Leaf green-white variegation is advantageous under N deprivation in Pelargonium × hortorum

Cyril Abadie, Marlène Lamothe, Caroline Mauve, Françoise Gilard and Guillaume Tcherkez

Abstract. Variegation (patchy surface area with different colours) is a common trait of plant leaves. In green-white variegated leaves, two tissues with contrasted primary carbon metabolisms (autotrophic in green and heterotrophic in white tissues) are juxtaposed. It is generally believed that variegation is detrimental to growth due to the lower photosynthetic surface area. However, the common occurrence of leaf variegation in nature raises the question of a possible advantage under certain circumstances. Here, we examined growth and metabolism of variegated Pelargonium × hortorum L.H. Bailey using metabolomics techniques under N deprivation. Our results showed that variegated plants tolerate N deficiency much better, i.e. do not stop leaf biomass production after 9 weeks of N deprivation, even though the growth of green plants is eventually arrested and leaf senescence is triggered. Metabolic analysis indicates that white areas are naturally enriched in arginine, which decreases a lot upon N deprivation, probably to feed green areas. This process may compensate for the lower proteolysis enhancement in green areas and thus contribute to maintaining photosynthetic activity. We conclude that under our experimental conditions, leaf variegation was advantageous under prolonged N deprivation.

Introduction

Most Angiosperms families have species or varieties with variegated leaves, that is, patchy leaves with green and non-green (sometimes white) areas. Variegated species are often found amongst understory herbs in temperate and tropical forests (for a review, see Evenari 1989), but also include crops such as alfalfa (Medicago sativa L.), sugar beet (Beta vulgaris L.), and parsnip (Pastinaca sativa L.) (Pringsheim and Schwarz 1933). The fact that variegation occurs in nature raises the question of the possible mechanisms (selective advantage) that maintains such a leaf trait. Amongst possible advantages are (Smith 1986): (i), leaf variegation may mimic leaf miners damage and thus dissuade female miners to deposit eggs; (ii), white areas have a larger reflectance and may thus decrease leaf temperature; (iii), leaf variegation may play the role of an aposomatic coloration. Alternatively, Givnish (1990) has proposed that leaf variegation serves to camouflage the foliage to colour-blind herbivores. The latter hypothesis would agree with the cost in nitrogen of leaf damage, since forest herbes have relatively high elemental N content and furthermore, white areas of green-white variegated leaves are particularly N-rich (see below). However, there is presently little evidence in favour of any of these hypotheses, except, maybe, for data from field experiments on Byttneria aculeata Jacq. (Malvaceae) that showed a lower prevalence of miner attack in variegated than plain morph, for any given frequency of the variegated morph (Smith 1986).

Nevertheless, possible ecological advantages of leaf variegation (e.g. against herbivory) cannot be divorced from metabolic imperatives. On the one hand, the absence of photosynthesis or the prevalence of respiration in white (chlorophyll-deprived) areas is detrimental to the plant carbon balance (Ivanova and Sherstneva 1999) despite a generally lower respiration rate in white compared with green areas (Toshoji et al. 2012). Indeed, biomass production is lower in variegated than plain (non-variegated) plants (Downton and Grant 1994; but see Vaughn and Stewart 1978 for an opposite case in Hosta) and white areas strictly depend upon the sugar input from green areas (Jones and Eagles 1962; Madore 1990). On the other hand, white areas have been shown to be N-rich (higher elemental N content), with considerable amounts in free amino acids (such as Arg, Asn and Lys) and polynitrogenous compounds (e.g. ornithine).
(Seltmann 1955; DeKock and Morrison 1958; Tcherkez et al. 2012). In the variegated mutant immutans of Arabidopsis, white sectors exhibit a higher expression of genes associated with Asn synthesis (Asp-aminotransferase and Asn synthetase) (Aluru et al. 2009). It has been argued that white and green areas undergo metabolic exchanges, with green areas providing a carbon source to white areas that may, in turn, redistribute nitrogenous compounds (Tcherkez et al. 2012) and thus, leaf variegation has been suggested to exemplify ‘a kind of successful parasitism’ between two leaf parts (Evenari 1989).

