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Title

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Permalink

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Journal

The Plant journal : for cell and molecular biology, 105(6)

ISSN

0960-7412

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Publication Date

2021-03-01

DOI

10.1111/tpj.15129

Peer reviewed

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Article type : Original Article

TITLE: *Phaseolus vulgaris* MIR1511 genotypic variations differentially regulate plant tolerance to aluminum toxicity

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Running Title: miR1511 regulates aluminum toxicity in plants

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/TPJ.15129](https://doi.org/10.1111/TPJ.15129)

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Keywords: Aluminum, genotypic variations, microRNAs, miR1511, *Phaseolus vulgaris*, root development.

SUMMARY

The common bean (*Phaseolus vulgaris*), a widely consumed legume, originated in Mesoamerica and expanded to South America, resulting in the development of two geographically distinct gene pools. Poor soil condition, including metal toxicity, are often constraints to common-bean crop production. Several *P. vulgaris* miRNAs, including miR1511, respond to metal toxicity. The *MIR1511* gene sequence from the two *P. vulgaris* model sequenced genotypes revealed that, as opposed to BAT93 (Mesoamerican), the G19833 (Andean) accession displays a 58-bp deletion, comprising the mature and star miR1511 sequences. Genotyping-By-Sequencing data analysis from 87 non-admixed *Phaseolus* genotypes, comprising different *Phaseolus* species and *P. vulgaris* populations, revealed that all the *P. vulgaris* Andean genotypes and part of the Mesoamerican (MW1) genotypes analyzed displayed a truncated *MIR1511* gene. The geographic origin of genotypes with a complete vs. truncated *MIR1511* showed a distinct distribution. The *P. vulgaris* *ALS3* (*Aluminum Sensitive Protein 3*) gene, known to be important for aluminum detoxification in several plants, was experimentally validated as the miR1511 target. Roots from BAT93 plants showed decreased miR1511 and increased *ALS3* transcript levels at early stages under aluminum toxicity (AIT), while G19833 plants, lacking mature miR1511, showed higher and earlier *ALS3* response. Root architecture analyses evidenced higher tolerance of G19833 plants to AIT. However, G19833 plants engineered for miR1511 overexpression showed lower *ALS3* transcript level and increased sensitivity to AIT. Absence of miR1511 in Andean genotypes, resulting in a diminished *ALS3* transcript degradation, appears to be an evolutionary advantage to high Al levels in soils with increased drought condition.

INTRODUCTION

The legume family (Leguminosae) is very large, comprising *ca.* 700 genera and grouped into 40 tribes. The tribe *Phaseolae* is by far the economically most important group; it includes common beans (*Phaseolus vulgaris*), yard-long-beans/cowpea (*Vigna unguiculata*) and soybeans (*Glycine max*). The use of genetic, genomic and biotechnological tools has clarified the phylogenetic relationships between *Phaseolus* species in general and *P. vulgaris* in particular (Broughton *et al.*, 2003). The *Phaseolus* genus includes five domesticated species: *P. vulgaris* (common bean), *P. lunatus* (lima beans), *P. acutifolius* (tepary beans), *P. coccineus* (scarlet runner beans) and *P. dumosus* (year bean) (Delgado-Salinas *et al.*, 1999).

P. vulgaris is characterized by a particular evolutionary history. Recent analyses based on sequence data presented clear evidence of the Mesoamerican origin of common bean, which was most likely located in México (Bitocchi *et al.*, 2012). The expansion of this species to South America (Ariani *et al.*, 2018) resulted in the development of two ecogeographic distinct genetic pools with partial reproductive isolation (Koinange and Gepts, 1992). After the formation of these genetic pools -between 500,000 and 100,000 years ago (Ariani *et al.*, 2018)- domestication took place, independently in the Mesoamerican (northern Mexico to Colombia) and the southern Andean (from southern Peru to Argentina) regions of the American continent (Kwak and Gepts, 2009, Bitocchi *et al.*, 2013). Genome analysis of BAT93 (Mesoamerican) and G19833 (Andean), *P. vulgaris* sequenced model genotypes, has initially revealed interesting differences, for example between their genome size and number of annotated genes (Schmutz *et al.*, 2014, Vlasova *et al.*, 2016).

The common bean is the most important legume for human consumption. In less favored countries from Latin America and Africa, common bean are staple crops serving as the primary source of protein in the diet. Soil acidity in these tropical regions is a major constraint for crop productivity, usually resulting in a combination of nutrient deficiency (e. g., phosphorus) and metal toxicity (Broughton *et al.*, 2003, Graham *et al.*, 2003). In acidic soils, aluminum (Al) toxicity (AIT) is the primary factor of growth restriction, resulting in the inhibition of root growth and function, as well as in the increased risk of plants to perish of drought and mineral deficiencies, thus decreasing crop production (Kochian *et al.*, 2004). High Al levels mainly affect roots causing an arrest of the growth of the principal and lateral roots (Larsen *et al.*, 2005). In Arabidopsis, the regulation of root growth is modulated by an ABC transporter-like protein, annotated as ALUMINUM SENSITIVE PROTEIN 3 (*ALS3*), which is localized in the tonoplast, suggesting a role in Al vacuolar sequestration (Dong *et al.*, 2017). The LOW PHOSPHATE ROOT 1 (*LPRI*) ferroxidase, an *ALS3*-downstream protein of the phosphate-deficiency signaling pathway, is involved in root growth inhibition, by modulating iron homeostasis and ROS accumulation in root apical meristem and elongation zone (Wang *et al.*, 2019). In root cells, AIT can affect multiple areas, as the plasma membrane, the cell wall and symplastic components (Zhang *et al.*, 2018).

Common bean is known to be highly sensitive to AIT but this sensitivity is genotype-dependent (Blair *et al.*, 2010). In 2010, the evaluation of the root morphological traits related to AIT of 36 *P. vulgaris* genotypes revealed that Andean genotypes were more resistant to Al than Mesoamerican ones (Blair *et al.*, 2010). Mendoza-Soto *et al.* (2015) reported that Mesoamerican (Negro Jamapa) common-bean plants subjected to high Al levels for short periods showed decreased root length as well as characteristic symptoms of AIT, such as ROS accumulation, callose deposition, lipoperoxidation and cell death in roots.

