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ORIGINAL ARTICLE

# ALTERED MERISTEM PROGRAM 1 promotes growth and biomass accumulation influencing guard cell aperture and photosynthetic efficiency in Arabidopsis

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## Abstract

ALTERED MERISTEM PROGRAM 1 (AMP1) encodes a putative glutamate-carboxypeptidase important for plant growth and development. In this study, by comparing the growth of Arabidopsis wild-type, *amp1–10* and *amp1–13* mutants, and *AMP1-GFP/OX2*- and *AMP1-GFP/OX7*-overexpressing seedlings in vitro and in soil, we uncover the role of AMP1 in biomass accumulation in Arabidopsis. *AMP1*-overexpressing plants had longer primary roots and increased lateral root number and density than the WT, which correlated with improved root, shoot, and total biomass accumulation. *AMP1*-overexpressing seedlings had an enhanced rate of growth of primary roots, and accordingly, sucrose supplementation restored primary root growth and promoted lateral root formation in *amp1* mutants, while reproductive development, fruit size, and seed content were also modified according to disruption or overexpression of *AMP1*. We further found that AMP1 plays an important role for stomatal development, guard cell functioning, and carbon assimilation. These data help explain the pleiotropic functions of AMP1 in both root and shoot system development, possibly acting in a sugar-dependent manner for regulation of root architecture, biomass accumulation, and seed production.

**Keywords** Stomata · Sugars · Plant biomass · Root architecture · Carbon fixation

## Introduction

The coordination of growth among the diverse plant organs is critical for adaptation to the environment and supports productivity. Despite the knowledge gained in the past two decades on the regulation of cell division and elongation, two cellular processes critical for sustained growth, only a very few genes and proteins have been found to orchestrate overall plant biomass production (Demura and Ye, 2010).

Plant biomass accumulation relies on carbon fixation via photosynthesis, which occurs in the green plant organs, mainly mature leaves, where sucrose is produced and exported to non-photosynthetic tissues such as stems and roots for growth (Roldán et al. 1999; Puig et al. 2012; Dimitrov and Tax, 2018). $CO_2$ fixation may lead to increases in the sugar pool that upon demand or according to day/night fluctuations may be used to produce starch, a storing carbohydrate, and a carbon resource (Graf et al. 2010; Graf and Smith, 2011; Azoulay-Shemer et al. 2018). Growth and development from germination to senescence is coordinated by sugar availability, metabolism, and energetic signaling. Defects in these processes impair growth, reduce shoot and root apical dominance, and affect flowering and seed production (Kircher and Schopfer, 2012; Yang et al. 2013; Yu et al. 2013; Mason et al. 2014; Chen et al. 2015).

$CO_2$ uptake occurs via specialized leaf epidermal structures called stomata, whose guard cells open or close depending

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upon environmental conditions to allow gas exchange (Brodribb and McAdam, 2011). Stomatal activity tightly depends on the stress hormone abscisic acid (ABA), which cross-talks with sugar biosynthesis and/or metabolism (Roelfsema and Prins, 1995; Cheng et al., 2002; Yang et al. 2017; Joshi-Saha et al. 2011; Kang et al. 2018). For instance, *glucose insensitive 6* (*gin6*) and *sugar insensitive 5* (*sis5*) Arabidopsis mutants are defective in genes allelic to *aba deficient 3* (*aba3*) and *aba insensitive 4* (*abi4*), respectively (Arenas-Huertero et al. 2000; Leon and Sheen, 2003).

ALTERED MERISTEM PROGRAM 1 (AMP1) encodes a putative glutamate carboxypeptidase, an integral membrane protein associated with endoplasmic reticulum (Helliwell et al. 2001). AMP1 has been implicated in different growth and developmental processes including dormancy, germination, flowering time, shoot and root growth, and seed production (Chaudhury et al. 1993; Vidaurre et al. 2007; Griffiths et al. 2011; Huang et al., 2015; Kong et al. 2015; López-García et al. 2016). This protein negatively regulates the HD-ZIP III transcription factors implicated in vascular tissue differentiation (Li et al. 2013; Müller et al. 2016) and affects the translation rate of miRNA targets (Li et al. 2013); however its specific biochemical role is unclear and also to which extent the phenotypic changes are caused by this misexpression of miRNA targets.

The pleiotropic phenotype of *amp1* mutants has been explained by alterations in hormonal homeostasis, mainly involving ABA and cytokinins (Chin-Atkins et al. 1996; Shi et al., 2013a, b; Yao et al. 2014). Regarding root growth, a link between AMP1 and ABA has been established since *amp1* mutants are hypersensitive to growth repressing effects of ABA on primary roots, and conversely AMP1-overexpression confers ABA resistance. Despite the described ABA-related function of AMP1, any possible relation with sugar metabolism is unknown. In this work, we uncover the critical function of this protein for overall plant biomass and seed production, stomatal aperture, and carbon fixation.

