Identification of predominant genes involved in regulation and execution of senescence-associated nitrogen remobilization in flag leaves of field grown barley

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Abstract

The transcriptomes of senescing flag leaves collected from barley field plots with standard or high nitrogen supply were compared to identify genes specifically associated with nitrogen remobilization during leaf senescence under agronomically relevant conditions. In flag leaves collected in field plots with high nitrogen supply, the decline in chlorophyll content was delayed. By comparing changes in gene expression for the two nitrogen levels, it was possible to discriminate genes related to nitrogen remobilization during senescence and genes involved in other processes associated with the late development of leaves under field conditions. Predominant genes that were more strongly upregulated during senescence of flag leaves from plants with standard nitrogen supply included genes encoding the transcription factor HvNAC026, serine type protease SCPL51, and the autophagy factors APG7 and ATG18F. Elevated expression of these genes in senescing leaves from plants with standard nitrogen supply indicates important roles of the corresponding proteins in nitrogen remobilization. In comparison, the genes upregulated in both flag leaf samples might have roles in general senescence processes associated with late leaf development. Among these genes were the transcription factor genes HvNAC001, HvNAC005, HvNAC013, HvWRKY12 and MYB, genes encoding the papain-like cysteine peptidases HvPAP14 and HvPAP20, as well as a subtilase gene.

Key words: Barley, field experiment, flag leaf, Hordeum vulgare L., nitrogen remobilization, nitrogen supply, leaf senescence, HvNAC026.

Introduction

Leaf senescence is a highly regulated complex developmental process terminated by death of the organ. The purpose of senescence is to remobilize nutrients, in particular nitrogen, from the senescing organs to still growing plant parts, and finally to the developing seeds. In crop plants such as cereals, senescence is a slow process, whereas in other plants, in particular Arabidopsis, senescence might occur quickly, and hence has been discussed as a cell-death process (Jansson and Thomas, 2008).

In crop plants, leaf senescence is of pivotal importance for yield (Gregersen et al., 2013). It has been estimated that during seed filling of most annual crop plants more than 70% of the leaf nitrogen is exported from the senescing leaves (Peoples and Dalling, 1988). Chloroplast proteins are the major source for grain nitrogen (Hörtensteiner and Feller, 2002). Up to 70% of the chloroplast nitrogen is assembled into ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Another major fraction is contained in the...
chlorophyll-protein complexes of the thylakoid membrane. As long as chlorophyll is bound to these proteins, they are not accessible to proteases (Hörtensteiner and Feller, 2002). The decline in chlorophyll content preceding the degradation of the membrane proteins is therefore an excellent marker of leaf senescence (Humbeck et al., 1996).

Senescence can be accelerated under situations of low nitrogen supply (Schulze et al., 1994), or it can be delayed or even reversed by excess nitrogen supply (Diaz et al., 2005; Schildhauer et al., 2008). A low nitrogen nutrition status during senescence is also known to promote proteolysis (Masclaux et al., 2000). Despite their importance for plant productivity, knowledge of the genes encoding the major proteases involved in senescence-associated degradation of chloroplast proteins and the factors regulating protein breakdown remains scarce. Based on gene expression studies, several proteases that are encoded by genes upregulated during senescence were identified, but their substrates are not known (Bhalerao et al., 2003; Gepstein et al., 2003; Guo et al., 2004a; Buchanan-Wollaston et al., 2005; Gregersen and Holm, 2006).

The intention of this study was to identify genes in barley encoding proteins that might be important for nitrogen remobilization during leaf senescence under agronomically relevant conditions in the field. For this purpose the transcriptomes of flag leaves collected from field plots supplied with two different levels of nitrogen were analysed. Flag leaves have been chosen because their senescence is known to be most important for seed filling (Stoy, 1973; Stamp and Herzog, 1976). Senescence symptoms in flag leaves of plants grown at high nitrogen supply were suppressed compared with flag leaves from plots with standard nitrogen supply. To identify key factors involved in regulation of nitrogen remobilization the first goal of this microarray analysis was to identify genes encoding transcription factors. The second goal was to identify genes encoding enzymes putatively involved in protein degradation.

