Abstract: Plant roots are essential organs for absorbing nutrients from the soil or medium. Sucrose functions as a vital carbon source in root development, and sucrose starvation interferes with the redox state of plant cells. However, the mechanism of root growth at sucrose starvation remains unclear. Here, we report that SHMT1 (serine hydroxymethyltransferase 1) plays a crucial role in primary-root growth. SHMT1 mutation caused decreased sugar levels, excessive H$_2$O$_2$ accumulation, and severe root-growth arrest at sucrose-free conditions, whereas plants with SHMT1 overexpression had increased sugar and decreased H$_2$O$_2$ levels, and longer primary roots. Sucrose supply fully restored root growth of shm1-2, but CO$_2$ alone could not, and SHMT1 is much more stable in roots than shoots at sucrose conditions, suggesting that SHMT1 accumulation in roots is critical for sucrose accumulation and root growth. Further ROS scavenging by GSH application or ROS synthesis inhibition by apocynin application or RBOHD mutation reduced H$_2$O$_2$ levels and partially restored the root-growth arrest phenotype of shm1-2 at low-sucrose conditions, suggesting that SHMT1 modulates root growth via sucrose-mediated ROS accumulation. Our findings demonstrated the role of SHMT1 in primary-root growth by regulating sucrose accumulation and ROS homeostasis in roots.

Keywords: sucrose accumulation; root growth; ROS homeostasis; GSH; RBOHD

1. Introduction

The plant roots are the first and direct organs that sense and respond to the rapid changes of the surrounding environment in the soil, in addition to absorbing nutrients and water from the soil [1]. Plant roots can be roughly divided into the meristem, elongation, and differentiation (maturation) zones [2]. The root length is mainly determined by the cell division within the meristem zone and the final cell length within the differentiation zone [3]. Plant hormones are essential for root development, such as auxin, cytokinin, abscisic acid, brassinosteroids, ethylene, and jasmonate [4]. Jasmonate suppresses cell division and elongation in roots [5], and ethylene enhances inhibition of root-cell elongation through promoting auxin biosynthesis and modulating auxin transport [6,7]. Unfavorable growth conditions, such as salt and osmotic stresses, also inhibit root growth and development mainly by disruption of auxin biosynthesis and redistribution of auxin in the root zones [8,9].

Carbon sources are also vital for root growth and development. Glucose functions as a signaling molecule in primary-root development, and the mechanisms are well-known [10,11]. Sucrose is a main product of photosynthesis, which plays an important role in energy supply during plant growth and development [12,13]. Recently, sucrose has also been proposed as a signal molecule to regulate the development of various plant organs, such as roots, leaves, stems, and flowers [14,15]. Exogenous application of sucrose can partially restore the root-growth inhibition caused by nutrient insufficiency or waterlogging, and induce the emergence of adventitious roots so as to better absorb nutrients from the
soil [16,17]. Loss of function of Arabidopsis MEDIATOR (MED) complex altered auxin homeostasis and inhibited primary-root growth, and sucrose supply induced auxin-responsive gene expression to reactivate both cell division and elongation in primary roots [18]. Because sucrose can break down into glucose, fructose, and trehalose 6-phosphate (T6P), it is difficult to distinguish whether the effect of sucrose on root development is sucrose-specific or not. In Arabidopsis, sucrose increases the root length by enhancing root-meristem activity, and its effect is greater than that of equimolar glucose [19], suggesting that sucrose promotion of root growth is not only due to the hydrolysis of sucrose. Recent studies indicated that sucrose acts as a shoot-derived signaling molecule to trigger root morphogenesis in sunflower-seedling development [20], and as a cotyledon-derived long-distance signal to control root growth during early seedling development in Arabidopsis [21], supporting the view that transport of sucrose synthesized endogenously in shoots into the root tips is essential for root elongation, by integrating plant phytohormone-signaling pathways [21,22]. These studies suggest that sucrose plays a vital role in root growth. However, how plants regulate root growth, especially in sucrose-starvation conditions, is not clear.

Reactive oxygen species (ROS) are continuously produced during plant photosynthesis, respiration, and photorespiration, and play vital roles in plant growth and development, as well as in response to abiotic and biotic stresses [23]. Excessive quantities of ROS reduce the root-meristem size by inhibiting the expression of cell-cycle genes during cell division [24]. Among all kinds of ROS, hydrogen peroxide (H$_2$O$_2$) and superoxide (O$_2^-$) are two stable forms produced in chloroplasts, mitochondria, peroxisomes, and apoplasts of plant cells [25,26], and are essential for root growth [27–30]. O$_2^-$ accumulates in the meristem zone, while H$_2$O$_2$ mainly accumulates in the elongation zone [28]. Their spatial-distribution changes disturb the root-tip transition from a zone of proliferation to a zone of elongation and differentiation, thereby affecting root length [31].

The production of ROS is closely related to photosynthetic metabolites [32]. Soluble sugars not only regulate the photosynthetic activity via influencing the expression of photosynthesis-related factors, but also modulate ROS balance [33]. Excessive sugar accumulation or sugar starvation in plants leads to excessive ROS accumulation [33]. Glucose treatment reduces the levels of O$_2^-$ and H$_2$O$_2$ generated by abiotic stresses in plants [34]. Exogenous sucrose supply enhances the resistance of Arabidopsis seedlings to the herbicide atrazine by reducing the levels of singlet oxygen (1O$_2$) and H$_2$O$_2$ [35,36]. Sucrose supply also alleviate NH$_4^+$ excess stress responses and ROS burst, which promote the assimilation and conversion of carbon and nitrogen, thereby promoting the growth of roots and shoots [37]. Additionally, sucrose starvation in plants induces the expression of ROS oxidative stress genes to produce excessive ROS, and increases the activities of ACX4 enzymes in oxidation of short-chain fatty acids and CAT3 enzymes to scavenge the over-accumulated H$_2$O$_2$ [38]. However, little is known about the specific relationship between sucrose starvation and ROS in modulating root growth.