## Materials and methods

### Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 (Col-0), the mutant lines *amp1–10* (SALK_021406) and *amp1–13* (SALK_22988), and the transgenic lines *AMP1-GFP/OX2* and *AMP1-GFP/OX7* (Shi et al. 2013b) were used for the experiments. Seeds were surface sterilized with ethanol 96% and sodium hypochlorite 20% for 5 and 7 min, respectively, and then washed five times with 1-ml sterilized distilled water and kept 2 days at 4 °C. The seeds were plated on 0.2x solidified MS medium containing basal salts (Murashige and Skoog Basal Salts Mixture, Sigma-Aldrich, St Louis MO), 1% agar (Phytagar, Gibco-BRL), and 0, 0.6, or 4.8% sucrose (Sigma-Aldrich, St Louis MO) and placed into a plant growth chamber (Percival AR-95 L) at 22 °C with a photoperiod of 16 h light/8 h darkness under light intensity of 105 μmol/m-2/s-1.

### Growth analysis

Arabidopsis root systems were analyzed 6 days after germination (DAG) with a stereoscopic microscope (Leica MZ6). For soil experiments, plants at 10 DAG were transferred to pots and placed into a plant growth chamber to examine weekly overall growth, developmental transitions, and biomass accumulation, until plant life cycle completion. The siliques and seeds were collected and measured using a Leica MZ6 microscope.

### Stomatal analysis

Stomata number and aperture were assessed in 10 DAG seedlings grown on 0.2x MS medium, supplemented with the solvent or with 1 μM ABA overnight. Images were acquired using a confocal microscope (Olympus FV1200). The stomatal aperture was measured in the ImageJ program.

### Propidium iodide staining

For fluorescent staining with propidium iodide (PI), plants were transferred from the growth medium to 10 mg $mL^{-1}$ of PI solution for 1 min. Seedlings were rinsed with deionized water and mounted on microscope slides. The same sample was recorded separately at wavelengths specific to both PI fluorescence with a 568-nm excitation line and emission window of 585–610 nm and GFP emission with 500–523 nm emission filter (488-nm excitation line), using a confocal microscope (Olympus FV1200). Finally, the three images were merged.

### Detection of starch

For starch detection, plants were cleared and fixed with 0.24 N HCl in 20% methanol (v/v) and incubated for 60 min at 62 °C. The HCl solution was substituted by 7% NaOH (w/v) in 60% ethanol (v/v) for 20 min at room temperature. Then, plants were dehydrated with ethanol treatments at 40, 20, and 10% (v/v) for a 24-h period each and immersed in concentrated Lugol solution 1 min, washed with deionized water, and mounted on microscope slides. The samples were analyzed and photographed using a Leica DM500B microscope.

### Preparation of $CO_2$ traps

Seeds of WT, *amp1*, and *OX7* lines were germinated in MS 0.2x medium and transferred 2 DAG to divided Petri plates. In each side of the plate, the agar solidified 0.2x MS medium was supplied either with 0.6% sucrose or 0.1 M $Ba(OH)_2$ solution. The primary root length was analyzed 10 DAG.

## Results

### *AMP1* overexpression improves growth and biomass production of Arabidopsis seedlings in vitro

In a previous report, *amp1* mutant seedlings were found to produce short primary roots, which correlated with an altered response to ABA (López-García et al. 2016). To further clarify the phenotypical alterations arising upon AMP1 dysfunction, the growth and development of WT Arabidopsis (Col-0), the single mutant *amp1–10* (SALK_021406), and the transgenic line *AMP1-GFP/OX7* (Shi et al. 2013b) were compared in experiments in which seedlings from all three genotypes were grown side by side over the surface of Petri plates containing agar solidified 0.2x Murashige and Skoog (MS) medium. Consistently with a positive role of AMP1 in root growth, *AMP1*-overexpressing plants (*OX7*) had longer primary roots than the WT and *amp1* mutants and had increased lateral root number and density (Fig. 1a–c). A similar trend occurred when the root system architecture was compared among the WT and an additional mutant allele, namely, *amp1–13* and overexpression line *AMP1-GFP/OX2* (Supplementary Fig. S1). Root and shoot fresh weight determinations indicated that *OX7* seedlings grow faster and accumulate more shoot (Fig. 1d), root (Fig. 1e), and total biomass (Fig. 1f) than the WT and *amp1* mutants. These results indicate that AMP1 is critical for plant biomass accumulation.

### *AMP1* overexpression enhances root growth rate

The root length effects in the seedling stage are quite intriguing. However, in this context, it was helpful to resolve, to which extent the effect is driven by an altered germination behavior of the used lines, since *amp1* is ABA hypersensitive and *OX7* has been shown to be ABA resistant (Shi et al., 2013a, b). To clarify if the root growth effect might be to a significant extent mediated by altered timing of germination, we next determined the actual growth rate of the root, once it is fully emerged after germination. WT, *amp1–10*, and *AMP1-GFP/OX7* seeds were germinated, and at time of radicle protrusion, the primary root growth was measured daily during 8 days. The data show that *amp1–10* and *AMP1-GFP/OX7* lines have an opposite behavior, with reduced or enhanced growth rate, respectively, when compared to the WT (Supplementary Fig. S2). These data indicate that AMP1 plays a critical role in determining growth of the primary root.

