Cytokinin Dynamics during the Response to Nitrogen in Two Contrasting *Plectranthus* Genotypes

Ellen T. Paparozzi³ and Jazbaat K. Chahal¹

Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE 68583-0724

Petre Dobrev

Institute of Experimental Botany AS CR, Rozvojova 263, 165 02 Prague 6, Czech Republic

Elizabeth A. Claassen²

Department of Statistics, University of Nebraska-Lincoln, Lincoln, NE 68583-0963

Walter W. Stroup

Department of Statistics, University of Nebraska-Lincoln, Lincoln, NE 68583-0963

Radomira Vankova

Institute of Experimental Botany AS CR, Rozvojova 263, 165 02 Prague 6, Czech Republic

ADDITIONAL INDEX WORDS. abscission, chlorophyll, cytokinin, leaf regreening, swedish ivy, nitrogen stress

ABSTRACT. Nitrogen response was compared in two *Plectranthus* species, *Plectranthus parviflorus* and *Plectranthus ambiguus*, which differ substantially in their phenotypic reaction to nitrogen limitation. The leaves of the former species gradually yellow during the nitrogen stress but are retained on the plant. This species copes with nitrogen deficiency also by gradual hydrolysis of starch grains. The latter species, *P. ambiguus*, responds by abscission of lower leaves. As plant hormones cytokinins are involved in nitrogen response as well as in regulation of the chlorophyll content, their dynamics were followed to explore the fast responses as well as the impact of nitrogen treatment. Both plant species responded to nitrogen deficiency/supplementation in similar ways, by down-regulation and upregulation, respectively, of active cytokinins. However, the different phenotypic reactions imposed by nitrogen limitation were associated with specific regulation of cytokinin pool accompanying the stress response by the two species. The increase of the active cytokinins on nitrogen resupply was faster and stronger in the regreening species, *P. parviflorus* than in *P. ambiguus*. However, the *P. ambiguus* plants maintained higher basal levels of all cytokinin metabolites as well as chlorophyll content when compared with *P. parviflorus*. Nitrogen deficiency was associated in this species with accumulation of *cis*-zeatin-type cytokinins, which preceded abscission of lower and later on of middle leaves. The achieved results indicate that phenotypic variations in the response to nitrogen deficiency/supplementation are associated with significant quantitative and qualitative differences in the cytokinin pool.

Nutrient availability strongly affects plant growth and development. To adapt to environmental conditions, which may cause considerable fluctuation in nutrient levels and accessibility, plants have evolved the ability to sense nutrients, regulate their uptake and assimilation as well as modulate root architecture to proliferate in nutrient-rich zones (Ruffel et al., 2011). One of the key growth-limiting nutrients is N and plants have adopted different strategies to cope with its limitation. Recently, we described that *P. parviflorus* (swedish ivy) responds to N deficiency by yellowing of leaves and cessation of growth to such an extent that the plant appears dormant (Korus et al., 2010). However, no leaves are lost.

On reintroduction of N to the system, the existing leaves regreen and new growth will ensue within 4 weeks. The regreening can happen repeatedly. However, another species of this genus, *P. ambiguus*, responds differently and more typically to N deficiency. Within 4 weeks of N limitation, leaves will abscise and reintroduction of N will then cause development of new leaves from terminal meristems and axillary buds.

Plant responses to N deficiency are mediated by plant hormones, especially by cytokinins (Argueso et al., 2009; Kiba et al., 2011). As cytokinins, often referred to as the leaf regreening hormone, also exhibit positive effects on chlorophyll concentration in leaves we decided to compare the dynamics of the response to N deficiency and subsequent N resupply by *P. parviflorus* and *P. ambiguus* at the level of chlorophyll and endogenous cytokinin metabolites.

Materials and Methods

PLANT MATERIAL AND EXPERIMENTAL SETUP. Four experiments were conducted from Fall 2008 through Spring 2010. The first

Received for publication 4 Dec. 2015. Accepted for publication 19 Feb. 2016. This research was supported in part by The Fleming Research Fund and the University of Nebraska Agricultural Research Division Hatch Project NEB-22-341. The authors thank Dr. Paul Staswick and Dr. Gautam Sarath for critical review of this manuscript.

¹Former graduate research assistant.

²Former graduate assistant Department of Statistics; current address SAS Institute, Cary, NC.