Along other regulators, plant response to metal toxicity involves also microRNAs (miRNA) as part of the regulatory mechanisms. These molecules are a class of non-coding small RNAs of about 21 nucleotides in length, regulating gene expression at post-transcriptional level, guided by sequence complementarity, inducing cleavage or translational inhibition of the corresponding target transcript (Rogers and Chen, 2013). The relevance of miRNA regulation in heavy metal tolerance is well documented; it has been demonstrated that heavy metal-responsive miRNAs show differential expression according to the toxicity level. Target genes of these miRNAs generally encode transcription factors that transcriptionally regulate networks relevant for the response to heavy metals. Additionally these encode transcripts for proteins that participate in metal absorption and transport, protein folding, antioxidant system, phytohormone signaling, or miRNA biogenesis and feedback regulation (Ding *et al.*, 2020). High-throughput small RNA sequencing analyses have identified miRNAs that respond to AIT in roots of different plants species, however their function in response to AIT is largely unknown. Some of the target genes cleaved by AIT-responsive miRNAs encode disease resistance proteins, transcription factors or auxin signaling proteins (Huang *et al.*, 2018, Song *et al.*, 2019). Our previous research indicated that *P. vulgaris* is no exception to this phenomenon. We identified common-bean miRNAs that respond to Al, these include conserved miRNAs that are Al-responsive in other plant species -i.e. miR319, miR390, miR393- and also miR1511 (Mendoza-Soto *et al.*, 2015, Huang *et al.*, 2018, Song *et al.*, 2019).

miRNAs from the miR1511 family have been identified in non-legume plants like strawberry (*Fragaria vesca*) (Šurbanovski *et al.*, 2016) and poplar tree (*Populus trichocarpa*), although in the latter its nature as a miRNA has been discussed as it has been considered as part of a retrotransposon (Klevebring *et al.*, 2009). Regarding legumes, miR1511 has been identified in *Medicago truncatula* (Formey *et al.*, 2014) and soybean (Luo *et al.*, 2012, Dong *et al.*, 2013). Also, miR1511 was identified in Mesoamerican common-bean cultivars, being more abundant in flowers and roots (Valdés-López *et al.*, 2008, Peláez *et al.*, 2012). However, this miRNA was not identified when analyzing the Andean G19833 reference genome (Formey *et al.*, 2015). Genetic variation in *MIR1511* has been reported in a comparative genotyping analysis of different Asian accession of domesticated soybean (*G. max*) as well as its wild type progenitor *Glycine soja*. While sequences of mature miR1511 and miR1511* were found in *G. max* accessions, the sequences of annual wild *G. soja* showed insertion/deletion (InDels) in the stem-loop region of *MIR1511* that included complete or partial deletions of mature miR1511 sequence (Htwe *et al.*, 2015).

Updated research indicates that the miR1511 target gene is not conserved in the different plants where it has been identified. In strawberry, the miR1511 targets an LTR retrotransposon gene (Šurbanovski *et al.*, 2016). Inconsistencies about the nature of miR1511 target gene also hold for legume species. For instance, different targets have been proposed for soybean ranging from genes coding for proteins involved in the

regulation of nitrogen metabolism (Dong *et al.*, 2013) to proteins relevant in plant cell development (Luo *et al.*, 2012). While in other species such as *M. truncatula* target genes have been searched but have not been identified. The *SPILL* transcript has been proposed as the common-bean miR1511 target (Arenas-Huertero *et al.*, 2009, Mendoza-Soto *et al.*, 2015), however despite several efforts from our and other groups this prediction could not be experimentally validated. These results suggested a species-specific selection of the corresponding target thus it was essential to experimentally validate the nature and possible function of the miR1511 target gene in common bean. Recent analyses led us to predict an ABC-2-type transporter-related gene, annotated as *Aluminum Sensitive Protein 3 (ALS3)*, as the target for miR1511. In this work we present its experimental validation. In addition, we genotyped *MIR1511* in ecogeographically different common-bean cultivars and investigated the role of miR1511 and its corresponding target in the regulation of plant response to AIT.

RESULTS

MIR1511 display genotypic variations among Phaseolus species

The comparison of *MIR1511* sequence from BAT93 (Mesoamerican) vs. G19833 (Andean) *P. vulgaris* reference sequences showed a 58-bp deletion in the G19833 genotype. Such deletion comprised around 57% of pre-miR1511 sequence and included 7-bp (35%) and 10-bp (50%) of mature and star miR1511, respectively (Figure 1a).

To explore this phenomenon at a larger scale within the *Phaseolus* genus, we analyzed Genotyping-By-Sequencing (GBS) data from 87 genotypes originated from a single genetic population (without gene flow between genetically distinct population), called non-admixed genotypes. These included genotypes from three *Phaseolus* species (*P. vulgaris*, *P. coccineus* and *P. dumosus*) and different populations of wild *P. vulgaris*: three populations from the Mesoamerican (MW1, 2 and 3), one from the Andean (AW), and one from the Northern Peru–Ecuador (PhI) gene pools (Ariani *et al.*, 2018) (Table S1). All the genotypes belonging to the Andean gene pool (AW) and part of the Mesoamerican genotypes displayed a truncated *MIR1511*, in contrast to the Northern Peru–Ecuador (PhI) genotypes and the other *Phaseolus* species that presented a complete version of the *MIR1511* in their genome. A population clustering of *P. vulgaris* genotypes confirmed these results and showed that in the three Mesoamerican populations only a part of the MW1 cluster presented the *MIR1511* deletion (Figure 1b).