### *AMP1* mutation or overexpression critically influences biomass accumulation in soil

*AMP1*-overexpressing seedlings develop a more robust root system that could efficiently take up water and nutrients and concomitantly may account to overall productivity. To test this possibility, the growth and development of the WT, *amp1–10*, and *AMP1-GFP/OX7* lines were analyzed in soil until life cycle completion. When compared to the WT, *amp1* mutants show decreased rosette and leaf sizes, flowered earlier, and had decreased apical dominance. On the other hand, *OX7* plants displayed an increased plant height, rosette, and leaf sizes (Fig. 2a–e). Reproductive development was also modified according to the AMP1 status, with the corresponding mutants producing shorter siliques with a reduced seed number compared to the WT, while *OX7* plants produced bigger fruits with much more seeds than the WT and heavier seeds (Fig. 3a–d). Thus, AMP1 regulates overall vegetative and reproductive development, which improves seed production.

### Sucrose supplementation restores primary root growth and promotes lateral root formation in *amp1* mutants

The reduced growth caused by loss-of-function of *AMP1* is similar to recently identified *MEDIATOR med12* and *med13* mutant phenotypes, which could be rescued by sugar supplementation (Raya-González et al. 2017). To test if sugars could support more growth in WT, *amp1*, and *OX7* seedling, sucrose was applied to agar drops, where the aerial tissues were placed and primary root growth and lateral root formation assessed later on. Primary root growth was induced from 1.2 to 4.8% sucrose in the WT, this induction being higher in *OX7* seedlings, which attained the maximum response from all three genotypes analyzed (Fig. 4a). Noteworthy, in *amp1* mutants, as the sugar levels increase, primary root growth and lateral root formation reached similar values to the WT (Fig. 4a–d). These data could be confirmed in experiments that analyzed primary root growth and lateral root formation in WT, *amp1–13,* and *AMP1-GFP/OX2*, where sucrose supplementation normalized root growth of *amp1–13* (Supplementary Fig. S3). These results suggest a novel function AMP1 in a sugar-dependent pathway for regulation of root architecture and biomass accumulation.

