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Research Article

Interactions Between Temperature and Sugars in the Regulation of Leaf Senescence in the Perennial Herb *Arabis alpina* L.

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Abstract

Annual plants usually flower and set seed once before senescence results in the death of the whole plant (monocarpic senescence). Leaf senescence also occurs in polycarpic perennials; even in "evergreen" species individual leaves senesce. In the annual model *Arabidopsis thaliana* sugars accumulate in the senescent leaves and senescence is accelerated by high sugar availability. Similar to *A. thaliana*, sugar contents increased with leaf age in the perennial *Arabis alpina* grown under warm conditions (22 °C day/18 night). At 5 °C, sugar contents in non-senescent leaves were higher than at a warm temperature, but dependent on the accession, either sugars did not accumulate or their contents decreased in old leaves. In *A. alpina* plants grown in their natural habitat in the Alps, sugar contents declined with leaf age. Growth at a cold temperature slightly delayed senescence in *A. alpina*. In both warm and cold conditions, an external glucose supply accelerated senescence, but natural variation was found in this response. In conclusion, sugar accumulation under warm conditions could accelerate leaf senescence in *A. alpina* plants, but genotype-specific responses and interactions with growth temperature are likely to influence senescence under natural conditions.

Keywords: Perennial; polycarpic; senescence; sugar signaling.

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Introduction

Leaf senescence is required for the recycling of nutrients, such as nitrogen, from leaves. In annual plants, it is possible to differentiate between sequential senescence that results in nitrogen recycling from old to young leaves during the vegetative phase, and monocarpic senescence that supplies nitrogen for reproduction during the flowering stage (Diaz et al. 2008). Ultimately, all leaves senesce and the whole plant dies. In contrast, perennial plants can live for several years or even longer. For example, whole-organism senescence was not detected in the long-lived mountain herb *Borderea pyrenaica* (García et al. 2011; Oñate et al. 2012). However, individual organs such as leaves also senesce in perennial species. In some perennials, all leaves senesce at the end of the growing season, but nutrients recycled from the leaves can be stored, for example, in the trunks of trees, or in the case of *B. pyrenaica*, in underground organs until the next season.

In annual species, vegetative growth often stops during reproduction (determinate development), which results in a reduced carbon requirement for growth. If, as in *Arabidopsis thaliana*, the inflorescences and even the fruits are photosynthetic, carbon is not likely to be limiting during the reproductive stage, as long as enough nitrogen is mobilized for the formation of the photosynthetic apparatus in the reproductive organs. This can result in excess carbon accumulating in the leaves

in the form of sugars, especially glucose and fructose (Quirino et al. 2001; Stessman et al. 2002; Pourtau et al. 2004; Wingler et al. 2012). In turn, sugar accumulation can serve as a signal to accelerate senescence-dependent nitrogen recycling (Masclaux et al. 2000; Pourtau et al. 2006; Parrott et al. 2007; Parrott et al. 2010).

In plants with indeterminate development, more carbon may be required during flowering to maintain growth, which means that sugars may not accumulate or serve as signals for senescence. This is suggested by experiments in which carbon availability was increased by growth in elevated CO_2 concentrations. While plants with determinate growth, such as wheat, respond with accelerated senescence (Nie et al. 1995; Zhu et al. 2009), senescence is delayed in plants with indeterminate growth, for example trees (Tricker et al. 2004; Taylor et al. 2008). However, once growth ceases in autumn, sugars may still accumulate in the leaves of trees. For example, sugar contents were shown to increase temporarily during early senescence in aspen (Keskitalo et al. 2005).

Growth is also reduced under stress conditions, which can result in carbon accumulation despite lower rates of photosynthesis (Muller et al. 2011). During cold stress, sugars accumulate as part of the cold acclimation response (Cook et al. 2004). However, growth of *A. thaliana* plants in cold temperatures (Masclaux-Daubresse et al. 2007) and overexpression of the *CBF2* transcription factor which is involved in the cold acclimation pathway (Sharabi-Schwager et al. 2010) both result in delayed senescence. In addition, in *A. thaliana,* the senescence response to an external sugar supply was shown to be deactivated at cold temperatures (Masclaux-Daubresse et al. 2007). Earlier work by Strand et al. (1997) has demonstrated that cold acclimation releases the suppression of photosynthesis that is usually caused by sugar accumulation. Interactions between sugars and temperature therefore play an important role in the regulation of photosynthesis and senescence in *A. thaliana*, but such interactions have not been studied in perennial plants or in alpine species which are adapted to growth in cold temperatures.

Senescence of individual leaves also occurs in “evergreen” perennials, such as *Arabis alpina*. *A. alpina* forms vegetative and flowering branches, and it is likely that in the vegetative branches, sequential senescence provides nutrients for continued growth. *A. alpina* is polycarpic (meaning that it can flower repeatedly) and has an obligate vernalization requirement. The molecular basis of polycarpic flowering has been analyzed at the molecular level: vernalization results in the silencing of the floral repressor *PEP1*, which is an orthologue of the *A. thaliana FLC* gene. In contrast to winter-annual *A. thaliana* lines where vernalization leads to the stable silencing of the floral repressor *FLC*, silencing of *PEP1* in *A. alpina* is only transient, allowing the plant to revert to vegetative development (Wang et al. 2009). In addition, *TFL1* inhibits flowering in young plants and keeps axillary shoots in the vegetative state, thereby supporting the perennial lifecycle (Wang et al. 2011). Importantly, this control of flowering also results in indeterminate growth which can have consequences for carbon availability and the role of sugars in the regulation of leaf senescence.