Thanks to GPS coordinates associated with the origin of each accession, we determined the geographical distribution of the *Phaseolus* genotypes analyzed. As shown in Figure 2, genotypes with a truncated *MIR1511* are located at the North and South ends (North Mexico and South Peru / Bolivia / North Argentina) of the wild common-bean geographical distribution, whereas the genotypes with a complete *MIR1511* are localized between these two groups.

miR1511 induces ALS3 transcript cleavage specifically in Phaseolus species

Predicted target genes for *P. vulgaris* miR1511 include *SP1L1-like* (Phvul.004G038000) (Phytozome 12, annotation v2.1) and isopentyl-diphosphate delta-isomerase (Phvul.008G054025), previously reported (Arenas-Huertero et al. 2009), and a protein with unknown function (Phvul.002G164100) and the *Aluminum Sensitive Protein 3* (*ALS3*, Phvul.001G172900), from our recent bioinformatic analysis. From these predicted targets, *ALS3* is the only one possibly related to ALT, as reported for Arabidopsis (Larsen et al., 2005), and showing an adequate binding-site penalty score (less than 5), thus the 5'RLM-RACE assay was used to experimentally validate the *ALS3* mRNA cleavage site. As shown in Figure 3a, a significant number (five out of seven) independently cloned transcripts mapped to the predicted site of cleavage, between the nucleotides at positions 457 and 458 of the transcript, which corresponds to position 9 and 10 of the predicted miR1511 binding site, thus confirming a miR1511-induced degradation. The other two degradation events mapped to 7 nucleotides upstream and 17 nucleotides downstream of the miRNA-associated degradation site, suggesting random degradation. An additional action of miR1511 to induce translation inhibition of *ALS3* mRNA in common bean, cannot be excluded.

miR1511 target genes differ among plant species (Luo et al., 2012; Dong et al., 2013; Šurbanovski et al., 2016). In order to evaluate the specificity of the miR1511/*ALS3* regulatory node in common-bean, we analyzed the miR1511/*ALS3* binding site sequence alignment from eight model plant species, including five legumes, which contain a precursor gene of miR1511 in their genome (Figure 3b). Because of the deletion in *MIR1511* from the G19833 genotype, we used the mature miR1511 and the corresponding *ALS3* binding site sequences from the BAT93 Mesoamerican genotype, as representative of *P. vulgaris*. Among plant species analyzed, *P. vulgaris* was the only one that showed a binding-site penalty score lower than 5, corresponding to a score recommended to consider a small

RNA-target binding as probably functional (Jones-Rhodes and Bartel, 2004)). For other species, the high penalty scores, ranging from 7.5 to 9, indicate a very low probability for the existence of a functional miR1511/ALS3 regulatory node (Figure 3b).

miR1511/ALS3 expression is modified by aluminum toxicity in common bean roots

A previous study about the response of different Andean and Mesoamerican common-bean cultivars to AIT showed that Andean genotypes are more tolerant to this abiotic stress, as compared to Mesoamerican genotypes (Blair *et al.*, 2010). Our phylogenetic analysis revealed that all the Andean genotypes present a deleted version of the *MIR1511* that would result in the absence of functional mature miR1511 (Figure 1). Previous work from our group showed that common-bean miR1511 expression responds to AIT stress (Mendoza-Soto, *et al.*, 2015). Here we analyzed the regulation of miR1511 and ALS3, as well as the early effects of AIT in roots of common-bean plants from the Mesoamerican BAT93 genotype vs. Andean G19833 genotype, with a deleted *MIR1511* (Figure 1).

Common-bean plantlets from BAT93 and G19833 genotypes were grown in hydroponic conditions either in control (nutrient sufficient) or AIT (70 μ M AlCl₃) treatments, for up to 48 hrs. First, we performed the expression analysis of miR1511 and *ALS3* target gene, using qRT-PCR (Figure 4). In AIT-stressed BAT93 plants, the transcript accumulation level of mature miR1511 gradually decreased, reaching more than half at 24 hours post-treatment (hpt), while at 48 hpt it returned to values close to those of time 0 (Figure 4a). As expected, G19833 plants did not express mature miR1511 (Figure 4a). The transcript level of *ALS3* target gene increased in AIT treatment. The *ALS3* transcript accumulation was significantly higher in G19833 roots, which lack miR1511, compared to BAT93 roots (Figure 4b). At 6 hpt, *ALS3* expression in G19833 roots almost doubled and remained unchanged up to 48 hpt, when transcript accumulation in BAT93 and G1988 roots reached similar levels (Figure 4b).

miR1511/ALS3 node regulates plant root growth in aluminum toxicity condition

To further study the role of miR1511/ALS3 in the physiological reaction of common-bean plants to high Al levels, we aimed to overexpress the miR1511 precursor in transgenic roots. As long as stable transformation of *Phaseolus vulgaris* plants is, to date, nearly impossible (very low

frequencies using particle bombardment-mediated transformation of meristematic tissues (Kim and Minamikawa, 1996)), we chose to use BAT93 and G19833 composite plants -with untransformed aerial organs and transgenic roots (Estrada-Navarrete *et al.*, 2009). As long as common bean is recalcitrant to stable transformation, this method is an alternative to demonstrate miRNA functionality (Formey *et al.*, 2019). The miR1511-overexpressing (OEmiR1511) composite plants as well as control plants, transformed with empty vector (EV), were grown in ALT and control treatments. The expression level of miR1511 and *ALS3* were determined by qRT-PCR in roots from composite plants harvested at 48 hpt (Figure 5). A two-fold accumulation of miR1511 transcript was observed in BAT93 OE1511 roots from plants grown in either treatment, compared to EV (Figure 5a). In G19833 EV roots, the absence of miR1511 was confirmed, however a significant accumulation of miR1511 mature transcript was observed in OE1511 roots, albeit at lower levels than the level from BAT93 OEmiR1511 roots (Figure 5a). In control treatment, both genotypes showed lower expression level of *ALS3* in OEmiR1511 vs. EV roots. In addition, increased *ALS3* transcript levels were observed in ALT stressed roots from both genotypes, as compared to control treatment (Figure 5b).