³Corresponding author. E-mail: etp1@unl.edu.

two preliminary experiments allowed determination of the pattern and differential response of the two species over the 6-week period. In addition, previous research showed that N concentration in leaves was $0.02\% \pm 0.01$ when N was absent and 5.98% to 7.26% when N was present. For each of the two final experiments, 24 pencil-sized cuttings of similar length with a five nodes from each of the two species, *P. parviflorus* and *P. ambiguus*, were rooted in vermiculite for 3–4 weeks. Stock plants of both species were well fertilized and showed no deficiencies. The cuttings were treated with rooting powder containing 0.3% indole-3-butyric acid (Hormex; Brooker Chemicals, Chatsworth, CA) before sticking and flats were placed under intermittent mist which was set to a 4-s burst every 20 min from 1 h after sunrise to 1 h before sunset. Supplemental incandescent light (75 W) was applied regularly from 2200 to 0200 HR to prevent flowering. The cuttings received no supplemental nutrients during rooting.

On rooting, one or two plants were set into a hydroponics setup which consisted of 24 7.6-L containers, tightly closed to prevent light transmittance. Each container had a lid of expanded foam insulation with two holes to hold the plant and the air hose. A large gauge hypodermic needle at the end of each air hose was attached to a center hose to provide a continuous aeration. The greenhouse temperature was 21–27 °C day and 16–18 °C night. Supplementation of incandescent light from 2200 to 0200 HR continued. The plants were in hydroponics for a total of 6 weeks (two experimental periods of 3 weeks each).

The duration of treatments and number of plants per pot was optimized in two preliminary experiments. For color, the color readings from the Royal Horticultural Society Color Chart (RHS, London, UK) were used so that when the plants that received 0 mg·L⁻¹ N exhibited a change in color to yellow-green (RHS Color Chart 144A) and plants that

Fig. 1. Changes in chlorophyll concentration in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The three-way interaction of species by treatment by week was statistically significant indicating that over the 6-week period each species responded differently to the presence or absence of N. The error bars indicate the error associated with mean for the chlorophyll measurements for a given week. The SE of the difference in leaf chlorophyll concentration between weeks for each species = 0.078 mg·g⁻¹ FW. While leaf chlorophyll concentrations for either cultivar did not return to initial values, both species looked visually green as measured using the Royal Horticultural Society Color Chart (RHS, London, UK).

Fig. 2. Changes in chlorophyll concentration in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The three-way interaction of species by treatment by week was statistically significant indicating that over the 6-week period each species responded differently to the presence or absence of N with a decrease in leaf chlorophyll concentration happening 1 week earlier in *P. ambiguus* as compared with *P. parviflorus*. The error bars indicate the error associated with mean for the chlorophyll measurements for a given week. The SE of the difference in leaf chlorophyll concentration between weeks for each species = 0.078 mg·g⁻¹ FW.

received $150 \text{ mg} \cdot \text{L}^{-1} \text{ N}$ were 137A (green), the one-time switch of treatments was done. This occurred after 3 weeks. The number of plants per pot depended on whether the plants received N or not. Any plant receiving N, had lower axillary bud growth such that plants had multiple terminals for sampling. If N was withheld extensively, two plants were needed to have pairs of leaves to sample each week—one plant for the first 3 weeks (period 1) and one plant for the next 3 weeks (period 2).

The $0 \text{ mg} \cdot \text{L}^{-1} \text{ N}$ solution consisted of $48.5 \text{ mg} \cdot \text{L}^{-1} \text{ Ca}$ ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$); $24.3 \text{ mg} \cdot \text{L}^{-1} \text{ Mg}$ and $32 \text{ mg} \cdot \text{L}^{-1} \text{ S}$ ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$); $75 \text{ mg} \cdot \text{L}^{-1} \text{ P}$ and $94.7 \text{ mg} \cdot \text{L}^{-1} \text{ K}$ (KH_2PO_4). The composition of the $150 \text{ mg} \cdot \text{L}^{-1} \text{ N}$ solution was $53 \text{ mg} \cdot \text{L}^{-1} (\text{KNO}_3)$ plus $96 \text{ mg} \cdot \text{L}^{-1} \text{ N}$ (NH_4NO_3); $48.5 \text{ mg} \cdot \text{L}^{-1} \text{ Ca}$ and $75 \text{ mg} \cdot \text{L}^{-1} \text{ P}$ [$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$]; $24.3 \text{ mg} \cdot \text{L}^{-1} \text{ Mg}$ and $32 \text{ mg} \cdot \text{L}^{-1}$ ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and $150 \text{ mg} \cdot \text{L}^{-1} \text{ K}$ (KNO_3). Thus, concentrations of all macroelements except N and K were the same. Both solutions contained the same microelement concentration: $0.50 \text{ mg} \cdot \text{L}^{-1} \text{ B}$, $0.02 \text{ mg} \cdot \text{L}^{-1} \text{ N Cu}$ (Cu EDTA), $1 \text{ mg} \cdot \text{L}^{-1} \text{ Fe}^{3+}$ (Fe EDTA), $0.50 \text{ mg} \cdot \text{L}^{-1} \text{ Mn}$ (Mn EDTA), $0.05 \text{ mg} \cdot \text{L}^{-1} \text{ Zn}$ (Zn EDTA), and $0.01 \text{ mg} \cdot \text{L}^{-1} \text{ Mo}$ ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$). The pH of the solutions was adjusted to 6.0 using 1 M KOH . All solutions were changed when either the pH changed by 1 unit or when the level of the solution dropped (at least one change per week).