In a previous study, we showed that in variegated leaves of *Pelargonium × hortorum* L.H.Bailey (Geraniaceae), white areas were N-rich, with a typical accumulation of Arg (Tcherkez et al. 2012). Metabolomic analyses further suggested the enhancement of alkaloid and Arg biosynthesis in white areas, and leaf-part specific isotopic labelling demonstrated that white and green areas of the same leaf exchanged nitrogenous molecules (with nitrogen export from green areas being quantitatively much more important). That is, $^{15}$N-nitrate deposited in the green area caused a clear $^{15}$N-enrichment in the white tissue, thereby suggesting that N was reduced in green areas (which have a nitrate reductase activity) and subsequently translocated and metabolised in white areas. However, the rationale of such a functional division of metabolism between white and green areas in variegated leaves remains unclear, and the possibility that nitrogen can be exported from white areas under certain circumstances cannot be excluded. That is, there might be some advantages of accumulating nitrogenous compounds in white areas: for example, under N-limited conditions, the remobilisation of nitrogenous compounds from white leaf parts could contribute to sustaining N-requirements for plant growth and development.

To test this hypothesis, we conducted N-deprivation experiments under greenhouse conditions on the same species, *P. × hortorum*. Using a combination of metabolomics and physiological measurements, we found that variegated morphs performed better than plain morphs, with a higher living biomass and larger photosynthetic rate, after 9 weeks of N deprivation. This was accompanied by a decrease in both elemental N and amino acid (such as Arg) content in white leaf areas, indicating that nitrogen remobilisation from white tissue took place. Our results suggest that leaf variegation was beneficial to plant growth under N-deprived conditions in this species.

**Materials and methods**

**Plant material**

The variety used here was *Pelargonium × hortorum* var. Panaché Sud L.H.Bailey, a periclinal chimera in which white areas are devoid of photosynthetic chloroplasts in both L2 (hypodermis) and L3 (mesophyll) cell layers. Two morphs (variegated and plain) were grown here. Plantlets were generated from cuttings planted in peat for 2 weeks for rooting and then transferred to potting mix. Plants were then grown in the greenhouse under 22/18°C, 60/55% RH, 16/8 h photoperiod (day/night). Plants were automatically watered five times a day with 1 g L$^{-1}$ nutrient (KNO$_3$-containing) solution Plant Prod 14-12-32 (Plant Prod, Puteaux, France; representing a final concentration of 7 mmol L$^{-1}$ NO$_3$ and 1.5 mmol L$^{-1}$ urea) supplemented with 20 μL L$^{-1}$ fertoligo L (Fertil, Boulogne-Billancourt, France; this nutrient solution is devoid of N). The experiment (N-deprivation) was initiated when plants reached the 15-leaf stage and lasted for 9 weeks. Plants were divided into two sets: a control set (normal nutrient solution) and a deprived set (nutrient solution without N). The N-deprived nutrient solution was based on the same composition without urea, and KNO$_3$ + K$_2$HPO$_4$ replaced by KHCO$_3$ + K$_2$HPO$_4$ to yield exactly the same P/K composition, and the solution was adjusted at the same pH. During the N-deprivation period, time was incremented in weeks, from 0 to 9.

**HPLC analysis of amino acids**

The quantitation of amino acids was carried out as in Tcherkez et al. (2012) by HPLC with the method by Godel et al. (1984) using o-phthalaldehyde (OPA) derivatisation.

**GC-MS analyses**

Metabolomics (GC-MS) analyses were conducted as described by Tcherkez et al. (2009) using gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF-MS). Briefly, leaf samples (20 mg of powder from freeze-dried material) were ground in a mortar in liquid nitrogen, and then in 2 mL of methanol 80%, in which ribitol (100 μmol L$^{-1}$) was added as an internal standard. After centrifugation and spin-drying, extracts were derivatised with methoxyamine (in pyridine) and N-methyl-N(trimethyl-silyl) trifluoroacetamide (MSTFA). Before loading into the GC autosampler a mix of a series of eight alkanes (chain lengths: C$_{10}$ to C$_{36}$) was included. Analyses were performed by injecting 1 μL in splitless mode at 230°C (injector temperature). The chromatographic separation was performed in helium as a gas-carrier at 1 mL min$^{-1}$ in the constant flow mode and using a temperature ramp ranging from 80 to 330°C between minute 2 and 18, followed by 6 min at 330°C. Ionisation was made by electron impact at 70 eV and the MS acquisition rate was 20 spectra s$^{-1}$ over the m/z range of 80–500. Peak identity was established by comparison of the fragmentation pattern with MS available databases (NIST) and in-house database obtained with standards, using a match cut-off criterion of 700/1000, and by retention time using the alkane series as retention standards. The integration of peaks was performed using the software Pegasus (Leco Corporation, Garges-les-Gonnesses, France). Peak integration was verified manually for each compound in all analyses. In both HPLC and GC-MS analyses, quantitativity was achieved using both an internal standard to check that the response of the detector (fluorescence for HPLC, mass signal for MS) was reproducible and calibration curves with standards (purchased at Sigma) were done. For data analysis, metabolite contents were expressed relative to leaf surface area, unless otherwise stated.