In this study, we analyzed the effect of growth temperature and sugar supply on senescence in the perennial herb *A. alpina*. In addition, sugar contents were determined in senescent and non-senescent leaves of plants grown at warm and cold temperatures and in their natural habitat in the field. We found that changes in sugar contents that occur as leaves age depend on the growth temperature, and thus conclude that the extent to which sugar signalling may be involved in senescence regulation depends on the environmental conditions and natural genetic variation.

Results

A. alpina accessions from two sites in the Écrins region of the French Alps were selected for the experiments. One accession originated from a rocky slope in the valley formed by the Romanche river, the other from a rocky outcrop near the Col du Lautaret (see Materials and Methods for detailed locations). Measurements were conducted in the field, and seeds were collected during August 2010. Plants were then grown from seed for experiments under controlled conditions. Leaves were counted from the base (old leaves) to the apex (young leaves) of a branch (**Figure 1**).

Effect of temperature on senescence

The senescence-dependent decline in chlorophyll content was determined in non-vernalized plants grown under warm (22 °C during the day/18 °C during the night) or cold (5 °C) conditions. Chlorophyll content, determined as a chlorophyll content index using a CCM-200 chlorophyll content meter, declined with leaf

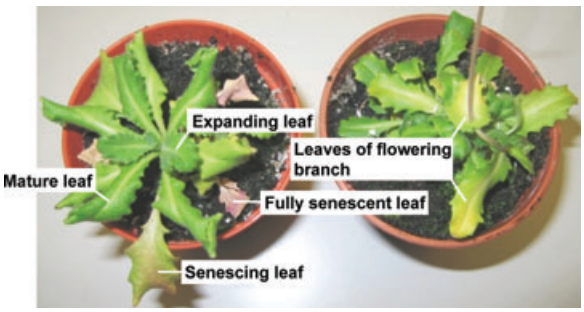

Figure 1. Leaf senescence in a non-flowering plant (left) and a flowering plant (right) from the Lautaret site.

For further analysis, leaves were counted from the base (old leaves) to the apex (young leaves) of a branch.

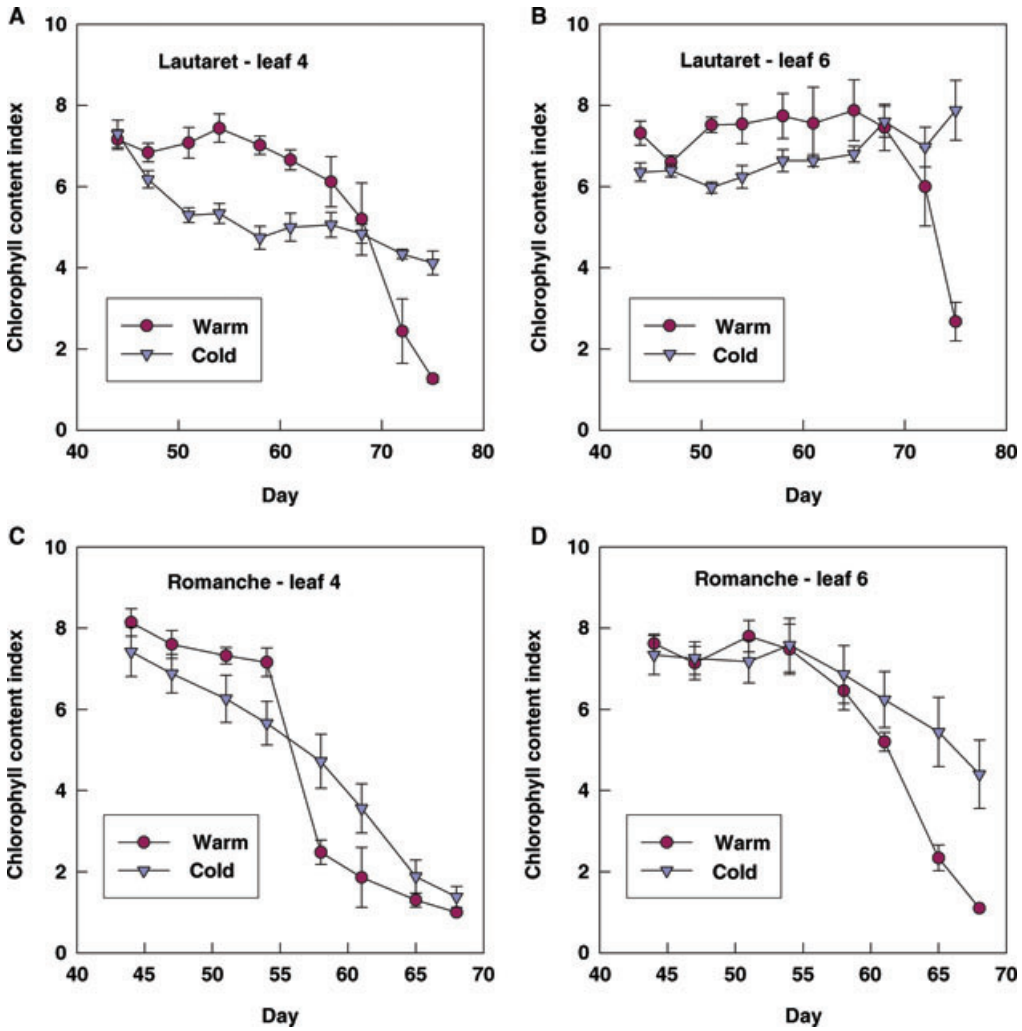

Figure 2. Effect of temperature on leaf senescence of *Arabis alpina* leaves.

