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REVIEW PAPER

Senescence, nutrient remobilization, and yield in wheat and barley

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Abstract

Cereals including wheat and barley are of primary importance to ensure food security for the 21st century. A combination of lab- and field-based approaches has led to a considerably improved understanding of the importance of organ and particularly of whole-plant (monocarpic) senescence for wheat and barley yield and quality. A delicate balance between senescence timing, grain nutrient content, nutrient-use efficiency, and yield needs to be considered to (further) improve cereal varieties for a given environment and end use. The recent characterization of the *Gpc-1* (*NAM-1*) genes in wheat and barley demonstrates the interdependence of these traits. Lines or varieties with functional *Gpc-1* genes demonstrate earlier senescence and enhanced grain protein and micronutrient content but, depending on the environment, somewhat reduced yields. A major effort is needed to dissect regulatory networks centred on additional wheat and barley transcription factors and signalling pathways influencing the senescence process. Similarly, while important molecular details of nutrient (particularly nitrogen) remobilization from senescing organs to developing grains have been identified, important knowledge gaps remain. The genes coding for the major proteases involved in senescence-associated plastidial protein degradation are largely unknown. Membrane transport proteins involved in the different transport steps occurring between senescing organ (such as leaf mesophyll) cells and protein bodies in the endosperm of developing grains remain to be identified or further characterized. Existing data suggest that an improved understanding of all these steps will reveal additional, important targets for continued cereal improvement.

Key words: Barley (*Hordeum vulgare* L.), *Gpc-1*, grain protein content, *NAM-1*, nitrogen metabolism, nitrogen transport, protein degradation, Rubisco degradation, senescence regulation and timing, wheat (*Triticum aestivum* L.).

Introduction

Senescence is the last developmental stage of plant cells, tissues, organs, and, in the case of monocarpic species, the entire plant. Most humans are (knowingly or not) familiar with the visual aspects of plant senescence. Striking examples where large-scale plant senescence processes paint entire landscapes include autumn colouring in deciduous trees, and senescence of monocarpic (cereal) crops, where large agricultural surfaces turn from green to yellow within a few weeks (Fig. 1). While chlorophyll degradation and (when red hues are also observed) anthocyanin biosynthesis are responsible for this glorious display, numerous additional molecular and biochemical processes contribute to the senescence syndrome. Once senescence has been initiated, it typically leads to a massive remobilization of phloem-mobile nutrients from the senescing plant parts to developing sinks, such as seeds and grains of monocarpic crops. In this context, nitrogen holds a special position. It is the quantitatively most important plant mineral nutrient, and nitrogen-containing macromolecules

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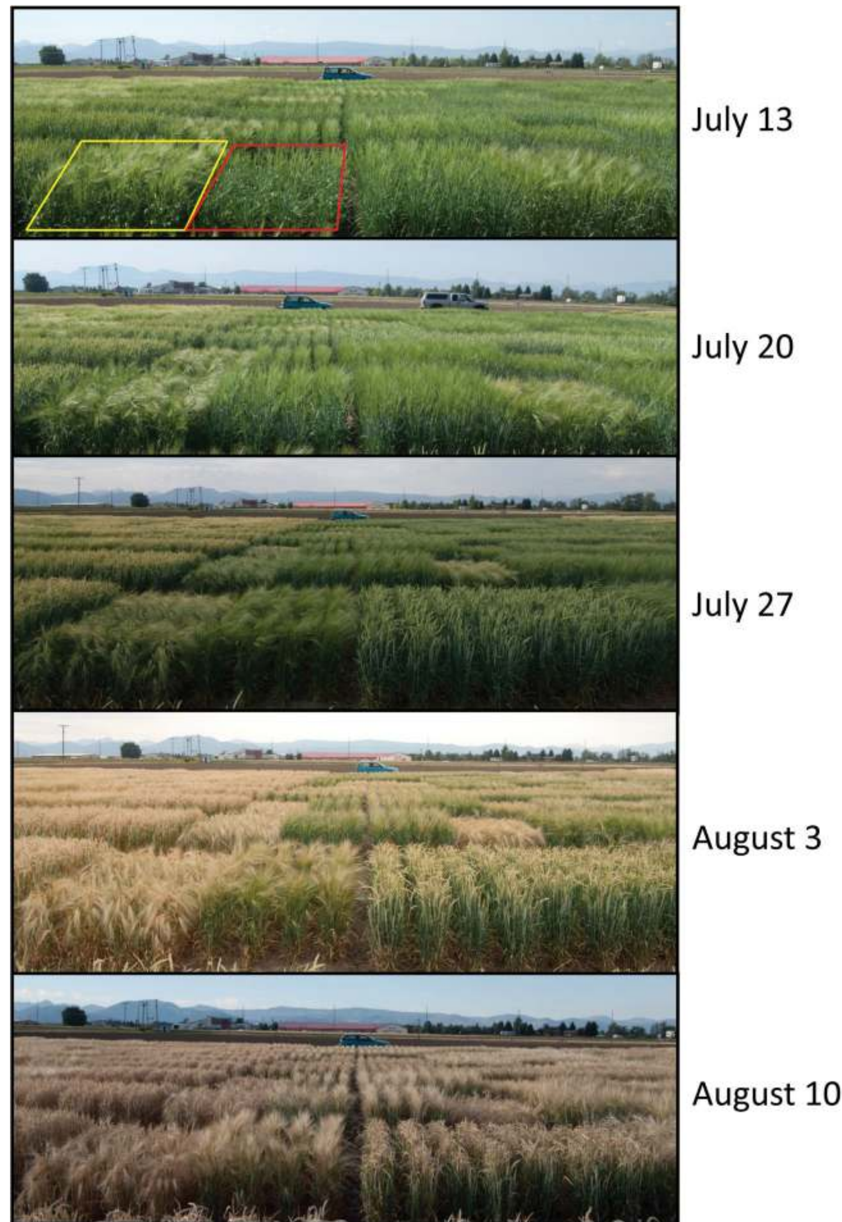

Fig. 1. Monocarpic senescence in barley. A field experiment analysing two different series of near-isogenic barley germplasm varying in the allelic state of a chromosome six locus controlling grain protein content and senescence is shown. Compare development and senescence of plants outlined in yellow (line '10_11') and in red (variety 'Karl'). See text and Jukanti *et al.* (2008) for details. Pictures were taken by A.M.F. at the Arthur Post Research Farm near Bozeman, Montana, USA.

(proteins and nucleic acids) have to be hydrolysed and converted to glutamate, glutamine and (to a lesser extent) other amino acids prior to phloem loading and transport to developing sinks.

It is noteworthy that the world's most important crops, including corn, wheat, and rice, are monocarpic species, in which resources from senescing organs are remobilized to the grains. Senescence timing (both onset and, once initiated, rate of the process) influences key agronomic traits including nutrient-use efficiency, yield (with late-senescing or 'stay-green' varieties often, but not always exhibiting higher yields; Thomas and Howarth, 2000; Gregersen *et al.*, 2013), and quality. Early and/or efficient nutrient remobilization is associated with higher grain protein concentration, but also with higher concentrations of desirable micronutrients such as Fe and Zn (Uauy *et al.*, 2006b; Heidlebaugh *et al.*, 2008; Jukanti *et al.*, 2008; Waters *et al.*, 2009). Analysis of the senescence process, both in model species and in crops, has therefore aimed at (a) understanding the internal (genetic, epigenetic) and environmental factors regulating the senescence process and (b) understanding the cellular and molecular details of nutrient (especially nitrogen) remobilization and transport. Due to their economic importance, much of this research has been performed in monocarpic crops, including wheat and barley.