SHMT1 encodes a key enzyme in the photorespiration process, which catalyzes the conversion of glycine into serine [39,40]. Mutation of SHMT1 clearly exhibited chlorotic and lethal phenotypes under ambient growth conditions [41,42], which were rescued by elevated CO$_2$ [43,44]. SHMT1 is also reported to participate in biotic or abiotic stress responses [45,46]. In this study, we demonstrated the crucial role of SHMT1 in primary-root growth via linking the sucrose with the redox state in Arabidopsis. shm1-2 mutant exhibited a severe root-retardation phenotype at low-sucrose conditions with increased H$_2$O$_2$ levels, and plants with SHMT1 overexpression had longer roots with reduced H$_2$O$_2$ levels. Further sugar-feeding assays, ROS scavenging by GSH, or reduction of RBOHD expression partially restored the root-growth inhibition phenotype at low-sucrose conditions (0%, 0.5% sucrose), demonstrating that SHMT1 participates in primary-root growth by sucrose-modulated redox state.
2. Results
2.1. Mutation of SHMT1 Caused Reduced Primary-Root Growth under Sucrose-Free Conditions

SHMT1 is reported to be involved in abiotic and biotic stress responses, such as salt, drought, and ionic lead (Pb) stresses [45–48]. Our experiments and microarray data from other labs showed that SHMT1 was induced by sucrose (Supplementary Figure S1), indicating that SHMT1 may be also involved in sucrose-mediated plant development or responses. We then determined the root growth of shm1-2 and Col-0 on 1/2 MS medium without sucrose for 8 days. The root length of shm1-2 was extremely shorter than that of Col-0, and the shm1-2 mutant had smaller and chlorotic cotyledons at sucrose-free conditions (Figure 1A,B), consistent with the previous studies where mutation of SHMT1 exhibited smaller plant size in soil [40,49]. When the full-length CDS of SHMT1 driven by the UBQ10 promoter was introduced into the shm1-2 mutant, the primary-root-growth arrest and cotyledon chlorotic phenotypes of shm1-2 were all rescued to the similar levels as Col-0 in the SHMT1-expression complementation lines (COM) (Figure 1A,B and Supplementary Figure S2A), suggesting that SHMT1 is the causative gene for the root-growth retardation observed in shm1-2 at sucrose-free conditions. To further explore the role of SHMT1 in root growth, we overexpressed SHMT1 in Col-0. Interestingly, the primary root length of SHMT1 overexpression lines (OE) was longer than that of Col-0 (Figure 1C,D and Supplementary Figure S2B). These results revealed the correlation of SHMT1 expression with primary-root length, and suggested that SHMT1 was involved in the primary-root-growth regulation at sucrose-free growth conditions.

Figure 1. SHMT1-promoted primary-root growth at sucrose-free conditions. (A) The root-growth phenotype of Col-0, shm1-2 and SHMT1 complementation lines (COM) grown on sucrose-free 1/2 MS medium for 8 days. (B) The primary-root length was statistically analyzed, as shown in (A). (C,D) The root-growth phenotype (C) and primary-root length (D) of SHMT1 overexpression lines (OE) grown on sucrose-free 1/2 MS medium for 8 days. Bars = 1 cm. Data presented were means ± SEM, n = 3 experiments; each experiment had 10 roots per genotype. (E–G) The contents of sucrose (E), glucose (F), and fructose (G) in Col-0, shm1-2 and SHMT1 OE seedlings grown on sucrose-free 1/2 MS medium for 8 days. Data presented were means ± SEM, n = 3 biological replicates. Different letters above error bars indicate significant difference at p < 0.05, using one-way ANOVA with Tukey’s test.
SHMT1 is an enzyme in the photorespiratory pathway, whose mutation causes decreased carbon recycling during photorespiration [42], and finally reduces photosynthesis and carbohydrate accumulation. We then determined the sucrose, glucose, and fructose contents in 8-day-old Col-0, shm1-2 and SHMT1 OE seedlings grown at sucrose-free conditions. The measurements showed that the levels of sucrose, glucose, and fructose were all significantly lower in the shm1-2 mutant and greater in the SHMT1 OE seedlings compared to Col-0 at sucrose-free conditions (Figure 1E–G). The primary root length positively correlated with these carbohydrate contents in these genotypes, indicating that the primary-root-growth differences in the SHMT1 mutant or overexpression plants might be caused by different levels of these carbohydrates. Reduced levels led to shorter primary roots, and greater levels resulted in longer ones.