CHLOROPHYLL ANALYSIS. Weekly, one fully expanded leaf starting from the first pair down from the terminal whorl was sampled for chlorophyll determination, which was done immediately, using acetone extraction (Arnon, 1949). In plants which did not grow, the second and third pairs were sampled and then the second plant was used. About 100–200 mg of the leaf tissue without the midvein were ground in 20 mL of 80% acetone using a mortar and pestle for 5 min. The solution was then filtered into a test tube. The filtered extract was then poured into a fresh cuvette and placed in the spectrophotometer to record the absorbance at 663 nm. The chlorophyll a and b contents were

Fig. 3. Changes in biosynthetic precursor content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The three-way interaction of species by treatment by week was statistically significant indicating that over the 6-week period each species responded differently to the presence or absence of N as evidenced by a stable level of precursors in *P. parviflorus* when no N was present vs. a reduction in precursors in leaves of *P. ambiguus*. Upon addition of N, precursor content increased in both species. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf precursor content between weeks for each species = $0.623 \text{ pmol} \cdot \text{g}^{-1} \text{ FW}$.

Fig. 4. Changes in biosynthetic precursor content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The three-way interaction of species by treatment by week was statistically significant indicating that over the 6-week period each species responded differently to the presence or absence of N in that upon removal of N a dramatic drop in precursor content occurred in leaves of *P. ambiguus* occurred at week 5. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf precursor content between weeks for each species = $0.662 \text{ pmol} \cdot \text{g}^{-1} \text{ FW}$.

evaluated spectrophotometrically at 663 and 645 nm. Chlorophyll a, chlorophyll b, and total chlorophyll concentrations were calculated using Arnon's equation (Arnon, 1949).

CYTOKININ ANALYSIS. For cytokinin analysis, the other fully expanded leaf from the first pair down from the terminal whorl was cut into thin strips 2–3 mm wide along the length of the leaf. Each sample was weighed, flash frozen with liquid N and stored in pre-cooled plastic tubes at -80°C . Samples were lyophilized. Cytokinins were extracted and purified according to Dobrev and Kaminek (2002). Samples (≈ 300 mg FW) were homogenized and extracted twice with methanol/water/formic acid (15:4:1, by volume). Deuterium-labeled standards (50 pmol of each) were added. The active cytokinins were *trans*-zeatin, [$^2\text{H}_5$]Z; *trans*-zeatin riboside, [$^2\text{H}_5$]Z9R; isopentenyladenine, [$^2\text{H}_6$]iP; isopentenyladenosine, [$^2\text{H}_6$]iP9R; dihydrozeatin, [$^2\text{H}_3$]DHZ; dihydrozeatin riboside, [$^2\text{H}_3$]DHZ9R. The conjugates were a) N-glucosides: *trans*-zeatin N7-glucoside, [$^2\text{H}_5$]Z7G; *trans*-zeatin N9-glucoside, [$^2\text{H}_5$]Z9G; isopentenyladenine N7-glucoside, [$^2\text{H}_6$]iP7G; isopentenyladenine N9-glucoside, [$^2\text{H}_6$]iP9G; dihydrozeatin N9-glucoside, [$^2\text{H}_3$]DHZ9G and b) O-glucosides: *trans*-zeatin O-glucoside, [$^2\text{H}_5$]ZOG; *trans*-zeatin riboside O-glucoside, [$^2\text{H}_5$]Z9ROG; dihydrozeatin O-glucoside, [$^2\text{H}_7$]DHZOG (Apex Organics, Honiton, UK). The *cis*-zeatin derivatives were determined from the retention time and the mass spectra of unlabeled standards and response ratio of their *trans*-zeatin counterparts. To get rid of some hydrophobic compounds, extracts were purified using a SPE-C18 column (SepPak-C18; Waters, Milford, MA), evaporated under vacuo into water phase, diluted with formic acid and separated on a reverse phase-cation exchange SPE column (Oasis-MCX; Waters). The cytokinin nucleotides were eluted with 0.35 M NH_4OH in water, cytokinin bases, ribosides and glucosides were eluted with 0.35 M NH_4OH in 70% methanol. Nucleotide fraction was treated with alkaline phosphate and evaporated. Cytokinin

Fig. 5. Changes in the physiologically active cytokinin content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The interaction of treatment by week was statistically significant indicating that both species reacted similarly to the presence or absence of N but how both species responded was different over the 6-week period. Leaves of *P. parviflorus* showed an increase in physiologically active cytokinin content after only 1 week of N and then did not increase, while leaves of *P. ambiguus* showed an increasing level over time. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf active cytokinin content between weeks for each species = $1.11 \text{ pmol}\cdot\text{g}^{-1} \text{ FW}$.