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Wheat and barley: ancient crops with high importance for the 21st century

In terms of total yield and calories produced, corn, wheat, and rice are the world's three most important crops (FAOSTAT, accessed at http://faostat.fao.org/site/291/default.aspx). While wheat yield per surface area is not as high as corn or rice yields, wheat can grow under drier and colder conditions than rice or corn; a substantial fraction of the world's wheat is therefore cultivated in rain-fed environments. Wheat is the world's most widely cultivated cereal, with over 220 million ha planted (for comparison: a large country, India, has a land surface of ~300 million ha), and it contributes about 20% of the dietary calories and proteins available worldwide (Shiferaw *et al.*, 2013). It should be remembered, however, that the term 'wheat' refers to several species. 'Common' or 'bread wheat' (*Triticum aestivum* L.) is the most widely cultivated form; it has a large (~17 Gb, about 5-times the size of the human genome) allohexaploid genome (AABBDD; $2n = 6x = 42$) reflecting its complex ancestry (Dubcovsky and Dvorak, 2007). 'Durum wheat' (*Triticum durum* L.) is the only tetraploid wheat (AABB, $2n = 4x = 28$) whose use is widespread today; accordingly, it has also been used for the analysis of leaf senescence and nutrient remobilization (e.g. Spano *et al.*, 2003; Rampino *et al.*, 2006).

Compared to the 'Big Three', barley is cultivated on substantially less surface (approximately 56 million ha; Newton *et al.*, 2011; comparable to the land surface of a large Eastern European country, Ukraine). Based on production, it is ranked fourth amongst the cereals and 11th overall, but it has a wide geographic range, both in terms of northern/southern latitudes and altitudes at which it can be successfully grown (Newton *et al.*, 2011). Although, like wheat, it is one of the world's oldest crops, it is no longer primarily used as a food source and is mainly grown for the production of alcoholic drinks and as an animal feed. Due to the fact that it has a smaller (~5.1 Gb) and simpler genome ($2n = 2x = 14$) than durum and bread wheats, and that a whole-genome shotgun assembly and an integrated physical map have recently become available (Mayer *et al.*, 2012), it is a useful model system for the study of temperate cereal crops, and it has extensively been used for the study of plant nitrogen metabolism and senescence (e.g. Jukanti *et al.*, 2008; Parrott *et al.*, 2010, Kohl *et al.*, 2012, Krupinska *et al.*, 2012).

Senescence timing in wheat and barley: *Gpc-B1/NAM-1*, yield, and quality

Senescence processes of monocarpic crops have been studied for decades (see Thomas and Stoddart, 1980 for an overview of the older literature), using physiological and biochemical methods. Molecular aspects of the process have been analysed more recently; as in other fields of modern plant science, these studies have profited from the use of *Arabidopsis* as a model system (e.g. Buchanan-Wollaston *et al.*, 2005; Guo and Gan, 2005; Kim *et al.*, 2007; Lim *et al.*, 2007; Fischer, 2012). In parallel, molecular and '–omics' techniques have also been applied to the analysis of senescence in wheat, barley and other crops (e.g. Gregersen and Holm, 2007; Parrott *et al.*, 2007; Jukanti *et al.*, 2008).

Senescence timing: organ vs. terminal senescence

When discussing the senescence syndrome, it is useful to distinguish between organ (e.g. leaf) senescence on the one side and whole-plant (monocarpic, terminal) senescence on the other side (Leopold, 1961). Organ senescence may occur throughout the life cycle of a plant. During vegetative growth, senescing organs translocate nutrients to younger plant parts (Gan and Amasino, 1997). This process is associated with leaf age, but also with environmental factors. During the reproductive stage of monocarpic plants, an overall senescence process is initiated, during which all tissues and organs of the parental plant die, and the nutrients remobilized from the senescing tissues are remobilized to the developing seeds (see Davies and Gan, 2012 for a thorough discussion of whole-plant senescence). In fact, when looking at a mature cereal field, it is useful to remember that the only living tissues present are those of the following generation, specifically the embryo, scutellum, and aleurone layer of the grain (see http://www.gramene.org/species/index.html for an overview of cereal anatomy).

The extent of organ senescence is highly dependent on environmental factors. Under controlled (mostly 'stress-free' conditions), vegetatively growing plants have a larger number of photosynthetically active (nonsenescing) leaves than under water- or nutrient-limiting conditions. The ratio of senescing to nonsenescing biomass is typically quite small in vegetatively growing wheat and barley plants kept under well-watered and well-fertilized conditions (Fig. 2), and developing plant parts

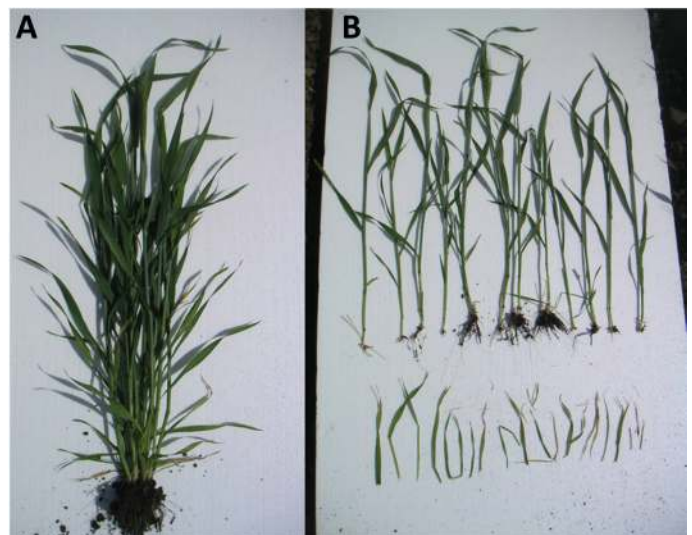

Fig. 2. (A) Wheat plant at the beginning of the reproductive stage (booting) grown under field conditions in Tel Aviv, Israel. (B) The same plant separated into two groups: the upper group represents green tillers without any sign of senescence while the lower group represents leaves that show (organ) senescence. Since most of the biomass is not senescing, organ senescence can be considered as a minor process compared to terminal senescence.

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obtain a larger fraction of their nutrients from root uptake than through remobilization from senescing plant parts. In contrast, post-anthesis (monocarpic) senescence affects the whole plant, with organs closest to the developing grains (flag leaves, glumes) generally senescing last (Feller *et al.*, 2008). Developing wheat grains obtain a large fraction of mobile micro- and macronutrients from the senescing organs of the parental plant, with a smaller contribution from root uptake (Kichey *et al.*, 2007). Therefore, the processes of senescence and nutrient remobilization taking place at the reproductive stage are more important for understanding the mechanisms that control plant productivity than organ senescence (sequential leaf senescence) occurring during vegetative plant development.

Nitrogen availability has a well-studied effect on terminal senescence timing (Martre *et al.*, 2006). Low N levels induce early senescence; this is associated with earlier (plastidial) protein degradation due to sink demand (Gan and Amasino, 1997). High N levels can delay senescence (Martre *et al.*, 2006), as sink demand may be satisfied to a higher extent from stored inorganic or organic nitrogen, allowing higher levels of ongoing photosynthesis.

Whole-plant senescence timing and yield

Barley and wheat productivity is measured by the total grain yield per area. It is determined by several components including number of spikes per plant, number of spikelets per spike, number of grains per spikelet, and grain weight. These components are under the control of environmental and genetic factors that are active during different stages of plant development:

1. The number of spikes per plant is mainly affected by the duration of the vegetative phase, which is regulated by the *VRN* and *PPD* genes (reviewed by Distelfeld *et al.*, 2009; Greenup *et al.*, 2009).
2. The number of spikelets per spike is determined during the period between floral meristem induction and the formation of the terminal spikelet (Sreenivasulu and Schnurbusch, 2012). Wheat has a nondeterminate spikelet meristem allowing the development of several flowers per spikelet, unlike barley for which only two-rowed and six-rowed varieties exist (Bonnet, 1966; Komatsuda *et al.*, 2007; Houston *et al.*, 2013; Koppolu *et al.*, 2013).
3. The number of grains per spikelet is regulated during spike development until anthesis (Arisnabarreta and Miralles, 2008; Sreenivasulu and Schnurbusch, 2012).
4. Grain weight is regulated throughout the reproductive stage, from spike development to maturity, and is affected by sink and source characteristics such as the flower capacity (glume size; Millet and Pinthus, 1984) and grain cell number (cell division during early endosperm/grain development; Brocklehurst, 1977; Jenner *et al.*, 1991). To a lesser extent, especially under stress conditions, the capacity and duration of photosynthesis, and mobilization of assimilates to the grain also affect grain weight although it appears that sink limitation is more prominent (Bingham *et al.*, 2007; Serrago *et al.*, 2013).