2.2. Exogenous Sucrose Rescued the Root-Growth-Arrest Phenotype of shm1-2 Mutant, but Elevated CO₂ Alone Could Not

To determine that the root-growth arrest of the shm1-2 mutant at sucrose-free conditions was mainly due to the deficiency of sucrose, glucose, or fructose, we grew Col-0 and shm1-2 plants on the 1/2 MS medium supplemented with different concentrations of sucrose, glucose, or fructose. Sucrose supply promoted primary-root growth of both Col-0 and shm1-2 plants, and the reduced shm1-2 primary-root length was fully rescued by 2% sucrose (Figure 2A,B). Glucose or fructose supply only partially recovered the root-growth retardation of shm1-2 compared to the effect of the same concentrations of sucrose (Figure 2A,B). The sucrose had greater effect than glucose and fructose on restoration of shm1-2 root growth, indicating that the shorter root length in shm1-2 was not only caused by the deficiency of sugar for energy supply, and that sucrose may also act as a metabolite in root growth. The effects of exogenous sucrose on root growth were also tested in the SHMT1 OE plants, which consistently showed longer primary-root length compared to Col-0 at all concentrations of sucrose conditions (Figure 2C,D). Additionally, the sucrose, glucose, and fructose contents were determined in the shm1-2 and SHMT1 OE seedlings supplemented with various concentrations of sucrose. Sucrose supply not only increased the sucrose level, but also the glucose and fructose levels in Col-0, shm1-2, and SHMT1 OE seedlings (Figure 2E–G), supporting the view that sucrose functions as a metabolite, which breaks down into glucose and fructose in plants. The levels of sucrose, glucose, and fructose were all significantly lower in the shm1-2 mutant and greater in the SHMT1 OE plants than those of Col-0 at low-sucrose conditions (Figure 2E–G); and at 2% sucrose conditions, Col-0, shm1-2, and SHMT1 OE had comparable levels of these three sugars. These feeding assays demonstrated that root-growth arrest in shm1-2 was mainly caused by the lower level of sucrose, and that longer roots in SHMT1 OE mainly resulted from the greater sucrose level at low-sucrose conditions. These results suggested that sucrose also acts as a signal molecule in SHMT1-mediated root growth in addition to being a metabolite for energy supply.

CO₂ acts as a donor for carbohydrate synthesis, and high CO₂ treatment represses photorespiration and increases sucrose synthesis in plants. A previous study reported that high CO₂ concentrations could restore the growth arrest and chlorotic phenotypes of another shm1 mutant allele, shm1-1, grown in soil [44]. To test whether the reduced primary-root growth can be rescued by high CO₂, shm1-2 together with Col-0 was grown on 1/2 MS medium without sucrose at ambient (500 ppm) or elevated CO₂ (2000 ppm) conditions. Elevated CO₂ promoted primary-root growth of Col-0 at sucrose-free conditions; however, in the shm1-2 plants, the primary-root growth was not accelerated by elevated CO₂ (Figure 3A,B). We then also tested the effect of elevated CO₂ on the primary-root growth of shm1-2 at 0.5% or 2% sucrose conditions. Interestingly, the primary-root growth of both shm1-2 and Col-0 was accelerated by elevated CO₂ at 0.5% or 2% sucrose conditions compared to that under ambient CO₂ conditions (Figure 3A,B). Moreover, elevated CO₂ increased the levels of sucrose, glucose, and fructose in Col-0 compared to ambient CO₂ conditions at all sucrose concentrations (Figure 3C–E). However, in shm1-2, elevated CO₂
had no impact on sucrose and fructose accumulation at low-sucrose concentrations (0%, 0.5%) compared to ambient 500 ppm CO₂ conditions. At 2% sucrose conditions, elevated CO₂ further promoted sucrose, glucose, and fructose accumulation in shm1-2 and there were no significant differences in the levels of these three sugars between Col-0 and shm1-2 (Figure 3C–E), consistent with their primary-root growth (Figure 3A,B). These results suggested that SHMT1 modulated primary-root elongation by affecting sucrose accumulation, and also indicated that during cotyledon development, a suitable level of sucrose, which might act as a signal, was required to activate early seedling development and photosynthesis.

Figure 2. Exogenous sucrose supply rescued the root-growth-arrest phenotype of shm1-2 mutant. (A,B) The root-growth phenotype (A) and root length (B) of shm1-2 and Col-0 on 1/2 MS medium containing 0.5% or 2% sugars for 8 days. (C,D) The root-growth phenotype (C) and root length (D) of Col-0, shm1-2 and SHMT1 OE grown on 1/2 MS medium containing 0%, 0.5%, or 2% sucrose for 8 days. Bars = 1.5 cm. Data presented were means ± SEM, n = 3 experiments; each line had 10 roots per experiment. (E-G) The content of sucrose (E), glucose (F), and fructose (G) in Col-0, shm1-2 and SHMT1 OE seedlings in (C). Data presented were means ± SEM, n = 3. Different letters above error bars indicated significant difference at p < 0.05, using two-way ANOVA with Tukey’s test.

To further explore the role of SHMT1 on modulation of primary-root growth, the expression level of SHMT1 was detected in Col-0 shoots and roots grown on 1/2 MS medium with different concentrations of sucrose at ambient CO₂ conditions (500 ppm) or with 1% sucrose at 500 and 2000 ppm CO₂. SHMT1 expression in shoots was increased by the increase in sucrose concentration; however, its expression in roots showed the opposite response. At 1% sucrose condition, SHMT1 was suppressed by 2000 ppm CO₂ either in shoots or in roots compared to 500 ppm CO₂ (Supplementary Figure S3A,B). In addition, we also detected the SHMT1 protein levels in shoot and root tissues of SHMT1-YFP expressing transgenic plants, the protein levels gradually increased with the increasing sucrose concentration in roots, but in shoot tissues SHMT1 levels were significantly reduced at 0.5% and 2% sucrose conditions, indicating that SHMT1 is much more stable in roots at
relatively high concentrations of sucrose. CO2 has no significant effect on SHMT1 protein levels, neither in roots nor shoots (Figure 3F,G). Together with the increase in root growth by sucrose addition and SHMT1 overexpression (Figure 2C,D), these results implied that SHMT1 modulation of root growth mainly depended on its level in roots, and the role of SHMT1 in photorespiration contributed less to root growth.

