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Cover page

Title

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Running title

Maize root response to nitrogen and strigolactones

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Figures: 4 BW, 4 colour

Supplementary Data: 2 Tables, 3 Figures

Title page

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The Control Of Zealactone Biosynthesis and Exudation Is Involved In The Response To Nitrogen In Maize Root

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Maize root response to nitrogen and strigolactones

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Abbreviations

AMF, Arbuscular Mycorrhizal Fungi;
ISH, *In Situ* Hybridization;
LC-MS/MS, Liquid Chromatography-quadrupole/time-of-flight tandem Mass Spectrometry;
LR, lateral root;
LRP, lateral root primordia;
N, nitrogen;
NO, nitric oxide;
PR, primary root;
SLs, strigolactones.

Abstract

Nitrate acts as a signal in regulating plant development in response to environment. In particular nitric oxide (NO), auxin and strigolactones (SLs) were supposed to cooperate to regulate the maize root response to this anion. In this study, a combined approach based on LC-MS/MS and on physiological and molecular analyses was adopted to specify the involvement of SLs in the maize response to N.

Our results showed that N deficiency strongly induces SL exudation, likely through stimulating their biosynthesis. Nitrate provision early counteracts and also ammonium lowers SL exudation, but less markedly. Exudates obtained from N-starved and ammonium-provided seedlings stimulated *Phelipanche* germination, whereas when seeds were treated with exudates harvested from nitrate-provided plants no germination was observed. Furthermore, our findings support the idea that the inhibition of SL production observed in response to nitrate and ammonium would contribute to the regulation of lateral root development. Moreover, the transcriptional regulation of a gene encoding a putative maize WBC transporter, in response to various nitrogen supplies, together with its mRNA tissue localization, supported its role in SL allocation. Our results highlight the dual role of SLs as molecules able to signal outwards a nutritional need and as endogenous regulators of root architecture adjustments to N, thus synchronizing plant growth with nitrogen acquisition.

Key words

Ammonium, LC-MS/MS, Maize, Nitrate, Root, Strigolactones

Introduction

Nitrogen (N) plays a vital role for plants. Globally, during 1961–2010, maize, rice and wheat received a total of 1594 Tg of N-fertilizer (Ladha *et al*., 2016), but more than 50% of the available N was lost due to the low Nitrogen Use Efficiency (NUE) of crops (Li *et al*., 2017). Improving crop NUE is essential to limit the impact of nitrogenous fertilization and to improve sustainability. Plants can uptake N in the soil in different forms, but nitrate and ammonium are the most common inorganic compounds. However, soluble nitrate (NO_3^-) is the major N source for crops in aerobic environments (Wang *et al*., 2012). It acts both as nutrient and signal, regulating many developmental processes (Bouguyon *et al*., 2012; Undurraga *et al*., 2017).

In maize primary root, NO_3^- early perception seems to involve the fine-tuning control of NO production and scavenging (Manoli *et al*., 2014; Trevisan *et al*., 2014), which likely regulates auxin levels and its transporter PIN1 re-localization in the transition zone (TZ) cells (Manoli *et al*., 2016). The TZ, which is located between the meristem and the elongation zone, plays a key role in sensing the external environment and in translating it into suitable developmental responses (reviewed by Baluška *et al.,* 2010). Furthermore, a subsequent study hypothesized that besides NO and auxin also Strigolactones (SLs) could take part to complex pathway governing the maize root adaptation to different N availabilities (Trevisan *et al*., 2015). Since NO and auxin act synergistically to control multiple aspects of root biology (Fernández-Marcos *et al*., 2011; Sanz *et al*., 2015) the role of SLs in the pathway where NO could act as coordinator of nitrate and auxin signalling to control the overall root response should be further studied, even in light of the existing interplay between SLs and NO (Kolbert, 2018).

SLs are a new class of carotenoid-derived phytohormones which act as both endogenous and exogenous signaling molecules in response to various environmental stimuli (Matusova *et al*., 2005; Pandey *et al*., 2016; Waters *et al*., 2017). They were identified as stimulants for germination of parasitic weeds in the *Orobanchaceae* family (Cook *et al*., 1966) and for mycorrhization initiation (Akiyama *et al*., 2005), but they also play multiple roles in regulating plant development (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008; Brewer *et al*., 2013). Moreover, soil nutrient deficiencies trigger enhanced SL biosynthesis, which in turns seem to influence root architecture (Kapulnik and Koltai, 2014; Kohlen *et al*, 2012, Koltai, 2015; Ito *et al.*, 2016). In fact, strigolactones have been described to have an impact on lateral root and root hair formation (Kapulnik *et al*., 2011; Mayzlish-Gati *et al*., 2012). On the whole, the effect of SLs on modifying RSA (Root System Architecture) in response to nutrient deprivation would appear dependent on auxin levels in root (Waters *et al*., 2017).

SLs occur in very small concentrations, both in plant tissues and in their exudates and they may be unstable, thus making not easy their detection and purification (Boyer *et al*., 2012). Recently Boutet-Mercey *et al.,* (2018) developed a method for SL quantification by LC-MS/MS in root tissues.

SLs are synthesized via all-trans-β-carotene isomerization, sequential oxidative cleavage of 9-*cis*-β-carotene by two carotenoid cleavage dioxygenases (CCD7 and CCD8), carlactone oxidations by cytochrome P450 monooxygenases MAX1, and yet to be characterized downstream conversions (Ruyter-Spira *et al*. 2013) giving birth to two sub-groups, strigolactones strictly speaking and strigolactones-like. Strigolactones-like are non-canonical SLs that do not include the classical ABCD skeleton, but still contain the D-ring, which mediates strigolactones perception and activity (de Saint Germain, 2016).

The functional annotation of transcripts isolated by RNA-sequencing in the TZ of nitrate-supplied maize root identified a set of genes likely involved in SL biosynthesis and transport (Trevisan *et al*., 2015). Transcriptomic data demonstrated that 2h of nitrate are enough to strongly inhibit the expression of *ZmCCD7* and *ZmCCD8* in TZ cells. Moreover, three genes encoding ABC (ATP-binding cassette) transporter proteins, (*ZmPDR1*, *ZmPDR3* and *ZmWBC33*), isolated from

accessions classified among the term "drug transporter activity" were highly co-expressed with the carotenoid cleavage dioxygenase genes, suggesting that they could putatively take part to the SL transport and/or exudation.