Fig. 6. Changes in the physiologically active cytokinin content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The interaction of treatment by week was statistically significant indicating that both species reacted similarly to the presence or absence of N but how both species responded was different over the 6-week period in that, over time, once N was removed leaf active cytokinin content in *P. ambiguus* decreased. *P. parviflorus* leaf content showed a fluctuating response before week 6 where the final level of active cytokinin content was similar to that of *P. ambiguus* leaves. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf active cytokinin content between weeks for each species = $1.08 \text{ pmol}\cdot\text{g}^{-1} \text{ FW}$.

precursors were then determined in separate run as ribosides. Both fractions were separated by high-performance liquid chromatography (Ultimate 3000, Dionex; Thermo Fisher Scientific, Waltham, MA); and the cytokinins were quantified using a hybrid triple quadrupole/linear ion trap mass spectrometer (TSQ Quantum Ultra AM triple-quad high resolution mass spectrometer, Thermo Fisher Scientific) operated in selected reaction monitoring mode. Liquid chromatography–mass spectrometry analysis was performed as described by Dobrev et al. (2002) using an auto sampler with cooled sample stack (HTS-Pal; CTC Analytics, Zwingen, Switzerland), a quaternary high-pressure liquid chromatography pump (Rheos 2200; Flux Instruments, Basel, Switzerland), a column oven (Delta Chrom CTC 100; Watrex, Prague, Czech Republic), and a triple-quad high-resolution mass spectrometer (TSQ Quantum Ultra AM). Ternary gradient elution (water/acetonitrile/acetic acid) was used. The mass spectrometer (MS) was operated in the positive MS/MS mode (SRM = single reaction monitoring) with monitoring of two to four transitions for each compound. The most intensive ion was used for quantification and the remainder for identity confirmation. Multi-level calibration graphs with [^2H] labeled cytokinin internal standards were used for quantification. Detection limits of different cytokinins varied from 0.05 to 0.1 pmol/sample. Two independent experiments were done. Each sample was injected at least twice.

STATISTICAL DESIGN AND ANALYSIS. For each experiment, the design was a split plot with repeated measures. The whole plot treatment factor was species. There were four whole plot units per bench. Two benches total. Each species was assigned to two of the four whole plot experimental units per bench. The split plot factor was the sequence of the four N treatments over two experimental periods (3 weeks per period; 6 weeks total). Technically, the split plot factor is a two-treatment

Fig. 7. Changes in *cis*-zeatin derivative content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to N deprivation over a 6-week period. The three-way interaction of species by treatment by week was statistically significant indicating that over the 6-week period of the study each species responded differently to the lack of N with leaves of *P. parviflorus* showing no change in *cis*-zeatin derivative content. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf *cis*-zeatin derivative content between weeks for each species = 6.89 pmol·g⁻¹ FW.

Fig. 8. Changes in *cis*-zeatin derivative content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The three-way interaction of species by treatment by week was statistically significant indicating that over the 6-week period of the study each species responded differently to the presence or absence of N with the leaves of *P. parviflorus* showing no change in *cis*-zeatin derivative content. However, *cis*-zeatin derivative content fluctuated in *P. ambiguus* leaves particularly when N was introduced. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf *cis*-zeatin derivative content between weeks for each species = 6.61 pmol·g⁻¹ FW.

two-period change-over design (Milliken and Johnson, 2009). There were four N treatment sequences: 0 N for both experimental periods (6 weeks total); 0 N in the first period (3 weeks) and then the switch to 150 mg·L⁻¹ N for the second period (3 weeks); 150 mg·L⁻¹ N in the first period (3 weeks) and then the switch to 0 N for the second period (3 weeks); and 150 mg·L⁻¹ N for both experimental periods (6 weeks). However, in any given whole plot unit, only three of the four N treatment sequences were included making it an incomplete block design. The chosen sequences optimized the precision of the measurements associated with the switch between 0 and full strength N. Thus some measurements were not taken. The split plot unit was the pot which contained one or two plants. Samples for chlorophyll and cytokinin determination were taken at multiple designated times with the emphasis on the regreening treatments (0 N–150 mg·L⁻¹ N and 150 mg·L⁻¹ N–0 N) to ensure that the response profiles could be estimated over weeks 1–3 for period 1 and weeks 4–6 for period 2. Data were analyzed using mixed model methods appropriate for split plot and repeated measure experiments (Littell et al., 2006). SAS[®] PROC GLIMMIX (SAS Institute, Cary, NC) was used to implement the analyses. The actual model (free of math notation) was: response = variety + whole-plot error + sequence + sequence × variety + split-plot error + week + week × variety + week × sequence + week × sequence × variety + repeated-measures error. It is necessary to use a mixed model procedure to adequately handle the multiple error terms, incomplete block and week features (as above) of these experiments. PROC GLIMMIX was used rather than PROC MIXED to take advantage of its advanced mean comparison features and plotting capabilities.