![Figure 3](image-url)

**Figure 3.** Elevated CO2 alone did not recover the growth-retardation phenotype of shm1-2 at sucrose-free conditions. (A) The root-growth phenotype of shm1-2 and Col-0 grown on 1/2 MS medium containing different concentrations of sucrose at 500 or 2000 ppm CO2 for 8 days. (B) The root length was statistically analyzed as shown in (A). Data presented were means ± SEM, n = 3 experiments, each with 10 roots per genotype. Bars = 1.5 cm. (C–E) The contents of sucrose (C), glucose (D), and fructose (E) in shm1-2 and Col-0 seedlings grown on 1/2 MS medium containing 0%, 0.5%, or 2% sucrose at 500 or 2000 ppm CO2 for 8 days. Data presented were means ± SEM, n = 3 experiments. Different letters above error bars indicated significant difference at p < 0.05, using two-way ANOVA with Tukey’s test. Western blot analyses of SHMT1 protein levels in shoots (F) and roots (G) of 35S-SHMT1-YFP expressing transgenic plants grown on 1/2 MS medium containing 0%, 0.5%, 2% sucrose at 500 ppm CO2 or 1% sucrose at 500, 2000 ppm CO2 for 8 days. Actin was used as a loading control.

2.3. Root-Meristem Activity in the shm1-2 Mutant Was Reduced at Low-Sucrose Conditions

We next measured root zone length, cell numbers, and cell length of MZ (meristem zone), TZ (transition zone), and EZ (elongation zone) in Col-0 and shm1-2 roots at 0%, 0.5%, and 2% sucrose conditions. At sucrose-free conditions, shm1-2 exhibited extremely shorter MZ, TZ, and EZ length, and fewer cell numbers in each zone than those of Col-0. When 0.5% sucrose was supplemented, the zone length and cell numbers of MZ, TZ, and
EZ in Col-0 were significantly increased. Although the zone length and cell numbers in these three zones in shm1-2 were still lower than those of Col-0 at 0.5% sucrose conditions, the difference was narrowed compared to the sucrose-free conditions. At 2% sucrose conditions, the zone length and cell numbers of MZ, TZ, and EZ in shm1-2 were comparable with those of Col-0 (Figure 4). There were no significant differences in the cell length of these three zones between Col-0 and shm1-2 at different sucrose conditions (Supplementary Figure S4A–C). These results indicated that SHMT1 controlled root length at low-sucrose conditions by regulating cell division rather than cell elongation in these zones.

![Figure 4](image)

**Figure 4.** Root-meristem activity in the shm1-2 mutant was reduced at low-sucrose conditions. (A) The root-meristem and transition-zone size of Col-0 and shm1-2 at 0%, 0.5%, and 2% sucrose conditions. (B–D) The MZ, TZ, and EZ lengths of Col-0 and shm1-2 at 0%, 0.5%, 2% sucrose conditions. (E–G) The corresponding cell number in the MZ, TZ and EZ at 0%, 0.5%, and 2% sucrose conditions. MZ, meristem zone; TZ, transition zone; EZ, elongation zone. Data presented were means ± SD, n = 3 experiments, each with 10 roots per experiment. Bars = 200 μm. Different letters above error bars indicated significant difference at p < 0.05, using two-way ANOVA with Tukey’s test.

To further explore whether the reduced cell number in the shm1-2 mutant was caused by the reduced cell-division activity, the expression levels of cell-cycle marker genes, CYCB1;1 (AT4G37490), CYCB1;2 (AT5G06150), and CYCB3;1 (AT1G16330) [50,51], were detected. The expression levels of these three genes were significantly downregulated in the shm1-2 mutant compared to Col-0 at low-sucrose conditions; 2% sucrose supply promoted the expression of these three genes and led to comparable levels in shm1-2 and Col-0 (Supplementary Figure S4D), consistent with the cell numbers and root-length phenotypes at different concentrations of sucrose conditions (Figures 2 and 4). These results implied that the inhibition of cell-division activity in the shm1-2 mutant at low-sucrose conditions were caused by the reduced expression levels of cell-cycle genes.
2.4. Accumulation of H$_2$O$_2$ in the Primary Roots Was Altered by SHMT1 Expression Changes at Low-Sucrose Conditions

Sucrose starvation/limitation in plants disturbs the oxidative state, thereby causing excessive accumulation of ROS [38]. To determine whether ROS accumulation was altered in the shm1-2 mutant or SHMT1 OE plants, the levels of superoxide (O$_2^-$, by NBT staining) and H$_2$O$_2$ (by H$_2$DCF-DA and DAB staining) in roots were detected at 0%, 0.5%, and 2% sucrose conditions. With the increase in sucrose concentration, the accumulation of O$_2^-$ in the roots of Col-0, shm1-2 and SHMT1 OE increased greatly, and had no significant differences among these genotypes (Figure 5A,D). The shm1-2 mutant obviously accumulated more H$_2$O$_2$ than Col-0 at low-sucrose conditions, as indicated by H$_2$DCF-DA and DAB staining, which was reduced to the similar level as Col-0 at 2% sucrose conditions. Moreover, the SHMT1 OE roots had reduced H$_2$O$_2$ levels (DAB staining) (Figure 5B,C,E,F), in accordance with their primary-root-length phenotypes at low-sucrose conditions (Figure 2C,D). These results suggested that change of SHMT1 expression influenced the accumulation of H$_2$O$_2$, but not superoxide, in roots at low-sucrose conditions, and SHMT1 modulated root length possibly through regulation of H$_2$O$_2$ levels.