The present study was aimed at better understanding the involvement of SLs in the maize response to N availability. A LC-MS/MS method was applied to try to identify already known SLs in maize root exudates obtained by seedlings grown with different N availabilities and to characterize their profile and the extent of their exudation in response to the nutrient treatment.

The transcriptional regulation of genes encoding SL biosynthesis components and putative SL transporters was also evaluated and *in situ* hybridization experiments were performed to study their mRNA localization. Finally, phenotypical analyses, based on both *Phelipanche* germination assays and lateral root (LR) development assessment were performed to gain new insights into the regulation of SL's endogenous and exogenous effects mediated by N availability in this crop.

Results

N-starvation, nitrate and ammonium provision differently affect SL exudation

To assess the effective presence and content of SL in the exudates obtained by plants grown without N or with NO_3^- or NH_4^+, a LC-MS method was developed.

SLs are usually screened by the LC-MS/MS in precursor ion scan mode, searching for ions undergoing the specific loss of Cycle D (-97 Da) (Xie *et al.*, 2010), but this strategy lacks sensitivity. MRM mode, being the most sensitive mode in LC-MS/MS, was then used for screening and quantification purposes, listing every MRM of SLs from literature. This allowed us to check the presence or absence of 31 SLs (20 canonical SLs, 5 non-canonical SLs and 6 unknown) in root exudates from different nutritional conditions and blanks, and quantify them if they were present. All chromatographic peaks with an area above 1000 were integrated. However, the common peaks between medium blank and exudates samples were considered as false positives and ignored. A peak was also ignored if it was found in one experiment (in one transition and one retention time) but not in the other. In order to obtain positive control samples (e.g. expected to contain SLs produced by maize), some maize roots were let to exudate in P starvation conditions, e.g. ideal conditions for SL production (Lopez-Raez *et al*., 2008a). Finally, one putative zealactone isomer eluting at the retention time of 10.8 min was detected as a SL, quantified and confirmed in both experiments (**Supplementary Table S1**). We found a number of additional signals but none except this compound could be confirmed matching our criteria. This compound exhibited typical characteristics of strigolactones. It was detected at MRM channels *m/z* 399>302 and 345>248, with characteristic losses of cycle D. Five MRM transitions with precursor ions *m/z* 399, 377 and 345 (**Table 2**) showed a response at that retention time, suggesting the putative SL would have a mass of 376. Accordingly, *m/z* 399 would correspond to the sodium adduct, *m/z* 377 would be the proton adduct and *m/z* 345 could be a fragment produced in the source of the mass spectrometer from a neutral loss of methanol. The two main MRM arising from *m/z* 399 et 377 corresponded to expected or published transitions for zealactone 1a and 1b (**Supplementary Fig. S1**), the 3 other arising from *m/z* 345 had been putatively attributed to didehydroorobanchol isomers (Lopez-Raez *et al*., 2008b) or didehydrostrigol isomers (Xie *et al*., 2007), suggesting that this *m/z* 345 fragment still bears strigolactone structure. The mass of the putative M376 SL corresponds to the mass of zealactones 1a and 1b as presented in Charnikhova *et al.* (2017). However, no signal was confirmed at the main MRM transition (377>97) presented for zealactones 1a and 1b in Charnikhova *et al,* (2017), and no standard was available to confirm the zealactone identity. So, the compound was hereafter referred to as putative zealactone isomer.

In the quantification of strigolactones in maize exudates this putative zealactone isomer was detected at a significant level (1.2 ng equivalent GR24 per g exuding root) in samples obtained from phosphate-starved seedlings, which were utilized

as a positive control for SL exudation (**Fig. 1**). Surprisingly this compound was detected at a much higher level (13.2 ng eq GR24/g root) in nitrogen-starved samples. In contrast, nitrate-supplied samples contained no detected zealactone isomer, indicating a clear inhibitory effect of nitrate on zealactone production. However, the effect of ammonium supply on SL content in exudates showed a decrease of the SL level but weaker than with nitrate supply.

N and P regulation of SL-related gene transcription in the primary root

The TZ of the root apex is very responsive to a short (2 h) nitrate treatment, which rapidly triggers the down regulation of genes involved in SL production and action (Trevisan *et al*., 2015). Here the transcript levels of *ZmCCD7*, *ZmCCD8*, encoding components of SL biosynthesis were measured in the first cm of root apex (including M, TZ, EZ and MZ) of seedlings grown without N for 24 hours and then transferred to a similar solution (negative control) or to two different solutions containing NO_3^-, 1 mM, NH_4^+, 1 mM for additional 24 (T1), 48 (T2) and 72 (T3) hours (**Fig. 2**) The transcription of both *ZmCCD7* and *ZmCCD8* was clearly up-regulated by N-deficiency, with an increasing trend with the increase of time of permanence in –N. On the contrary, when NO_3^- was supplied the level of their expression didn't change during the experiment and it was always significantly lower (1,5-4,5; 8-15 and 4-6 fold changes for *ZmCCD7* and *ZmCCD8* at T1, T2 and T3 respectively). As far as the NH_4^+ supply was concerned, a different trend was observed for *ZmCCD7* and *ZmCCD8* expression. In fact, *ZmCCD7* was transcribed at very low levels in all the time-points, while *ZmCCD8* expression was still up-regulated after 24 hours of ammonium supply (T1), and decreased thereafter (T2 and T3) to levels lower than those measured for N-depleted roots.

The expression of two genes putatively involved in SL transport (*ZmPDR1* and *ZmWBC33*) was then assessed in the same nutritional condition and also in the presence of TIS108 (Fig. 3). *ZmPDR1* was expressed at very low levels almost in all treatments, with a significant increase of its expression observed only after 72 hours (T3) both in N-starved and TIS108-supplied roots. *ZmWBC33* displayed a different profile with a significantly higher expression in N-deprived roots (10, 17 and 61 fold changes at T1, T2 and T3 respectively) in comparison to NO_3^--supplied plants. NH_4^+ provision also clearly down-regulated *ZmWBC33* expression, even though less rapidly in comparison to NO_3^-. In fact, after 24 hours of NH_4^+ supply *ZmWBC33* transcription was still six times higher than that observed in the presence of NO_3^-. The provision of TIS108 to N-starved roots negatively affected the transcription of *ZmWBC33*.