**Elemental N content**

The %N was measured using an elemental analyser Flash-EA (Thermo Scientific; Courtabeuf, France) on samples (3 mg of lyophilised leaf material) weighted in tin capsules (Courtabeuf Analyses Service, Mont Saint Aignan, France). Glutamic acid (USGS-40, IAEE Vienna, Austria) of known elemental composition was used to calibrate analyses.
Nitrate and protein content

The nitrate content was determined after the method by Cataldo et al. (1975). Briefly, frozen leaf material (1.6 cm²) were ground at 0°C with 200 μL of deionised water. After centrifugation, 20 μL of the supernatant were mixed with 40 μL salicylic acid 20 mmol L⁻¹ (acidified with H₂SO₄) and after 20 min at ambient temperature, it was basified with 950 μL NaOH 2 mol L⁻¹. The absorbance was read at 410 nm. The protein content was determined using the method of Bradford.

Photosynthesis

Photosynthetic gas exchange measurements were carried out with an open system Li-6400 (Li-Cor, Lincoln, NE, USA) the inlet of which was connected to the outlet of the dew point generator Li-610 (Li-Cor) to fix humidity. Measurements were done 4 h after the onset of light in the greenhouse (light-adapted leaves). Standard net photosynthetic rate (A) was measured at 380 μmol mol⁻¹ CO₂, 500 μmol m⁻² s⁻¹ PAR (21% O₂, 21°C, 70% RH). Maximal carboxylation velocity (V_max) was computed from A/cᵋ curves using the Excel macro by Sharkey et al. (2007).

Statistics and graphical representations

Pair wise statistics were performed using heteroscedastic Student-Welsh tests and two-factor analyses were performed with a 2-way ANOVA (results labelled as significant when P<0.05). Fisher post-hoc analysis of the data validated the ANOVA, with an acceptable (<5%) false discovery rate (FDR) of 4.3%. Metabolomics networks were constructed from correlation matrixes (cosine correlation) generated by MeV (Eisen et al. 1998), with the 2D-Fruchterman-Meignold algorithm, using the software Pajek (Batagelj and Mrvar 2004).

Results

Plant morphology and biomass

Plants at week 4 (mid-course) and 8 are shown in Fig. 1a–d. Plain morphs grew better than variegated morphs in the first phase of the N-deprivation period and then stopped stem and leaf growth, with the appearance of dead leaves and therefore, after 9 weeks, variegated morphs kept more functional leaves (Fig. 1c). The time course of plant aerial biomass is shown in Fig. 1e. Variegated morphs were always smaller (lower leaf biomass) by ~40% until week 6 at which plain morph leaf biomass started to decline. After 9 weeks, leaf biomass of variegated morphs was ~30% larger than that of plain morphs. There was a progressive change in the proportion of white vs green surface area in variegated morphs, from ~40% initially to ~10% after 9 weeks. Both morphs had dead leaves from week 7: this remained minimal in variegated morphs but in plain morphs, this represented up to 27% of total leaf biomass. When grown under control conditions (no N deprivation), leaf biomass represented 7.8 ± 0.5 and 9.1 ± 0.5 g DW plant⁻¹ after 9 weeks in variegated and plain morphs respectively (data not shown in Fig. 1), that is, three times higher than under N-deprivation.