**Materials and methods**

**Plant material**

The winter barley cultivar Lomerit (Hordeum vulgare L., cv. Lomerit), introduced in 2001, has been one of the most cultivated crops. During crop rotation rapeseed and oat were consistently cultivated on one large field. The field used in 2010 was adjacent to the field used in 2009. For both fields, rapeseed, wheat, and oat were used as previous described (Kohl et al., 2009) and the HarvEST 35 assembly, both available at HarvEST (http://www.harvest-web.org, last accessed 24 February 2014), as well as by a BlastN search of a recently generated transcriptome assembly (Kohl et al., 2012). Initially, functional classification and annotation of genes was performed using the Mercator software (Usadel et al., 2009). Results were subsequently checked by BlastX searches of the non-redundant database from NCBI (http://www.ncbi.nlm.nih.gov, last accessed 24 February 2014) and the MEROPS...
peptidase database (Rawlings et al., 2012). To generate heat maps, the Cluster 3.0 Software and TreeViewer 1.1.3 were used. For simplicity, sequences included on the microarray or in the qRT-PCR were designated in this work as genes.

Quantitative real-time PCR

RNA samples from leaves collected in 2010 were used for cDNA synthesis using the SuperScript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. All reactions were done in three biological replicates, and for each of the biological replicates three technical replicates were measured. Primer design and quantitative real-time PCR were performed as described previously (Christiansen et al., 2011) using the ABI Prism 7900HT Sequence Detection System and the Power SYBR Green PCR master Mix (Applied Biosystems, Foster City, USA). As references the gene encoding the 18S ribosomal RNA (AK251731) and the gene encoding the splicing factor 2 (SP2) (AK249101) (Fig. 3A) were used. The primer sequences are listed in Supplementary Table S2 (available at JXB online).

Ct values and fold changes were calculated by the R software (R developmental core team, 2005) using the HTqPCR package (Dvinge and Bertone, 2009).

Results

Senescence of flag leaves in the field at standard nitrogen supply

For characterization of senescence of flag leaves from the 2009 field plots, the relative chlorophyll contents and protein contents were determined (Fig. 1A, B). Although the relative chlorophyll content of leaves from standard-nitrogen plots (SN) in the sampling period from 4 until 20 June 2009 declined by 80%, the chlorophyll content of leaves from high-nitrogen plots (HN) declined by only 24% during the same period. By 12 June 2009, the decrease in chlorophyll content was accelerated in leaves collected from plots of both nitrogen supplies. This date was defined as the day of senescence onset (Krupinska et al., 2012). The protein content decreased in SN leaves during the whole sampling period until 16 June 2009 to a level of 42% compared with the initial protein content. In HN leaves the protein content decreased from 27 May 2009 until 4 June 2009 to a level of 55% compared with the initial protein content, and stayed almost constant thereafter (Fig. 1B). Grain filling was monitored by ear dry weight, which increased continuously during the sampling period and reached a maximum at 18 June 2009 (Fig. 1C). No significant differences were observed between the dry weight of SN ears and HN ears indicating that the standard nitrogen supply did not limit the filling of the kernels.

In comparison to 2009 the development of the plants in 2010 was delayed by about 20 days (days of flowering were 11 May 2009 and 31 May 2010). This was compensated partly by a shorter period between the heading date and the onset of senescence (Fig. 1; Supplementary Fig. S1, available at JXB online).

Gene expression analyses by microarrays

To identify the predominant genes important for nitrogen-remobilization processes, senescing flag leaves from SN field plots were compared with flag leaves collected at the same time from HN field plots. Changes in gene expression during senescence were analysed using the 4x44k barley microarray. Raw datasets generated by scanning of the hybridized arrays are available on ArrayExpress (E-MTAB-2202).