The homeostasis of ROS in plants is balanced by production and scavenging [52]. Oxygen (O$_2$) is reduced by NADPH oxidase to superoxide (O$_2^-$) using NADPH as an electron donor [53]. The superoxide is released into the apoplastic space and dismutated into H$_2$O$_2$ by superoxide dismutase (SOD) [54]. Peroxidases facilitates the conversion of H$_2$O$_2$ into H$_2$O and O$_2$ [55]. To determine whether H$_2$O$_2$ overaccumulation in the shm1-2 mutant was due to the defect of H$_2$O$_2$ production or scavenging, the expression levels of NADPH oxidase genes (RBOHD/F, Respiratory Burst Oxidase Homologue D/F), SOD1, and catalase genes (CAT1, CAT2 and CAT3) were detected in Col-0 and shm1-2 seedlings grown on 1/2 MS medium with 0% and 0.5% sucrose. Expression levels of RBOHD, RBOHF, and SOD1 were significantly upregulated in shm1-2 compared to Col-0 at low-sucrose conditions, and those of CAT1, CAT2, and CAT3 were obviously downregulated (Figure 5G). The activities of SOD (which catalyzes superoxide into H$_2$O$_2$) and CAT (which scavenges H$_2$O$_2$) were measured in the 8-day-old seedlings of Col-0 and shm1-2 supplied with different concentrations of sucrose. SOD activity was greatly increased and CAT activity was significantly decreased in shm1-2 compared to Col-0 at low-sucrose conditions (0%, 0.5%) (Supplementary Figure S5A), while at 2% sucrose conditions, shm1-2 and Col-0 had comparable SOD and CAT activities (Supplementary Figure S5). These results were consistent with the expression levels of H$_2$O$_2$-producing and scavenging genes (Figure 5G), and suggested that the increased H$_2$O$_2$ accumulation in the shm1-2 mutant was caused by the increased H$_2$O$_2$ production and reduced H$_2$O$_2$ scavenging. Taken together, our results revealed that SHMT1-expression-level changes influenced H$_2$O$_2$ accumulation in the primary roots and indicated that the inhibition of primary-root growth in shm1-2 at low-sucrose conditions was possibly due to the high oxidative status caused by H$_2$O$_2$ overaccumulation.

2.5. GSH Treatment Partially Rescued the Reduced Root Growth of shm1-2 during Sucrose Starvation

Previous studies reported that H$_2$O$_2$ could be scavenged by GSH antioxidant [30,56]. To further explore whether H$_2$O$_2$ scavenging could restore cell division of root tips in shm1-2, the plants were grown on the 1/2 MS medium with low sucrose plus 200 µM GSH or not for 8 days. As expected, GSH treatment greatly rescued the primary-root-growth retardation of shm1-2 at low-sucrose conditions (Figure 6A,B). H$_2$O$_2$-level determination of these roots showed that application of GSH greatly reduced the accumulation of H$_2$O$_2$ in shm1-2 roots at sucrose-free conditions, and reduced to the similar level as Col-0 at 0.5% sucrose conditions (Figure 6C,D and Supplementary Figure S6A,B), suggesting that GSH was able to scavenge H$_2$O$_2$ in the roots and H$_2$O$_2$ accumulation was a major cause of primary-root arrest in shm1-2 at low-sucrose conditions. The zone length and cell numbers in MZ, TZ, and EZ of these roots at low-sucrose conditions with or without 200 µM GSH
were also analyzed. The length and cell numbers of MZ, TZ, and EZ zones in shm1-2 were greatly rescued at sucrose-free conditions, and completely restored at 0.5% sucrose conditions by GSH application (Figure 6E and Supplementary Figure S6C–H). Excessive accumulation of ROS inhibits the expression levels of cell-cycle genes [30]. In the shm1-2 plants, the cell-cycle genes (CYCB1;1 and CYCB1;2) were significantly decreased at low-sucrose conditions (Supplementary Figure S4); we then detected the expression levels of cell-cycle genes CYCB1;1 and CYCB1;2 in these GSH treated or untreated seedlings. qPCR analyses showed that GSH application increased the levels of these cell-cycle genes in both Col-0 and shm1-2, and the expression levels in the mutant were restored to the similar levels as in Col-0 at 0.5% sucrose conditions (Supplementary Figure S7). These results suggested that GSH treatment reduced the H2O2 level in the shm1-2 roots, which then activated the cell-cycle gene expression and alleviated the arrest of root growth during sucrose starvation.

Figure 5. SHMT1-expression changes altered H2O2 accumulation in roots at low-sucrose conditions. (A–C) NBT staining for superoxide (O2-) (A), H2DCFDA (B) and DAB staining (C) for H2O2 in the primary roots of 8-day-old Col-0, shm1-2 and SHMT1 overexpression lines at 0%, 0.5%, and 2% sucrose conditions. Bars = 200 μm. (D–F) The NBT-staining intensity (D), fluorescence intensity (E), and DAB-staining (F) intensity in Col-0, shm1-2, OE-1 and OE-2 at 0, 0.5%, and 2% sucrose conditions by Imagej in (A–C), respectively. The intensity in Col-0 roots at sucrose-free conditions was set as 100%. In (D–F), data presented were means ± SEM, n = 3 experiments, each with 10 roots per experiment. Different letters above error bars indicated significant difference at p < 0.05, using two-way ANOVA with Tukey’s test. (G) Quantitative PCR analyses of H2O2-producing genes (RBOHD, RBOHF, SOD1) and scavenging genes (CAT1, CAT2, CAT3) in 8-day-old seedlings of shm1-2 and Col-0 at 0%, 0.5% sucrose conditions. Data presented were means ± SEM, n = 3.
Figure 6. GSH treatment partially rescued the growth-arrest phenotype of the shm1-2 mutant at low-sucrose conditions. (A) The primary-root-growth phenotype of Col-0 and shm1-2 at low-sucrose conditions plus 200 μM GSH or not for 8 days. Bars = 1 cm. (B) Statistical analyses of the root length in (A). Different letters above error bars indicated significant difference at p < 0.05, using two-way ANOVA with Tukey's test. (C,D) H2DCFDA (C) and DAB (D) staining for H2O2 in primary roots of 8-day-old Col-0 and shm1-2 seedlings at low-sucrose conditions plus 200 μM GSH or not. Bars = 200 μm. (E) The root-meristem and transition-zone size of Col-0 and shm1-2 at low-sucrose conditions plus 200 μM GSH or not for 8 days. Bars = 200 μm.