Nitrogen content

The nitrogen elemental content is shown in Fig. 2a. Plain morphs and green areas of variegated morphs had similar %N and white areas of variegated morphs were enriched in nitrogen by ~1%, regardless of the time considered. The %N declined progressively

---

Fig. 1. Morphology (a–d) and leaf biomass (e) in Pelargonium × hortorum subjected to N deprivation. (a–d) Photographs of both variegated and plain morphs taken at week 4, (a, b) and week 9 (c, d) of the N deficiency experiment, (e), biomass (in g DW plant⁻¹) of alive leaves of plain (closed discs) and variegated (total of green and white areas; half-filled discs), with green and white areas (triangles). The amount of dead leaves that appeared at each time is indicated with black (plain) and grey (variegated) bars.
during N-deprivation, from ≈3% initially to ~1.5% (week 9) in green tissues, and from ~5% to ~3% in white tissue. Such a decline matches the expected dilution effect due to growth (Fig. 1): with a relative growth rate of ~0.015 g g$^{-1}$ day$^{-1}$, a 2-fold dilution in the N content at week 9 can be anticipated, as observed in Fig. 2a. The relative metabolic N : C ratio computed from metabolite content (obtained by GC-MS) (Fig. 2b) was considerably larger in white tissue due to the prevalence of N-containing compounds such as Arg (see below) and declined from ~10% (week 0) to 6% (week 9). This ratio remained roughly constant in green areas of variegated leaves and declined slightly in plain morphs (from ~3.5 to 2%). In other words, N-deprivation was accompanied by a clear disappearance of nitrogenous metabolites in white areas while this effect was less marked in green areas.

**Amino acid pattern**

Changes in amino acid composition and content are shown in Fig. 3. Changes in individual amino acid content from the beginning to the end of the N-deprivation period were rather large in white parts of variegated morphs (black bars), with a clear decrease in Arg (80 mmol N g$^{-1}$ DW), Ala and Glu. Asn, Glu and Ser tended to accumulate. Changes were modest in green areas and in plain morphs, despite an accumulation in Glu and, in plain
morphs, of Gln. The contrasted behaviour of different amino acids suggests that in white areas, there was a remobilisation (disappearance) of Arg and import of nitrogen in the form of Asn. By contrast, the build-up of some amino acids (and the lack of clear decrease of others) in green parts and plain morphs rather suggest that proteolysis occurred, thereby feeding the pool of free amino acids: there was a global decrease in amino acid N in white tissue while plain morphs exhibited a clear increase (Fig. 3b) and in green areas of variegated leaves, there was a small increase only. Accordingly, changes in amino acid content (expressed per gram DW) did not appear to be similar when expressed in % of total N (Fig. 3c). In fact, there was no significant change in white areas, suggesting that all of nitrogenous fractions declined concomitantly. By contrast, in both green areas and plain morphs, total amino acid content expressed in % of total N increased significantly (as compared with control conditions), suggesting that amino acids specifically accumulated, amongst nitrogenous compounds.

Metabolomics

GC-MS analyses led to the detection and quantitation of 81 metabolites (87 analytes), among which 63 were differentially abundant in variegated-white/variegated-green/plain tissues and 30 were significantly affected by N-deprivation/time (P < 0.05, ANOVA) (no metabolites in interaction; thus several metabolites were significantly affected by both factors in an independent [non-interacting] manner) (Fig. S1). It should be noted that here, significant changes with time (see Fig. S1b, available as Supplementary Material to this paper) encompass both the effect of N-deprivation itself and development (9 week period). Nevertheless, the effect of development appeared to be small (see Fig. 3c for amino acids).

Generally, metabolites that differed significantly between leaf tissues were similar to that found previously by Tcherkez et al. (2012), with white areas enriched in amino acids such as Asp, Ser, Met, Asn and Arg and green tissues enriched in carbohydrates and their derivatives (e.g. ascorbate, citramate and threonic lactone). Among metabolites significantly changing with time, some displayed consistent patterns in all of the tissues considered (general increase in polyols, oxalate, shikimate; general decrease in malate, fumarate) whereas others had tissue-specific patterns. To make these more obvious, Fig. S2 represents ranked correlation coefficients (R) between time and metabolite content (positive values represent an increase with time: negative values, a decrease). Both green areas of variegated morphs and plain morphs had similar patterns, with best positive correlation with quinate, glucarate and galactosylglycerol, and best negative correlation with tartaric acid. In white areas, however, the best positive correlation was with phosphate and galactonate and the best negative correlation was with Arg and Ala. The co-ordination of metabolite change was examined by drawing a correlation network, in which spatially closest metabolites co-vary most tightly (Fig. 4). In both plain morphs and green areas of variegated morphs, sugars (and their derivatives) co-varied (red arc) and so did organic acids of the tricarboxylic acid pathway (green arc) whereas in white areas, there was no consistent co-variational cluster for both sugars and organic acids (Fig. 4c). Key nitrogenous compounds were widely scattered in the network in plain morphs, while they appeared to be well clustered in white areas, except for Asn (blue lines). We noted that Arg was not closely related to other nitrogen-containing compound (including its precursor Glu) in plain morphs (Fig. 4a), but appeared to co-vary with Gln (and other amino acids) in the white area (Fig. 4c) of variegated leaves. The green area of variegated morphs showed an intermediate pattern (Fig. 4b).