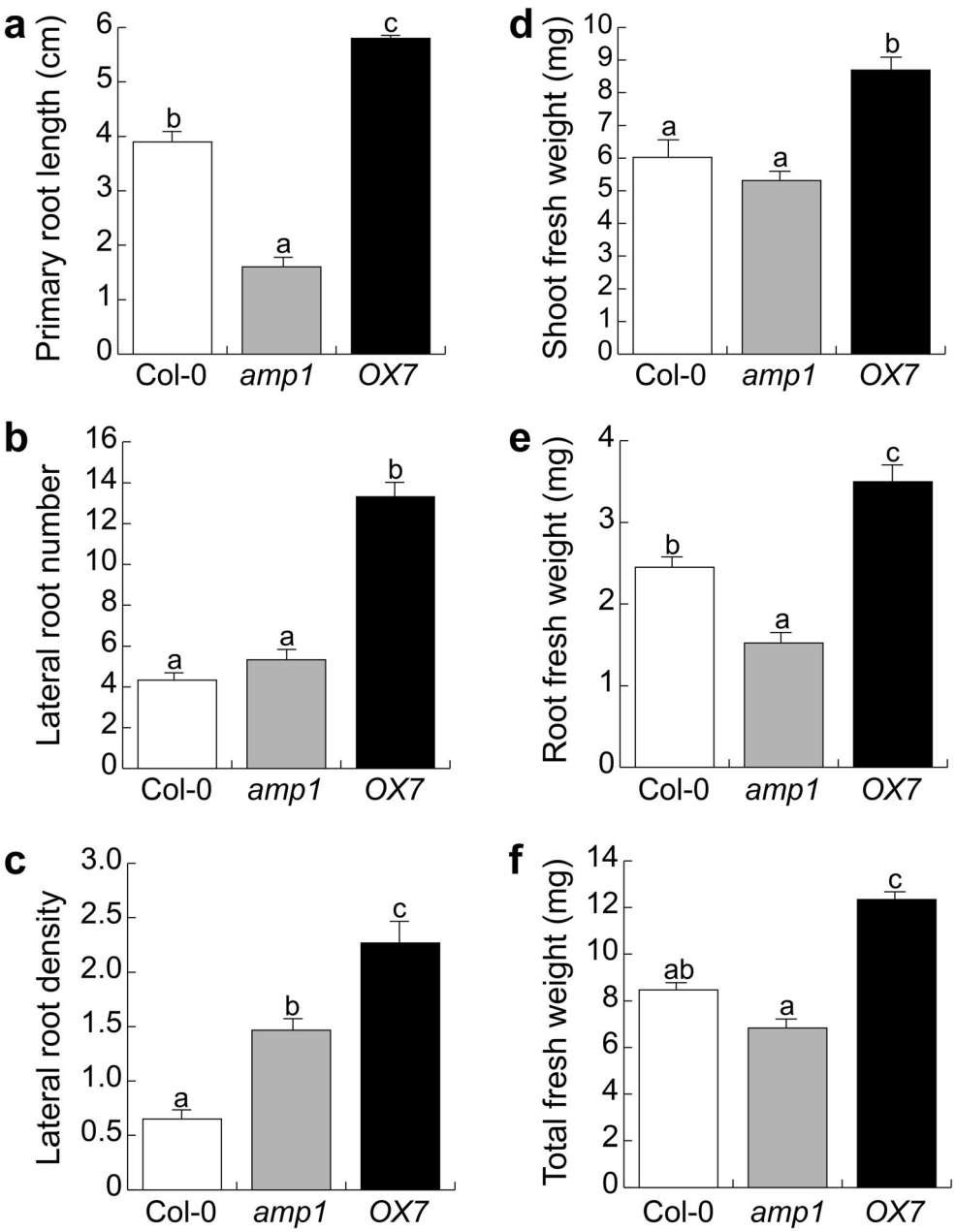


**Fig. 1** AMP1 influences root growth and biomass-related traits. Arabidopsis WT, *amp1–10* mutants, and *AMP1-GFP/OX7*-overexpressing seedlings were grown in vitro for 6 days and root architecture and biomass analyzed. (a) Primary root length, (b) lateral root number, (c) lateral root density, (d–f) shoot, root, and total plant fresh weight. Error bars represent SD from 20 seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results

## AMP1 is required for stomata development and mediates guard cell aperture in an ABA-dependent manner

To analyze a possible role of AMP1 in guard cells and stomata dynamics, we next quantified stomata and measured guard cell aperture with or without ABA in WT, *amp1*, and *OX7* seedlings. The number of stomata in *amp1* was lower than in the WT, while in *OX7*, it was increased (Fig. 5a). The stomatal aperture in *amp1* mutants is similar to the WT in control conditions, and ABA induces its closure in both plant genotypes. Interestingly, *OX7* seedlings had an increased guard cell aperture in control conditions irrespective of ABA treatments (Fig. 5b–c). Additionally, starch content increases in guard cells in *amp1* mutants and diminished in guard cells of *OX7* line when compared to the WT in control conditions, whereas ABA treatment induced starch accumulation in all three genotypes (Fig. 6). These data suggest that the stomatal development required AMP1, and at the same time, this gene mediates guard cell aperture and starch accumulation in an ABA-dependent manner.

## AMP1 modulates $CO_2$ uptake

The altered number of stomata and aperture dynamics suggests that photosynthetic activity could be modulated by

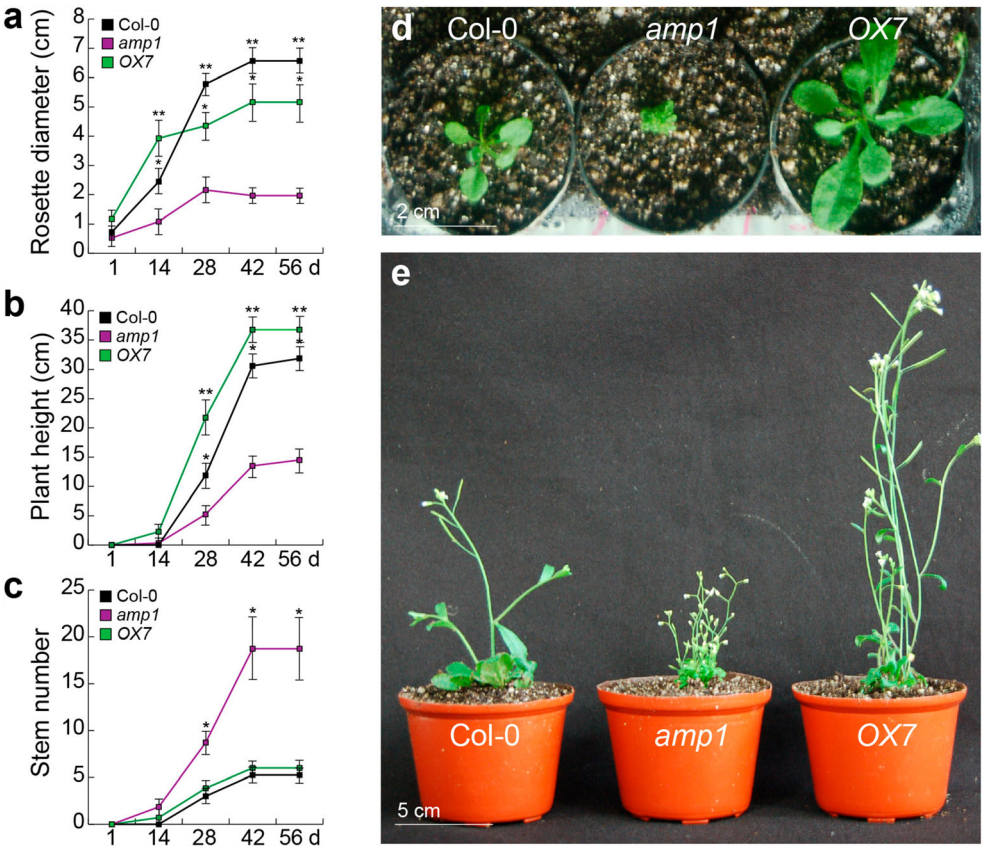


**Fig. 2** AMP1 affects rosette size, stem number, and plant height. Arabidopsis wild-type, *amp1–10* mutants, and *AMP1-GFP/OX7*-overexpressing seedlings were germinated and grown in MS 0.2x medium for 10 days and then transferred to soil pots to analyze growth and development during their life cycle. (a) Rosette size, (b) plant height, (c) stem number. Representative images of rosettes (d) and plants at reproductive stage (28 days) (e) showing the differences in soil phenotypes between the three lines. Error bars represent SD from 20 seedlings analyzed, and stars indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results