The expression of *ZmCCD8*, *ZmPDR1* and *ZmWBC33* was measured also in P-depleted and Pi-supplied maize root after 24 hours of treatment (T1) for comparison. The expression of both *ZmCCD8* and *ZmWBC33* was significantly induced by P-starvation, but no differences were observed for *ZmPDR1*. Moreover, when TIS108 was supplied to P-starved seedlings an appreciable decrease of *ZmCCD8* and *ZmWBC33* transcription was noticed (**Fig. 4**). All together these results seem to suggest that *ZmWBC33*, and not *ZmPDR1*, could take part to the transport of SL. A structural and phylogenetic analysis of ZmWBC33 is reported in the supplementary data (**Supplementary Fig. S2, Supplementary Table S2**).

Spatial pattern of ZmWBC33 and ZmCCD8 in primary root and in shoot

To further identify the particular tissues in which SL-related transcripts accumulate, *ISH* experiments were performed for *ZmWBC33* and *ZmCCD8* in root and shoots. *ISH* allowed detection of target mRNAs in both tissues (Fig. 5). A reliable expression was consistently observed for both the antisense probes, but no labelling with the sense probe was recorded (**Fig. 5A-B and Supplementary Fig. S3**). A relatively uniform distribution of signals was observed for the transcripts of these two genes, revealing that both are predominantly expressed throughout the vascular parenchyma of the primary root, even though *ZmWBC33* showed a higher mRNA accumulation than *ZmCCD8*. In root apex longitudinal sections, comprising the root cap and meristematic area, a clear hybridization signal for *ZmWBC33* and *ZmCCD8* was detected

also in the epidermis and in 1–2 longitudinal files of cells immediately inside of it (hypodermis) (**Fig. 5A-B, panels I-II**). A more diffuse signal was also detected in the outermost layers of the stele, including the pericycle. Moreover, expression was detected in the initials of the epidermis and cortex, in the potential metaxylem tissues, in cortex cells surrounding lateral root primordia (LRP). (**Fig. 5A-B, panels I-II**). Apart from hypodermis, faint staining was seen in root tip cells. This was particularly evident for *ZmCCD8* probe, which signal was completely absent in the quiescent centre cells, but it started to accumulate in their immediate daughters and in the proximal meristem (**Fig. 5B, panel I**). As cellular differentiation progressed, mRNAs of these SL-related genes accumulated in the region where the xylem would develop (Fig. **5A-B, panels I-II**), with the expression domain of *ZmWBC33* larger than those of *ZmCCD8*.

ZmCCD8 transcript levels appeared to decrease in all distal tissues as they progressively elongated and/or differentiated (**Fig. 5B, panels I-II**), while *ZmWBC33* transcripts accumulated also in elongation zone and in the closest part of differentiated root tissue (**Fig. 5A, panels I-II**). At the late stage of vascular development, when cellular differentiation was being completed, expression of these genes continued in the cells between the metaxylem elements (**Fig. 5B, panels II)**. Cross-sections of roots gave the same patterns of *ZmWBC33* and *ZmCCD8* signals throughout the root apex (**Fig. 5A-B, panels VI-VII**).

Interestingly, *ZmWBC33* and *ZmCCD8* expression was patchy detected in young lateral root primordia and became evident as the lateral root tips start to emerge from the primary root (**Fig. 5A-B, panel III**). The signal was not present at detectable levels in lateral root founder cells or in lateral root initials.

As already mentioned, SL biosynthesis is not restricted to the roots, thus the tissue specific distributions of *ZmWBC33* and *ZmCCD8* were carried out also in aerial tissues (**Fig. 5A-B, panels IV, V**). The cross sections of the vegetative shoot apexes show that they are expressed also in young leaves, with only slight differences between their patterns. Their expression was limited to the adaxial surface and the vascular bundle of young leaves (**Fig. 5A-B, panels IV, V**). In the aerial tissues, probe signal for *ZmCCD8* (**Fig. 5B, panels IV, V**) is more intense than *ZmWBC33* (**Fig. 5A, panels IV, V**), but *ZmWBC33* seems to be more localized in the phloem, xylem and vascular bundle. Hybridization signal was not detected in epidermis and mesophyll cells.

Exudates differently affect* Phelipanche ramosa *seed germination

To evaluate the effects of SL exuded by root on the rhizosphere an indirect assay was performed. Root exudates obtained from N-depleted, P-starved and ammonium-supplied roots triggered an appreciable induction of the germination of *P. ramosa* seeds (85%, 80% and 75% higher than in the negative control, respectively) **(Fig. 6A and B).** A similar effect was observed when seeds were supplied with GR24 (positive control). In contrast, when seeds were treated with exudates obtained from nitrate supplied plants only a slight (less than 25%) germination rate was observed respect to the control. Furthermore, when TIS108 was provided to both N- and P-depleted roots, thus presumably inhibiting SL biosynthesis and exudation, only a weak germination (20%) of *P. ramosa* seeds was observed. As expected, no spontaneous germination could be observed when *P. ramosa* seeds were incubated only with nutrient solution as a control (–data not shown).

N deficiency inhibition of LR development seems to involve SL signalling

The effects of N-deficiency, nitrate and ammonium supply in the presence of a SL biosynthesis inhibitor (TIS108) and of a synthetic SL analogue (GR24) on lateral root density (number of LRP/mm primary root) were evaluated (**Fig. 7**). When seedlings were moved from a N-free solution to a nitrate-supplied medium, the LRP density showed a significant increase (+10% already after 2 h and +20% after 24 h of nitrate supply, respectively). The length of primary roots (PR) showed an

increase in the first 2 hours of nitrate supply (+15%) and a decrease in response to a more prolonged treatment (24 hours, -12%). Moreover, when TIS108 was supplied to N-deprived seedlings a significant increase of LRP density (+25%) and a slight decrease of primary root length (-7.5%) were observed, likely re-establishing the phenotype observed for nitrate supplied plants. A pattern similar to that observed after 24 hours of nitrate provision was noticed in response to ammonium, with a reduction of PR length (-8.5%) and a parallel increase of LRP density (+15%). On the contrary, seedlings supplied with a synthetic analogous of SL (GR24) (in the presence of nitrate) showed a lower LRP density (-15%) and a slightly longer PR length (+5%), thus resembling to N-deprived plants.