(A) and **(B)** Leaves 4 and 6 of plants from the Lautaret site.

(C) and **(D)** Leaves 4 and 6 of plants from the Romanche site. Chlorophyll was monitored in plants grown at 22 °C during the day and 18 °C at night (warm conditions) throughout the experiment, and plants transferred from the warm conditions to 5 °C (cold conditions) on day 44. Data are means of 3–5 plants ± *SE*.

age indicating the progress of senescence (**Figure 2**). In the older leaves (leaf 4) from plants from the Lautaret site, chlorophyll content initially dropped faster in the cold temperature condition, but later on senescence progressed more rapidly at warm temperatures. Data for leaf 6 also indicate that senescence was delayed by the cold treatment. A similar effect, albeit less obvious, was observed in plants from the Romanche site. A temporary decrease in maximum photosystem II efficiency (F_v/F_m) in plants from the Lautaret site indicated an initial stress response to transfer to the cold temperature condition, but photosynthetic function then recovered (Supplemental Figure S1), supporting the view that transfer to cold temperatures results in stress, but does not accelerate senescence.

At the end of the experiment, chlorophyll content was determined (**Figure 3**) in the same leaves harvested for sugar analysis (**Figures 4** and **5**), with leaf positions selected to represent senescence stages, from mature (leaf 8 at the Lautaret site, leaf 12 at the Romanche site) to senescent leaves (leaf 4 at the Lautaret site, leaf 6 at the Romanche site). Plants from the Lautaret site contained more chlorophyll after growth in the cold temperature than in warm temperatures. This difference was significant in leaves 8 and 4. Since the chlorophyll content index represents chlorophyll on a leaf area basis, it is possible that the higher content in cold-grown plants reflects increased leaf thickness. However, **Figure 2** does not suggest that chlorophyll content increased after transfer to the cold temperature

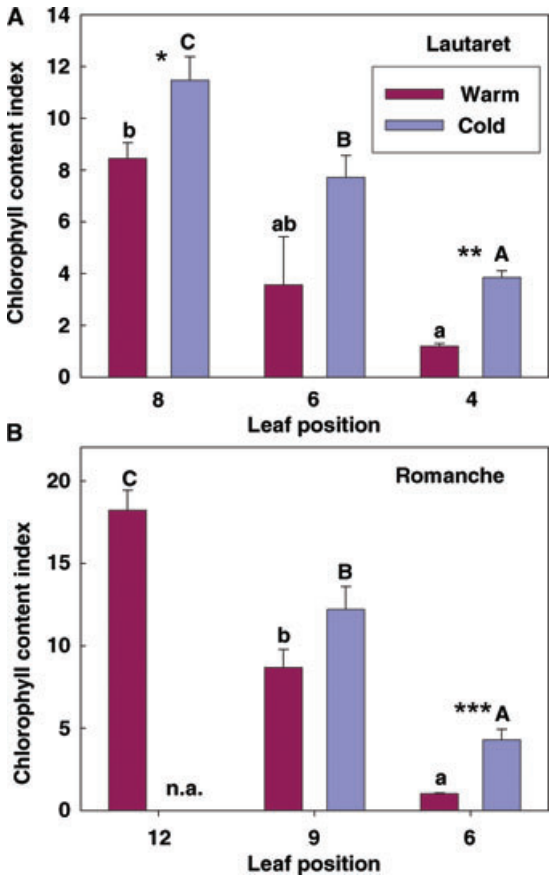

Figure 3. Chlorophyll content in the leaves of non-vernalized plants grown at 22 °C during the day and 18 °C at night (warm conditions) or at 5 °C from day 44 (cold conditions).

(A) Plants from the Lautaret site (means of 2–5 plants ± *SE*) after 76 days of growth.
(B) Plants from the Romanche site (means of 5 plants ± *SE*) after 69 days of growth. n.a. = not available. Asterisks indicate significant differences between the warm and cold treatments (*t*-test); $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Different letters indicate significant differences ($P \leq 0.05$) between leaf positions within a temperature treatment (ANOVA and Tukey's pairwise comparison).