The primary and earliest symptoms of plants subjected to ALT stress is an inhibition of lateral root formation and root growth due to the alteration of root cell expansion and elongation, inhibiting cell division (Frantzios *et al.*, 2001). On this basis, we analyzed the root architecture phenotype of BAT93 and G19833 OEmiR1511 and EV plants, grown under ALT or control treatments for 48 h (Figure 6). The growth rate of root length, width and area as well as the number of lateral roots, was calculated from the difference of each value at 48 hpt vs. time 0. The BAT93 EV plants under ALT showed decreased rates (*ca.* 50%) of each root parameter (Figure 6a-d), thus indicating the drastic effect of high Al on root development. In contrast, G19833 EV plants showed higher tolerance to ALT evidenced by similar rate of the root length, area, width and lateral root number in stress vs control treatments (Figure 6a-d). These results are in agreement with those previously reported indicating a higher tolerance to ALT of Andean common-bean genotypes compared to Mesoamerican genotypes (Blair *et al.*, 2010). Surprisingly, in G19833 plants (OEmiR1511) genetically engineered for the expression of mature miR1511, the effect of root phenotype was evident. The rate of root length, area, width and lateral root number of G19833 OEmiR1511 ALT-stressed plants significantly decreased as

compared to EV plants, showing reduced levels similar to those from BAT93 stressed plants (Figure 6 a-d).

Expression of ALS3-epistatic gene LPR1 is modulated by the miR1511/ALS3 node

In *A. thaliana*, primary root growth inhibition under phosphate limitation or ALT is mediated by ALS3 and LPR1, a ferroxidase (Ruíz-Herrera *et al.*, 2013). LPR1 acts downstream of ALS3 and its expression is epistatic to *ALS3* expression (Dong *et al.*, 2017). To determine if LPR1 could be involved in the different response to ALT of BAT93 vs. G19833 plants, we measured the accumulation of *LPR1* transcripts in similar ALT conditions as those from Figure 4. The transcript level of *LPR1* gene decreased in ALT treatment. In ALT BAT93 roots, the transcript level of *LPR1* gradually decreased reaching half of the initial expression at 48 hpt. In ALT G19833 roots, the *LPR1* expression was significantly lower compared to BAT93 roots from 6 hpt to 24 hpt (Figure 7a). At 48 hpt, *LPR1* transcript reached similar levels in roots from both genotypes (Figure 7a). The LPR1 expression pattern was opposite to the *ALS3* expression profile in ALT-stressed roots (Figures 4a and 7a), indicating an epistatic relation between these two genes and the possible participation of LPR1 together with ALS3 in the control of common-bean root growth under ALT.

In order to determine if miR1511 indirectly controls *LPR1* expression *via* the regulation of *ALS3* transcript, we evaluated the *LPR1* transcripts accumulation in transgenic roots from OEmiR1511 and EV (control) composite plants, growing in Alt vs control conditions. In both BAT93 and G19833 roots, a significant increase of *LPR1* transcript accumulation was observed in OEmiR1511 roots from plants grown in either treatment, compared to EV roots (Figure 7b). In ALT treatment, roots from both genotypes showed significant lower *LPR1* transcript level compared to roots from control condition. Again, *LPR1* expression pattern was the opposite compared to that of *ALS3* in the same transgenic root samples (Figure 5b), thus indicating the probable epistatic relationship between these two genes and the indirect regulation of miR1511 on *LPR1* expression.

DISCUSSION

Common bean MIR1511

In plants, microRNA genes have a higher birth and death rates than protein-coding genes (Nozawa *et al.*, 2012). For various authors, the miRNAs' evolution rate generates a reservoir of adaptability for gene regulation (Fahlgren *et al.*, 2007, Axtell *et al.*, 2011). Due to this high evolutionary turnover rate, new miRNA families and members emerge, while others lose their regulatory role and disappear from genomes of phenotypically close species or genotypes. In soybean, *MIR1511* is subjected to this mechanism. Htwe *et al.*, (2015) reported two altered versions of *MIR1511* alleles (a 147 bp deletion including the miR1511 complete mature sequence and a 71 bp deletion that includes parts of both the mature miR1511 and the miR1511* sequences) in some annual wild soybean (*G. soja*) genotypes, whereas no deletion was found in *G. max* and perennial wild soybean *MIR1511*. Here, we report a similar phenomenon for *P. vulgaris MIR1511* genotypic variations. Only part of the MW1 subgroup of *P. vulgaris* Mesoamerican genotypes and all the Andean genotypes analyzed displayed a 58 bp-deletion in the miR1511 precursor gene compared to the corresponding sequence of *P. dumosus*, *P. coccineus*, the PhI gene pool and the rest of *P. vulgaris* Mesoamerican genotypes (Figure 1). As *MIR1511* is present in non-legume species, the most parsimonious hypothesis to explain the evolution process associated with this event is to consider a deletion of part of miR1511 precursor sequence. In contrast to soybean, where probably two deletion events were required for the generation of two alternative *MIR1511* alleles, our results suggest a single deletion event in the common ancestor of a part of MW1 Mesoamerican subgroup and the Andean genotypes for the generation of a different allele of miR1511 precursor gene. This single *MIR1511* deletion event hypothesis supports the Mesoamerican model proposed by Ariani and colleagues (2018) where the Mesoamerican gene pool is the ancestral population from which the other gene pools (PhI and AW) have derived. The fact that the PhI gene pool contains an untruncated version, as do the other closely-related *Phaseolus* species included in this analysis, further confirms the sister-species status of the PhI gene pool, now known as *P. debouckii* (Rendón-Anaya *et al.* 2017a & b). *P. debouckii* also contains ancestral, *i.e.*, non-derived, sequences for phaseolin seed protein and chloroplast DNA (Kami *et al.*, 1995; Chacón-Sánchez *et al.*, 2005, 2007). Based on the *MIR1511* phylogenetic history presented here (Figure 1), we propose an *addendum* to this model where AW gene pool genotypes derived from one, or more, member(s) of the MW1 Mesoamerican subgroup.