Photosynthesis

Net CO2 assimilation (A) was followed during N-deprivation in green areas of variegated leaves and in plain morphs (Fig. 5a). The maximal carboxylation velocity (V_{max}) was obtained by fitting A/C<sub>I</sub> response curves (Fig. 5a, inset). There was a decline in both A and V_{max} under N-deprivation (and a slight decline only under control conditions). The observed decline upon N-deprivation...
was large, with \( A \) values of zero at week 7 for plain morphs and 2.4 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for green areas of variegated morphs. Indeed, assimilation rates were always larger in variegated compared with plain morphs, under N-deprivation. \( V_{\text{max}} \) followed the same pattern.

**Protein and nitrate content**

The content in total soluble proteins was measured each week and the rate of change along the experiment is shown in Fig. 5b. Initial protein content (i.e. just before the onset of N-deprivation) was 550 ± 110, 640 ± 140 and 500 ± 50 mg g \( \text{DW} \)^{-1} in green plain morph, and green and white areas of variegated morph respectively. Under control conditions, there was a visible decrease in protein content (Fig. 5b, black bars). The decrease was higher under N-deficiency (white bars), although not significantly (except in the plain morph at \( P < 0.10 \)). The protein content in the white tissue was quite variable (with no significant change along the experiment). There was no clear difference in nitrate content between control and N-deprived conditions (Fig. S3): the nitrate content was quite variable and remained within a range of ± 50% of the initial content (dotted lines in Fig. S3) with no significant change during the experiment.

**Discussion**

To our knowledge, the question of possible advantages of leaf variegation under N-restriction has never been addressed. Some relationships have been hypothesised between nitrogen deprivation and leaf variegation in *Arabidopsis* mutants (*immutans, spotty, var1* and *var2*), in which nitrogen deficiency could be anticipated to increase electronic excitation pressure due to energy imbalance between photochemistry and cellular utilisation, thereby promoting the appearance of white sectors (Rosso et al. 2009). In the mutant *immutans*, transcriptomics analyses suggest that white sectors have an altered nitrogen metabolism (lower nitrate and nitrite assimilation but higher Asn synthesis) and a huge change in the proteasic profile (50-fold increase in cysteine protease *At3 g19390* and 110-fold increase in trypsin protease inhibitor *At1 g73260*) (Aluru et al. 2009), suggesting modifications in N-remobilisation capacity. Here, we conducted controlled experiments in which the performance and the metabolism of variegated morphs under N-deficiency were examined.