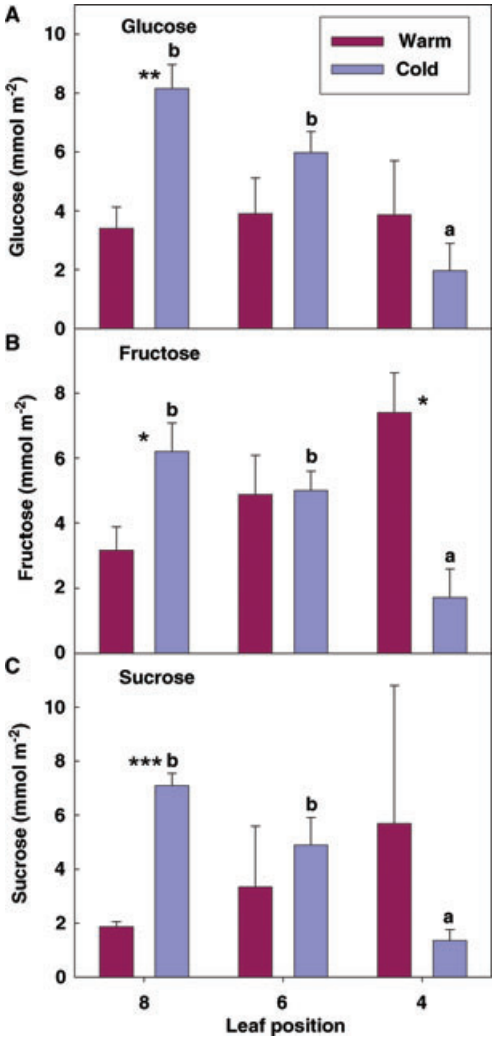

Figure 4. Sugar contents in the leaves of non-vernalized plants from the Lautaret site after 76 days of growth.

Plants were grown at 22 °C during the day and 18 °C at night (warm conditions) or at 5 °C from day 44 (cold conditions). Data are means of 2–5 plants ± *SE*. Asterisks indicate significant differences between the warm and cold treatments (*t*-test); $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Different letters indicate significant differences ($P \leq 0.05$) between leaf positions within a temperature treatment (ANOVA and Tukey's pairwise comparison).

condition, and indicates that the higher chlorophyll content at the end of the experiment is instead due to delayed senescence. A similar pattern was also found in plants from the Romanche site, where chlorophyll content in leaf 6 was significantly higher in the cold temperature than in warm temperatures. Leaf 12 was only harvested after growth in warm temperatures, as leaves grown in the cold were too small for analysis. At the time of transfer to the cold temperature, plants from the Lautaret site had on average 9.2 leaves, while those from the Romanche site had 11.4 leaves. The older leaves were therefore formed before transfer to the cold condition, and the differences in chlorophyll content in these leaves thus indicate delayed senescence and not delayed leaf formation.

Effect of temperature on sugar contents

Sugar contents (**Figures 4** and **5**) were determined in the same leaves that were analyzed for chlorophyll content (**Figure 3**). There were clear effects of temperature on sugar contents

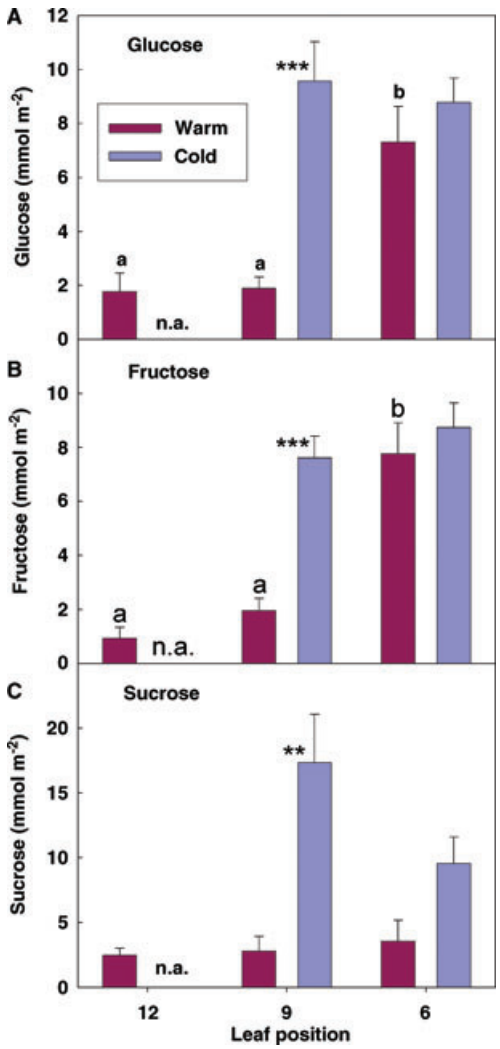

Figure 5. Sugar contents in the leaves of non-vernalized plants from the Romanche site after 69 days of growth.

Plants were grown at 22 °C during the day and 18 °C at night (warm conditions) or at 5 °C from day 44 (cold conditions). Data are means of 5 plants ± *SE*. n.a. = not available. Asterisks indicate significant differences between the warm and cold treatments (*t*-test); $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Different letters indicate significant differences ($P \leq 0.05$) between leaf positions within a temperature treatment (ANOVA and Tukey's pairwise comparison).

that were dependent on the leaf position. In warm conditions, glucose remained stable, while fructose and sucrose contents increased slightly, but not significantly, in senescing leaves of plants from the Lautaret site (**Figure 4**). In the cold temperature condition, this pattern changed. In the non-senescent leaf 8, sugar contents were higher than in warm-grown plants, but all three sugars decreased with leaf age. This resulted in a lower fructose content of the senescent leaf 4 in cold compared to warm conditions.