Results

The N concentration (150 mg·L⁻¹) as well as the experiment duration

Fig. 9. Changes in *cis*-zeatin derivative content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The three-way interaction of species by treatment by week was statistically significant indicating that over the 6-week period of the study each species responded differently to the presence or absence of N in that once N was removed from the system *P. ambiguus* leaves showed an increase in active derivatives at week 6. *Cis*-zeatin derivative content in leaves of *P. parviflorus* decreased when N was present and then did not respond further when N was removed from the system. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf *cis*-zeatin derivative content between weeks for each species = 6.97 pmol·g⁻¹ FW.

Fig. 10. Changes in *cis*-zeatin derivative content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to a set N concentration over a 6-week period. The three-way interaction of species by treatment by week was statistically significant indicating that over the 6-week period of the study each species responded differently to the presence of N with leaves of *P. ambiguus* showing an increase in *cis*-zeatin derivative content. *Cis*-zeatin derivative content in leaves of *P. parviflorus* decreased when N was present and then stabilized. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf *cis*-zeatin content between weeks for each species = 7.02 pmol·g⁻¹ FW.

(3 + 3 weeks) were optimized in preliminary experiments, to ensure complete regreening of *P. parviflorus* plants. Cuttings of both *Plectranthus* species were rooted in vermiculite with no nutrients supplied and appeared yellowish and slightly deficient when placed in the hydroponic solutions. Once in solution, they received either all nutrients or all except of N. Full N supplementation ($150 \text{ mg}\cdot\text{L}^{-1} \text{ N}$) ensured regreening of *P. parviflorus* plants within 3 weeks. The N concentration of plants that received no nitrogen during the experiments were $0.02\% \pm 0.01\%$, whereas plants receiving $150 \text{ mg}\cdot\text{L}^{-1} \text{ N}$ ranged from 5.98% to 7.26%.

CHLOROPHYLL CONCENTRATION.

Chlorophyll content of fully expanded leaves from the second pair down from the terminal whorl was followed in the absence of N or after its supplementation. Both species showed a decrease in chlorophyll concentration within 1 week of N deficiency. This trend continued in both species as long as N was withheld (concentrations at 6 weeks ≈ 0.3 and $0.2 \text{ mg}\cdot\text{g}^{-1} \text{ FW}$, respectively) (data not shown). Statistical analysis revealed that the three-way interaction of species by treatment by week was significant indicating that each species responded differently to the presence or absence of N over the 6-week period. Initially, *P. ambiguus* plants contained about double the basal concentration of chlorophyll than *P. parviflorus*, despite both having yellow-green leaves after the rooting period. After 3 weeks of N deficiency, abscission of lower and later on of middle leaves was observed in *P. ambiguus* plants. When N was added to the solution after sampling at week 3 [0 – $150 \text{ mg}\cdot\text{L}^{-1} \text{ N}$ treatment (Fig. 1)], chlorophyll concentration of *P. ambiguus* plants started increasing, but within 3 weeks did not regain the initial concentration level. Three-week N supplementation caused only a little change in chlorophyll concentrations in *P. parviflorus* plants. This is despite the fact that leaves appeared visually green. This was validated by scoring 137A green using the RHS Color Chart.

When *P. ambiguus* received $150 \text{ mg}\cdot\text{L}^{-1} \text{ N}$ for the first 3 weeks

Fig. 11. Changes in the cytokinin N-glucoside content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The three-way interaction of species by treatment by week was statistically significant indicating that over the 6-week period each species responded differently to the presence or absence of N in that *P. ambiguus* leaves showed a dramatic increase N-glucoside content after 2 weeks of N supplementation. Leaves of *P. parviflorus* showed only a slight increase in N-glucoside content once N was added back to the system. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf cytokinin N-glucoside content between weeks for each species = $2.44 \text{ pmol}\cdot\text{g}^{-1} \text{ FW}$.

Fig. 12. Changes in the cytokinin N-glucoside content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The three-way interaction of species by treatment by week was statistically significant indicating that over the 6-week period each species responded differently to the presence or absence of N. While both species showed a decrease in N-glucoside content, the decrease was greater in leaves of *P. ambiguus*. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf cytokinin N-glucoside content between weeks for each species = $2.41 \text{ pmol}\cdot\text{g}^{-1} \text{ FW}$.