2.6. Mutation of RBOHD Partially Restored the Root-Growth Arrest of shm1-2 during Sucrose Starvation

H2O2 accumulation in shm1-2 was caused by H2O2 overproducing and less scavenging, and NADPH oxidase genes RBOHD and RBOHF were dramatically upregulated in shm1-2 at low-sucrose conditions (Figure 5G). To confirm that H2O2 overaccumulation in the shm1-2 mutant was caused by the increase of NADPH oxidase activity at sucrose-limited conditions, Col-0 and shm1-2 seedlings treated with 10 μM apocynin (NADPH oxidase inhibitor) were used to observe the root growth and H2O2 level at low-sucrose conditions. Apparently, reducing the activity of NADPH oxidase partially restored the root-growth-arrested phenotype in shm1-2 at low-sucrose conditions, especially at 0.5% sucrose (Figure 7A,B). Moreover, the H2O2 level in shm1-2 detected by DAB staining was significantly reduced after apocynin treatment at low-sucrose conditions (Figure 7C and Supplementary Figure S8A). The expression level of RBOHD was increased more than RBOHF in shm1-2 (Figure 5G) and previous studies reported that mutation of RBOHD caused decreased H2O2 levels [57,58]. The shm1-2 and rbohD (CS9555) single mutants were then crossed to generate the double mutant. As expected, the root length of the shm1-2 rbohD double mutant was obviously longer than that of the shm1-2 single mutant at low-sucrose conditions, especially at 0.5% sucrose conditions (Figure 7D,E). Moreover, the mutation of RBOHD in shm1-2 dramatically decreased the H2O2 level in roots at low-sucrose conditions (Figure 7F and Supplementary Figure S8B). These results revealed that decreasing the H2O2 level by reducing NADPH oxidase activity, which was caused by...
RBOHD mutation, could partially restore the root retardation in shm1-2 at low-sucrose conditions, further suggesting the important role of ROS accumulation in regulation of root growth during sucrose starvation.

**Figure 7.** Application of NADPH oxidase inhibitor or mutation of RBOHD partially restored the root-arrest phenotype of shm1-2 at low-sucrose conditions. (A) The primary-root-growth phenotype of Col-0 and shm1-2 at low-sucrose conditions plus 10 μM apocynin (NADPH oxidase inhibitor) or not for 8 days. Bars = 1 cm. (B) Primary-root length was statistically analyzed, as shown in (A). (C) DAB staining for H₂O₂ in the primary roots of 8-day-old Col-0 and shm1-2 at low-sucrose conditions plus 10 μM apocynin or not. Bars = 200 μm. (D,E) The primary-root-growth morphology (D) and root length (E) of Col-0, shm1-2, rbohD and shm1-2 rbohD at low-sucrose conditions for 8 days. Bars = 1 cm. (F) DAB staining for H₂O₂ in primary roots of 8-day-old Col-0, shm1-2, rbohD, and shm1-2 rbohD at low-sucrose conditions. Different letters above error bars indicate significant differences at \( p < 0.05 \), using two-way ANOVA with Tukey’s test. Bars = 200 μm. (G) A proposed model for SHMT1 in primary-root-growth regulation. SHMT1 in roots promotes the accumulation of sucrose to inhibit H₂O₂ accumulation; the reduced H₂O₂ level then activates expression of cell-cycle genes and increases root-meristem activity, thereby promoting primary-root growth. The increased sucrose may also directly increase the expression of cell-cycle genes to promote primary-root growth.

3. Discussion

Sucrose, a main product of photosynthesis, controls various developmental and metabolic processes in plants [14]. Sucrose starvation causes enormous changes in gene expression, enzyme activity, and cellular morphology [59]. However, the mechanisms underlying the sucrose deficiency on plant-root elongation still remain unclear. Here, we demonstrate that Arabidopsis SHMT1, which encodes a key enzyme in the photosynthes-
tory pathway with mitochondrial serine hydroxymethyltransferase activity, is essential for primary-root growth at low-sucrose conditions.

SHMT1 mutation caused extremely short roots (Figure 1A,B), and SHMT1 overexpression resulted in longer roots in the sucrose-free growth medium (Figure 1C,D). The correlation of SHMT1 expression level and primary-root growth at sucrose-free conditions demonstrated the involvement of SHMT1 in root growth during sucrose starvation. Sugar measurements (Figure 1E–G) and feeding assays (Figure 2A,B) suggested that the alterations of root growth were due to the sugar-level differences. Sucrose supply had greater promotion on shm1-2 root growth than the same concentration of other sugars did (Figure 2A,B), such as glucose and fructose, suggesting that sucrose plays a more dominant role than other sugars in SHMT1-mediated primary-root growth. Moreover, sucrose can break down into fructose and glucose, and sucrose supply increased glucose and fructose levels in shm1-2 (Figure 2E–G). These results suggest that SHMT1 regulates primary-root growth mainly through modulation of sucrose accumulation, and sucrose not only acts as a metabolite, but also as a signal molecule in primary-root growth mediated by SHMT1.