Finally, seedlings grown without N and supplied with both TIS108 and GR24 also displayed a phenotype similar to that observed for –N plants, thus supporting previous results.

Discussion

Nitrogen is a key element for crop but its availability in agricultural soils is limited and plants have developed strategies to adapt to its fluctuations. Nitrate represents the principal N form for crop, and it acts both as nutrient and signal, regulating many aspects of plant metabolism and development. Previous works led to the hypothesis of a coordinated action of NO, auxin and SLs in regulating the early response of maize root apex to nitrate (Trevisan *et al.,* 2015; Manoli *et al*., 2016). In this paper, further evidences on SL involvement in the signalling pathway governing root maize response to N were gained.

Phosphate deficiency has been demonstrated to be the optimal condition for the stimulation of SL production (Kapulnik and Koltai, 2016). However, in our growth condition, nitrogen deficiency is much more effective than phosphorous deficiency in stimulating SL accumulation in the exudates and triggers the exudation of a significantly higher amounts of these compounds if compared with either nitrate or ammonium supplied plants (**Fig. 1, Table 2, Supplementary Table S1, Supplementary Fig. S1**).

However, while 24 hours of nitrate supply are sufficient to totally switch down SL exudation, ammonium is less effective or it needs more time to inhibit this process. This different behaviour of roots in response to different N forms could be motivated by the evidence that plants take advantages of mycorrhizal associations for NH_4^+ acquisition more than it does in the case of nitrate (Chalot *et al.,* 2016; Guether *et al*., 2009).

The trend of expression levels observed for Zm*CCD7* and Zm*CCD8* in response to N-starvation, or in the presence of either NO_3^- or NH_4^+ (**Fig. 2**) globally suggest that the accumulation of transcripts encoding *ZmCCD8* could represent a reliable and useful marker for SL biosynthesis also in maize, in accordance with the results obtained by Arite *et al*. (2007) in rice.

Until now, the only characterized SL transporters are the ABCG protein PDR1 from *Petunia axillaris* (Kretzschmar *et al*., 2012) and its close homologue PDR6 from *Nicotiana tabacum* (Xie *et al*., 2015). In contrast, no SL transporters have been isolated yet from Monocots or even from Arabidopsis. Among the transcripts expressed in TZ of maize roots and down regulated by 2h of nitrate provision two genes encoding a maize homolog of PDR1 and a WBC transporter (*ZmWBC33*) respectively were identified (Trevisan *et al*., 2015). ZmWBC33 (**Supplementary Fig. S2, Supplementary Table S2**) is a member of the WBC subfamily of maize ABCG transporters (Pang *et al.*, 2013), named after the identification of the canonical WHITE-BROWN complex of *Drosophila melanogaster* (Ewart *et al*., 1994). The present results show a marked induction of *ZmWBC33* transcription by nitrogen and phosphate deprivation, and a clear downregulation of its expression in the presence of nitrate and ammonium, whilst only slight regulation of the expression of *ZmPDR1* was observed in response to N supply or deprivation (**Fig. 3**). The expression profiles observed for *ZmCCD7*, *ZmCCD8* and *ZmWBC33* are consistent with the pattern of SL exudation detected by LC–MS/MS, and support the hypothesis that N deficiency triggers SL production and exudation, and

that both nitrate and ammonium act as a negative signal to inhibit or reduce SL exudation. Moreover, according to the expression pattern observed for *ZmWBC33*, nitrate is more effective and rapid, while roots seem to require a more prolonged presence of ammonium to down regulate its transcription, in accordance with the previously described trend of SL exudation. The transcription of both *ZmCCD8* and *ZmWBC33* was also strongly induced by P deprivation, which, in turn, did not affect *ZmPDR1* transcription.

SLs are synthesized in both the roots and the shoots and are transported outside as exudates or acropetally, presumably in the xylem, to repress bud activity (Borghi *et al*., 2016). In shoots like in roots, SL biosynthetic tissues are spread along the vasculature or localized in specific organs. In the present work a detailed *in situ* localization of *ZmWBC33* and of *ZmCCD8* mRNA has been performed (**Fig. 5**). *ZmWBC33* mRNAs were detected in all surveyed tissues but preferentially in roots. In primary root *ZmWBC33* was shown to be lightly expressed in the meristem of the root tip, and it starts to accumulate in the epidermis and in cortical cells along the vasculature, included the stele of the transition and elongation zones. The same localization pattern was observed for PaPDR1 in Petunia (Sasse *et al*., 2015) and for NtPDR6 in Nicotiana (Xie *et al*., 2015). PDR1 exhibits an asymmetrical localization in petunia root tips leading authors to suppose that at least in this region of the root active cell-to-cell transport occurs. An analogous hypothesis was supported by recent work using fluorescent-tagged SL (Prandi *et al*., 2014; Fridlender *et al*., 2015). In our experiments *ZmWBC33* transcripts co-localizes with those of the SL-biosynthesis gene *ZmCCD8* in stele, in the cortex and in epidermis. Sasse and co-authors (2015) also showed that *PDR1* co-localizes with *CCD8* in the root tip of Petunia, and a similar pattern was observed also for *CCD8/MAX4* in *Arabidopsis thaliana* (Sorefan *et al*., 2003). These findings support our hypothesis that ZmWBC33 could be involved in the SL cell-to-cell flux in maize root.

In differentiated zone *ZmWBC33* transcript was observed in root vascular tissues and in the apical meristem of LRP at different stages. *ZmCCD8* and *ZmWBC33* were localized in the vasculature also in shoots, confirming that SL synthesis takes place also in the aerial part (Lopez-Obando *et al*., 2015) and supporting the hypothesis that *ZmWBC33* could be involved in the transport of SL out of the leaves, either to the lateral buds or to the main stem.