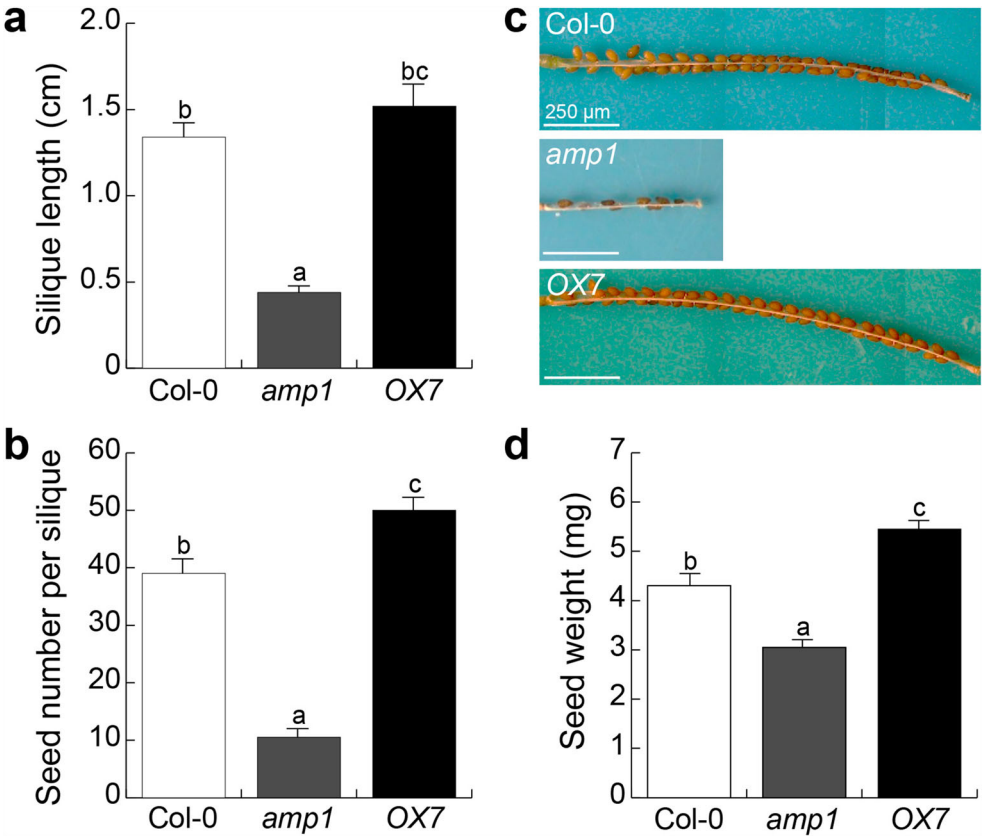


**Fig. 3** AMP1 regulates seed number and weight. Siliques of WT, *amp1–10*, and *OX7* lines were collected and dissected at maturity to analyze seed content. (a) Silique length. (b) Seed number per silique. (c) Representative images of opened mature siliques. (d) Seed weight. Error bars represent SD from 10 seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$