CO$_2$ is an indispensable source for plant photosynthesis to synthesize carbohydrates, and elevated CO$_2$ is widely reported to promote plant photosynthesis and suppress the photorespiration process [60,61]. Elevated CO$_2$ (2000 ppm) enhanced the primary-root elongation of Col-0 compared to the ambient CO$_2$ (500 ppm) at various concentrations of sucrose conditions; however, its effect on shm1-2 root growth at sucrose-free conditions was abolished (Figure 3A,B). These results implied that a minimum level of sucrose stored in seeds was required to ensure early seedling development and normal photosynthesis. SHMT1 protein was more stable in roots and unstable in shoots at sucrose conditions (Figure 3F,G), and SHMT1 OE plants had longer roots at sucrose-free conditions (Figure 2C,D), suggesting that the SHMT1 level in roots directly modulates sucrose accumulation, which is critical for primary-root growth. Sucrose supply decreased SHMT1 protein levels, and high CO$_2$ suppressed SHMT1 expression in shoots, suggesting that photorespiration contributes less in SHMT1-mediated primary-root growth. SHMT1 mutation caused sucrose accumulation lower than this minimum level, inhibiting early seedling development and primary-root growth. The measurement of sugar contents at these conditions supported this view (Figure 3C–E). The previous studies reported that the growth-arrest phenotype of shm1-1 grown in soil could be well-restored at high-CO$_2$ conditions [43,44], which could be explained by the organic fertilizers and microorganisms in the soil providing carbon sources, thus promoting root growth and seedling development. All these results suggested that SHMT1 modulated primary-root growth through positively regulating sucrose accumulation in roots. We speculated that in addition to being an enzyme in the photorespiration pathway, SHMT1 may also have a role in regulating the stability of sucrose synthesis-related proteins, or affecting the enzymatic activity of sucrose synthase, thus influencing sucrose accumulation in roots.

Sucrose starvation causes degradation of key cell-cycle proteins, such as CYCD3;1 and RBR1 in a proteasome-dependent manner [62,63] to inhibit cell proliferation during development in Arabidopsis. Our results showed that at low-sucrose conditions, SHMT1 modulated root growth mainly via regulating cell division by influencing the expression levels of key cell-cycle genes in roots. The zone length and cell numbers in each root zone were consistent with the expression levels of CYCB1;1, CYCB1;2, and CYCB3;1 in shm1-2 at low-sucrose conditions (Figure 4 and Supplementary Figure S4), suggesting that sucrose starvation led to the reduced expression of these cell-cycle genes, thereby causing the inhibition of root-meristem activity.

It is well known that excessive ROS inhibit the root elongation through suppressing the cell division [64–66]. Previous studies have shown that sucrose starvation disturbs ROS status. For example, H$_2$O$_2$ is produced more as a by-product in the roots of autophagy-related mutants [67], and CAT3 activity is increased to scavenge the over-accumulated H$_2$O$_2$ in Arabidopsis at sucrose-starvation conditions [38]. In this study, we found that ROS—mainly H$_2$O$_2$—were over-accumulated in the shm1-2 mutant during sucrose starvation, which was
caused by the increased H$_2$O$_2$ production and reduced scavenging process (reduced CAT activity) (Figure 5 and Supplementary Figure S5). The supplement of GSH or NADPH oxidase inhibitor in _shm1-2_ greatly reduced ROS accumulation, restored the reduced expression of cell-cycle genes, rescued the root-meristem activity and cell division, and partially recovered the root-growth retardation at low-sucrose conditions (Figure 6, Figure 7 and Supplementary Figures S6–S8A), providing several lines of evidence that the short root length was caused by overaccumulated ROS. Moreover, mutation of _RBOHD_ in _shm1-2_ also reduced the ROS level and partially restored the root-growth-inhibition phenotype of _shm1-2_ at low-sucrose conditions (Figure 7D–F and Supplementary Figure S8B). These results at pharmacological and genetic levels demonstrated that the primary-root-growth retardation in _shm1-2_ mutant during sucrose starvation is directly linked to the redox state of root cells.

We proposed a model for SHMT1 in primary-root-growth regulation. Under low-sucrose conditions, SHMT1 promotes sucrose accumulation in roots, which inhibits H$_2$O$_2$ accumulation in roots. The reduced H$_2$O$_2$ level activates expression of cell-cycle genes and increases root-meristem activity, thereby promoting primary-root growth. The increased sucrose level may also directly increase the expression of cell-cycle genes to promote primary-root growth through modulation of sucrose accumulation and ROS status. Furthermore, our findings also shed light on the role of sucrose and ROS in primary-root growth. Moreover, our study also suggests a potential role of SHMT1 in improvement of plant tolerance to drought stresses and survival in barren soil by increasing root length.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

All Arabidopsis lines used in this study were in the Columbia background (Col-0). The mutant line _shm1-2_ (SALK_083735, a T-DNA insertion mutant that insert in the last exon of _SHMT1_) was obtained from Dr. Yongfei Wang (Shanghai Institute of Plant Biotechnology, Shanghai, China) and _rbohD_ was kindly provided by Dr. Shunping Yan (Huazhong Agricultural University, Wuhan, China). Plants were grown on half Murashige and Skoog (1/2 MS) medium containing different supplements in CO$_2$-controlled growth chambers (Percival), in which CO$_2$ concentrations can be accurately and well-controlled in a range of 100–2000 ppm with controlled conditions (22°C, 16 h light/8 h night regime, 100 µmol photons m$^{-2}$ s$^{-1}$ light intensity, and 70% relative humidity).

4.2. Phenotype Identification and Root-Length Measurement

Seeds were surface-sterilized and grown on vertical 1/2 MS plates with different concentrations of sugars (0%, 0.5%, 2% m/v) for 8 days. Roots were imaged with a Nikon camera and the root length was measured with ImageJ software.