Whole-plant senescence overlaps with grain filling, and the synchronization of these two processes is highly important in determining yield, particularly through the grain weight component (the other yield components are largely determined before the initiation of terminal senescence). Indeed, stay-green mutants in various cultivars have increased grain weight and yield as a result of delayed senescence (Spano *et al.*, 2003; Christopher *et al.*, 2008; Chen *et al.*, 2010) and also perform better under water stress (Gous *et al.*, 2013). However, other studies with stay-green mutants and lines (e.g. Derkx *et al.*, 2012; Avni *et al.*, 2013; Emebiri, 2013) indicate that sink capacity may become a limitation to yield when senescence is delayed.

Whole-plant senescence, grain protein content, and grain mineral content

Whole-plant senescence affects not only yield, but also quality parameters such as grain protein content (GPC) and micronutrient (Fe, Zn) content. In wheat and barley, there exists a well-established negative correlation between yield and GPC (Kibite and Evans, 1984; Simmonds, 1995) that can be explained by two hypotheses. The first hypothesis suggests that, in varieties or lines with delayed senescence, grain proteins and micronutrients are 'diluted' by prolonged carbohydrate accumulation leading to increased grain weight and yield (Slafer *et al.*, 1990; Gregersen, 2011). Depending on both genetics and environment, late senescence may be associated with less efficient nutrient remobilization, possibly further lowering grain nutrient content. On the other hand, early senescence may lead to more efficient or complete N and micronutrient remobilization. The term 'dilemma of senescence' has been utilized in a recent review to draw attention to this situation (Gregersen, 2011). A second hypothesis revolves around the fact that the synthesis of storage proteins consumes more carbon per mass unit than starch synthesis (reflecting the 'cost' of nitrogen uptake, assimilation, and transport) (Munier-Jolain and Salon, 2005). The delicate balance between grain weight, nutrient content and senescence is demonstrated by the effect of the *Gpc-1* genes in wheat and barley on these parameters.

The Gpc-B1 *(*NAM-B1*) gene*

Gpc-B1 (also called *NAM-B1*) was the first gene responsible for variation in GPC that was identified by a map-based cloning approach in wheat. The allele for high GPC was identified in a wild emmer accession (*Triticum turgidum* ssp. *dicoccoides* (Körn.) Thell) (DIC) collected in Israel (Avivi, 1978). This accession was crossed with durum wheat (cv. Langdon) (LDN) (*T. turgidum* ssp. *durum* (Desf)) and used to create a full set of chromosome substitution lines each containing a different DIC chromosome in the background of tetraploid wheat (Joppa and Cantrell, 1990). These lines were later used to identify a major QTL for GPC on chromosome 6BS (Joppa

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et al., 1991, 1997), which was demonstrated to also influence senescence timing (Uauy *et al.*, 2006a) and grain Fe and Zn concentrations (Cakmak *et al.*, 2004; Distelfeld *et al.*, 2007). The map-based cloning of *Gpc-B1* from wild emmer revealed that the gene encodes a NAC transcription factor (Uauy et. al., 2006b; Distelfeld and Fahima, 2007). Sequence comparison between modern wheat varieties and wild emmer accessions indicated that, in most modern durum and bread wheat varieties, a frame shift mutation within the first intron and a thymine residue insertion at position 11, or a complete deletion, results in a nonfunctional protein (Uauy *et al.*, 2006b; Hagenblad *et al.*, 2012).

Tetraploid wheat *Gpc-B1* has a functional orthologous copy on chromosome 6A designated *Gpc-A1*. One paralogous copy is present on chromosome 2B, designated *Gpc-B2*. Hexaploid wheat *Gpc-1* has two functional orthologous copies on chromosomes 6A (*Gpc-A1*) and 6D (*Gpc-D1*) and two paralogous *Gpc-2* copies that are present on chromosomes 2B (*Gpc-B2*) and 2D (*Gpc-D2*) (Uauy *et al.*, 2006b). Silencing of all *Gpc* copies using RNA interference (RNAi) in hexaploid wheat cv. Bobwhite resulted in a 24–30-day delay in senescence and a more than 30% decrease in grain protein and zinc and iron content compared to control plants (Uauy *et al.*, 2006b). Analysis of the individual effects of the *Gpc-A1* and *Gpc-D1* genes in hexaploid wheat using knockout mutants showed that both genes have similar functions in the regulation of monocarpic senescence and nutrient remobilization in wheat (Avni *et al.*, 2013). The expression pattern of the *Gpc* genes supports their role as early regulators of terminal senescence since they are induced in flag leaves around spike heading (when the leaves are still green) and their mRNA levels continue to increase in the leaves until complete yellowing (Uauy *et al.*, 2006b).

The introgression of the wild emmer *Gpc-B1* allele into modern wheat cultivars resulted in near-isogenic lines with up to 20% higher GPC, but this can result in slight yield penalties (due to reduced grain size), depending on the genetic background and the environment (Brevis and Dubcovsky, 2010; Kumar *et al.*, 2011; Carter *et al.*, 2012; Tabbita *et al.*, 2013). In the opposite case, when the *Gpc-1* genes were knocked out, the plants showed delayed senescence and reduced GPC, but this did not affect grain weight, suggesting that sink capacity may have been limiting in this instance (Uauy *et al.*, 2006b; Avni *et al.*, 2013).

Gpc-B1 *orthologous genes in barley*

Phylogenetic analysis indicates the presence of orthologous genes in rice, barley, *Aegilops*, maize, *Arabidopsis*, and *Brachypodium* (Uauy *et al.*, 2006b; Distelfeld *et al.*, 2008; Distelfeld *et al.*, 2012). It is worth noting that the *Gpc* orthologue in rice was shown to be predominantly expressed in stamens and have a function in anther development; it affects fertility but not terminal senescence (Distelfeld *et al.*, 2012). In barley, a RIL population from a cross between a high-GPC variety ('Lewis') and a low-GPC variety ('Karl') was used to map a GPC QTL on chromosome 6H (See *et al.*, 2002; Mickelson *et al.*, 2003). Barley lines with the high-GPC locus from 'Lewis' were backcrossed to 'Karl' to create near-isogenic line '10_11'; this line showed higher GPC and accelerated senescence when compared to 'Karl' (Heidlebaugh *et al.*, 2008; Jukanti and Fischer, 2008; Jukanti *et al.*, 2008; Fig. 1), a similar phenotype to that of the *Gpc-B1* wild emmer introgression lines (Uauy *et al.*, 2006a). Using the wheat *Gpc-B1* sequence, two barley genes with 98% similarity were identified and designated *HvNAM-1* and *HvNAM-2*. These genes were mapped to chromosomes 6H and 2H, respectively (Uauy *et al.*, 2006b). The barley 6HS and wheat 6BS GPC QTLs showed a high degree of conserved colinearity which, combined with the phenotypic similarities between the two, suggested that *HvNAM-1* is the orthologue of *Gpc-B1* in wheat (Distelfeld *et al.*, 2008). The 'Karl' allele (low GPC) and the high GPC allele from cv. 'Lewis' differ in two SNPs that translate to two amino acid changes (Distelfeld *et al.*, 2008). To further compare the functions of the *Gpc-1* and *HvNAM* genes, the expression patterns of *HvNAM-1* and *HvNAM-2* in flag leaves were measured (Fig. 3). The results showed that the expression pattern of *HvNAM-1* is similar to that of *Gpc-1* (Uauy *et al.*, 2006b), with high expression levels after anthesis and throughout terminal senescence.