The leaves collected on 4 June 2009 from SN and HN plots (SN1, HN1) were chosen as non-senescent control leaves. Expression changes between senescent SN leaves and SN control leaves that were not senescing (SN2 vs. SN1) were...
SN1  100%  SN2  61%
HN1  100%  HN2  86%  HN3  76%

Fig. 2. (A) Microarray design used to analyse differences between non-senescing and senescing samples from plants grown under standard nitrogen (SN) or high nitrogen (HN) supply. Relative chlorophyll contents (Fig. 1A) are given in percentages. Each line represents one comparison. (B) Filtering of the microarray results. Conditions for each filtering step are given. The procedure resulted in two groups of genes: genes only differentially expressed in the comparison SN2 vs. SN1 and genes differentially expressed in both comparisons SN2 vs. SN1 and HN3 vs. HN1.

leaves it was reduced by only 14%. Importantly, both samples were collected at the same time of the day. It is expected that changes in gene expression caused by environmental factors such as light and temperature occur simultaneously in both samples and are eliminated by the subtraction of the datasets (Fig. 2B). To distinguish between genes involved in nitrogen remobilization and other developmentally regulated genes, expression changes during senescence of SN leaves (SN2 vs. SN1) were compared with changes in gene expression in the oldest HN leaves (HN3 vs. HN1), which were collected at 20 June 2009 (Fig. 2B). At this third sampling time point (HN3) the chlorophyll content in HN3 flag leaves was reduced by only 24% compared with the first time point (HN1).

Reduction of redundancies of the probes on the 44k barley microarray and statistical analysis in limma resulted in 12,894 genes that appeared to be unique and had an adjusted $P$-value smaller than 0.05 in the combined analysis for differentially expressed genes (Fig. 2B). Of the differentially expressed genes, 750 showed at least 2-fold (log$_2$) changes in expression in the comparison of SN2 vs. SN1 (Fig. 2B; Supplementary Table S1, available at JXB online) and these were chosen for further analysis. To eliminate expression changes due to environmental factors, the 61 genes, which were also differentially expressed during the same period in HN leaves (HN2 vs. HN1), were subtracted from this gene pool (Fig. 2B; Supplementary Table S1, available at JXB online).

Among the remaining 689 genes, 117 (93 up- and 24 down-regulated) were also differentially expressed during further development of HN leaves (HN3 vs. HN1) and 572 (376 up- and 196 downregulated) genes were differentially expressed in senescing SN leaves (SN2 vs. SN1) (Fig. 2B; Supplementary Table S1, available at JXB online). 75 of the 117 genes differentially expressed in both SN and HN leaves (SN2 vs. SN1 and HN3 vs. HN1) as well as 330 of the 572 genes with altered expression levels in SN leaves (only in SN2 vs. SN1) could be assigned with high accuracy to a total of 27 functional categories called BIN codes (Thimm et al., 2004) (Supplementary Fig. S2; Supplementary Table S1, available at JXB online). Overall, the genes identified as differentially expressed belong mainly to the categories transport, development, signalling, protein, and RNA (Supplementary Table S1, available at JXB online). With regard to nitrogen remobilization, the analysis was focused on genes involved in degradation and regulation processes (Table 1).

The 93 genes upregulated at late developmental stages from both SN (SN2 vs. SN1) and HN (HN3 vs. HN1) plots might play roles in age-dependent processes occurring independent of nitrogen supply. Among these 93 genes, five genes were identified encoding transcription factors and 13 genes encoding proteins putatively involved in processes associated with protein degradation (Table 1A). The transcription factors included MYB, HvNAC013, HvNAC005 and HvWRKY12. Among the genes associated with protein degradation, four encoded peptidases including HvSAG12, seven encoded factors of the ubiquitin-proteasome-system and one was the barley orthologue of the Arabidopsis autophagy-related gene ATG8. HvSAG12 and one of its orthologues in Arabidopsis, encode a cysteine protease which is used as a senescence marker gene (Lohman et al., 1994). Its senescence-associated upregulation was previously shown in barley (Jukanti et al., 2008; Parrott et al., 2010) and wheat (Gregersen and Holm, 2006; Ruuska et al., 2008).