A clear distinct geographical distribution pattern was observed among the *P. vulgaris* genotypes featuring the *MIR1511* deletion and the ones with an unaltered allele (Figure 2). *MIR1511* deletion occurred in genotypes originating from the northern and southern extreme limits of the common-bean distribution in Latin American area. Such distribution pattern correlates with the annual precipitation pattern reported for the American continent (Ariani *et al.*, 2018, Harris *et al.*, 2020), indicating that genotypes with *MIR1511* deletion originated from areas with significantly less precipitation as compared to areas where genotypes with unaltered *MIR1511* originated (Ariani *et al.*, 2018, Berny Mier y Teran *et al.*, 2020, Harris *et al.*, 2020). Drought makes soil not suitable for agriculture; it tends to increase soil concentration of different compounds that would result in plant toxicity, including aluminum toxicity, which is an important plant growth-limiting factor (Schier and McQuattie, 2000, Ramankutty *et al.*, 2002, Kochian *et al.*, 2004, Yang *et al.*, 2012). The harsh soil conditions of areas where *P. vulgaris* genotypes lacking of *MIR1511* originated probably forced these common-bean populations to adapt and favored selection of genotypes with higher AlT tolerance.

In this work, we showed the experimental validation of a target gene for *P. vulgaris* miR1511. We validated the miR1511-induce cleavage of *ALS3* transcript, an ABC transporter participating in Al detoxification in plants (Figure 3). However, additional action of miR1511 by translation repression of *ALS3* cannot be excluded. Other proposed target genes for *P. vulgaris* miR1511 are not related to plants AlT response and show high binding-site penalty score, thus improbable to be considered as functional in the AlT response.

Here we provided evidence of the role of the miR1511/*ALS3* node in the common-bean response to AlT (Figures 4-7). We interpret that the *MIR1511* deletion resulting in lack of mature miR1511 allowed common-bean adaptation to high Al in the soils by eliminating the negative regulation of *ALS3* transcript and the accumulation of LPR1, in the first 48 hpt, thus increasing its tolerance to AlT and favoring plant growth. Interestingly, similar characteristics hold for the soybean *MIR1511*-deletion case where the origin of soybean genotypes featuring a *MIR1511*-altered allele is geographically correlated with areas susceptible to high Al concentration in soil due to presence of drought in these regions (IGBP-DIS-SoilData, 1998, Huang and Li, 2011).

Regulatory role of miR1511 in aluminum toxicity

High aluminum levels in soil mainly affect plant roots; aluminum can be allocated to different subcellular structures thus altering the growth of the principal root and the number of lateral roots (Larsen *et al.*, 2005, Zhang *et al.*, 2018). In this sense, it has been observed that AlT-stressed plants favor the transport of chelated Al to vacuoles and from roots, through the vasculature, to aerial tissues that are less sensitive to Al accumulation (Ma *et al.*, 1997, Larsen *et al.*, 2005, Dong *et al.*, *et al.* 2017). In Arabidopsis and other plants, ALS1 and ALS3, from the ATP-binding cassette transporter family, are involved in Al detoxification and enhance tolerance to AlT (Xia *et al.*, 2010, Hwang *et al.*, 2016, Bojórquez-Quintal *et al.*, 2017). ALS3 is located in the tonoplast, the plasma membrane of root cortex epidermal cells, and in phloem cells throughout the plant (Ryan *et al.*, 2011, Bojórquez-Quintal *et al.*, 2017). It has been shown that Arabidopsis *als3* mutants are more sensitive to AlT exhibiting extreme root growth inhibition, compared to wild type plants (Larsen *et al.*, 2005). Recent studies on the role of Arabidopsis ALS3 in root growth inhibition revealed its regulation via the inhibition of the STOP1-ALMT1 and LPR1 pathways, which indirectly control ROS accumulation in roots via the modulation of Fe accumulation (Wang *et al.*, 2019). Furthermore, Arabidopsis *ALS3* expression is induced by excess Al (Eticha *et al.*, 2010), a phenomenon we observed in common-bean plants as well (Figure 4, 5). Common-bean *ALS3* expression doubled after 6 hours under AlT in roots from G19833 (Andean) plants, while in stressed roots from BAT93 (Mesoamerican) plants a similar level was reached only after 48 h of treatment (Figure 4). The opposite expression profile was found for the *ALS3*-epistatic gene *LPR1*, in the same samples (Figure 7a). Our data on the different *ALS3* and *LPR1* expression level from both genotypes indicate that the absence of the negative regulator miR1511 in G19833 plants allows a faster response to AlT. Although the level of mature miR1511 decreased in BAT93 roots up to 24 h of after exposure to high Al, this level seems sufficient to induce degradation of *ALS3* transcript, which showed reduced levels compared to G19833 roots, and an increase of *LPR1* expression (Figure 4 and 7).

Our analysis of root architecture (root elongation, width and area and number of lateral roots) in common-bean plants showed that G19833 Andean genotype plants are more tolerant to AlT as compared to Mesoamerican BAT93 plants (Figure 6). These data agree with those reported by Blair *et al.* (2010). The miR1511 overexpression in transgenic BAT93 roots increased the root growth sensitivity to Al and, moreover, an increased sensitivity to AlT was observed in G19833 composite

plants engineered for miR1511 expression (Figure 6). These data support the hypothesis that miR1511 induces degradation of *ALS3* transcripts thus delaying the adequate root response to ALT stress. Therefore, absence of miR1511 resulting in diminished *ALS3* transcript degradation appears to be an evolutionary advantage to Al contamination in soils, leading to an inhibition of the LPR1 pathways, a faster relocation of chelated Al to vacuole and Al-tolerant aerial tissues and a lesser effect on root growth, a phenomenon that partially explains why *P. vulgaris* Andean genotypes are more resistant to ALT than Mesoamerican ones (Figure 8) (Blair *et al.*, 2010).

Overall, the current results about ALT in arid environments, combined with previous results by other authors, illustrates the complexity of adaptation to drought conditions. Tolerance of these conditions encompass mechanisms of growth and development like root depth and reshaping of the root profile, persistent growth despite drought conditions (Berny Mier y Teran *et al.*, 2019a), continued translocation of photosynthesis from pod walls into seeds resulting in a high pod harvest index (Klaedtke *et al.*, 2012, Polania *et al.*, 2016, Berny Mier y Teran *et al.*, 2019b), and Al detoxification under unfavorable edaphic conditions (current results). In turn, this knowledge helps designing and interpreting experiments in the introgression of genetic diversity for drought tolerance from wild to domesticated common-bean (Berny Mier y Teran *et al.*, 2020) and breeding drought tolerant common bean, in general (Beebe *et al.*, 2008, Hoyos-Villegas *et al.*, 2017, Mukankusi *et al.*, 2019).