**Is leaf variegation beneficial under N-deprivation?**

Our results show that leaf variegation had an effect on shoot biomass under control conditions (total leaf dry mass of variegated morphs was ~15% lower than in plain morphs) and this was more pronounced under N-deprivation (~40% reduction) for 6 weeks. This effect was not observed thereafter, plain morphs having eventually a lower biomass (Fig. 1e). It should nevertheless be noted that N-deprivation did not cause an arrest of plant growth (at least until week 6 in plain morphs), and in variegated morphs, growth continued until the end of the N-deprivation period, despite the appearance of dead leaves. We noted that there was a progressive decrease in leaf N content (Fig. 2). Nitrogen exported by leaves was likely used to sustain growth. The average rate of N-disappearance in mature leaves computed from Fig. 2 was 0.48 and 0.24 mg N g \( \text{DW} \)^{-1} day \( ^{-1} \) in variegated and plain morphs, respectively. Plant shoot growth (Fig. 1) represented a demand of 0.16–2.2 (week 9 to week 0) and 0.16–0.63 (week 6 to week 0) mg N g \( \text{DW} \)^{-1} day \( ^{-1} \) in variegated and plain morphs, respectively. This imbalance is well reflected by the fact that elemental N content declined with time (N in plant matter progressively more ‘diluted’), at least up
to week 6 (Fig. 2). Nevertheless, the decrease in N content in leaves of variegated morphs, mostly due to the loss of N in white tissues, represented a larger amount (the double) of N available for growth as compared with plain morphs. This effect was accompanied by a slightly lower proteolysis in green areas of variegated morphs (Fig. 5b), and the larger N-availability in variegated morphs was reflected by larger photosynthetic rates and maximal velocity (Fig. 5) which both depend (amongst other things) upon Rubisco content. Therefore, under our conditions, variegated morphs appeared to cope better with N-deprivation than plain morphs, by maintaining photosynthesis and growth for up to 9 weeks. The apparent advantage of variegation likely came from N-remobilisation from white tissues, which may play the role of N reserve (see below for the associated metabolism), and a change in leaf development (the proportion of white tissue declined with time (Fig. 1) and the white tissue produced was less and less N-enriched), thereby optimising leaf N-requirements.

Metabolic effects of N-deprivation

The metabolic basis of the resistance of variegated morphs to N-deprivation appeared to be a combination of the remobilisation of free amino acids in white tissues (Fig. 3) and, maybe, proteolysis (Fig. 5). Proteolysis seemed to occur mainly in green tissue, as revealed by the build-up in several amino acids (including Arg or Gln, Fig. S2) whereas the remobilisation of free amino acids occurred in the white tissue, causing a typical decrease in Arg content. It is likely that N was transferred from white to green tissues in the form of Asn since that metabolite increased a lot (Fig. 3a) and did not behave as other nitrogenous compounds in white tissues (Fig. 4c). Asn has indeed been shown to play a role in N-exchange between plant organs (e.g. Gauffinon et al. 2013). It should also be noted that Gln increased in plain morphs (Fig. 3a) whereas it decreased in variegated morphs. Gln accumulation in plain morphs could have come from N remobilisation via proteolysis and ammonium (re)assimilation (by glutamine synthetase).

Leaf protein content tended to decrease even under control conditions (Fig. 5b) probably reflecting the change in the plant total N-requirement to N-provision ratio as plants aged and grew (‘N-dilution’ effect). However, the progressive decrease in protein content under control conditions was not associated with an alteration in carboxylation capacity (Fig. 5a). By contrast, N-deprivation caused a substantial decrease in both photosynthetic rate and maximal carboxylation velocity (for similar effects of nitrogen restriction, see Seemann and Sharkey 1986; Cruz et al. 2003; Jia and Gray 2003). It is therefore plausible that N-deprivation was associated with the remobilisation of nitrogen contained in proteins involved in photosynthesis (such as the Rubisco), a common senescence-like response to N deficiency (Hörtensteiner and Feller 2002; Feller et al. 2008). Although there was no significant change (P < 0.05) in protein disappearance rate upon N-deprivation (Fig. 5b), it should be noted that a minimal increase of 1% in protein degradation rate already represents an equivalent N-remobilisation rate of ~0.12 mg N g⁻¹ DW day⁻¹ in green tissues, that is, 50 and 25% of the N growth requirement (see above) in plain and variegated morphs respectively.

Non nitrogenous compounds were also affected by N-deprivation. There was a general increase in some carbohydrates, polyols, ascorbate and its derivatives (threonic acid and threonolactone) (Figs 4, S2). The build-up in these metabolites is a rather common response to N-deficiency (Urbanczyk-Wochniak and Fernie 2005) and reflects the partitioning of the carbon source to non-nitrogenous compounds. It should also be noted that the ascorbate pathway is already very active in Pelargonium under control conditions (Tcherkez et al. 2012). The accumulation of quinate and shikimate indicated the enhancement of the aromatic (shikimate) pathway, likely reflecting the synthesis of flavonoids (anthocyanins), which accumulated visibly (content not measured here) as a well-known response to N-deficiency (Lea et al. 2007). In white areas of variegated morphs, there was a decrease in putrescine and Pro, in addition to Arg (Fig. S2). This effect was likely linked to Arg degradation, since Arg can be easily converted to ornithine (by arginase), which may, in turn, yield putrescine (by ornithine decarboxylase). Pro is also probably linked to Arg metabolism since it can be synthesised easily from glutamate-semialdehyde via ornithine transaminase and pyrroline-5-carboxylate reductase. Interestingly, there was a modest but significant increase in urea content in leaf tissues (Figs. S1, S2). Urea was perhaps involved in N export from leaves or its increase was a consequence of protein degradation in green tissues. In white tissues, it could have come from Arg remobilisation via the urea cycle by the action of arginase (Witte 2011). However, the present data do not supply direct information on metabolic N-fluxes, since metabolomics analyses provide only metabolite content. Unfortunately, isotopic labelling at each time point (each week) with 15N would have complicated N-deprived conditions. It is thus certainly desirable to carry out similar experiments with a variegated line of a plant species with genetic resource available, such as Arabidopsis, so as to further explore metabolic responses with genomic tools (Masclaux et al. 2001).