4.3. Generation of SHMT1 Complementation and Overexpression Transgenic Plants

The coding sequence (CDS) of _SHMT1_ was amplified by the primer pairs listed in Supplementary Table S1. The purified PCR product was digested with _BamHI_ and _SpeI_, and cloned into the corresponding sites of the BarII-UBQ10-MCS vector (Tang et al., 2020). The _UBQ10:SHMT1_ construct was transformed into _shm1-2_ and Col-0 via the floral-dipping method [68] to generate the complementation and overexpression plants, respectively. To generate the _35S:SHMT1-YFP_, the full length CDS of _SHMT1_ was amplified and recombined into the vectors of pEarleygate101, then the _35S:SHMT1-YFP_ construct was transformed into Col-0 to obtain _SHMT1-YFP_ overexpression plants. The transgenic plants were all screened on soil with basta spraying.

4.4. Semiquantitative PCR and Quantitative Real-Time PCR

Total RNA was extracted from whole seedlings using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. The
cDNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the provided protocols. Actin7 was used as an internal control for RT-PCR. Quantitative RT-PCR was performed by using a Universal SYBR® Green kit and the C1000 Touch Thermal Cycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The EF1α gene was used as a reference for qRT-PCR. The primers used are listed in Supplementary Table S1.

4.5. Measurements of Sucrose, Glucose, and Fructose Contents

Sucrose, glucose, and fructose were extracted from 8-day-old seedlings and their contents were measured using Colorimetric/Spectrophotometric Assay kits (Comin Biotechnology Co., Ltd., Suzhou, China) for these three sugars as described [69]. Absorbances were measured at 340 nm (A340) by a Mithras LB 940 microplate analyzer.

4.6. Western Blot Analyses

Proteins were extracted from 10-day-old 35S-SHMT1-YFP expressing transgenic plant roots. Crude extracts were centrifuged at 12,000 rpm for 10 min at 4 °C and supernatant proteins separated by 10% SDS-PAGE gel. The proteins were transferred onto a PVDF membrane. Membranes were blocked in 5% skim-milk TBS-T buffer for 1 h, and then incubated with antibodies against GFP (ABclonal, Wuhan, China) overnight at 4 °C. The membranes were then washed three times with TBS-T buffer, and incubated with diluted 1:10,000 antimouse HRP secondary antibodies for 2 h at room temperature. The detection was performed by Thermo Scientific Pierce ECL Kit (Thermo Fisher Scientific, Waltham, MA, USA).

4.7. Analyses of Zone Length, Cell Numbers, and Cell Length in Root Tips

Seedlings were grown vertically on 0%, 0.5%, or 2% sucrose containing 1/2 MS medium for 8 days, supplemented with or without 200 µmol GSH. Roots were incubated in 10 µmol propidium iodide (Sigma-Aldrich, St. Louis, USA) for 3 min, which was used to visualize the root cells, and were then imaged with a Leica TCS SP8 confocal microscope. The zone length, cell numbers, and cell length of meristem zone (MZ), transition zone (TZ), and elongation zone (EZ) in the cortex were measured and analyzed by ImageJ software as described [30]. The experiments were repeated at least three times, and in each measurement at least 10 roots per genotype were used.

4.8. Determination of ROS Levels

H2DCF-DA (2′,7′-dichlorodihydrofluorescin diacetate) (Sigma-Aldrich) and DAB (3′,3′-diaminobenzidine) were used for H2O2-level detection in roots as described [30]. Briefly, for H2DCF-DA staining, the roots of 8-day-old seedlings grown on 0%, 0.5%, or 2% sucrose containing 1/2 MS medium were immersed in 20 µmol H2DCF-DA buffer for 10 min and then washed by water. The fluorescence was determined by a laser scanning confocal (TCS SP8, Leica, Westzlar, Germany) microscope with excitation at 488 nm and emission at 525 nm. The relative fluorescence was determined, and the fluorescence in Col-0 roots without sucrose was set as 100%. DAB staining (1 mg/mL) and NBT staining (nitroblue tetrazolium, 1 mg/mL) for superoxide were performed according to the published methods [30]. The roots of 8-day-old seedlings grown on 0%, 0.5%, or 2% sucrose containing 1/2 MS medium plus 200 µmol GSH or 10 µmol apocynin or not were used.

4.9. Analyses of Antioxidant Enzyme

The 8-day-old seedlings grown on 0%, 0.5%, or 2% sucrose 1/2 MS medium were harvested and total proteins were extracted at 4 °C. Superoxide dismutase (SOD) and catalase (CAT) activities were measured with SOD and CAT assay kits (Comin Biotechnology Co., Ltd., Suzhou, China) as described [30]. A spectrophotometer (DU730, Beckman Coulter, Pasadena, CA, USA) was used to measure the absorbances at 560 nm and 240 nm for SOD and CAT, respectively.
4.10. Statistical Analysis

The data were statistically analyzed. Differences among different treatments or genotypes were assessed by one-way or two-way ANOVA with Turkey’s test. The p values less than 0.05 were considered statistically significant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23094540/s1.

Author Contributions: Experimentation, data analysis, writing—original draft, Y.Y.; conceptualization, supervision, writing—original draft, D.X. (Danyun Xu); experimentation, writing—original draft, D.X. (Denghao Xiang). and L.J.; project administration, writing—original draft, review and editing, H.H. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by grants from the National Natural Science Foundation of China (31970730 and 32170721), and the Foundation of Hubei Hongshan Laboratory (2021HSZD011).

Data Availability Statement: No large datasets were generated as part of this study.

Acknowledgments: We are grateful to Yongfei Wang (Shanghai Institute of Plant Biotechnology, China) for providing shm1-2 mutant seeds and Shunping Yan (Huazhong Agricultural University) for providing the rbohD mutant seeds.

Conflicts of Interest: The authors declare no conflict of interest.

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