In plants from the Romanche site grown at warm temperatures (**Figure 5**), there was a significant increase in both glucose and fructose content along with leaf age, while sucrose content did not change. For leaf 9 (early senescence), sugar contents were higher in cold-grown than warm-grown plants. In cold-grown plants, there was, however, no significant difference in sugar contents between leaf 9 and the senescent leaf 6. These results show a clear effect of growth temperature on the change in sugar contents with leaf age.

Plants from the Romanche site were also vernalized (**Figure 6**) to test whether the general increase in sugar contents found in non-vernalized plants also occurs in plants that are flowering. In addition, this enabled comparison of sugar contents between leaves from vegetative and flowering branches. In this case, glucose, fructose and sucrose all accumulated with leaf age and were higher in senescent leaves from vegetative branches than in leaves from flowering branches which had an equally low chlorophyll content.

Senescence-dependent changes in sugar contents in the field

Samples for sugar analysis were also taken in the field. Only leaves from vegetative branches were analyzed, but all plants also had flowering branches. At the Lautaret site, the decline in maximum photosystem II efficiency (F_v/F_m) was monitored as an indicator of senescence (**Figure 7**). With senescence of the older leaves, glucose and sucrose contents declined, while there was a small increase in fructose.

At the Romanche site, a decline in chlorophyll content indicates progressive senescence towards the lower leaves (**Figure 8**). In this case, contents of all three sugars decreased with leaf age. The decline in chlorophyll and sugar contents towards the older leaves resulted in a positive correlation between chlorophyll (determined as SPAD units) and total sugar content of individual leaves (Supplemental Figure S2).

Senescence response to sugar treatment and temperature

Under warm conditions, plants from the Romanche site showed an accelerated decline in F_v/F_m in response to treatment with glucose on low-nitrogen medium (**Figure 9**), indicating accelerated senescence. Cold treatment led to a decrease in F_v/F_m before values stabilized. While plants grown in the absence of glucose showed some recovery, F_v/F_m declined further in plants in the presence of glucose, suggesting that glucose also induced senescence in the cold.

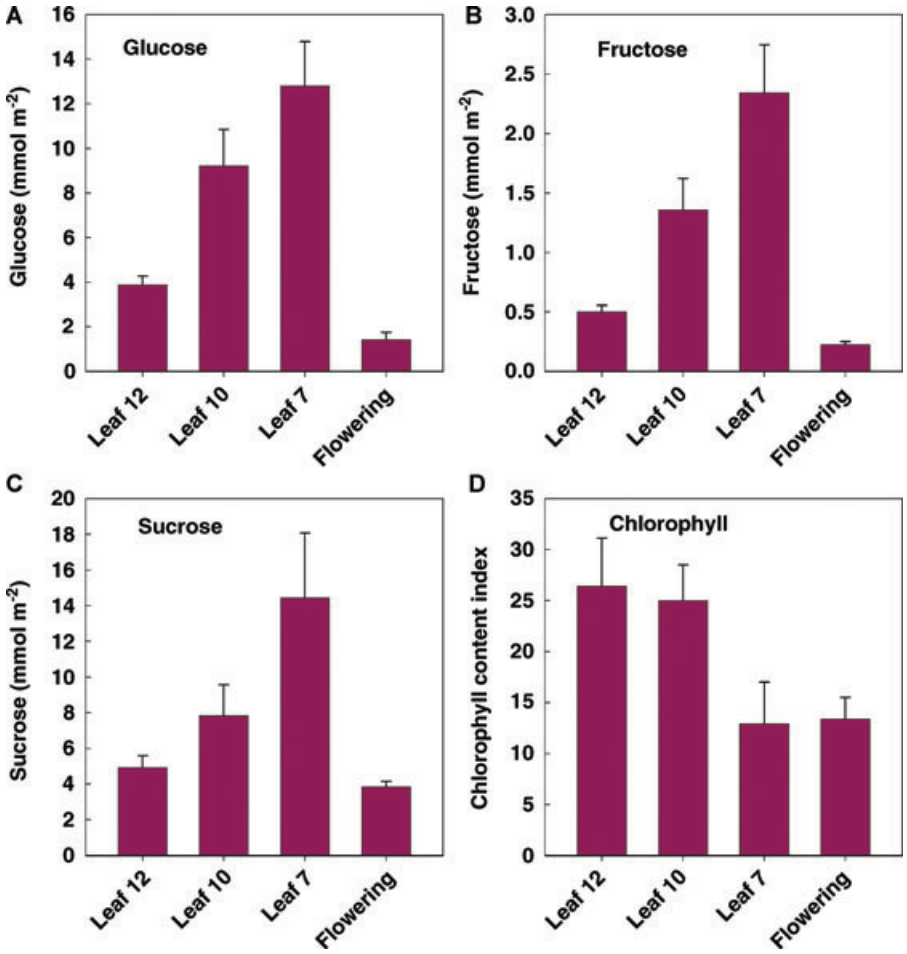

Figure 6. Sugar and chlorophyll contents in the leaves of vernalized plants from the Romanche site.

Plants were grown at 22 °C during the day and 18 °C at night. Leaves 7, 10 and 12 were harvested from vegetative branches and compared to leaf 1 from the flowering branch ("Flowering"). Data are means of 6–8 plants ± *SE*.