Leaves of plants with standard nitrogen supply (SN) showed typical symptoms of senescence. The genes strongly upregulated in these leaves (SN2 vs. SN1) hence might play roles in proteolysis and nitrogen remobilization. The two genes with highest upregulation during senescence of SN leaves were HvPAP20, encoding a cysteine peptidase, and HvNAC026, encoding a NAC transcription factor (Table 1B). Furthermore, 13 genes encoding putative transcription factors and 29 genes putatively involved in protein degradation processes were identified. Most of the transcription factors can be assigned to the NAC, C2C2(Zn), C2H2, and IAA families. The other genes encode eight peptidases, 11 factors of the ubiquitin-proteasome-system (UPS), seven autophagy-related factors, and three other proteins putatively involved in protein degradation.
Table 1. Fold changes in expression as determined by the microarray hybridization of genes assigned to the categories 'RNA/regulation of transcription' and 'protein/degradation'.

(A) Genes upregulated (min. 2-fold log₂) in both senescing flag leaves of plants from plots with standard (SN2 vs. SN1) and excess (HN3 vs. HN1) nitrogen supply. (B) Genes exclusively upregulated (min. 2-fold log₂) in senescing flag leaves of plants from plots with standard nitrogen supply (SN2 vs. SN1). If possible, gene names are indicated for either barley or for the orthologous genes in *Arabidopsis*. Genes tested by qRT-PCR are marked by asterisks.