In the root, *ZmWBC33* could regulate SL accumulation in the meristem, which was suggested to be highly sensitive to alterations in strigolactones concentration (Ruyter-Spira *et al*., 2011). Out of the root tip, the *ZmWBC33* localization in the vasculature seems to suggest that it might contribute to loading SL into the xylem thus contributing to translocation to shoot and to coordination of shoot and root growth. Moreover, *ZmWBC33* specific localization in the epidermis of the root tip and root elongation zone may suggest its involvement in the root secretion processes.

In the LRP and the aerial tissues ZmWBC33 could redistribute the SL produced *in loco* by ZmCCD8, changing SL homeostasis and participating in lateral root and shoot development.

Major questions for the future concern ZmWBC33 structure-function relationships, which will require the elucidation of its three-dimensional structures and multisubstrate binding properties. Specifically, it will be interesting to investigate if ZmWBC33 directly transports SLs, identify the specific substrate(s), recognize the identity of a putative dimerization partner and test how ZmWBC33 activity is regulated in response to environmental conditions that prompt changes in growth.

To try to better decipher how N availability affects few SLs-mediated functions the effects of root exudates on the germination rate of *Phelipanche ramosa* were observed (**Fig. 6**). Exudates obtained by N-deprived and NH_4^+-supplied roots significantly induced this species to germinate, signifying that even if ammonium considerably constrain SL production (**Fig. 1, Table 2, Supplementary Table S1, Supplementary Fig. S1)**, the amount of these compounds is still sufficient to exert their stimulatory action on *Phelipanche* germination and conceivably on mycorrhizal partners, confirming the already known extraordinary sensibility for SLs of parasitic species (Boari *et al.,* 2016). On the contrary, upon nitrate supply, that drastically

inhibits SL exudation, and when TIS108 was supplied to N-deprived roots, very low or no germination was observed, further indirectly confirming the presence of SLs in the exudates harvested from N-deprived plants.

SLs are crucial molecules not only for root-soil communication but also as endogenous signals in regulating whole plant development (Koltai and Beveridge, 2013; Agusti *et al*., 2011; Ueda and Kusaba, 2015). Lateral root development has a considerable biological and agronomical relevance in the overall response to nitrogen (York *et al*., 2016). Few discoveries highlighted the complexity of the molecular networks modulating the plasticity of LR formation in response to nutrients in cereals (reviewed in Yu *et al*., 2016). SLs seem to control lateral root development (Kapulnik and Koltai, 2014, Koltai *et al*., 2010) depending on auxin levels (Ruyter-Spira *et al*., 2011) and possibly also through a cytokinin-auxin feedback loop (Jiang *et al*., 2016). However, until now no evidence on the SL participation to the pathway through which N influence LR development have been reported. In Arabidopsis nitrate and ammonium seem to promote LR proliferation in a different and complementary way, with ammonium increasing lateral root branching and nitrate promoting lateral root elongation (Remans *et al.*, 2006; Lima *et al.*, 2010).

The present results indicate that nitrate and ammonium supply to maize seedlings previously grown in a minus N solution noticeably stimulate LR development (**Fig. 7**). Moreover, thanks to the use of GR24 and TIS108, it would seem that this stimulation could be linked, at least in part, to the complete or partial inhibition of SL production observed in response to nitrate and ammonium respectively.

The present results indicate that nitrate and ammonium supply to maize seedlings previously grown in a minus N solution noticeably stimulate LR development (**Fig. 7**). Nevertheless, when GR24 was supplied together with nitrate, the -N phenotype was re-established. This phenotype could not be attributed entirely to an SL effect, because rac-GR24 is known to activate responses that are specific to naturally occurring SLs and responses that are not, such as KAI2 pathway (Scaffidi *et al*., 2014). However, provision of TIS108 (which inhibits SL biosynthesis) to N-starved plants, completely restored the +N phenotypes. In addition, GR24 was also used to complement the root phenotype with TIS108, showing that the complementation assay led to a phenotype similar to N-deprived plants. Even if maize produces in large amount non-canonical SLs (Charnikhova *et al*., 2017) that can contribute to different responses *in vivo*, while GR24 is a canonical SL, GR24 itself is still the most widely used synthetic SL for bioassay (Zwanenburg *et al*., 2016). Nevertheless, non-canonical SLs such as zealactones would be the most appropriate choice, even though they are still scarcely available. Taken together, these data seem to suggest that the stimulation of lateral root development could be linked, at least in part, to the complete or partial inhibition of SL production observed in response to nitrate and ammonium respectively.

In a recent study Koltai and co-authors (2015) reported that the strigolactone-signalling pathway affects auxin transport, cellular trafficking and PIN polar localization in the plasma membrane. Moreover, PDR1 overexpression was demonstrated to influence auxin transport/allocation in several tissues of Petunia (Liu *et al*., 2018). In maize roots of seedlings grown in a N-deprived solution for 24 hours a reallocation of PINs by cytoskeleteton remodelling was observed already after two hours of nitrate provision (Manoli *et al*., 2016). This study suggests that nitrate induces fast NO burst, which impairs SLs levels resulting in both PIN-dependent auxin re-distribution and cell elongation, thus providing an hypothetical model of how NO, auxin and SLs may cooperate in regulating the early response of maize root apex to nitrate.

The interplay existing between SLs and NO has been deeply reviewed by Kolbert (2018) and many papers reported the link between auxin and nitrate in the control of root development in Arabidopsis (for example Krouk *et al*., 2010; Mounier *et al*., 2014), but only few information is available for maize (Sun *et al*., 2017). The present results allow to include also SLs among the key components of the response of maize root to N, but further evidences need to be provided to precisely clarify the exact interaction among N, auxin and SLs in the regulation of lateral root development.

In conclusion (**Fig. 8**), this study demonstrates that N-deficiency strongly induce SL exudation in maize roots and that nitrate rapidly switches off SL exudation. Moreover, ammonium reduces SL exudation by roots but less markedly in comparison to nitrate, thus likely allowing root to continue to establish mycorrhizal associations. However, the decrease of SL production observed in response to both these ions would seem to contribute to the signalling pathway underlying lateral root development in response to N.