Discussion

In *A. thaliana*, the hexoses glucose and fructose accumulate strongly in senescing leaves. This has been shown by analyzing development over time, for example under different light conditions (Wingler et al. 2006) or by comparing mature leaves to those that had lost 75% or more of their chlorophyll content (Quirino et al. 2001). For tobacco leaves on different positions along the stem it was shown that hexoses peaked just before the initiation of senescence-dependent nitrogen recycling (Masclaux et al. 2000). A general increase in sugar contents with leaf age was also found in leaves of the vegetative branches of vernalized and non-vernalized *A. alpina* plants grown under standard (warm) condition (**Figures 4, 5** and **6**). However, the senescence-dependent sugar increase was smaller than typical for *A. thaliana*. This less pronounced sugar accumulation in *A. alpina* may be related to indeterminate growth: in contrast to *A. thaliana* where vegetative growth more or less stops when the plants start to flower, carbon utilization for growth continues in *A. alpina*. This may result in reduced sugar accumulation.

In the field, sugar contents declined in senescing leaves (**Figures 7** and **8**). Differences between the laboratory and field conditions may be caused by differences in growth temperature as indicated by the effect of cold temperature on sugar contents under controlled conditions in the laboratory. Although the samples were harvested under relatively warm conditions (15.2 °C at the Lautaret site; 27.0 °C at the Romanche site), sugar contents may reflect conditions during the previous growth period. Meteorological data from the nearby Station Alpine Joseph Fourier show that the respective weeks prior to harvest of the sugar samples were warm: average maximum temperatures were 15.9 °C and 15.3 °C during the week before harvest of the Lautaret and Romanche samples, respectively; average

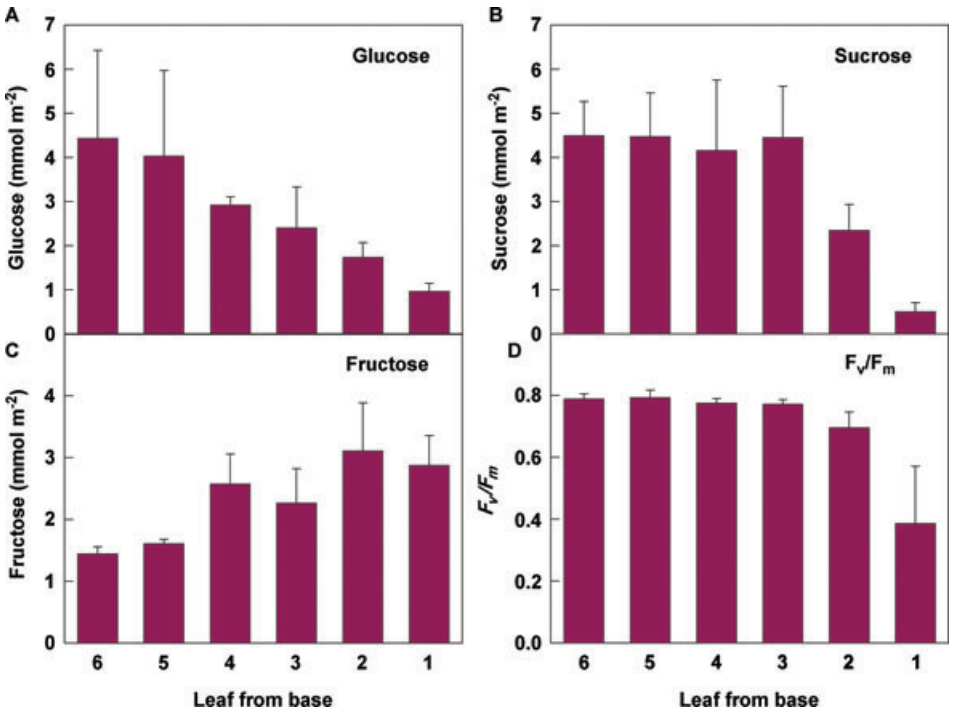

Figure 7. Senescence-dependent decline in F_v/F_m and sugar contents in leaves from non-flowering branches of plants growing at 2090 m in the field (Lautaret site).

Data are means of 3 plants ± *SE*.