| Accession  | Name                  | BIN Description | SN 2 vs. 1 | HN 2 vs. 1 | 3 vs. 1 | SN vs. HN 1 vs. 1 | 2 vs. 2 | 2 vs. 3 |
|------------|-----------------------|-----------------|------------|------------|---------|------------------|---------|---------|
| **(A) protein.degradation** |                       |                 |            |            |         |                  |         |         |
| CA019173   | ATG8                  | autophagy       | 2.60       | 1.93       | 3.32    | 2.02              | 2.69    | 1.30    |
| TA40019_4513| HvSAG12               | cysteine protease| 2.59       | 1.38       | 2.18    | 0.92              | 2.13    | 1.33    |
| AM941123* | HvPAP14               | cysteine protease| 5.26       | 1.17       | 2.92    | 0.55              | 4.64    | 2.89    |
| BF256720   | RD21                  | cysteine protease| 2.18       | 1.06       | 2.17    | 0.35              | 1.47    | 0.35    |
| TA42918_4513| Subtilase             | subtilases      | 6.13       | 1.91       | 4.03    | 0.43              | 4.66    | 2.53    |
| TA36132_4513| UBC5                  | ubiquitin.E2    | 3.76       | 1.28       | 2.71    | 1.18              | 3.66    | 2.23    |
| BG414942   | ubiquitin.E3.RING     |                 | 3.65       | 1.28       | 2.46    | 0.72              | 3.09    | 1.92    |
| TA46919_4513| HvSAG12               | cysteine protease| 3.57       | 1.53       | 2.20    | 0.67              | 2.72    | 2.04    |
| CA008354   | HvSAG12               | cysteine protease| 2.99       | 1.28       | 2.10    | 0.87              | 2.58    | 1.76    |
| TA37012_4513| RGLG2                 | ubiquitin.E3.RING| 2.22       | 1.03       | 2.04    | 0.17              | 1.36    | 0.34    |
| TA35901_4513| UBC5                  | ubiquitin.E3.RING| 3.84       | 1.58       | 2.57    | 0.57              | 2.82    | 1.84    |
| TA35023_4513| UBC5                  | ubiquitin ubiquitin.protease| 2.61| 0.65 | 2.78 | -0.13 | 1.83 | -0.30 |
| **RNA.regulation of transcription** |                       |                 |            |            |         |                  |         |         |
| TA48006_4513| MYB                   | MYB-related transcription factor family| 3.91 | 0.77 | 2.10 | 0.27 | 3.42 | 2.08 |
| TC163636* | HvNAC013              | NAC domain transcription factor family| 3.32 | 1.45 | 2.90 | 0.77 | 2.64 | 1.18 |
| AK251058* | HvNAC005              | NAC domain transcription factor family| 2.15 | 1.88 | 2.34 | 1.15 | 1.43 | 0.96 |
| TC164038  | putative transcription regulator| 3.48 | 1.02 | 2.32 | 0.98 | 3.45 | 2.14 |
| BM370440* | HvWRKY12              | WRKY domain transcription factor family| 3.24 | 1.64 | 2.72 | 0.40 | 2.00 | 0.91 |
| **(B) protein.degradation** |                       |                 |            |            |         |                  |         |         |
| BF258397   | AAA type              | AAA type        | 2.16       | 0.58       | 1.17    | 0.49              | 2.07    | 1.48    |
| TA38933_4513| APG7                  | autophagy       | 3.80       | -0.66      | 0.50    | -1.06             | 3.40    | 2.23    |
| BF622783   | ATG3                  | autophagy       | 3.16       | 0.59       | 1.66    | 0.43              | 3.00    | 1.93    |
| BE216622   | APG8A                 | autophagy       | 3.12       | 0.94       | 1.99    | 0.58              | 2.77    | 1.72    |
| BG471466   | autophagy             |                 | 2.56       | 0.61       | 1.25    | 0.58              | 2.53    | 1.88    |
| AM085509   | APG9                  | autophagy       | 2.40       | 0.64       | 1.43    | 0.42              | 2.19    | 1.39    |
| TA56628_4513| ATG18F                | autophagy       | 2.35       | 0.44       | 1.19    | 0.92              | 2.83    | 2.08    |
| BF628472   | ATG8C                 | autophagy       | 2.13       | 0.86       | 1.08    | 0.18              | 1.45    | 1.23    |
| AM941127* | HvPAP20               | cysteine protease| 4.27       | 0.84       | 1.69    | -0.10             | 3.32    | 2.48    |
| AM941122  | HvCPR3               | cysteine protease| 3.66       | 1.14       | 1.98    | -0.02             | 2.50    | 1.65    |
| AM941116  | HvCPR3               | cysteine protease| 2.37       | 0.62       | 1.01    | 0.42              | 2.18    | 1.79    |
| TA45222_4513| protease              | protease        | 2.36       | 0.44       | 0.97    | 0.31              | 2.23    | 1.71    |
| EX594583  | protease              |                 | 2.24       | 0.78       | 1.38    | 0.52              | 1.98    | 1.38    |
| CD663018  | RD19                 | protease        | 2.17       | 0.72       | 1.32    | 0.38              | 1.82    | 1.22    |
| AK251038* | SCPL51               | protease        | 3.56       | 1.14       | 1.63    | 0.30              | 2.72    | 2.23    |
| TA57593_4513| protease              | protease        | 2.61       | 0.15       | 0.48    | -0.13             | 2.33    | 1.99    |
| BY686809  | UBC11                | ubiquitin.E2    | 2.24       | 0.66       | 0.96    | 0.32              | 1.91    | 1.61    |
| TA53580_4513| ubiquitin.E3.BTB/POZ| Cullin3.BTB/POZ| 3.93       | 0.10       | 1.64    | 0.50              | 4.33    | 2.79    |
| BY686762  | ubiquitin.E3.BTB/POZ| Cullin3.BTB/POZ| 2.43       | 0.49       | 0.86    | 0.07              | 2.01    | 1.63    |
| TA44869_4513| ubiquitin.E3.RING    |                 | 2.92       | 1.28       | 1.60    | 0.22              | 1.86    | 1.54    |
| CA008421  | ubiquitin.E3.RING    |                 | 2.35       | 0.72       | 1.49    | 0.38              | 2.01    | 1.25    |
| BM816188  | ubiquitin.E3.RING    |                 | 2.13       | 0.56       | 1.11    | 0.38              | 1.96    | 1.40    |
Quantitative analyses of the expression of selected genes

Microarray analysis showed that 376 genes were highly upregulated mainly in senescing SN leaves (SN2), whereas 93 genes were upregulated in both senescing SN (SN2 vs. SN1) and HN (HN3 vs. HN1) flag leaves. To investigate whether the differences in expression levels of genes selected by microarray hybridizations are indeed caused by different quantities of nitrogen supply in the field, barley plants were grown in the following year (2010) in field plots supplied with the same two quantities of nitrogen. Again, flag leaves were collected from SN and HN plots. Expression of selected genes was analysed by quantitative real-time PCR (qRT-PCR) (Table 1, marked by asterisk; Fig. 3B, C).