Other studies have also demonstrated the association between *HvNAM-1* and GPC in barley (Jamar *et al.*, 2010; Cai *et al.*, 2013). These studies point to additional variation in GPC that is not caused by variation in the *HvNAM-1* sequence, suggesting differential regulation of this gene or a role for *HvNAM-2* and other loci on nearly all barley chromosomes (all except 4H) in controlling GPC in barley.

The *GPC* locus in barley was shown to influence plant development and leaf senescence before anthesis, but only after floral transition at the shoot apex had occurred (Lacerenza *et al.*, 2010; Parrott *et al.*, 2012). High-GPC line '10_11' was found to flower ~5 d earlier than its near-isogenic low-GPC parent, 'Karl'. Similarly, Asplund *et al.* (2013), comparing 40 Swedish spring wheat varieties varying in the functional state of *Gpc-B1*, found that the presence of a functional gene was

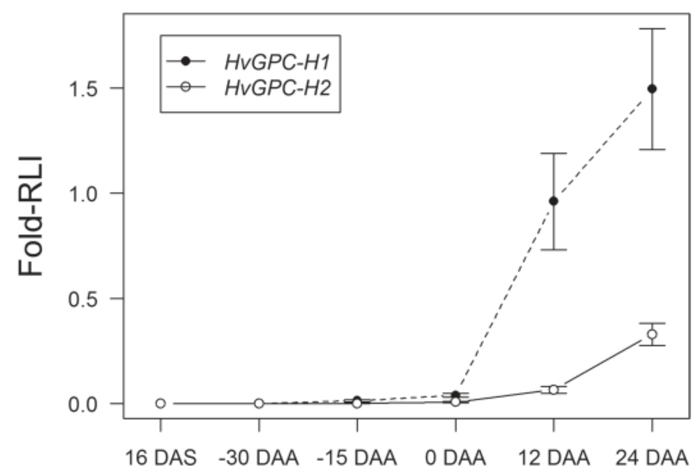

Fig. 3. Relative expression levels of *HvGpc-1* and *HvGpc-2* in barley cv. 'Golden Promise' across a senescence time course, analysed by quantitative real-time RT-PCR. Y-axis values represent the fold-*RLI* (RNase L inhibitor-like protein; Gimenez *et al.*, 2011) level of expression calculated as $1/(2^{Ctsample - CtRLI})$. DAS = d after sowing; DAA = d after anthesis.

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associated both with earlier anthesis and with faster whole-plant senescence. In other wheat germplasm, no differences in preanthesis development or senescence were observed (Uauy *et al.*, 2006b; Avni *et al.*, 2013). These contrasting results could be caused by the effect of linked gene(s), depending on the used barley or wheat germplasm. A possible candidate is the gene coding for barley glycine-rich RNA-binding protein 1 (HvGR-RBP1) which is a functional homologue of *Arabidopsis* glycine-rich RNA-binding protein 7 (AtGRP7); importantly, a role in the promotion of flowering has previously been demonstrated for the *Arabidopsis* gene/protein (Streitner *et al.*, 2008). The recent sequencing of the barley genome (Mayer *et al.*, 2012) localizes *HvGR-RBP1* to chromosome 6H, supporting the interpretation that it may be linked to *HvNAM-1*. Differences in preanthesis development of germplasm carrying functional *Gpc-B1* genes may also be related to culture conditions (e.g. vernalization abolishes such differences; Parrott *et al.*, 2012) or epistatic interactions depending on the germplasm used for the studies. We are currently developing near-isogenic lines that carry smaller introgressions from '10_11' in the 'Karl' background in order to further dissect the barley GPC QTL region and study its pleiotropic effects.

Senescence timing in wheat and barley: other regulatory aspects

At the molecular level, senescence regulation by *Gpc* genes in barley and wheat currently represents the most thoroughly investigated case study. However, additional molecular analyses and numerous physiological and biochemical studies contribute to a more complete understanding of cereal senescence processes.

Source–sink interactions and sugar signalling

It has been known for over 30 years that source–sink interactions, due to their importance for the redistribution of nutrients and signalling compounds, influence leaf as well as whole-plant senescence. This fact has been demonstrated through experiments physically removing either source (such as fully developed leaves) or sink structures (developing cereal ears, legume pods, young leaves, or shoot tips), through local phloem interruption (see below) or through molecular approaches interfering with long-distance transport (see Feller and Fischer, 1994 for a review of the older literature). Experiments performed with detached organs can be viewed as a special situation of source–sink modification. Due to the ease with which large numbers of developmentally and genetically identical detached leaves, leaf segments, or leaf discs can be subjected to a range of experimental conditions (e.g. permanent darkness, all thinkable combinations of light intensity and duration, application of nutrients, and signalling compounds), numerous studies investigating (cereal) leaf senescence have benefited from this approach (e.g. Dungey and Davies, 1982; Weidhase *et al.*, 1987; Fischer and Feller, 1994; Herrmann and Feller, 1998; Schlüter *et al.*, 2011). To illustrate this point, it is helpful to remember that segments excised from primary barley leaves were instrumental in the elucidation of chlorophyll catabolism (Matile *et al.*, 1988). However, senescence of detached plant shoots or organs may differ from developmental senescence of intact plants (e.g. Becker and Apel, 1993).

Phloem export from mature (source) cereal leaves can be interrupted through 'steam girdling' at the base of the leaf blade. Steam is directed through a hypodermic needle, which is pressed for a few seconds against the leaf, killing all tissues in a narrow zone. This method has been used for the analysis of both wheat (Feller and Fischer, 1994) and barley leaf senescence (Parrott *et al.*, 2005, 2007, 2010). Steam girdling leads to a rapid and strong accumulation of soluble carbohydrates (including fructose, glucose, and sucrose) from ongoing photosynthesis in the symplastically isolated leaves and to rapid leaf senescence. Degradation of chlorophylls and proteins and accumulation of free amino acids occur after carbohydrate accumulation, suggesting that they are a consequence of increased sugar levels.

Studies in *Arabidopsis* and other species indicate that sugar accumulation leads to senescence initiation (Pourtau *et al.*, 2004, 2006; Wingler *et al.*, 2009), at least partially through hexose/hexokinase signalling (Dai *et al.*, 1999; Wingler *et al.*, 2009; Swartzberg *et al.*, 2011). The described girdling experiments indicate that soluble sugars are also important for senescence induction in barley and wheat. Profiting of the girdling approach, Parrott *et al.* (2007) have performed a comprehensive analysis of the barley senescence transcriptome, identifying numerous genes putatively involved in senescence regulation and nitrogen remobilization. Genes specifically induced in girdled vs. control leaves included several *Senescence-Associated Genes* (*SAGs*), genes coding for WRKY and MYB transcription factors, for enzymes involved in nitrogen metabolism (glutamine synthetase, glutamate synthase, asparagine synthetase, glutamate dehydrogenase, and several aminotransferases) and for several amino acid transporters potentially involved in nitrogen retranslocation. Additionally, 50 genes coding for peptidases from several catalytic classes were upregulated at least 2-fold in girdled leaves, providing a list of candidate genes/enzymes which may be important for senescence-associated nitrogen remobilization (discussed in a later section of this article).

Signalling compounds in wheat and barley senescence regulation

The fact that 'classical' phytohormones contribute to the regulation of plant senescence processes are among the earliest results in senescence research, and detached plant material was instrumental in obtaining these data (see Thomas and Stoddart, 1980, for an overview of the older literature; see Gan, 2010; Fischer, 2012; Jibran *et al.*, 2013 for recent reviews).