However, there was no significant change in nitrate leaf content either along the experiment or between control and N-deprived conditions (Fig. S3). In other words, leaf nitrates did not appear to have been utilised under nitrogen restriction in our experimental conditions. Minimal nitrate utilisation under such circumstances has also been observed in Arabidopsis thaliana Col 0 (but nitrate consumption does occur in other ecotypes) (Masclaux-Daubresse and Chardon 2011). In addition, it should be noted that here, leaf nitrate (within the range 10–40 μmol g DW⁻¹) represented only a very small fraction of total leaf N (~1% only), that is, an unimportant N source. Alternatively, nitrate may have been rapidly assimilated into amino acids so that residual plant nitrate in soil was low. Furthermore, under our growth conditions, the nutrient solution contained not only nitrate but also urea (see ‘Materials and methods’), which is used by Pelargonium with the same efficiency as ammonium-nitrate under greenhouse conditions (Amberger-Ochsenbauer et al. 2010).

Rationale and perspectives

Our results are consistent with a metabolic advantage of variegated morphs under N-deprivation. However, there is
presently no evidence that N-restriction has played a role in maintaining variegation in natural populations. It is likely that the enhanced resistance to N-deprivation is ‘exaptational’, that is, represents a corollary selective advantage that accompanies a specific selected trait (e.g. leaf motting favouring camouflage, see ‘Introduction’). We further recognise that here, uncertainty remains as to: (i) N-deprivation had effects on root growth and metabolism (in this study, roots were disregarded). That said, plant here were cultivated in pots that effectively limited root development for all morphs; (ii) other elements such as sulfur may be of importance. In fact, our data (and our previous data in Tcherkez et al. 2012) suggest that there is a larger % S in white tissues (higher Met, Cys and lanthionine content) and as such, a better resistance of variegated morphs to S-deprivation is plausible; (iii) variegated morphs are more common in N-limited areas in nature. Present data on North-American variegated species do not support a correlation between soil N-content and variegation prevalence (Givnish 1990). Nevertheless, there might be some indirect relationship with nitrogen. Under the camouflage hypothesis, the advantage of avoiding herbivory might relate to the associated cost in nitrogen of leaf damage. Indeed, white areas of green-white variegated leaves are N-rich, at least in the species investigated so far (particularly in Pelargonium). A larger sampling of variegated species would be necessary to test whether white tissues are consistently enriched in nitrogen. Such a wide-spectrum botanical analysis will be conducted in a subsequent study. More generally, when explored in species where there are molecular tools and genomic information available, the physiology of variegation could have interesting implications in understanding how leaves maintain their amino acid pools, reduce proteolysis and delay leaf senescence under N deprivation.

Acknowledgements
The authors thank Dr Linda De Bont for nitrate determinations. CA and GT acknowledge the financial support by the Agence Nationale de la Recherche through a grant Jeunes Chercheurs, under contract no. 12-0001–01.

References
expression of speci
masclaux-daupressse c, tcherkez g, reisdorf-cren m, sakakibara y, hase t, clément g, avice jc, granjean o, marmagne a, boutet-mercy s, azzopardi m, soulav f, suzuki a, (2013) arabidopsis thaliana asn2 encoding asparagine synthetase is involved in the control of nitrogen assimilation and export during vegetative growth. plant, cell & environment 36, 328–342. doi:10.1111/j.1365-3040.2012.02576.x