qRT-PCR was done with RNA from flag leaves collected in 2010 at five stages of development. These stages were characterised by the relative chlorophyll (Supplementary Fig. S1A, available at JXB online) and protein contents (Supplementary Fig. S1B, available at JXB online) of the leaves. In addition, ear dry weight was determined (Supplementary Fig. S1C, available at JXB online). The relative chlorophyll content in HN and SN flag leaves at 12 June was set to 100%. Typically for senescing leaves, the transcript level of the gene coding for the small subunit of Rubisco (HvRBCS) declined in SN leaves during the sampling period, whereas in HN flag leaves its level was rather stable (Fig. 3A). Inversely, the transcript level of the senescence marker gene HvSAG12 (Jukanti et al., 2008; Parrott et al., 2010) increased during development in both SN and HN leaves (Fig. 3A). Genes showing senescence-associated upregulation in both SN and HN leaves such as the SAG12 gene (Fig. 3A) might have functions in other senescence-associated processes than in nitrogen remobilization. Additional genes, which showed upregulation during leaf development independent of the nitrogen supply, encode the transcription factors MYB, HvNAC001, HvNAC005, HvNAC013, and HvWRKY12, as well as the two papain-like cysteine peptidases HvPAP14 and HvPAP20, and a gene encoding a subtilase (Fig. 3B). Among the genes showing highly increased expression during senescence of SN leaves were the genes encoding the transcription factor HvNAC026, a gene encoding an orthologue of the serine protease SCPL51 in Arabidopsis as well as two genes homologous to the genes encoding autophagy-associated proteins ATG7 and ATG18F in Arabidopsis (Fig. 3C).

Most of the genes selected by microarray hybridizations (Table 1) showed comparable tendencies in the expression profiles by qRT-PCR analysis (Fig. 3). Exceptions were the genes HvNAC001 and HvPAP20, which in 2010 showed again a strong upregulation in senescing SN leaves but also in the elder HN leaves. The expression of the subtilase gene in SN leaves differed in 2010 from the previous year.

Discussion

Characterization of senescence of flag leaves collected from field-grown barley plants and determination of kernel weight during the time of leaf sampling showed that the standard
nitrogen supply provided in this study did not limit the filling of the kernels (Fig. 1; Supplementary Fig. S1, available at JXB online). The high nitrogen supply in HN plots was in excess and hence could diminish/suppress the development of senescence symptoms such as chlorophyll degradation. This situation allowed identification of genes putatively involved in protein degradation and regulation processes important under nitrogen-limiting conditions using the pool of genes upregulated mainly in SN (SN2 vs. SN1) flag leaves.

For this study flag leaves were chosen, because their senescence is of pivotal importance for grain filling (Stoy, 1973; Stamp and Herzog, 1976). The most prominent changes in gene expression were detected in the SN treatment (SN2 vs. SN1). To identify genes specifically involved in nitrogen remobilization during senescence occurring at standard nitrogen supply (SN), genes upregulated during development of flag leaves independent of the nitrogen supply (HN) were excluded from this gene pool. The number of genes relevant for nitrogen remobilization was furthermore reduced by a very stringent minimum fold change of 2 (log2). By this approach 13 genes encoding transcription factors, and 29 genes encoding proteins putatively involved in protein degradation processes were identified as most strongly upregulated in senescing SN leaves (Table 1B). A qRT-PCR with similar samples collected
in 2010 further reduced the number of relevant genes. Among the genes selected by microarray hybridization only the genes encoding the transcription factor HvNAC026 as well as the genes SCPL51, APG7, and ATG18F were reproducibly upregulated specifically in leaves undergoing nitrogen remobilization. As these analyses were done on material grown in the fields and in addition in two consecutive years several environmental factors could influence the development and behaviour of the plants. Thus, the weather conditions in 2010 (Krupinska et al., 2012) led to a delayed flowering time point and shortened time between flowering and senescence (Fig. 1A; Supplementary Fig. S1, available at JXB online). However, by using two adjacent fields cultivated in the same crop rotation and fertilized with equal amounts for several years, differences of the initial nitrogen amount in the soil were reduced as much as possible.