Transcriptomic analysis of barley flag leaves indicated upregulation of genes coding for a putative 9-*cis*-epoxycarotenoid dioxygenase (a key enzyme in abscisic acid biosynthesis),

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for two ABA-responsive element-binding proteins, a cytokinin oxidase (involved in cytokinin degradation) and an ACC oxidase (involved in ethylene biosynthesis) in near-isogenic early (line '10_11') vs. late-senescing germplasm (variety 'Karl') at 21 d past anthesis, suggesting that barley senescence is favoured by low cytokinin, but enhanced abscisic acid and ethylene levels and by ABA-dependent signalling (Jukanti *et al.*, 2008). Several studies have directly analysed the influence of hormone levels on cereal senescence. Yang *et al.* (2003) demonstrated that water stress enhances leaf senescence and substantially increases ABA levels, but decreases cytokinin levels in wheat. Furthermore, ABA levels were positively correlated with the remobilization of carbon reserves and grain filling. Additional data indicate that, besides a role in (drought) stress-induced senescence, ABA is also involved in the control of developmental senescence. ABA levels increase in leaves of several species (including rice and maize) as they age, and transcript levels of genes associated with ABA synthesis and signalling are also enhanced with increasing leaf age (Jibran *et al.*, 2013). Furthermore, Zhang and Gan (2012) have recently demonstrated in *Arabidopsis* that stomatal movement and water loss during senescence are controlled through a regulatory chain consisting of (increased) ABA, the AtNAP transcription factor and a type 2C protein phosphatase, suggesting a mechanism through which ABA is involved in the regulation of developmental leaf senescence. Causin *et al.* (2009) established a correlation between shading (low blue light levels), low cytokinin levels, and enhanced chlorophyll and protein degradation rates in wheat. Cytokinin oxidase activity was increased ~15-fold in dark-incubated barley leaf segments after 4 d of treatment (Conrad *et al.*, 2007). The same laboratory (Schlüter *et al.*, 2011) demonstrated an even stronger increase in the activity of this enzyme in barley leaf segments incubated in a light/dark cycle, again accompanied by a substantial decrease in chlorophyll levels, and also established that cytokinin oxidase is important during late development of (attached) flag leaves of field-grown barley plants. These studies indicate that cereal leaf senescence, like senescence in other plants, is associated with lower cytokinin, but higher ABA and possibly ethylene levels.

Oxidative stress (i.e. enhanced levels of toxic oxygen-containing compounds such as singlet oxygen, superoxide radicals, hydrogen peroxide, and hydroxyl radicals) has long been implicated in the senescence process, specifically through oxidative modification of plastidial proteins leading to their degradation (Ishida *et al.*, 1997, 1999; Roulin and Feller, 1998). More recently, it has become clear that such compounds are also involved in senescence signalling. Expression of the senescence-regulating transcription factor *AtWRKY53* is induced by hydrogen peroxide; additionally, leaf hydrogen peroxide levels were demonstrated to increase during plant development exactly at the moment when plants started to bolt and *WRKY53* was highly induced (Hinderhofer and Zentgraf, 2001; Miao *et al.*, 2004). The NAC transcription factor JUNGBRUNNEN1 (JUB1; German for 'Fountain of Youth'), as suggested by its name, was demonstrated to be a negative regulator of senescence (Wu *et al.*, 2012). Overexpression of this transcription factor increased plant tolerance to abiotic stress and led to reduced levels of hydrogen peroxide, whereas increased H_2O_2 levels were observed in *jub1-1* knockdown plants, linking hydrogen peroxide signalling and senescence regulation. In wheat, removal of reproductive sinks (spikelets) from ears at anthesis was associated with higher ratios of reduced/oxidized glutathione (GSH/GSSG), higher activities of antioxidant enzymes, and delayed flag leaf senescence (Srivalli and Khanna-Chopra, 2009). Similarly, senescence was delayed in the wheat *tasg1* stay-green mutant under two different water regimes, and levels of two reactive oxygen species (superoxide radicals and hydrogen peroxide) were consistently lower in *tasg1* flag leaves (Tian *et al.*, 2013). These results indicate that ROS-mediated signalling is also important for the regulation of wheat leaf senescence, but genes/proteins involved in signal transduction (such as functional homologues of AtWRKY53 or JUB1) have not been identified yet.

The 'stay-green' trait, quantitative trait locus analysis and stress resistance

Senescence is a highly visual phenomenon (Fig. 1). Profiting of this fact, a number of studies in different species have performed biochemical and/or genetic analyses of so-called 'stay-green' lines or varieties. However, when working with germplasm that visibly retains chlorophylls longer than a corresponding control or wild-type line, 'functional' stay-green types (where extended green-ness is associated with an extension of photosynthesis) have to be distinguished from 'cosmetic' types, which may have a mutation in a gene important for chlorophyll degradation, but otherwise catabolize essential components of the photosynthetic machinery at the same rate as controls (see Thomas and Howarth, 2000 for a discussion of this important point). The benefits of stay-green phenotypes for plant yield, especially under drought conditions, have been thoroughly investigated in sorghum and in corn (e.g. Tollenaar and Daynard, 1978; Borrell *et al.*, 2000a,b), sparking an interest in the analysis of delayed leaf senescence in other crops including wheat and barley.

In contrast to the experiments leading to the discovery of wheat/barley *Gpc-1* genes (initiated through the identification of a QTL important for GPC), several studies have used QTL mapping to identify loci important for extended photosynthetic functionality. Kumar *et al.* (2010) identified three QTLs associated with extended greenness on wheat chromosomes 1AS, 3BS, and 7DS, but only the QTL on chromosome 1A was detectable in both years of their field studies. Naruoka *et al.* (2012) performed an analysis of 'green leaf duration after heading' (GLDAH) and identified QTLs on chromosomes 2D, 4A, and 4B which were present in more than one of the environments used for their investigation. Importantly, their study indicated a positive correlation between GLDAH and test weight, seed weight, and seed diameter when plants were exposed to heat and drought conditions during grain filling, but not under cooler, well-watered conditions. These data suggest that in wheat, as in sorghum, delayed senescence may be important for abiotic stress resistance. Joshi *et al.* (2006), analysing 1407 spring wheat lines, found an association

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between the stay-green trait and spot blotch (*Bipolaris sorokiniana*) resistance, indicating that this trait may also have value for the selection of germplasm with improved performance under biotic stress conditions. Tian *et al.* (2012, 2013) and Hui *et al.* (2012) analysed a wheat mutant (*tasg1*) with a conspicuous stay-green phenotype (mentioned in connection with ROS signalling, above) and found that it is associated with extended photosynthesis, improved stability of thylakoid membrane proteins, and enhanced antioxidant competence, clearly establishing its status as a functional stay-green mutant line. Similarly, Luo *et al.* (2013) characterized a strong functional stay-green wheat variety (termed CN17) and found extended photosynthetic performance, probably due to considerably delayed loss of chloroplast function. However, in all these studies, the genes responsible for delayed senescence are unknown. While molecular markers associated with 'stay-green QTLs' may be valuable for breeding efforts aimed at increasing wheat yield under terminal drought conditions, identification of underlying genes could provide important basic information on the regulation of the wheat senescence process, as illustrated by the analysis of the *Gpc* genes.

Transcription factors and senescence signalling

Several NAC and WRKY transcription factors are among the best-studied *Arabidopsis* genes involved in senescence regulation (Guo and Gan, 2006; Zentgraf *et al.*, 2010; Balazadeh *et al.*, 2011; Hickman *et al.*, 2013). The role of NAC transcription factors was discovered almost simultaneously in *Arabidopsis* and wheat (see discussion of *Gpc* genes, above), based on completely different motivations and experimental approaches (Guo and Gan, 2006; Uauy *et al.*, 2006b). An overview of 48 barley *NAC* genes was recently provided by Christiansen *et al.* (2011); these authors demonstrated that many (but by far not all) of these genes are strongly expressed in senescing barley flag leaves. Among the WRKYs, the most detailed studies have been performed on AtWRKY53, a positive regulator of the senescence process, establishing a regulatory network centred on this particular transcription factor (Hinderhofer and Zentgraf, 2001; Miao *et al.*, 2004; Zentgraf *et al.*, 2010).