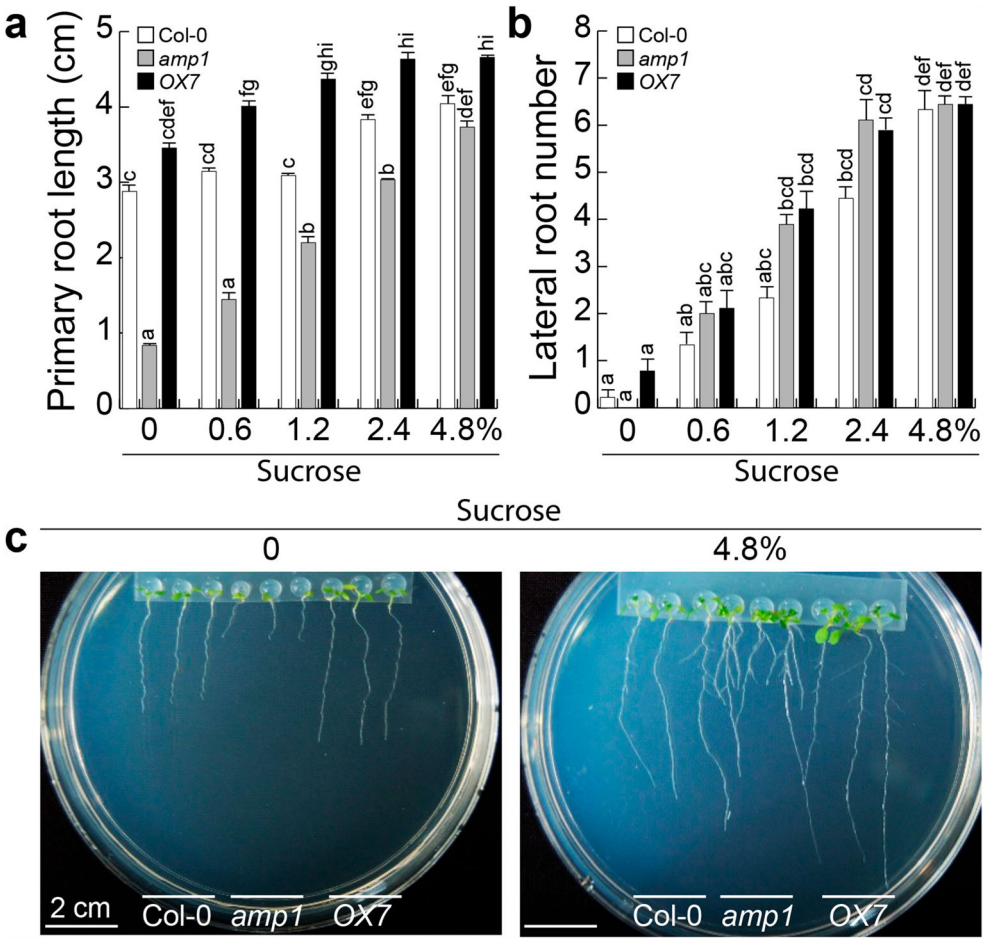


**Fig. 4** Sucrose supplements restore normal growth in *amp1* primary roots. Arabidopsis WT, *amp1–10* mutants, and *AMP1-GFP/OX7*-overexpressing seedlings were germinated and grown in MS 0.2x medium and 2 days after germination were transferred to fresh medium with the shoot placed over a drop of medium enriched with 0, 0.6, 1.2, 2.4, and 4.8% sucrose, and 4 days later, the primary root length (a) and lateral root number were analyzed (b). Representative images of seedlings grown side by side at 0 and 4.8% sucrose treatments are shown (c). Error bars represent SD from 36 seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results

AMP1. To examine the importance of $CO_2$ assimilation in the phenotype conferred by AMP1 modulation to plants, experiments with the $CO_2$ scavenger $Ba(OH)_2$ were performed in medium lacking sucrose or supplemented with 0.6% sucrose. Photosynthesis is determinant for root growth; thus, the primary root growth of WT, *amp1*, and *OX7* seedlings was compared under these conditions. Our results showed that primary root growth of plants from all three lines diminished with $Ba(OH)_2$ in medium without sucrose, with the greater inhibition observed in *OX7* seedlings (Fig. 7). In WT or *OX7* plants grown in 0.6% sucrose, $Ba(OH)_2$ inhibited the primary root in a similar fashion observed in plants grown without sucrose. These data evidenced the requirement of $CO_2$ to support the enhanced root growth in AMP1-overexpressing seedlings.

## Discussion

Plants integrate the energetic status in root and shoot systems to optimize growth and development. Sugars are translocated from the shoot to the root for active growth, and conversely roots provide mineral nutrients and water for optimal reproduction. Our detailed comparison of several traits in WT, *amp1*, and *OX7* seedlings suggested the critical role of AMP1 in overall plant fitness.

Following germination, the primary root adapts the plant to the substrate, explores the environment, and branches via de novo organogenesis, typically through formation of lateral roots. Both primary root growth and branching were promoted in *OX7* seedlings, and this correlates with more root and shoot biomass being accumulated. In response to nutrient deficiency, toxic pollutants, heavy metals, and/or biotic and abiotic stress, the primary root growth halts and instead the formation of lateral roots are stimulated, and this architectural readjustment is believed to help plants to avoid the stress imposed (Gaedeke et al. 2001; López-Bucio et al. 2002; Giehl et al. 2014; Verbon and Liberman, 2016; Veerappa et al. 2019). Noteworthy, only a very few papers have reported an increased branching potential without compromising growth of the main root axis as it certainly occurs in *OX7* seedlings. Thus, the positive correlation in root growth traits with superior plant biomass production was certainly expected and might be relevant toward future agricultural applications.

Photosynthesis occurs in mature leaves, also known as source organs that supply sugars to demanding or sink tissues, principally young leaves, flowers, and roots (Zakhartsev et al. 2016). The optimal sink to source