In conclusion, our study reports an original case of the gene evolution of a single MIRNA the *MIR1511*, within *Phaseolus vulgaris* and close relatives, allowing adaptation to the aluminum toxicity abiotic stress.

EXPERIMENTAL PROCEDURES

Phylogenetic and geographic analysis of Phaseolus accessions.

Phaseolus vulgaris G19833 and BAT93 *MIR1511* sequences were obtained from the *Phaseolus vulgaris* release v2.1, from Phytozome 12 database, and from NCBI Whole Genome Shotgun database (accession number LPQZ000000000), respectively. Sequences for the phylogenetic tree display in Figure 1 were obtained from the SNPhylo analysis performed by Ariani *et al.* (2018), based on the collection of representative SNPs located in sequences at least 5 kb from an annotated

feature (presumably neutral variants). Only sequences from non-admixed genotypes were selected for the analysis (Table S1), constituting a total of 87 sequences from three *Phaseolus* species (*P. dumosus*, *P. coccineus* and *P. vulgaris*), including three populations for the Mesoamerican (MW1, 2 and 3), one for the Andean (AW), and one for the Northern Peru–Ecuador (PhI) *P. vulgaris* gene pool. Sequence alignment was performed thanks to MUSCLE algorithm (Edgar, 2004). Phylogenetic tree was inferred using the Maximum Parsimony method with 1000 bootstrap replicates. Accession PI430191 (*P. coccineus*) was used as outgroup for rooting the phylogenetic tree. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

MIR1511 deletion analysis

GBS sequence reads from the 87 selected genotypes were preprocessed as previously described (Ariani *et al.*, 2018) and mapped to the BAT93 *MIR1511* sequence using the BWA-MEM algorithm as described by Ariani *et al.* (2018). *MIR1511* was considered complete if at least one read was mapped to the miR1511 mature sequence and at least another read mapped to another part of the miR1511 precursor sequence. When at least one read was mapped to another part of the miR1511 precursor sequence and none on the miR1511 mature sequence, *MIR1511* was considered deleted.

For further supporting this analysis we performed an additional validation for the samples with a complete BAT93 *MIR1511* sequence. In brief, the reads aligning to the BAT93 *MIR1511* sequence were re-mapped to the G19833 reference genome. Alignment results showed a partial alignment to these reads in the homologous region containing the truncated *MIR1511* in the G19833 reference, up to the deletion visible in Figure 1a (i.e. where the mature miR1511 sequence is truncated in G19833).

The common-bean distribution map was designed thanks to HamsterMap using the GPS coordinates from each genotype (Tables S1), available from the Genetic Resources Unit of the Centro Internacional de Agricultura Tropical (<https://genebank.ciat.giar.org/genebank/language.do?collection=bean>).

miR1511 Target Gene Prediction and Experimental Validation

The miR1511 target gene candidates were determined using default parameters of the prediction-algorithm softwares: psRNATarget (<http://plantgrn.noble.org/psRNATarget/analysis>) (Dai

et al., 2018) and WMD3 version 3.1 (<http://wmd3.weigelworld.org/>) (Ossowski *et al.*, 2009). Based on its lowest psRNATarget score, the *ALS3* gene was selected as the best candidate. To experimentally validate the cleavage site of *ALS3* target transcript we used a modified 5'RLM-RACE approach. Total RNA (3 µg) isolated from BAT93 roots using mirVana™ miRNA Kit (Ambion) was subjected to 5'RACE reaction using FirstChoice RLM-RACE kit (Ambion), omitting calf intestine alkaline phosphatase and tobacco acid pyrophosphatase treatments. Specific oligonucleotides designed downstream of predicted miR1511 cleavage site (Table S2) were used for PCR amplification. The nested PCR products were cloned into the vector pTZ57R/T using the InsTAcloning™ PCR cloning kit (Thermo Scientific) and verified by sequencing.

Analysis of miR1511/*ALS3* binding site evolution

ALS3 transcript (Medtr7g100160.1, Glyma.03G175800, Glyma.10G047100, Phvul.001G17290, Prupe.6G245600, Fve-mrna18124.1-v1, Cucsa.307440.1 and Lj1g3v4578730.1) and miR1511 mature sequences were obtained from *Medicago truncatula* Mt4.0v1, *Glycine max* Wm82.a2.v1, *Phaseolus vulgaris* v 2.1, *Prunus persica* v2.1, *Fragaria vesca* v1.1, *Cucumis sativus* v1.0 and *Lotus japonicas* MG20 v3.0 databases from Phytozome v 12 and *Lotus* Base (Mun *et al.*, 2016). Alignment and penalty score were determined thanks to psRNATarget (<http://plantgrn.noble.org/psRNATarget/analysis>; Dai, Zhuang, & Zhao, 2018) and the default parameters. For the evolutionary tree construction, virtual sequences simulating three types of base pairing (pairing, mismatch and G:U pairing) between miR1511 and *ALS3* transcripts, were generated for each species. Sequence alignment was performed thanks to MAFFT online service v7.467 (Kato *et al.*, 2017), with L-INS-i option set. Construction of evolutionary tree was based on the average linkage (UPGMA) method.

Plant Material and Growth Conditions

Seeds from the common bean (*P. vulgaris* L.) Mesoamerican cv BAT93 and Andean accession G19833 were surface sterilized in 10% (v/v) commercial sodium hypochlorite for 5-10 min and finally rinsed 5–6 times in sterile distilled water. Subsequently seeds were germinated on moist sterile paper towels at 30°C for 2-3 days in darkness. Plants were grown in hydroponic system under

controlled environmental conditions (25 - 28°C, 16h photoperiod) as previously described (Valdés-López *et al.*, 2010). For control treatment, the hydroponic trays contained 8 L of full-nutrient Franco & Munns solution (Franco and Munns, 1982). For the ALT stress treatment, plantlets were allowed to adapt to the hydroponic culture for 4 days, then the nutrient solution was supplemented with 70 µM AlCl₃. For both treatments the pH of the solution was adjusted to 4.5 and was controlled throughout the experiment. Common-bean composite plants with transgenic roots (Estrada-Navarrete *et al.*, 2007) were generated as described below and grown in similar control or ALT conditions as those described for un-transformed plants.