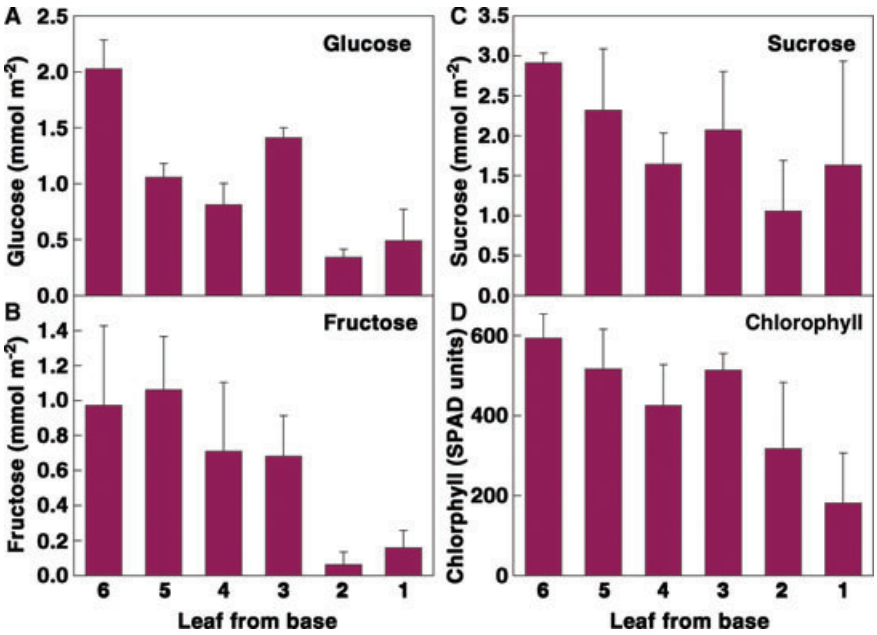

Figure 8. Senescence-dependent decline in chlorophyll and sugar contents in leaves from non-flowering branches of plants growing at 1684 m in the field (Romanche site).

Data are means of 3 plants ± *SE*.

minimum temperatures were 6.6 °C and 7.4 °C during the week before harvest of the Lautaret and Romanche samples, respectively. However, low night-time minimum temperatures of 2.2 °C and 2.1 °C occurred about a week before both harvests.

In non-senescent leaves, sugar contents were higher at cold than at warm temperatures, which is in agreement with the literature for *A. thaliana* and other plants where sugar accumulation is involved in the cold acclimation process (Cook et al. 2004). However, this was no longer the case in senescent leaves, and in plants from the Lautaret site fructose content was even higher at warm temperatures. Senescence therefore clearly affects to what extent sugars accumulate under cold conditions.

The effect of cold temperatures on sugar contents in senescing leaves has not been explored for *A. thaliana*. In cold conditions, both photosynthesis and growth are inhibited. If growth is more strongly inhibited than photosynthesis, this can result in excess carbon availability as for example during drought stress (Muller et al. 2011). Gorsuch et al. (2010) found that glucose and starch contents peak a few days after exposure of pre-existing *A. thaliana* leaves to cold temperatures, but then slowly decline. This has been ascribed to increasing carbon demand as growth recovers. In the experiment presented here, all leaves were cold treated for the same duration, suggesting that the decrease in sugar contents with leaf age in plants from the Lautaret site (**Figure 4**) was more likely to be related to the senescence-dependent decline in photosynthesis rather than the duration of the cold treatment.

The decline in sugar contents with leaf age under field conditions (**Figures 7** and **8**) makes it unlikely that sugar accumulation serves as a signal for leaf senescence in *A. alpina* growing under natural conditions. It is, however, conceivable that during continued growth at warm temperatures, increased sugar contents could signal high carbon availability and induce senescence-dependent nutrient recycling to promote growth. In plants from the Romanche site, glucose treatment accelerated senescence both under warm and cold conditions (**Figure 9**). There was, however, no effect of glucose on senescence in plants from a separate site using sorbitol as an osmotic control (Supplemental Figure S3). Addition of the sugar analogue 2-deoxyglucose, which is phosphorylated by hexokinase but is not further metabolized at a significant rate, resulted in senescence in plants in the absence of glucose. A further accession from Norway did not show any acceleration of senescence based on glucose supply (Supplemental Figure S4). Natural variation was also found in the extent to which senescence is induced by glucose in *A. thaliana* (Wingler et al. 2006), but overall our results suggest that sugar signalling during senescence may be less important in the perennial *A. alpina* than in *A. thaliana*.

The effect of temperature on chlorophyll content indicates that senescence is slightly delayed in response to cold treatment in leaves that already existed when the plants were transferred to a cold temperature (**Figures 2** and **3**). Leaves of plants that had developed in cold conditions showed a clear delay in the decline in F_v/F_m (data not shown), suggesting that leaf senescence is delayed in leaves that are newly formed in the cold. However, the chosen conditions were quite mild and, if combined with high light, even above-freezing temperatures could result in stress-induced senescence in the natural growth environment. The interactions between leaf senescence, temperature, and sugars are summarized in **Figure 10**.

The senescence response to cold temperature is likely to affect survival, growth, and fitness, thereby determining the altitudinal or latitudinal range of plants. For *A. alpina,* genetic loci that are related to temperature have been identified (Manel et al. 2010; Poncet et al. 2010). Differences were found here in the senescence-dependent changes in sugar contents between two *A. alpina* accessions from the same region. Such natural variation in metabolite content may reflect adaptations to the growth conditions. For example, differences in sucrose and fructose contents were reported for populations of *Arabidopsis lyrata* ssp. *petraea* (Davey et al. 2008). Kunin et al. (2009) found temperature to be associated with genetic profiles in *A. lyrata* ssp. *petraea*. However, metabolomic profiles were not related to genetic similarity, so the adaptive value of variation

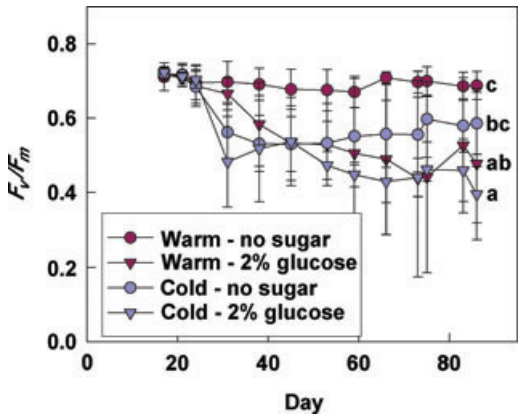

Figure 9. Interactive effects of glucose and temperature on whole-rosette senescence in plants from the Romanche site.