Furthermore, a putative novel component of the maize SL transport machinery has been identified, even though further functional studies are mandatory to gain new insight in the WBC33 actual role.

A more precise knowledge of the SL involvement in the integration of information on N availability and hormonal signalling to regulate the maize root plasticity to nutritional stresses could be of great interest both for root biology research and for the possible applications of these molecules in agriculture.

Material and methods

Maize growth conditions

Seeds of the maize inbred line B73 (*Zea mays* L.) were germinated as described by Manoli *et al*. (2014). After germination seedlings were grown for 24h in a N-deprived solution and then transferred to: -N (negative control), NO_3^- 1 mM or NH_4^+ 1 mM. The expression analyses were performed after 24 h (T1), 48 h (T2) or 72 h (T3). To test the effect of phosphate availability a second experiment was performed by growing seedlings in a P-deprived solution (-P) for 24 h and then transferring them for further 24 h in a similar –P solution or in a PO_4^{3-}supplied medium (40 µM). 6-phenoxy-1-phenyl-2-(1H-1,2,4-triazol-1-yl) hexan-1-one (TIS108) and *rac*-GR24 (Strigolab, Torino, Italy) were used at a 2 µM concentration as inhibitor of SL biosynthesis (Ito *et al*., 2011) and as synthetic SL analogue, respectively.

Lateral root primordia (LRP) analysis and exudates collection were carried out at T1. For exudates collection seedlings were transferred to a renewed solution and exudates were collected after 24 hours.

A growth chamber with a day/night cycle of 14/10 h at 25/18°C air temperature, 70/90% relative humidity, and 280 µmol $m^{-2} \cdot s^{-1}$ photon flux density was used.

Unless stated otherwise, all chemicals were obtained from Sigma Chemicals (Sigma, St Louis, MO, USA).

SL identification and quantification in exudates

Exudates were obtained by two independent experiments in three biological replicates. The extraction of root exudates was based on the protocol of Gomez-Roldan *et al*. (2008). Each exudate was prepared with at least 1 g of root fresh weight (each sample had an accurate weight of root). All volumes of root exudates and the corresponding blank samples were extracted with an equivalent volume of ethyl acetate and 10 ng of GR24 were added as an internal standard. All the extracts were evaporated to dryness and finally dissolved in 100 µL of acetonitrile before LC-MS/MS analysis. Chromatographic conditions were similar as in Boutet-Mercey *et al.* (2018).

Ninety transitions MRM (Multiple Reaction Monitoring) of the literature were monitored using Waters Xevo TQ-S equipped with an ESI source in positive or negative mode. The **Supplementary Table S1** shows the monitored transitions for 31 SLs including 20 canonical SLs, 5 non canonical SLs et 6 unknown, according to bibliography. The source parameters for the MRM mode were similar as in Boutet-Mercey *et al*. (2018). The relative quantification of the putative SL was carried out by a ratio between area of the chromatographic peak of the putative SL and area of internal standard GR24 (MRM transition 321 > 224) multiplied by the amount of added internal standard, relative to the mass of exuding roots.

Experiments with three biological replications were repeated twice (two cultures) to confirm the results. The data are presented as means ± standard errors (n = 3) from a typical single experiment. Exuded amounts of SL were compared statistically by using Student's t test ($P < 0.05$).

RNA extraction and cDNA synthesis

One cm of root apices from the root tip cap were sampled from 15 to 20 pooled seedlings, in three independent biological repetitions, and immediately frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA) as previously described by Trevisan *et al.* (2011). RNA was quantified with a Nanodrop1000 (Thermo Scientific, Nanodrop Products, Wilmington, DE, USA) and reverse transcribed to cDNA as described by Manoli *et al.* (2012).

Quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was performed using the StepOne Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) as described by Nonis *et al*. (2007). SYBR Green reagent (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) was used in the reaction, according to the manufacturer's instructions. Melting-curve analysis confirmed the absence of multiple products and primer dimers. Target gene relative expression was determined according to the Livak and Schmittgen (2001) method, using *MEP* (membrane protein PB1A10.07c, Zm00001d018359) as reference gene, according to Manoli *et al.* (2012). Primers were designed using Primer3 web tool (version 4.0.0; http://bioinfo.ut.ee/primer3/; Rozen and Skaletsky, 2000) and further verified with the PRATO web tool (Nonis *et al*., 2011). The list of genes and of the primers used are reported in **Table 1.**

Three technical replicates were performed on three independent biological repetitions.

RNA In situ hybridization of ZmCCD8 and ZmWBC33

In situ hybridization of maize primary root with digoxigenin (DIG)-labeled probes was performed as described by Trevisan *et al.* (2011). *ZmWBC33* and *ZmCCD8* antisense probes were amplified in PCR using the primers listed in Table 1. The fragment was cloned into the T-easy vector (Madison, WI, USA) for labelling. The sense and antisense probes were synthesized in vitro using T7 and SP6 RNA polymerases (Roche, Basel, Switzerland) and labelled with digoxigenin RNA labelling mix (Roche) following the manufacturer's protocol. Roots were fixed, dehydrated, infiltrated with paraffin and sectioned (7 µm) as described by Trevisan *et al*. (2011). Histo Clear II (National Diagnostics, Atlanta, GA, USA) was used to remove paraffin from sections. Slides were hydrated in a decreasing ethanol series. Hybridization was conducted as described by Trevisan *et al*. (2011). After staining, slides were observed with an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan). Images were captured with an Axiocam Zeiss MRc5 color camera (Carl Zeiss, Oberkochen, Germany), and processed with Adobe Photoshop 6.0.