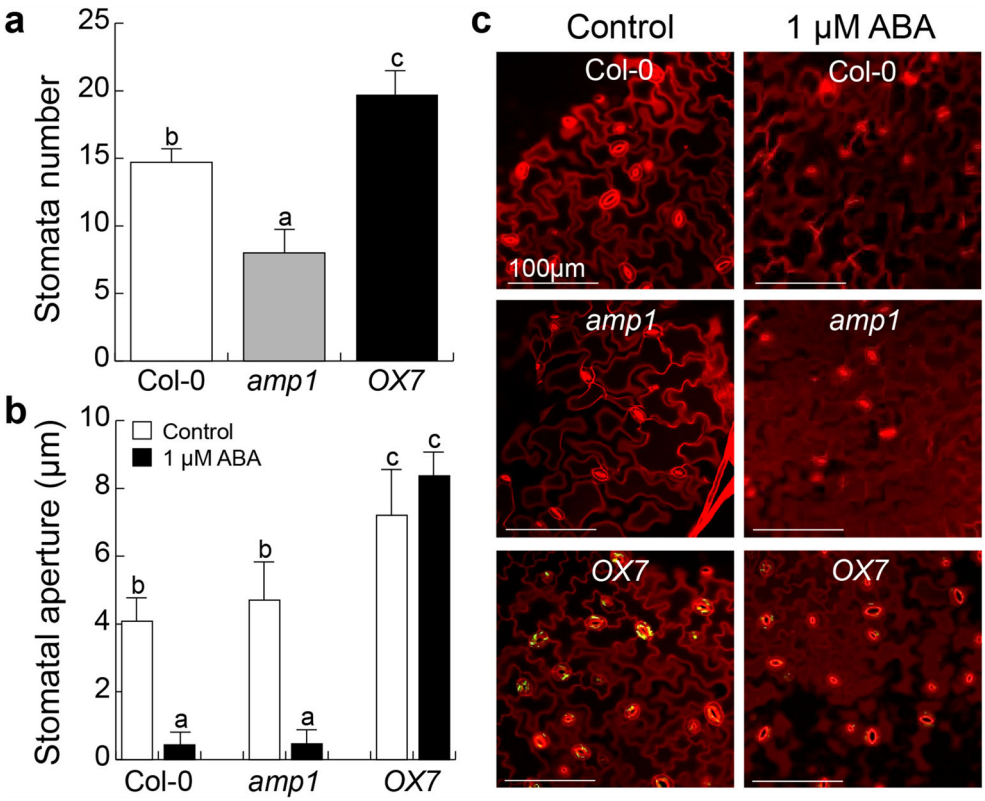


**Fig. 5** AMP1 determines stomatal functioning. Stomata number and guard cell aperture was analyzed in WT, *amp1*, and *AMP1-GFP/OX7* seedlings under standard growth conditions and in response to 1 μM ABA in 0.2x liquid medium overnight. (a) Stomata number. (b) Stomatal aperture with or without ABA supplementation. (c) Representative images of stomata in Arabidopsis leaves from plants treated or not with ABA. Error bars represent SD from 3 fields and 30 stomata analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results

relationships may help improve root growth and plant reproduction, two traits markedly affected in *amp1* mutants and improved in *OX7* overexpressing seedlings. Leaf and root growth is determined by genetic factors that drive cell proliferation and expansion, which rely upon an adequate supply of energy (Cookson et al. 2007; Hauben et al. 2009; Gonzalez et al., 2012). A correlation in the function of AMP1 with gibberellins (GA's) is possible, since the overexpressing lines of *GIBBERELLIN 20-OXIDASE 1* (*GA20OX1*) produced bigger leaves and blossom earlier (Huang et al. 1998; Gonzalez et al. 2010). Indeed, *amp1* was resistant to exogenous GA's application (Saibo et al., 2007) but oversensitive to ABA (López-García et al. 2016); such hormonal antagonism between ABA and GA's signaling may determine the pleiotropic functions of AMP1.

In soil both *amp1* and *OX7* lines had an early flowering phenotype, but at later times (28 days), rosette diameter of the *OX7* line decreased compared to the WT. This may be explained because stem height and number of stems increased with time, in particular stem number exacerbates after day 28 in the *OX7* line; thus, it is tempting to speculate that a change in biomass partitioning from rosette leaves occurred when stems develop their own leaves. Noteworthy, *amp1* mutants produced small and infertile flowers that yielded short siliques, in contrast to *OX7* plants that had bigger fruits with more seeds. This correlation may be explained because larger fruit contains either more or bigger seeds, and this was the case for *OX7* seedlings. *AMP1* mutation shortens the fruit length and the plant height, showing the opposite correlation between fruit length and seed content when compared to the WT and *OX7* line. Thus, AMP1 protein plays a fundamental role in fruit and seed harvest.

Through detailed analysis of *AMP1* mutation and overexpression in Arabidopsis, we unraveled its possible function in sugar production/metabolism. Plants with contrasting AMP1 levels had alterations in stomatal number and aperture, and this may lead to an increased or decreased gas exchange in the leaves, including $CO_2$ which is the main substrate for photosynthesis. Indeed, either *amp1* mutant plants or the *OX7* overexpressors had abnormal and contrasting amounts of starch in guard cells, supporting the idea that starch formation is modulated by AMP1. On the other hand, AMP1 prevented starch accumulation in guard cells in an ABA-dependent manner. The role of starch in guard cells and its relation with ABA signaling is a debatable topic; during starch synthesis, the stomata are closed in response to ABA (Santelia et al. 2011), but the opposite also occurs when starch is degraded to sugars and malate, since both act as osmotically active solutes and contribute to stomatal opening (Comparot-Moss et al. 2010; Fulton et al. 2008; Kötting et al. 2010; Graf et al. 2010). From our data, no correlation could be observed between stomatal aperture and starch accumulation in guard cells.