Besides NACs, only limited information on senescence regulation by transcription factors (TFs) is available for wheat and barley. Mangelsen *et al.* (2008) performed a detailed analysis of WRKY TFs in barley. These authors did not specifically characterize the importance of particular family members for the senescence process, although it may be noted that expression of *HvWRKY23* was significantly positively correlated with *AtWRKY53* when comparing transcript levels across a series of plant organs. HvWRKY23 should therefore be analysed for a role in cereal senescence regulation. It has been shown in *Arabidopsis* that AtS40-3 (*S40* was first discovered as a senescence marker gene in barley) is involved in *WRKY53* regulation; specifically, in a mutant line with a T-DNA insertion in the promoter of *AtS30-3*, low constitutive gene expression (which did not increase during senescence) was associated with a stay-green phenotype and lower *WRKY53* and *SAG12* expression at later developmental stages (Fischer-Kilbienski *et al.*, 2010). A detailed analysis of wheat *WRKY* gene function was recently performed by Niu *et al.* (2012). Based on sequence/phylogenetic comparisons, a group of wheat TFs related to AtWRKY53 was identified in that study; some of these genes may have functional roles in the control of wheat senescence. Additional data on the importance of TFs for wheat and barley leaf senescence stem from transcriptomic analyses of the process (Gregersen and Holm, 2007; Parrott *et al.*, 2007; Jukanti *et al.*, 2008; Cantu *et al.*, 2011). Similarly to the situation in *Arabidopsis*, numerous transcription factors from different families (including Myb, WRKY, AP2, zinc finger, GRAS, bZIP, and bHLH gene/proteins) are upregulated during wheat and barley senescence, but functional analyses of these genes have not been performed so far.

As for transcription factors, little functional information on genes/proteins involved in senescence regulation through signal transduction (phosphorylation/dephosphorylation processes, ubiquitination, and hormone signalling) is available for wheat or barley, but numerous genes with putative functions in these processes have been identified in the cited transcriptomic analyses (see also Krugman *et al.*, 2010 for drought-induced senescence in emmer wheat). Work in one of the authors' laboratories, comparing near-isogenic barley germplasm differing in senescence timing, has found strong (>10-fold) upregulation of genes coding for HvGR-RBP1 (discussed in connection with *Gpc* genes, above), two leucine-rich repeat transmembrane protein kinases, a polyubiquitin, two proteins involved in RNA splicing, a trehalose 6-phosphate phosphatase, and a 'mother of FT and TF1 protein' (MFT) in the early senescing line '10_11' as compared to the late-senescing parental variety 'Karl' (Jukanti *et al.*, 2008; see Fig. 1 for a visual comparison of the senescence behaviour of 'Karl' and '10_11'). Additionally, genes coding for numerous protein kinases, two additional glycine-rich RNA-binding proteins and several proteins with potential roles in flowering time regulation (including two FT proteins, an additional MFT protein, a CONSTANS-like protein) were upregulated at >2-fold. Functional characterization of these genes has the potential to identify additional regulatory networks controlling monocarpic senescence in cereals and may also uncover crosstalk with signalling pathways involved in flowering time control.

Nitrogen remobilization from senescing wheat and barley leaves

As already mentioned in this article, *Arabidopsis* has served as the primary model for the analysis of molecular mechanisms governing the senescence process. Contrasting with that situation, much of the information available on nutrient (especially nitrogen) remobilization during senescence has been obtained with monocarpic crops, particularly corn, wheat, rice, barley, and soybean. This may be explained by the fact that the analysis of senescence-associated nutrient remobilization and retranslocation has been a very active field of research even before the widespread use of molecular

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approaches and before the rise of *Arabidopsis* as the primary plant model species.

Degradation and remobilization of plastidial proteins

It has been known for over 30 years that most of the reduced nitrogen available for remobilization from senescing leaves to developing seeds or grains is present in chloroplasts. In wheat leaf mesophyll cells, about one-third of the total reduced N is present in one single enzyme, Rubisco, which is often referred to as the world's most abundant protein (Peoples and Dalling, 1988; Feller *et al.*, 2008). An even larger fraction can be found in additional plastidial proteins, including all other (besides Rubisco) Calvin cycle enzymes and proteins necessary for the photosynthetic light reactions, which are located in thylakoid membranes (Peoples and Dalling, 1988). Intriguingly, the biochemically most active proteases (particularly cysteine endopeptidases with acidic pH optima) present in senescing leaves are found in lytic vacuolar compartments, separated from most of their potential substrates by the tonoplast and by the chloroplast envelope. Two principal hypotheses of plastidial protein degradation have therefore been forwarded. One hypothesis suggests that plastidial proteases, albeit biochemically much less active than vacuolar enzymes, are mostly responsible for stromal and/or thylakoid protein degradation, with oxidative modifications initiating or facilitating the process (Mitsuhashi and Feller, 1992; Mitsuhashi *et al.*, 1992; Desimone *et al.*, 1996, 1998; Ishida *et al.*, 1997, 1998, 1999; Zhang *et al.*, 2007; Feller *et al.*, 2008). The second hypothesis is based on the possibility that chloroplasts, or vesicles containing chloroplast material, are transported to lytic vacuoles, enabling vacuolar serine and cysteine proteases to degrade plastidial proteins including Rubisco (Feller *et al.*, 2008; Ishida *et al.*, 2008; Martínez *et al.*, 2008; Prins *et al.*, 2008; Guiboileau *et al.*, 2012; Ono *et al.*, 2013). In this context, work by Chiba *et al.* (2003), using naturally senescing wheat leaves, has paved the way for more detailed molecular genetic analyses performed with *Arabidopsis* and tobacco (described below). There is ample evidence supportive of both hypotheses, suggesting that both plastidial and extra-plastidial pathways are used to achieve complete breakdown of the chloroplast proteome.

Evidence for the importance of plastidial proteolysis stems from the fact that these organelles contain numerous proteases, including members of the Clp, FtsH, Lon, Deg, and Spp families, as well as a complement of active aminopeptidases (Sinvany-Villalobo *et al.*, 2004; Huesgen et al, 2005; Adam *et al.*, 2006; Sakamoto, 2006; Ostersetzer *et al.*, 2007; Schuhmann and Adamska, 2012). Furthermore, a series of experiments have demonstrated that intact isolated chloroplasts incubated in light or darkness can at least initiate Rubisco degradation, leading to characteristic fragments stemming from hydrolysis of its large subunit (Mitsuhashi and Feller, 1992; Mitsuhashi *et al.*, 1992; Desimone *et al.*, 1998; Zhang *et al.*, 2007). Rubisco degradation has also been demonstrated in lysates of purified wheat chloroplasts (Kokubun *et al.*, 2002), and several manuscripts indicate that modification of this and possibly other proteins by oxidative stress may be important for the initiation of protein catabolism (Desimone *et al.*, 1996, 1998; Ishida *et al.*, 1997, 1999).