Maize root exudate collection and* Phelipanche ramosa *germination bioassay

Parasitic seeds (provided by prof. Antonio Elia, University of Foggia) were pre-conditioned under sterile conditions as reported by Pouvreau *et al*. (2013). After the preconditioning period, the GFFP (Glass Fiber Filter Paper) disks with parasitic seeds were treated with 50 µL of root exudates and incubated in darkness at 25°C for 6 days. To better contrast the radicle, seeds were also stained using 40 µL of Neutral Red solution (1:4000, w/v) for each disk (Guillotin *et al*., 2016). Germinated seeds were then counted using a stereo microscope (Olympus BX50 microscope, Olympus

Corporation, Tokyo, Japan). Images were captured with an Axiom Zeiss MRc5 colour camera (Carl Zeiss, Oberkochen, Germany), and processed with Gnu Image Manipulation Program (GIMP).
Three biological replicates for each treatment and an ANOVA statistic test were performed (n=30).

Lateral root density analysis

Seedlings were grown for 24 h in the N-deficient solution and then transferred in different nutrient solutions for 24 h, as described in the first M&M paragraph. The effect of NO_3^- was evaluated also after only 2 h of treatment.
To better visualize LRP an haematoxylin staining solution supplied with ferric ammonium sulphate was used, as described by Canellas *et al*. (2002). Root images were collected using a flatbed scanner. The lateral root and the primary root length were measured using the Image J Image Analysis Software and the LR density was expressed as percentage compared to the value observed for N-deprived roots. Three biological replicates for each treatment and an ANOVA statistic test were performed (n=30).

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Conflicts of interest: No conflicts of interest declared

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733 **Tables**

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Table 1: List of primers used in qRT-PCR experiments. Primers used to amplify ISH probes are evidenced in bold.

PRIMER	SEQUENCE	DESCRIPTION
Zm00001d002736_T01_For	AGTCCACACCCGTCTACCTG	***ZmCCD7***
Zm00001d002736_T01_Rev	GGTCCAGCTTCTTGTTCAGC	***ZmCCD7***
Zm00001d043442_T01_For	AGAAAGGTGTCTCTGCTGCT	***ZmCCD8***
Zm00001d043442_T01_Rev	CTATGGGCTCGCTCACATGA	***ZmCCD8***
Zm00001d043598_T01_For	GGAAACCCGATCAGCAGGT	***ZmPDR1***
Zm00001d043598_T01_Rev	GCAGTAAAGCCAGCCAACAC	***ZmPDR1***
Zm00001d019398_T01_For	CGCTAACACGGTCTCATCAA	***ZmWBC33***
Zm00001d019398_T01_Rev	ATCATCATCAGCCCTTCGAC	***ZmWBC33***

735

736
737 **Table 2**: **LC-MS/MS parameters for putative zealactone isomers detected in the samples**
738

Compounds	RT (min)	MRM transitions	CV (V)	CE (eV)	Q/C
Putative SL like Zealactone isomer $_{[M+ Na]+}$	10.86	399 > 302[a]	20	20	Q
Putative SL like Zealactone isomer $_{[M+H]+}$	10.86	377 > 345[b]	20	15	C
Putative SL like Zealactone isomer $_{[M+H-CH3OH]+}$	10.86	345 > 248[c]	20	15	C
Putative SL like Zealactone isomer $_{[M+H -CH3OH]+}$	10.86	345 > 203[d]	20	15	C
Putative SL like Zealactone isomer $_{[M+H -CH3OH]+}$	10.86	345 > 175[d]	20	15	C

739
740 RT (min): Retention time in minutes
741 Diagnostic transition MRM: characteristic precursor and product ions for multiple reaction monitoring
742 CV (V): cone voltage
743 CE (eV): collision energy
744 Q /C: transition used for quantification (Q) or confirmation purpose (C)
745 a. Putative specific MRM transition for D ring-containing ions [M+Na - D ring]+ (Xie et al., 2010).
746 b. Loss of a methanol group (Charnikhova et al., 2017).
747 c. Putative specific MRM transition for a D ring-containing ion (Xie et al., 2010) after in source loss of a methanol group.
748 d. Putative MRM transitions after loss a methanol group, analog to didehydro-Orobanchol transitions (Lopez-Raez et al.,
749 2008)
750
751

Figure legends

Figure 1: LC-MS/MS, MRM quantification of SL in maize root exudates

Quantitative analysis of the relative amounts of putative zealactone forms in maize root exudates [ng*(g root FW)$^{-1}$] of seedlings exposed to additional 24h of nitrate (NO_3^-), ammonia (NH_4^+) or N starvation (-N) after a 24h-pre-incubation under N-deficient conditions. Quantification in root exudates of phosphate-starved seedlings (-P) was included as positive control. The root exudates were collected after the treatment and then shock-frozen in liquid nitrogen immediately afterward. Following extraction, the analytes were quantified by analysis using of LC-MS/MS, MRM mode. The experiments were repeated twice and data were from a typical single experiment. Values are mean ± SE of three replicates. Asterisks indicate significant differences in SL levels between -N and N fertilization conditions according to Student's t test ($P < 0.05$).

nd: non-detected.

Figure 2: Real-time qRT-PCR expression profiles of SL biosynthesis *ZmCCD7*, *ZmCCD8* genes in maize roots.

Maize seedlings were grown in hydroponics media either under N-deprivation (-N) or subjected to 1 mM N-fertilization (NO_3^- or NH_4^+) after a 24h-pre-incubation period in N-deficient conditions. After 24 hours (T1), 48 hours (T2) and 72 hours (T3) of treatment 1 cm of root apices from the root tip cap were collected from every pool of plants (n=15 to 20) to detect relative mRNA levels for ZmCCD7 (a) and ZmCCD8 (b) by means of qRT-PCR analysis. Expression levels were normalized to MEP (Zm00001d018359, Manoli et al. 2012). Data are mean ± SE for three biological replicates.

Figure 3: Real-time qRT-PCR expression profiles of *ZmPDR1* and *ZmWBC33* genes in maize roots

Maize seedlings were grown 24 hours in a N-deprived nutrient solution and then transferred to a 1 mM N-supplied media (NO_3^- or NH_4^+), to a N-deprived solution (-N) or to a N-deprived solution supplied with 2 µM TIS108, for additional 24 hours (T1), 48 hours (T2) and 72 hours (T3). At each time point 1 cm of root apex from the root tip cap was collected from every seedling (n=15 to 20) and the relative mRNA levels for ZmWBC33 (a) and ZmPDR1 (b) were evaluated by means of qRT-PCR. Error bars represent the SEM for three biological replicates.