(Fig. 2), chlorophyll concentrations were stable. After N removal at week 3, chlorophyll concentrations of *P. ambiguus* leaves remained high until week 4 and then started decreasing. However, *P. parviflorus* plants showed little upregulation during 3-week N supplementation. When N was removed, only a mild response was observed and it took 3 weeks for chlorophyll concentration to drop. When both species received 150 mg·L⁻¹ N for the entire 6 weeks, chlorophyll concentration in leaves of *P. ambiguus* increased from 1.5 to 2.1 mg·g⁻¹ FW. *Plectranthus parviflorus* leaves showed chlorophyll elevation from 0.8 to 1.3 mg·g⁻¹ FW, mainly in the last 2 weeks (data not shown).

CYTOKININ BIOSYNTHETIC PRECURSORS (CYTOKININ PHOSPHATES). The relationship between N supplementation and endogenous cytokinin content was compared in *Plectranthus* species exhibiting differential response to the nutrient limitation. The initial N deficiency was associated with low levels of cytokinin phosphates (includes *trans*-zeatin riboside phosphate, isopentenyladenosine phosphate, and dihydrozeatin riboside phosphate), consistently in both species (Fig. 3). When N was introduced after 3 weeks of N deficiency, levels of cytokinin precursors were enhanced, to a higher extent, in *P. ambiguus* (Fig. 3). The statistical analysis (significant three-way interaction of species by N treatment by week) revealed different responses between the tested species over the 6-week period due to the presence or absence of N. Nitrogen supplementation for the first 3 weeks coincided with relatively high content of cytokinin phosphates (Fig. 4). Upon withdrawal of N after 3 weeks of full nutrition, there was a significant decrease in the cytokinin phosphates in leaves of both *P. ambiguus* and *P. parviflorus*. When no nitrogen was provided for 6 weeks, cytokinin phosphates were very low [values similar to week 6 in Fig. 4 (data not shown)]. When plants received full strength N for 6 weeks, cytokinin precursors for both species peaked at 6–7 pmol·g⁻¹ FW (data not shown).

ACTIVE CYTOKININS. When nutrient solution without N was supplied to plants for 6 weeks, the concentration of the active cytokinins (*trans*-zeatin, dihydrozeatin, isopentenyladenine, and their ribosides) in *P. parviflorus* and *P. ambiguus* declined (data not shown). Simultaneously, leaf yellowing and growth cessation were observed for *P. parviflorus*, whereas *P. ambiguus* began to lose leaves, starting from the bottom. Statistical analysis (interaction of N treatment by week) indicated that active cytokinins in both species responded to the presence or absence of N in a similar way in that they decreased in the absence of N and increased once N was supplied. However, different patterns of response by each species were observed over the 6-week period. When N was supplied after a 3-week N deficiency, *P. parviflorus* plants responded by an elevation of the active cytokinin

concentrations (Fig. 5). Response of *P. ambiguus* plants was similar, but milder. When plants of *P. ambiguus* received 150 mg·L⁻¹ N during the first 3 weeks of the experiment, the level of active cytokinins in leaves was significantly higher than in the absence of N (Fig. 6). Once N was removed from the solution, the concentration of active cytokinins fell as N deficiency increased. The response of *P. parviflorus* plants was delayed by 1 week. However, by week 5 the levels of active cytokinins were the same for both species.

CIS-ZEATIN DERIVATIVES. As *cis*-zeatin and its riboside are cytokinins often associated with stress responses, *cis*-zeatin-type cytokinins were evaluated separately from the other cytokinins. Originally, both *cis*-zeatin and *cis*-zeatin riboside were considered inactive products of transfer RNA degradation. However, when cytokinin receptors were identified and their affinity to different cytokinin bases and ribosides was compared, they started to be considered to be active cytokinins. Statistical analysis (significant three-way interaction of species by N treatment by week) showed significant differences in the *cis*-zeatin-type pool between the tested species. Overall, the levels of *cis*-zeatin and its riboside were lower in *P. parviflorus* than in *P. ambiguus*. In the latter, the levels of the most abundant *cis*-zeatin derivatives (i.e., *cis*-zeatin O- and N-glucosides) were comparable to the content of *trans*-zeatin-, isopentenyladenine-, and dihydrozeatin-type-O and N-glucosides. Nitrogen addition generally did not affect the concentration of *cis*-zeatin derivatives (9.8–15.9 pmol·g⁻¹ FW) in *P. parviflorus* leaves (Figs. 7–10). Their levels were maintained whether N was present or not. However, the *cis*-zeatin derivatives represented predominant

Fig. 13. Changes in the cytokinin O-glucoside content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The three-way interaction of species by treatment by week was statistically significant indicating that over the 6-week period each species responded differently to the presence or absence of N. Under N deprivation, both species showed a peak in O-glucoside leaf content. However, on reintroduction of N, only leaves of *P. ambiguus* showed a recovery to peak level. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf cytokinin O-glucoside content between weeks for each species = 2.41 pmol·g⁻¹ FW.