Transcription factors

In senescent leaves from plots with standard (SN2 vs. SN1) or excess (HN3 vs. HN1) nitrogen supply, genes encoding transcription factors could be identified that might play roles in age-related processes other than leaf nitrogen remobilization. This group of genes included genes encoding a MYB transcription factor, two NAC transcription factors (HvNAC013, HvNAC005) and the transcription factor HvWRKY12 (Table 1A). By qRT-PCR with samples collected in 2010 these results could be confirmed (Fig. 3B). Expression of the gene encoding the MYB factor was also shown to be upregulated in germinating seeds (Zhang et al., 2004), as well as in developing seeds 14–21 days post anthesis (Jukanti and Fischer, 2008). The HvNAC013 gene was shown to be elevated in response to abscisic acid (ABA) and methyl jasmonate (MeJA) (Christiansen et al., 2011). The HvNAC013 protein was shown to interact with the barley RADICAL-INDUCED CELL DEATH 1 protein (RCD1) (Kjaersgaard et al., 2011), which is involved in regulation of genes responsive to stress and regulated during development (Teotia and Lamb, 2011). HvNAC005 (Christiansen et al., 2011) and ANAC029/AtNAP, the orthologous gene in Arabidopsis (Guo et al., 2004a), were already described as upregulated during senescence. HvNAC005 was furthermore shown to be markedly upregulated by ABA treatment (Christiansen et al., 2011).

The hybridization of the microarray identified HvWRKY12 as the only gene encoding a WRKY factor upregulated during senescence. Its transcript level increased during development in both SN (SN2 vs. SN1) and HN (HN3 vs. HN1) leaves (Table 1A). This result was also confirmed by qRT-PCR using samples collected in the second year (Fig. 3B). Interestingly, the HvWRKY12 gene was not upregulated during senescence induced by carbohydrate accumulation as a result of stem girdling (Parrot et al., 2007). AtWRKY75, its orthologous gene in Arabidopsis (Mangelsen et al., 2008), was reported to be upregulated during senescence (Guo et al., 2004a) and in response to pathogens (Chen et al., 2013). OsWRKY72, its orthologous gene in rice, could be induced by treatment with ABA (Xie et al., 2005).

In previous studies on senescence-associated gene expression, genes encoding members of the NAC and WRKY transcription factors were shown to have high levels of expression in senescent leaves (Guo et al., 2004a; Buchanan-Wollaston et al., 2005; Balazadeh et al., 2008; Breeze et al., 2011; Fischer, 2012). Here, the two NAC genes HvNAC001 and HvNAC026 were shown to have highest upregulation in senescent leaves from SN plots in 2009 as analysed by microarrays (Table 1B). This result was similar for HvNAC026 during qRT-PCR using samples collected in 2010 even if an upregulation in HN plots was also shown (Fig. 3C). For the HvNAC026 gene an upregulation in senescing barley flag leaves and in the grain at the dough stage was shown (Christiansen et al., 2011). The orthologous gene in Arabidopsis, XND1, was described to negatively regulate lignocellulose synthesis and programmed cell death in the xylem (Zhao et al., 2007). Orthologues in poplar are involved in xylem formation, leaf growth, and development (Grant et al., 2010). It is likely that during leaf senescence, HvNAC026 acts as a suppressor of cell death processes, thereby allowing efficient nitrogen remobilization. Whether nitrogen remobilization is hampered in transgenic barley plants with reduced level of HvNAC026 remains to be investigated. The HvNAC001 gene was also upregulated in senescing leaves from SN plots but the transcript level was higher in leaves from HN plots (Fig. 3B). Thus, it is unlikely that HvNAC001 is a regulator of nitrogen remobilization. In other studies on HvNAC001, a slight upregulation in senescent leaves and in leaves treated with ABA, but an intensive upregulation in older grains was reported (Christiansen et al., 2011).

Genes encoding proteins putatively involved in protein degradation during senescence

The second focus of the microarray analyses was on genes encoding proteins involved in protein degradation, in particular proteases. Several proteases have been described to be associated with protein degradation during senescence (Gepstein et al., 2003; Guo et al., 2004a; Schaller, 2004; Buchanan-Wollaston et al., 2005; Parrott et al., 2005, 2007), but for most of them their functional roles in nitrogen remobilization remain to be shown.