Plants were grown on a low-nitrogen agar medium with or without addition of 2% glucose at 22 °C during the day and 18 °C at night (warm conditions) or at 5 °C from day 24 (cold conditions). F_v/F_m was monitored in the whole plant. Data are means of 3–20 plants ± *SD*. Different letters indicate statistically significant differences ($P \leq 0.05$) between the treatments at day 86 (ANOVA and Tukey's pairwise comparison).

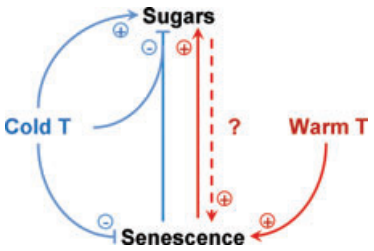

Figure 10. Model for the interactions between leaf senescence, temperature and sugars in *Arabis alpina*.

Blue lines indicate interactions at the cold temperature, red lines indicate those at warm temperatures. While cold treatment results in the accumulation of sugars, sugar contents remain stable or decline when leaves grown at the cold temperature senesce. Similarly, sugar contents decline with senescence in plants grown under natural conditions. However, senescence leads to sugar accumulation at warm temperatures. Compared to warm temperatures, cold treatment results in a slight delay in senescence. The effect of sugars on senescence in *A. alpina* is probably genotype-dependent.

in metabolism in response to temperature remains unclear. Detailed analysis of senescence regulation by temperature and carbon availability in accessions originating from various geographic locations, for example different altitudes, may reveal adaptations to local growth conditions.

Materials and Methods

Plant material and sampling sites

Seeds from *Arabis alpina* (L.) plants were collected in the Écrins region of the French Alps. The accessions used for this study originated from the Col du Lautaret (Lautaret; 2090 m altitude; 45° 01′ 57.6″ north; 006° 24′ 20.9″ east) and the Romanche valley (Romanche; 1684 m altitude; 45° 01′56.6″ north; 006° 21′22.1″ east). Further experiments presented in Supplemental Figure S3 were performed with an accession from near the Col du Lautaret (2064 m altitude; 45° 01′ 38.6″ north; 006° 23′ 11.0″ east). Measurements in the field were conducted on August 12th 2010 at the Lautaret site and on August 23rd at the Romanche site. Meteorological data were obtained from the SAJF Meteorological Station at the Col du Lautaret (http://sajf.ujf-grenoble.fr/).

Growth under controlled conditions

Seeds were suspended in 0.3% agar and pipetted onto Levington's cactus compost (The Scotts Miracle-Gro Company, Godalming, UK). Plants were grown at 12 h day length at a light intensity of 100 μmol m^{-2} s^{-1} at either 22 °C during the day and 18 °C at night (warm), or at 5 °C (cold). For growth on agar plates, seeds were sown onto low nitrogen (4.7 mM NO_3^-) agar medium with or without addition of 2% (w/v) glucose as described by Wingler et al. (2004), and incubated under the same warm and cold conditions as described above. For vernalization, plants were grown for four weeks in the warm conditions and then incubated for a further 12 weeks at 5 °C at 12 h day length and a light intensity of 40 μmol m^{-2} s^{-1}, before being transferred back to warm temperature.

Photosynthetic parameters

Chlorophyll content was determined non-destructively using hand-held devices that determine the absorbance of red light. For measurements in the laboratory, the chlorophyll content index was determined using a CCM-200 chlorophyll content meter (Opti-Sciences, Hudson, USA). Field-based measurements were performed with a Minolta SPAD chlorophyll meter (N-Tester, Hydro Agri, Immingham, UK). Maximum quantum yield of photosystem II (F_v/F_m) in individual leaves of plants growing in compost or in the field was analyzed using a FMS-2 pulse-modulated fluorometer (Hansatech, King's Lynn, UK), and whole rosette F_v/F_m in plants grown on agar plates was analyzed with a FluorCam 700MF kinetic imaging fluorometer (Photon Systems Instruments, Brno, Czech Rebublic) as described before (Wingler et al. 2004).

Sugar contents

Samples for sugar measurements of plants grown under controlled conditions were taken at mid-day. In the field, sugars were harvested at around 3 pm Central European Summer Time from plants exposed to the sun. Temperature at the Lautaret site was 15 °C and at the Romanche site 27 °C. Sugars (glucose, fructose and sucrose) were extracted in 80% ethanol at 80 °C and determined spectrophotometrically using coupled enzymatic assays (Stitt et al. 1989).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of temperature on leaf senescence of *A. alpina* leaves.

Figure S2. Correlation between chlorophyll and total sugar content (glucose, fructose and sucrose) in leaves of plants growing at 1684 m in the field (Romanche site).

Figure S3. Effect of glucose treatment on senescence of *A. alpina* plants from a location near the Col du Lautaret (2064 m altitude; 45° 01′ 38.6″ north; 006° 23′ 11.0″ east).

Figure S4. Effect of glucose treatment on senescence of *A. alpina* plants (Trolheimen, Norway).

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