Figure 4: Real-time qRT-PCR expression profiles of *ZmCCD8*, *ZmPDR1*, *ZmWBC33* genes in maize roots.

Seedlings were grown 24 hours in a P-deprived nutrient solution and then transferred to a 40 µM PO_4^{3-} (+PO_4^{3-}) solution, to a P-deprived solution (-P) or to a P-deprived solution supplied with 2 µM TIS108 (TIS108) for additional 24 hours. At the end of the treatment 1 cm of root apex from the root tip cap was collected from every seedling at each time point (n=15 to 20) and the relative mRNA levels for ZmCCD8, ZmPDR1, ZmWBC33 were evaluated by means of qRT-PCR. Error bars represent the SEM for three biological replicates.

Figure 5: *In situ* hybridization of *ZmWBC33 (A) and ZmCCD8 (B)* gene in primary root and emerging lateral roots of 3 days old maize seedlings exposed to nitrate depletion (72h).

Hybridization signal is visible as red – purple precipitate. Longitudinal (panels I-III) and transversal (IV-VII) sections from the primary root region (panels I-III, VI and VII) and shoot apex (panels IV and V) were reacted with antisense digoxigenin-labeled probe for *ZmWBC33* (A) and *ZmCCD8* (B). The expression of *ZmWBC33* and *ZmWBCCD8* in emerging lateral root primordia (longitudinal section of primary root) are reported in panels III, A and B respectively.

Hybridization with *ZmWBC33* and *ZmCCD8* gene-specific sense probes (negative control) are included in supplementary materials (**Supplementary Fig S3**).
Bars = 200 µm

Figure 6: *Germination of* P. ramosa *seeds induced by root exudates of maize seedlings*.
Maize seedlings were grown 24 hours in a N-deprived nutrient solution and then transferred to a 1mM N-supplied media ($+NO_3^-$ or $+NH_4^+$), to a N-deprived solution (-N) or to a N-deprived solution supplied with 2 µM TIS 108 (-N/TIS108), to a nitrate-supplied media plus GR24 (GR24) or water (H_2O) for additional 24 hours. Another pool of seedlings was grown in a phosphate deprived solution (-P) for 24 h and then transferred for additional 24 h in –P media (-P) or in a -P solution supplied with TIS108 2 µM (-P/TIS108). Root exudates were collected as reported by Pouvreau *et al*., (2013) and used to test the induction of germination in *Phelipanche ramosa* seeds. Each disk was treated with root exudates in triplicate. Germinated seeds were evidenced by Neutral Red staining and counted using a stereo microscope. The germination rate was expressed as mean percentage. Letters above the bars indicate different significance groups ($P<0.05$).

Figure 7: Lateral root primordia (LRP) density and primary root length of maize seedlings exposed to different nitrogen provision. Maize-seedlings were grown in a N-depleted solution for 24 h and then transferred for: 24h in a nitrogen depleted solution (-N), or 2 h in a 1 mM nitrate supplied solution and for the remaining 22 h in nitrogen depleted solution ($+NO_3^-$ 2h), or 24 h in a 1 mM nitrate supplied solution ($+NO_3^-$), or for 24 h in 1 mM NH_4^+ (NH_4^+), or for 24 h in a 1 mM NO_3^- supplied solution plus 2 µM GR24 ($+ NO_3^- +$GR24) or 24h in a N-depleted media plus 2 µM TIS108 (TIS108) or 24h in a N-depleted media plus both 2 µM TIS108 and 2 µM GR24 (TIS108 + GR24). An hematoxylin staining was used to evidence the mitotic sites associated with the earliest stages of lateral root development. Data are expressed as increment of LRP density respect to the control (grey blocks, left axis). For every thesis, -N treatment was the control (100%). Results are presented as mean ± SE from three biological replicates for each treatment and an ANOVA statistic test was performed (* indicates significant differences with $P<0.05$; ** indicate significant differences with $P<0.01$). Red circles (right vertical axis) represent the primary root length recorded after each treatment. Results are presented as mean ± SE from three biological replicates for each treatment and an ANOVA statistic test was performed. Letters above the bars indicate different significance groups ($P<0.05$).

Figure 8: Scheme of the proposed model for the role of SLs in the response of maize seedlings to different nitrogen sources. Maize seedlings were grown for 24h in a nitrogen depleted solution, and then they are moved to a different media, according to the absence (-N) or the presence ($+NO_3^-$ or NH_4^+) of nitrogen sources. To better decipher the role of strigolactones, an SL biosynthesis inhibitor (TIS108) or a synthetic SLs (GR24) were added to the growth media (N-depleted or NO_3^- -supplied respectively). LC-MS/MS, MRM SLs quantification showed a significantly higher content of a putative maize zealactone in exudates obtained by N-deprived roots, whilst nitrate and ammonium provision switches off SLs exudation, even though the ammonium effect appeared less incisive respect to nitrate. The presence of a minimal amounts of SLs in exudate of ammonium-treated roots would seem to enable plants to establish relationship with their neighbours, as confirmed by the *P. ramosa* germination rate, which was on the contrary almost completely inhibited in the presence of exudates derived from nitrate-supplied roots. Furthermore, LRP density in response to N-deprivation, nitrate or ammonium supply, TIS108 or GR24 provision led to the hypothesis that the decrease of SLs content observed in response to both nitrate and ammonium would contribute to the signalling pathway underlying lateral root development in response to N.

Supplementary data

Supplementary Table S1: MRM transitions monitored for the SLs screening according to bibliography or deduced of literature.

Supplementary Table S2: Bioinformatics analysis of *ZmWBC33* promoter.

Supplementary Figure S1: MRM chromatogram of root exudate from maize seedlings.

Supplementary Figure S2: Nucleotide and deduced aminoacid sequence information about ZmWBC33 gene and protein, with a phylogenetic tree of PDR1 protein sequence homologs.

Supplementary Figure S3: *In situ* hybridization of primary root and emerging lateral roots of 3 days old maize seedlings exposed to nitrate depletion (72h) with sense digoxigenin-labeled probe for *ZmWBC33* and *ZmCCD8.*