HvPAP20 and HvPAP14 are both cysteine peptidases of the papain type. HvPAP14 was one of the most upregulated genes during development of flag leaves from SN field plots (Table 1A). The gene encodes a HDEL-tailed papain cysteine peptidase. For the orthologous genes in Ricinus communis and Arabidopsis, an involvement in plant cell death was shown (Gietl and Schmid, 2001; Hierl et al., 2012). Expression patterns of both genes, HvPAP20 and HvPAP14, showed similarities to the expression pattern of the senescence-associated gene HvSAG12 (Fig. 3A, B), which codes for another papain-type cysteine peptidase. In time course experiments on Arabidopsis leaf senescence, AtSAG12 was upregulated at a later stage of senescence (Breeze et al., 2011). OsSAG12-1, the orthologous gene in rice was described as a negative regulator of cell death (Singh et al., 2013). Furthermore, the gene encoding a subtilase-type serine peptidase showed a high upregulation in senescing SN and HN leaves in the microarray experiment (Table 1A). qRT-PCR showed that the expression increased during development only in HN.
leaves (Fig. 3B). The orthologous gene in Arabidopsis codes for a protein identified in the vacuole (Carter, 2004). It was shown to be upregulated in mutants devoid of the microRNA miR159, which is involved in the regulation of programmed cell death (Alonso-Peral et al., 2010).

Eight genes encoding cysteine peptidases and two genes encoding serine peptidases were identified as highly upregulated in senescing SN (SN2 vs. SN1) leaves (Table 1B). Two of these genes were selected for further analysis by qRT-PCR with samples collected in the field in 2010. The first gene is HvPAP20 encoding a cysteine type peptidase, and the second gene is SCPL51 encoding a serine type protease (Table 1B). qRT-PCR confirmed a higher upregulation during senescence of SN leaves compared with HN leaves for SCPL51 (Fig. 3C), but not for HvPAP20 (Fig. 3B). In previous studies, SCPL51 was shown to be upregulated in germinating barley seeds (Druka et al., 2006; Sreenivasulu et al., 2008), and after anthesis in leaves of a high-grain-protein variety compared with the leaves of a low-grain-protein variety (Jukanti et al., 2008). These results are in accordance with the idea that SCPL51 is involved in nitrogen remobilization, which is likewise important for senescence and germination (Thomas and Smart, 1993; Thomas and Howarth, 2000; Palma et al., 2002; Schaller, 2004). Although HvPAP20 was upregulated during senescence of SN leaves, its transcript level was higher in the older HN leaves. HvPAP20 encodes a cathepsin-b-like cysteine protease (Martínez and Díaz, 2008) known to be also upregulated during germination of barley (Druka et al., 2006; Sreenivasulu et al., 2008).

The high upregulation of ATG7 and ATG18F in senescent SN leaves indicates that autophagy is also involved in nitrogen degradation and remobilization in barley flag leaves under field conditions (Table 1B, Fig. 3C). The protein encoded by ATG7 in barley is involved in the protein conjugation reaction (Mizushima et al., 1998). The autophagy-related gene ATG18F was also shown to be upregulated in Arabidopsis seedlings exposed to sucrose or nitrogen starvation (Xiong et al., 2005). Besides its role in accomplishment of protein degradation (Guiboileau et al., 2012; Lee et al., 2013), autophagy might suppress cell death by controlling the NPR1-dependent salicylic acid signalling process (Yoshimoto et al., 2009).

In conclusion, the gene expression analyses presented here indicate that the NAC transcription factor HvNAC026, the serine type protease SCPL51, and the autophagy factors APG7 and ATG18F might be major regulators and executors of nitrogen remobilization during barley leaf senescence in the field. Both HvNAC026 and autophagy might be important for remobilization through their negative impact on cell-death processes.

Supplementary Data
Supplementary data are available at JXB online.

Figure S1. Characterization of flag leaves and ears in 2010.

Figure S2. Heat map of the selected and annotated genes identified by the microarray experiment.

Table S1. Microarray expression values.

Table S2. Sequences of primers used for quantitative real-time PCR.

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