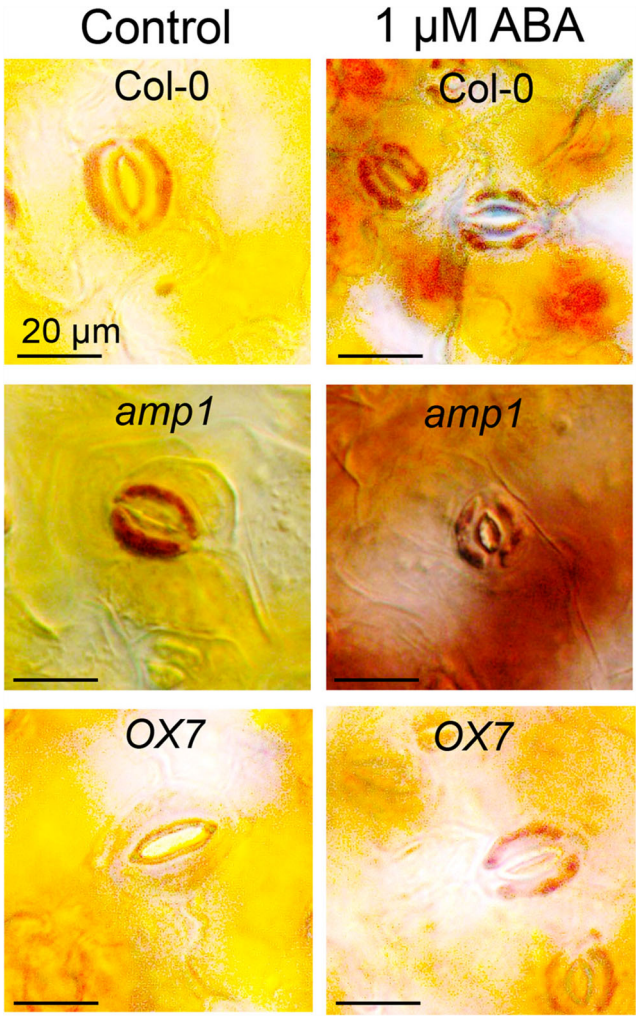


**Fig. 6** AMP1 influences starch accumulation in guard cells. Arabidopsis WT, *amp1–10* mutants, and *AMP1-GFP/OX7*-overexpressing seedlings were germinated and grown in MS 0.2x medium, and leaves were stained with Lugol to detect starch in guard cells in response to ABA treatment. Representative images from three independent fields on single leaves are presented, and at least six independent seedlings per line and treatment were analyzed

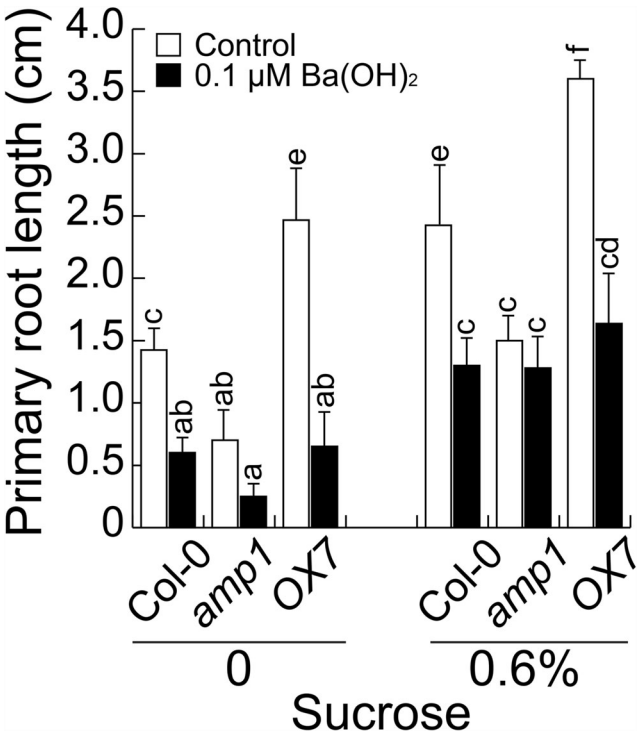


**Fig. 7** Enhanced primary root growth of *OX7* seedlings is dependent on $CO_2$ uptake. Arabidopsis WT, *amp1–10* mutants, and *AMP1-GFP/OX7*-overexpressing seedlings were germinated and grown in MS 0.2x medium with or without sucrose in presence or absence of $Ba(OH)_2$ which acts as a $CO_2$ trap. The *OX7* primary root was the longest in 0 and 0.6% of sucrose in the presence of $CO_2$. However, when $CO_2$ is trapped by $Ba(OH)_2$ the primary root length is similar between all three genotypes. Error bars represent SD from 20 seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results

Sucrose supplements rescued the *amp1*-stunted root phenotype and in the WT and *OX7* seedlings still induced overall plant growth. This suggests the participation of AMP1 in the carbon assimilation pathway and/or metabolism. In support of this possibility, the use of $CO_2$ traps clearly decreased the growth potential of all three lines, namely, the WT, *amp1* mutants, and *OX7* overexpressors. Perhaps AMP1 drives sucrose biosynthesis via $CO_2$ uptake and the improvement of photosynthesis, resulting not only in accelerated growth and development but also in more biomass being accumulated, which is a highly desirable trait in agriculture.

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