cytokinin forms in *P. ambiguus* plants, showing the highest concentrations at weeks 5 and 6 in case of the 6-week N deficiency [43.5–59.6 pmol·g⁻¹ FW (Fig. 7)]. When *P. ambiguus* plants received no N for the first 3 weeks, the concentration *cis*-zeatin derivatives rose and reached maximum at week 4, then it dropped precipitously by week 6 due to a consistent N supply (Fig. 8). Leaves of *P. ambiguus* plants that received 150 mg·L⁻¹ N for all 6 weeks had 4–15 pmol·g⁻¹ FW of *cis*-zeatin derivatives (Fig. 10); i.e., about one-third of the concentration of N deficient plants. This difference was mirrored in the 150-0 N treatment where low levels were maintained at N presence, but after 3-week N deficiency, *cis*-zeatin derivative concentration started increasing (Fig. 9).

CYTOKININ N-GLUCOSIDES. Statistical analysis (the three-way interaction of species by N treatment by week) indicated significant differences in cytokinin N-glucosides between the tested species over the 6-week period due to the presence or absence of N. When both species received no N but all the other elements, the levels of cytokinin deactivation products (N-glucosides of *trans*-zeatin, dihydrozeatin, and isopentenyladenine) were not significantly changed in either species for weeks 1–3 [7.4–13.7 pmol·g⁻¹ FW (Fig. 11)]. Addition of N after 3 weeks of deprivation resulted in upregulation of cytokinin N-glucosides in both species. *Plectranthus ambiguus* responded with a dramatic increase at week 5, doubling the cytokinin N-glucosides concentration. *Plectranthus parviflorus* plants showed a similar profile, but cytokinin N-glucoside increase at week 5 was only mild. However, it did coincide with the beginning of leaf regreening. However, by week 6 both species had concentrations similar to those observed in plants grown for 6 weeks without N (data not shown). When N was supplied during the first 3 weeks and then removed, cytokinin N-glucosides decreased in both species indicating downregulation of cytokinin N-glucosylation, together with the suppression of cytokinin biosynthesis, particularly in *P. ambiguus* plants (Fig. 12). When N was supplied for the whole period, a transient increase of cytokinin N-glucoside content was found in *P. ambiguus* plants after 2 weeks (data not shown).

CYTOKININ O-GLUCOSIDES. Statistical analysis (significant interaction of species by week) indicated significant differences in dynamics of cytokinin O-glucosides (includes *trans*-zeatin O-glucoside, *trans*-zeatin riboside O-glucoside, dihydrozeatin O-glucoside, and dihydrozeatin O-riboside glucoside) over the 6-week period between the species. Both species showed a peak in cytokinin O-glucosides: *P. ambiguus* after 2 weeks and *P. parviflorus* after 3 weeks (Fig. 13). On reintroduction of N, initially, both species showed a decrease in cytokinin O-glucosides. However, after 3 weeks, *P. ambiguus* leaves

showed a recovery of cytokinin O-glucoside content to peak level.

When N deficiency was induced after full N treatment, levels of cytokinin O-glucosides decreased between weeks 2 and 4 (Fig. 14). Decline of cytokinin O-glucosides, starting at week 2, was found in leaves of *P. ambiguus*. Prolonged N limitation resulted in a continued decrease of cytokinin O-glucosides in both species. For *P. ambiguus*, the 150 mg·L⁻¹ N treatment resulted in strong upregulation of cytokinin O-glucoside concentration, especially at the beginning of the experiment (week 2), the response being stronger than in *P. parviflorus*. Constant N treatment was associated with low cytokinin O-glucoside content [10–30 pmol·g⁻¹ FW (data not shown)] in both species.

Discussion

Different plant species or even individual genotypes may adopt a different strategy to adjust to N deficiency. We compared two *Plectranthus* species, *P. parviflorus* and *P. ambiguus*, which differ substantially in their response to N limitation. The leaves of the former species gradually yellow during the nutrient stress but are retained, while the latter one responds by abscission of lower leaves (Borman et al., 2013). Preliminary experiments on *P. parviflorus* demonstrated that this species can be exposed repeatedly to N deficiency conditions and repeatedly regreen on reintroduction of N without leaf senescence and loss (data not shown).

Fig. 14. Changes in the cytokinin O-glucoside content in leaves of *Plectranthus parviflorus* (P) and *Plectranthus ambiguus* (A) in response to changes in N concentration over a 6-week period. The interaction of species by week was statistically significant indicating that during the 6-week period each species responded differently regardless of N presence or absence. *P. ambiguus* leaf O-glucoside content showed a similar response to N presence and removal as N-glucoside content. However, *P. parviflorus* leaves showed an increase in O-glucosides when N was present and then a decrease after 2 weeks without N. The error bars indicate the error associated with mean for the cytokinin measurements for a given week. The SE of the difference in leaf cytokinin O-glucoside content between weeks for each species = 2.61 pmol·g⁻¹ FW.