Plasmid construction and generation of composite plants

miR1511 overexpression in common-bean transgenic roots was performed using the pTDTO plasmid (Aparicio-Fabre *et al.*, 2013). The expression cassette is driven by the 35S cauliflower mosaic virus (35SCaMV) promoter. A red fluorescent protein (tdTomato) was used as transformation-efficiency reporter gene. Precursor of miR1511 (170-bp PCR product) was obtained using common-bean root cDNA as template and the specific primers, which are listed in Table S2. After purification, PCR products were digested by XhoI and BamHI and inserted into the pTDTO vector. The resulting OEmiR1511 plasmid, and the corresponding empty vector (EV), were introduced into *Agrobacterium rhizogenes* K599 by electroporation. Then, these transformed bacteria were used as vector of plant transformation, as described previously (Formey *et al.*, 2019).

RNA Isolation and Analysis

Total RNA was isolated from 100 mg 48 hpt root tissue using mirVana™ miRNA Isolation Kit (Ambion), following the manufacturer's recommendations. Three biological replicates, from plants grown under the same conditions, were analyzed. For mature miRNA quantification, cDNAs were prepared from total RNA (1 µg) that was polyadenylated and reversely transcribed using the NCode miRNA First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For mRNA detection, cDNA was first obtained with Superscript III Reverse transcriptase (Invitrogen) and oligo-dT for priming cDNA preparation. Ten-fold dilutions of resulting cDNAs were then used for qRT-PCR experiments, using SYBR Green qPCR Master Mix

(Thermo Fisher Scientific, Waltham, MA, USA). The reactions were analyzed using Applied Biosystem 7300 real-time thermocycler (Foster City, CA, USA). qPCR cycles were set as follows: 94°C for 1 min, then 40 cycles of 94°C for 20 s and 60°C for 60s. Two technical replicates were performed for each reaction. The “comparative Ct method” and normalization by geometrical mean of three housekeeping genes (*HSP*, *MDH* and *UBQ9*) (Vandesompele, *et al.*, 2002) and U6 sRNA, were used for relative accumulation level of mRNA transcripts and mature miRNAs, respectively. The sequences of primers used for PCR amplification are listed in Table S2.. To assess the significance of differential expression of the mean values from three biological replicates for each condition, Mann-Whitney statistical tests were performed .

Analysis of root architecture phenotype

The architecture of transgenic roots from composite plants under control or AIT treatments was analyzed by determining the growth rate of root length, width, and area, as well as the number of lateral roots formed, using the ImageJ software. For both treatments root measurements were done at the beginning of the experiment ($t=0$, control condition) and after 48 hrs of growth. As mentioned before, AIT treatment plants were first adapted to hydroponic culture in control treatment (for 4 days). They were then taken out from this culture to be quickly photographed -for subsequent root architecture analysis- and were introduced ($t = 0$) into a hydroponic culture under AIT treatment for 48 hpt to be harvested and photographed again. Data of growth rate of each parameter represent the difference of the values at 48 and 0 hpt. Each root architecture parameter was determined in transgenic roots from 10 to 15 composite plants for each treatment. Statistical analyses were performed using the Mann-Whitney null hypothesis statistical test.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

ACKNOWLEDGEMENTS

We are grateful to Marisel Lliteras and Víctor Bustos for technical assistance. This work was supported in part by Dirección General de Asuntos del Personal Académico (DGAPA) – UNAM

(Grants nos. IN200816, IN203819, IA202620) and Consejo Nacional de Ciencia y Tecnología (Grants nos. A1-S-22570 and A1-S-1629). JAM-R received a postdoctoral fellowship from DGAPA-UNAM.

AUTHORS CONTRIBUTION

JAMR, GH and DF designed the project and wrote the manuscript. JAMR, AE, AL, SIF and MR performed experimental validation. AA performed *in silico* analysis. PG helped edit and revise the manuscript. GH and DF supervised this project.

CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no competing interests.

SUPPORTING INFORMATION

Table S1. Information regarding the wild common-bean genotypes analyzed with GBS.

Table S2. Primer sequences for qRT-PCR.

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FIGURE LEGENDS

Figure 1. Distribution of *MIR1511* gene deletion among *Phaseolus vulgaris* genotypes.

(a) Alignment of G19833 (top) and BAT93 (bottom) *MIR1511* sequences showing the nucleotides where the miR1511 mature (pink) and star (orange) sequences map. Dashes: deletion of the corresponding nucleotide. Vertical bars: nucleotide conservation (b) Phylogenetic tree of the non-admixed *Phaseolus vulgaris* genotypes built using neutral variants. *Phaseolus coccineus* PI430191 was used as outgroup for rooting the phylogenetic tree. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Colored fragments of the outer circle represent the different *Phaseolus* populations, as indicated. The IDs of genotype accessions displaying a deletion in their *MIR1511* sequence are highlighted in yellow.

Figure 2. Geographic distribution of the *Phaseolus* genotypes analyzed. The map depicts part of Latin America with the borders between different countries. The GPS coordinates where different *Phaseolus* genotypes were collected are indicated by dots or stars, following a similar color code as that from Figure 1b. Dots: genotypes displaying a complete *MIR1511* allele. Stars: genotypes displaying a truncated *MIR1511* allele. Brown dots represent the genotypes from *P. dumosus*.