Both correlative and direct evidence for the importance of vacuolar proteases for plastidial protein degradation has been obtained. Treatment of wheat leaf segments with E-64, an inhibitor of (vacuolar) cysteine proteases, delays degradation of some stromal proteins including Rubisco (Thoenen *et al.*, 2007). Transcriptomic analysis of barley leaf senescence (using both the girdling system and comparison of near-isogenic early vs. late-senescing germplasm) indicated induction of numerous serine and cysteine protease genes, including genes coding for proteins which are typically located in lytic vacuoles (Parrott *et al.*, 2005, 2007; Jukanti *et al.*, 2008); proteases which were strongly upregulated in both experiments are shown in Table 1). Growth of barley plants in a hydroponic system, using three different N supply regimes combined with steam girdling of leaves allowed the manipulation of leaf carbohydrate and N levels and C/N ratios. In these experiments, expression of a family C1A cysteine protease (see MEROPS peptidase database at http://merops.sanger.ac.uk/index.shtml; Rawlings *et al.*, 2012 for protease nomenclature) was specifically induced under conditions of high carbohydrate, but low to moderate nitrogen levels (i.e. high C/N ratios), conditions leading to fast leaf senescence (Parrott *et al.*, 2010). Prins *et al.* (2008) demonstrated that expression of a rice cysteine

Table 1. *Upregulation of peptidase genes during barley leaf senescence*

Gene designations based on Affymetrix barley1 DNA microarray contigs (Close *et al.*, 2004). Peptidase family and identifier according to MEROPS peptidase database (Rawlings *et al.*, 2012; http://merops.sanger.ac.uk/index.shtml). Tentative activity based on MEROPS clan/family information. Regulation girdling, gene upregulation (-fold) in girdled vs. control leaves at 4 (4d) or 8 (8d) d past treatment (Parrott *et al.*, 2007); Regulation 21 dpa, gene upregulation (-fold) in high grain protein/early senescence near-isogenic line '10_11' vs. low grain protein/late-senescence parent 'Karl' at 21 d past anthesis (Jukanti *et al.*, 2008); Xaa, any protein amino acid.

Gene/contig	MEROPS classification	Tentative activity	Regulation girdling	Regulation 21 dpa
Contig6013_at	A1B; A01.050	Endopeptidase	3.9 (4d)	7.9
Ema01_SQ002_F07_s_at	C1A; C01.010	Endopeptidase	70 (8d)	19
Contig9006_at	C1A; C01.UPA	Endopeptidase?	3.7 (8d)	2.6
Contig5661_at	M24B; M24.007	Exopeptidase; Xaa-pro dipeptidase	5.4 (8d)	2.0
Contig600_at	S10; S10.009	Carboxypeptidase	15 (8d)	3.2
Conti6685_at	S10; S10.UPW	Carboxypeptidase	7.1 (8d)	3.3

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protease inhibitor (the cystatin OC-1) in tobacco led to a substantial delay in the senescence-related decline of photosynthesis and Rubisco activity. Using an *Arabidopsis* autophagy mutant (a T-DNA insertion in the *ATG5* gene), Ishida *et al.* (2008) obtained data indicating that Rubisco and stroma-targeted fluorescent proteins can be transferred to vacuoles in an *ATG*-gene dependent manner, without destruction of the entire chloroplast. Autophagy and transport of stromal proteins to vacuoles were also observed in individually darkened *Arabidopsis* leaves (Wada *et al.*, 2009), and further studies have since confirmed the importance of autophagy for the transfer of stromal proteins to lytic vacuolar compartments during leaf senescence (Guiboileau *et al.*, 2012; Ono *et al.*, 2013), explaining how abundant vacuolar proteases may be involved in plastidial protein degradation. Martínez *et al.* (2008) used a similar approach to demonstrate the involvement of 'senescence-associated vacuoles' in plastidial protein degradation in senescing tobacco leaves; importantly, senescence-associated vacuoles contain abundant proteolytic activity. In all these studies, there was no evidence for transfer of thylakoid proteins to vacuolar compartments, indicating that different pathways may be utilized for catabolism of stromal and thylakoid proteins. This finding may also explain why the kinetics of stromal and thylakoid protein degradation have been found to be different in some studies, including those investigating cereals (Gregersen *et al.*, 2008). There remains a strong possibility that chloroplast-located proteases are important for (the initiation of) thylakoid protein degradation; additionally, plastidial proteases and/or oxidative modification of stromal proteins (inside intact chloroplasts) may prepare these substrates for complete degradation through autophagy.

While these briefly discussed studies have allowed major progress in the understanding of senescence-associated protein degradation, important gaps remain. Plant genomes contain hundreds of protease genes (678 have been identified in the rice genome, and 826 in the *Arabidopsis* genome; van der Hoorn, 2008), yet the *in vivo* substrates of only very few of them have so far been identified. Furthermore, while the degradation of Rubisco and other stromal proteins during senescence has been well described, and while it now appears very likely that vacuolar proteases are important for this process, the specific genes/proteins needed for plastidial protein catabolism remain to be identified. Modern mass spectrometry-based techniques such as those developed in the medical field (Huesgen and Overall, 2012), combined with the analysis of appropriate (protease knockdown or knockout) mutants, will be needed to achieve substantial progress in this important area. However, it is likely that a considerable level of redundancy exists in the pathways of senescence-associated proteolysis (particularly in crops with large, complex genomes such as wheat), and that multiple knockouts or RNAi lines suppressing several related proteases will initially be needed to obtain phenotypes amenable to mass spectrometric analysis.

Senescence-associated nitrogen metabolism

Nitrogen retranslocation from senescing leaves to developing seeds and grains has been analysed in some detail, but it is important to remember that retranslocation also takes place during vegetative plant development, particularly from older to younger leaves. Furthermore, even redistribution from a wheat or barley plant's topmost leaves to the developing grains may occur indirectly: for example, via the glumes (which finally senesce and retranslocate mobile nutrients to the developing seeds as well) (Lopes *et al.*, 2006; Feller *et al.*, 2008) or via the roots (Simpson *et al.*, 1983). It is well established that amino acids delivered from leaves to roots through phloem transport can be recycled to the shoot in the transpiration stream (xylem). However, as the transpiration rate of developing seeds/grains is typically low, xylem-to-phloem transfer in the peduncle has been demonstrated to be important for the delivery of reduced nitrogen and other solutes to seeds (Feller and Fischer, 1994).

Reduced nitrogen is exported from senescing barley and wheat leaves as glutamate, but also as aspartate, threonine, and serine (Winter *et al.*, 1992; Caputo *et al.*, 2001; Kichey *et al.*, 2006; Gregersen *et al.*, 2008). Glutamine also appears to be important, as its relative abundance increases during late senescence in wheat phloem (Simpson and Dalling, 1981); additionally, Fisher and Macnicol (1986) found that glutamine accounted for almost half the amino acids in sieve tube sap from wheat peduncles around midgrain filling. These data indicate that most amino acids derived from senescence-associated (plastidial) protein degradation undergo modification prior to phloem loading. Several studies demonstrate that the GS-GOGAT cycle is of primary importance in this context. Higher plants contain two major isoforms of glutamine synthetase, designated as GS1 (cytosolic isoform) and GS2 (plastidial isoform). GS2 is typically encoded by a single nuclear gene, while several genes are encoding GS1 subunits (Ireland and Lea, 1999; Coruzzi and Last, 2000). GS2 is the dominant isoform in mature leaves, but it is degraded early during leaf senescence and is unstable in isolated chloroplasts (Streit and Feller, 1983; Kamachi *et al.*, 1991; Mitsuhashi and Feller, 1992). In contrast, *GS1* genes are induced during late development. Available data therefore suggest that GS1, and not GS2, is important for N retranslocation from senescing cereal leaves. Using a combination of agronomic and physiological approaches, Kichey *et al.* (2006) have confirmed the central role of GS for wheat whole-plant nitrogen management. The role of GOGAT for N remobilization has been studied as well. Available data indicate that the NADH-dependent isoform plays an important role in N remobilization, but this enzyme appears to be important for N metabolism in sinks (young leaves and grains) rather than in senescing leaves (Obara *et al.*, 2001; Yamaya *et al.*, 2002).