The ability of plants to sense N and to regulate its uptake is a crucial part of plant adaptation to variable environmental conditions, including nutrient availability (Argueso et al., 2009). The role of cytokinins in these processes has been established (Kiba et al., 2011) and cytokinins were reported as long-distance signals which coordinate N demand and acquisition (Ruffel et al., 2011). Our results confirmed close N–cytokinin relationship. Nitrogen strongly affected cytokinin biosynthesis in both *Plectranthus* species, as indicated by the positive correlation between the presence of N in the medium and the levels of cytokinin precursors (cytokinin phosphates). This finding is in accordance with the stimulatory effect of nitrate on the expression of genes for cytokinin biosynthetic enzymes *IPT3* or *IPT5* (Takei et al., 2001a) as well as of *CYP735A2* (Wang et al., 2004) in arabidopsis (*Arabidopsis thaliana*).

Low levels of cytokinin phosphates, induced by N deficiency, corresponded well to the low levels of active cytokinins in both species. Simultaneously, cytokinin deactivation was suppressed, as indicated by the low levels of cytokinin O- and N-glucosides, which suggests tendency to maintain the levels of active cytokinins as high as possible, even under conditions of their suppressed biosynthesis. Conversely, N supplementation had a rapid positive effect on active cytokinin levels in both species. High increase in active cytokinin levels was followed by transient elevation of cytokinin O- and N-glucosylation, probably to establish cytokinin homeostasis. This positive effect of N on active cytokinin concentrations in *Plectranthus* species is in accordance with reports on number of other species; e.g., sunflower [*Helianthus annuus* (Salama and Wareing, 1979)], barley [*Hordeum vulgare* cv. Golf (Samuelson and Larsson, 1993)], maize [*Zea mays* (Takei et al., 2001b)], or *Festulolium* [cv. Felina (Pavliková et al., 2012)].

In spite of generally similar response to N deficiency/supplementation, *P. parviflorus* differed by accumulation of huge starch grains under control conditions (continual 150 mg·L⁻¹ N). This starch supply was slowly hydrolyzed when the plants were stressed by the lack of N (Korus et al., 2010). The starch hydrolysis resulted in the release of reducing sugars, which may serve as a sugar source/signal to maintain leaf integrity and prevent leaf abscission to diminish/tolerate the N stress. In contrast, *P. ambiguus* reacts to N stress by abscission of the lower leaves. It is interesting that N deficiency in the leaf abscising *P. ambiguus* is associated with an elevation of *cis*-zeatin type cytokinin content, the form of cytokinins reported to play a role during stress conditions (Gajdosova et al., 2011). The *cis*-zeatin and its riboside exhibit much lower physiological activity than *trans*-zeatin (Gajdosova et al., 2011), interacting with only a subgroup of cytokinin receptors (Spichal et al., 2004). This elevation of *cis*-zeatin in the upper leaves under these N stress conditions may enable the cytokinins to increase the sink strength of the upper leaves and influence the abscission of lower leaves and remobilization of nutrients from the abscising leaves to the upper leaves. In *P. parviflorus*, the regreening plant, N deprivation did not affect the concentration of *cis*-zeatin derivatives. Thus, another mechanism is involved.

As cytokinins have a direct link to chlorophyll concentration, stimulating its biosynthesis in dark, as well as slowing down its degradation during senescence, we compared the N response in *P. parviflorus* and *P. ambiguus* at the level of chlorophyll content as well. The chlorophyll concentration

followed the cytokinin dynamics in both plant species. However, the leaf regreening plant, *P. parviflorus*, exhibited a delay in chlorophyll downregulation by 1 week in comparison with *P. ambiguus*. The response was even slower in the case of N resupplementation. This may be in part because the range of chlorophyll concentration in the leaf regreening plant, *P. parviflorus*, was generally lower than in *P. ambiguus* even though the plants visually appeared green.

In conclusion, the link between cytokinins and nitrogen metabolism has long been established in other plants. In this research, there is a unique situation in that two species differ in their response to nitrogen loss in that one will shed lower leaves while the other does not. This phenotypic stress response has been associated with *cis*-zeatin-type cytokinin accumulation in *P. ambiguus*, the leaf abscising species, but not in *P. parviflorus*. While the underlying mechanism that allows *P. parviflorus* to retain its leaves during N deprivation and eventual regreening does not appear to be related to *cis*-zeatin-type cytokinin content, it may involve plant survival mechanisms such as starch hydrolysis.

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