Figure 3. Analysis the miR1511 target gene, *ALS3*. (a) Alignment of miR1511 (bottom) and *ALS3* transcript sequence (top), including the predicted target site. Number at the end of the sequences indicates the coordinates of the corresponding sequence. Vertical arrows and associated numbers indicate the first nucleotide position of 5'RACE cloned sequences from *ALS3* transcript and the ratio of independent clones that mapped the site vs. the total (7) cloned sequences, respectively. Vertical bars: bp matches. (b) Evolutionary tree of alignments of binding sites between miR1511 and its target *ALS3*, encoded in different plant species. *Glycine max* 1 and 2 represent each of the duplicated *ALS3*

genes from *G. max* genome (Glyma.03G175800, Glyma.10G047100). Number at the ends of the sequences indicates the coordinates of the corresponding sequence. Vertical bars: bp matches. Dots: G:U binding. Penalty score (Jones-Rhoades & Bartel, 2004) and alignments were obtained from psRNATarget analysis of the miR1511/*ALS3* binding site on the corresponding species.

Figure 4. Expression analysis of miR1511 and *ALS3* target gene in roots of BAT93 and G19833 common bean plants under Al toxicity. Common bean plantlets were grown under ALT treatment and harvested at the indicated time points (hours post treatment, hpt). Expression levels of miR1511 (a) and *ALS3* target gene (b) were determined by qRT-PCR in roots from plants under ALT. Expression levels are based on Ct value, normalized with the expression of the housekeeping genes for miR1511 and *ALS3*, respectively. Values represent mean $\pm$ sd from three biological replicates and two technical replicates each. The Mann-Whitney null hypothesis statistical test is relative to the different mean gene expression between genotypes, at each time (* represent a p -value < 0.05).

Figure 5. Expression analysis of miR1511 and *ALS3* target gene in roots of BAT93 and G19833 composite plants that overexpressing miR1511. BAT93 and G19833 were transformed via *Agrobacterium rhizogenes* bearing the OEmiR1511 or empty vector (EV) vectors. Generated composite plants, with transgenic roots, were grown under control or ALT treatments. Expression level of mature miR1511 (a) and its target gene *ALS3* (b) were determined by qRT-PCR in roots harvested at 48 hpt. The expression level values were based on Ct value, normalized with the expression of the housekeeping genes for miR1511 and *ALS3* transcript, respectively. Values were normalized to the value from BAT93 EV plants in control condition. Values represent mean $\pm$ sd from three biological replicates and two technical replicates each, and bars with the same letter are not significantly different (p -value < 0.05) according to the Mann-Whitney null hypothesis statistical test.

Figure 6. Root phenotype of BAT93 and G19833 composite plants (OEmiR1511 vs. EV) grown under Al toxicity. a) Images of representative transgenic roots from BAT93 and G19833 composite plants (OEmiR1511 vs. EV) grown under ALT or control conditions. Scale bar = 1 cm. The growth rate of root length (b), area (c) and width (d) as well as the rate of number of lateral roots formed (e)

were determined in transgenic roots from plants grown on control or AlT treatments for 48 hpt. Values were normalized to the value from EV plants in control condition, from each genotype separately. Values represent mean $\pm$ sd from roots of ten to fifteen independent composite plants each. The Mann-Whitney null hypothesis statistical test with respect to control conditions (* p -value < 0.05 and ** p -value < 0.01).

Figure 7. Expression analysis *LPR1* gene in roots of BAT93 and G19833 plants and in miR1511-overexpressing composite plants. a) Common bean plantlets were grown in AlT condition, similar as in Figure 4. b) Composite plants with transgenic roots, transformed with OEmiR1511 or EV, were grown in AlT or control conditions, similar as in Figure 5. Expression levels of the *LPR1* gene were determined by qRT-PCR in roots harvested at the indicated time points. Expression levels are based on Ct value, normalized with the expression of housekeeping genes. Values represent mean $\pm$ sd from three biological replicates and two technical replicates each. For statistical analysis the Mann-Whitney null hypothesis statistical test was used. In a) the statistical test is relative to the different mean gene expression between genotypes, at each time (* represent a p -value < 0.05). In b) bars with the same letter are not significantly different (p -value < 0.05).

Figure 8. Proposed model for miR1511 root growth regulation in common bean. Left: Common bean plants were subjected to aluminum (Al) toxicity (high concentration of Al^{3+} ion). Right: Regulatory pathway exerted by miR1511 in roots from BAT93 (top) and G19833 (bottom) AlT plants (separated by a horizontal dotted line). Transcription of the *MIR1511* locus (), indirectly downregulated by high Al, and precursor processing occur in the nucleus and generates mature miR1511 in BAT93 and not in G19833 plants, due to a deletion in its *MIR1511* locus. Once exported to cytoplasm, miR1511 induces *ALS3* transcript cleavage decreasing *ALS3* protein, that sequesters Al^{3+} ions in vacuole and indirectly inhibits the accumulation of *LPR1* (Ruíz-Herrera *et al.*, 2013, Dong *et al.*, 2017). The *LPR1* ferroxidase enzyme participates in iron ion ($\text{Fe}^{2+}/\text{Fe}^{3+}$) homeostasis imbalance and accumulation of reactive oxygen species (ROS) that would affect root growth (Wang *et al.*, 2019). The indirect stimulation of *LPR1* activity *via* miR1511 in BAT93 genotype is

responsible for the root growth inhibition in the first 48 hours of ALT. This phenomenon is delayed in G19833 genotype due to the absence of functional miR1511. Positive regulation is represented by arrows and negative regulation by perpendicular bars. Solid and dashed lines represent direct and indirect regulations, respectively.

(a)

TCATTCGTGGTATCAGG-----CTCTGATACCATGATGCTTATTCCTCCCAACCCAA

|||||

TCATTCG**TGGTATCAGGTCCTGCTTCA**CCAAATGATCTTGAGTTCAACTCCTACACAAGCACTTGGTT**AACCAGGCTCTGATACCAT**GATGCTTATT-----CCCAA

BAT93

G19833

(b)

- *P. coccineus*
- *P. vulgaris* MW1
- *P. vulgaris* MW2
- *P. vulgaris* MW3
- *P. vulgaris* PhI
- *P. vulgaris* AW

 Truncated *MIR1511*

(a)

miR1511

(b)

ALS3

(a)

miR1511

(b)

ALS3

EV Control EV AIT OEmiR1511 Control OEmiR1511 AIT

(a)

(b)