From glutamine and glutamate, nitrogen can be transferred to other amino acids through the actions of asparagine synthetase (AS) and several aminotransferases (transaminases). Upregulation of both aminotransferases and asparagine synthetase in senescing tissues has been demonstrated (Fujiki *et al.*, 2001; Yoshida *et al.*, 2001; Winichayakul *et al.*, 2004). In barley, transcriptomic comparison of near-isogenic early vs. late-senescing germplasm demonstrated a 2.4-fold upregulation of an *AS* gene in flag leaves at 21 d

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past anthesis (Jukanti *et al.*, 2008; supplementary data) and several aminotransferase genes were significantly upregulated as well, but these genes and their protein products have not been functionally characterized yet. Both *AS* and aminotransferases have been overexpressed in various crops in an attempt to improve overall plant nitrogen use efficiency and, at least in some instances, this approach has led to enhanced seed yield or seed protein content (see McAllister *et al.*, 2012 for a recent review).

Nitrogen transport to developing grains

Several short- and long-distance transport steps are needed to transfer nitrogen derived from (plastidial) protein degradation and amino acid metabolism in senescing leaf cells to the protein bodies of cereal endosperm. Membrane transporters are necessary for the release of amino acids or oligopeptides from vacuoles and chloroplasts. While transporters mediating the efflux of sucrose from mesophyll cells into the apoplast have recently been characterized in *Arabidopsis* (Chen *et al.*, 2011), little is known about amino acid efflux from senescing mesophyll cells. Within the leaf mesophyll, transport needs to occur towards vascular bundles. Loading of photosynthetic products and amino acids into the phloem has been well investigated and occurs from the apoplast in most species including wheat and barley. Uptake of sucrose and amino acids into the sieve tube/companion cell complex is mediated by specific transporters, coupling an electrochemical (proton) gradient with secondary, active accumulation of these compounds (Lalonde *et al.*, 2004).

High concentrations of carbon, nitrogen, and other compounds are present in phloem sap, allowing efficient transport of sucrose and amino acids to the developing sinks (Fisher and Wang, 1995; Marschner, 1995; Caputo *et al.*, 2001). Mechanisms for xylem-to-phloem transfer have been identified in upper stem tissues, and are important for the transport of root-borne amino acids, or indirect (*via* the root system) transport of leaf-generated amino acids, to the grains. In cereals with their small closed bundles, such transfer can occur within the bundle in an internode; it functions not only for amino acids, but also for alkali cations and other solutes (Feller and Fischer, 1994). For amino acids, transfer is selective and depends on the side chain. In cereals it was most efficient for basic and bulky hydrophobic amino acids and considerably less efficient for acidic amino acids (Feller and Hölzer, 1991; Feller and Fischer, 1994). These data may be useful in identifying responsible membrane transport proteins (see below).

Phloem unloading in developing wheat grains has been studied in detail by Wang and Fisher (1994a,b), but molecular mechanisms governing this process are less well understood than phloem loading in source leaves. In developing legume seeds, an apoplastic step is involved in assimilate transfer between the seed coat and the embryo, and nutrients are taken up by carrier-mediated transport in the embryo epidermis (McDonald *et al.*, 1996). In contrast, Wang and Fisher (1994a) found the evidence for an active transport step in nutrient transfer to cereal endosperm inconclusive. This suggests that the mechanisms used for assimilate transfer from maternal to filial tissues may be different in wheat and legumes.

Physiological and microscopic techniques have been successful in delineating the general steps involved in nutrient transport from source to sink tissues including seeds and grains. Considerably less information is still available at the molecular level, with important gaps in the knowledge of membrane transport proteins important for intracellular and intercellular/long-distance transport of amino acids. Most of the identified transporters have been characterized in *Arabidopsis*. So far, molecular knowledge has been obtained for amino acid import into the phloem, with the identification of a transporter important for xylem-to-phloem transfer (AtAAP2, *Arabidopsis* amino acid permease 2) (Zhang *et al.*, 2010). Progress has also been achieved with the identification of transporters important for seed import (particularly AtAAP1 and AtAAP8). In contrast, knowledge on amino acid exporters (such as those needed for export of reduced N from senescing mesophyll cells prior to uptake by phloem tissue) is almost completely lacking (see Tegeder, 2012 for a recent review). Similarly, little is known about transporters involved in intracellular amino acid transport (e.g. release from plastidial and vacuolar compartments to the cytosol), although one transporter (AtDiT2.1, a glutamate/malate exchanger exporting glutamate from plastids to the cytosol) has been characterized (Renné *et al.*, 2003). An RNA-seq approach was recently used to identify barley genes coding for amino acid transporters (and cysteine proteases) expressed in flag leaves, glumes, and developing grains with putative importance for senescence-associated N retranslocation (Kohl *et al.*, 2012). Among the analysed amino acid permeases, *HvAAP2* and *HvAAP6* were found to be highly expressed in vegetative organs, while *HvAAP3* was grain specific. Importantly, HvAAP3 is closely related to AtAAP1 and AtAAP8 which, as mentioned above, are important for N supply to developing seeds. Considering that data obtained so far indicate that amino acid transporters play central roles in plant N metabolism and source–sink interactions (Tegeder, 2012), it will be important to close existing gaps in the knowledge of whole-plant nitrogen partitioning; such processes, especially those associated with senescence, may well be important components of overall cereal nitrogen use efficiency.

Conclusions and outlook

Research performed over the last 40 years has led to a considerably improved basic understanding of cereal senescence. The application of molecular and '-omics' techniques has identified a number of processes and genes regulating senescence; some of these discoveries may be of value for molecular breeding and transgenic approaches aimed at improving parameters such as GPC, nutrient-use efficiency, and yield.

Additionally, this research has identified several important challenges for future research:

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1. The detailed characterization of the regulatory network surrounding the *Gpc* genes has a high potential to allow the targeted manipulation of the balance between yield, nutrient-use efficiency and grain nutrient content, profiting of some recent research in *Arabidopsis* (Guo and Gan, 2006; Zhang and Gan, 2012).
2. Identification of additional regulatory genes, based on data obtained (a) in model species, (b) by map-based cloning of genes responsible for cereal stay-green QTLs, and(c) by analysis of genes coregulated/coexpressed across a series of experimental conditions with known senescence regulators (Fischer, 2012) will lead to a more comprehensive understanding of cereal senescence and identify additional breeding targets.
3. Informed by recent advances in the understanding of the role of autophagic processes in plastidial protein degradation (Ishida *et al.*, 2008; Wada *et al.*, 2009; Guiboileau *et al.*, 2012; Ono *et al.*, 2013) and based on progress in mass spectrometric techniques (Huesgen and Overall, 2012), it will be essential to identify the protease genes responsible for the degradation of Rubisco and other soluble (stromal) and thylakoid proteins. Available data already indicate that inhibition of senescence-associated protein degradation has a profound influence on the entire senescence process (Prins *et al.*, 2008).
4. While the importance of (cytosolic) glutamine synthetase for N remobilization has been demonstrated, it is likely that additional genes involved in organic acid and amino acid metabolism await discovery and characterization, to complete the understanding of the conversion of protein degradation products into nitrogen transport forms.
5. It will be necessary to identify and characterize the amino acid transporters important for nitrogen remobilization to developing cereal grains and to more completely delineate the transport pathway between maternal and filial tissues. Similarly, to improve overall grain nutrient content, the transport proteins involved in micronutrient (re)translocation to developing grains should be identified and characterized.

With respect to points 3–5, it is not clear which of these steps are (most) rate-limiting in the overall process of nutrient remobilization or how they are influenced by source strength and sink capacity. An answer to this question will emerge from the analysis of the partial processes involved and from the combination of appropriate alleles and mutations in different genetic backgrounds.

While working towards the goal of a more complete understanding of plant senescence processes, it is important to interpret novel findings in a broader context: for example, with respect to source–sink relations during senescence, cross-talk between flowering time control and senescence regulation, and cross-talk between senescence control and plant reaction to biotic (pathogens) and abiotic (particularly drought and nutrient levels) factors. Such an approach will also make the contributions of senescence research to basic and applied whole-plant biology more valuable.

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