observations that regulation of nitrogen uptake also involves the rate of assimilation into amino acids (Devienne-Barret et al., 2000) and plant organs often accumulate specific amino acids in response to nitrogen supply (Mack and Schoerring, 2002). Nevertheless, it is suggested that the increased relative activity of isoform d at 2.5 mM nitrate compared with higher nitrate supplies reflects an acclimatory increase in a specific stress-responsive protease in white clover leaves to avoid premature senescence under sub-optimal nitrate supplies.

Plants acclimated to restricted nitrate supply were challenged further by the complete withdrawal of nitrate. Plants which had received greater than 2.5 mM nitrate prior to minus-N treatment showed a gradual decrease in leaf protein when supplementary nitrate was withdrawn. Over a period of 6 weeks, protein tended towards that of the 2.5 mM nitrate treatment, which was relatively unaffected by nitrate withdrawal. This suggests that the 2.5 mM nitrate treatment was already seriously limiting for growth. A significant interaction between duration of treatment and nitrate supply before withdrawal was identified for leaf protein concentration (Table 2), reflecting that both the endpoint and the rate of change were influenced by prior nutrition. Although there was no similar effect on total protease activity (Table 2), there did appear to be an effect of time and prior nutrition on isoform profile and abundance (Fig. 4). This is consistent with the proposed role for

<table>
<thead>
<tr>
<th>Growth NO₃ /C₂₅₅</th>
<th>Chlorophyll (mg g⁻¹ FW)</th>
<th>Protein (mg g⁻¹ FW)</th>
<th>Protease activity (units h⁻¹ g⁻¹ FW)</th>
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<td>6</td>
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</table>

**Fig. 4.** Effect of 6 weeks of nitrogen starvation on protease activity in leaves from plants previously grown at (A) 2.5, (B) 5.0, (C) 7.5, or (D) 10.0 mM nitrate. Molecular weight standards are shown in (A). Each track was loaded with the protein contained in 0.5 mg FW.

Table 2. Effect of withholding nitrate on the chlorophyll, protein, and protease content of destructively sampled leaves of non-nodulating white clover previously grown at different nitrate supplements

LSD, least significant difference between means.
isoform d in the feedback regulation mechanism of Barneix and Causin (1996); the combination of a high residual protein concentration (e.g. withdrawl of nitrogen from plants grown at 10 mM nitrate) and small changes in protease activity will result in increased amino acid production which will limit further proteolysis. Therefore, changes in protein concentration can be significant while changes in protease activity are not. Once the position is reached where only essential proteins remain and amino acid concentrations fall, it is speculated that this will trigger more extensive proteolysis, which will presumably involve a different range of proteases with different substrate specificities.

The observation that chlorophyll decreased over the 6 week period of nitrogen starvation implies that senescence had been induced by nutrient starvation and that the remobilization processes had been switched on (Thomas and Stoddart, 1980; Zaveleta-Mancera et al., 1999; Hortensteiner and Feller, 2002). Senescence-specific induction of cysteine protease activity has been observed previously (Morris et al., 1996; Li et al., 2000; Yamada et al., 2001; Chen et al., 2002; Hayden and Christopher, 2004) and during senescence of the non-nodulating white clover leaves there was a strong up-regulation of a cysteine protease at 42 kDa (isoform c) compared with activities in non-senescent leaves (Fig. 2B). Senescing white clover leaves also produced an aspartate or metalloprotease (isoform b) which was not detectable in mature leaves. This agrees with the growing body of evidence supporting senescence-specific expression of examples from most protease classes genes in leaves and petals (Panavas et al., 1999; Wagstaff et al., 2002; Buchanan-Wollaston et al., 2003; Breeze et al., 2004). Roberts et al. (2003) reported a serine protease activity during senescence of wheat leaves for which Rubisco was a target protein. In this study’s experiments, protease a was identified as a putative senescent protease, but its activity was less influenced by senescence than the cysteine proteases (isoforms c and d) were. The white clover serine protease (isoform a) does not appear to be the same as that in wheat leaves. Firstly, the Mr of isoform a was much lower than that of the holoenzyme (118 kDa) and greater than the subunits (59 kDa) identified by Roberts et al. (2003). Secondly, the protease identified by Roberts et al. (2003) had a neutral pH optimum, whereas staining of protease activity gels at neutral pH (data not shown) did not reveal increased or extra protease activities compared with the data shown here.

The use of non-nodulated legumes has demonstrated interesting relationships between protease profiles and rates of proteolysis under nutrient limitation and subsequent response to stress. Leaves of non-nodulating soybean, grown in the absence of nitrogen supplements, were observed to enter senescence earlier and had enhanced proteolytic activity during the initial period of senescence compared with a nodulating soybean line (Miceli et al., 2000). However, these authors concluded that as protease activity increased in both nodulating and non-nodulating lines, plant nitrogen status played a minor role in the regulation of proteases. By contrast, this work shows that it is possible to have similar protease activities but for the protease isoform composition to be quite different, suggesting the potential targeting of different substrates under different nutrient or stress regimes. During senescence, the greatest increase in activity was seen in isoform c, but isoform d also showed an increased contribution to the protease pool with senescence (Fig. 2). However, only isoform d increased in activity with the duration of the minus-N treatment. It has previously been demonstrated that protease activity can change in response to the onset and reversion of senescence (Zaveleta-Mancera et al., 1999). Despite visible symptoms such as loss of chlorophyll, it appears that the proteases involved in remobilization during nutrient limitation are not the same as those involved during senescence-related nitrogen mobilization. From these data it is suggested that protease activity in white clover also reacts to changes in plant nitrogen status. Thus, isoform d could reflect a reversible protease activation which protects against premature senescence during times of low nitrogen which, if not alleviated, are followed by increased isoform c activity, co-incident with the onset of senescence. It may be that the nitrogen limitation treatments imposed here resulted in only the very early stages of senescence, from which recovery is possible (Zavaleta-Mancera et al., 1999).

It is concluded that during acclimation to nutrient limitation, nitrogen remobilization involves the realignment of constituent proteases, presumably to facilitate degradation of storage or non-essential proteins and to promote plant survival or increase the chances of seed set. These results imply that protease expression in mature white clover leaves is responsive to the nitrogen status of the plant and that this response is modulated by nutritional history. These data support the hypothesis that plants acclimate to altered nitrate supply by increasing the relative contribution of specific proteases to the total protease activity. However, subtle changes in protease composition may be overlooked as they are not always accompanied by significant changes in total protease activity.

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