Running Title:
The Roles of SnRK2.6 in Hormone Signaling and Plant Metabolism

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The Protein Kinase SnRK2.6 Mediates the Regulation of Sucrose Metabolism and Plant Growth in *Arabidopsis*

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ABSTRACT

In higher plants three subfamilies of Snf1-related protein kinases have evolved. While the SnRK1 subfamily has been shown to share pivotal roles with the orthologous yeast Snf1 and mammalian AMPK in modulating energy and metabolic homeostasis, functional significance of the two plant-specific subfamilies SnRK2 and SnRK3 in these critical processes is poorly understood. We show here that SnRK2.6, previously identified as crucial in the control of stomatal aperture by abscisic acid (ABA), has a broad expression pattern and participates in the regulation of plant primary metabolism. Inactivation of this gene reduced oil synthesis in Arabidopsis seeds, whereas its overexpression increased sucrose synthesis and fatty acid desaturation in the leaves. Notably, the metabolic alterations in the SnRK2.6 overexpressors were accompanied by amelioration of those physiological processes that require high levels of carbon and energy input, such as plant growth and seed production. However, the mechanisms underlying these functionalities could not be solely attributed to the role of SnRK2.6 as a positive regulator of ABA signaling although we demonstrate that this kinase confers ABA hypersensitivity during seedling growth. Collectively, our results suggest that SnRK2.6 mediates hormonal and metabolic regulation of plant growth and development and that besides the SnRK1 kinases, SnRK2.6 is also implicated in the regulation of metabolic homeostasis in plants.
INTRODUCTION

Plants are constantly confronted by biotic and abiotic stress and nutrient deprivation that disrupt metabolic and energy homeostasis or diminish carbon and energy availability for maintaining cell vitality, growth and proliferation. It is believed that maintaining energy balance and availability at the cellular and organism levels is critical for optimizing plant growth and development. This underscores the cellular and physiological importance of energy sensors that control energy balance through regulating fundamental metabolic pathways in response to nutritional and environmental stresses.

At present, a prevailing view is that energy sensors are evolutionarily conserved in eukaryotes, which are represented by Snf1 (sucrose non-fermenting kinase 1) in yeast, AMPK (adenosine monophosphate-activated protein kinase) in mammals (Hardie and Carling, 1997; Hardie et al., 1998; Carling, 2004; Hardie, 2007; Hedbacker and Carlson, 2008) and SnRK1 (Snf1-related protein kinase 1) in plants (Halford et al., 2003; Baena-González et al., 2007; Polge and Thomas, 2007; Baena-González and Sheen, 2008; Halford and Hey, 2009). In the yeast Saccharomyces cerevisiae, Snf1 is a serine/threonine protein kinase that is required for derepression of the transcription of glucose-repressible genes and plays a key role in the diauxic shift from fermentative to oxidative metabolism in response to glucose deprivation. The mammalian ortholog AMPK is a master metabolic regulator protecting mammalian cells against metabolic stresses that inhibit ATP synthesis or accelerate ATP consumption (Hardie, 2007). AMPK is also implicated in the control of the whole-body energy level (Lage et al., 2008; Dzamko and Steinberg, 2009). However, in plants, a large family of the Snf1-related protein kinases (SnRK) has evolved to comprise not only SnRK1 but also the two additional plant-specific subfamilies SnRK2 and SnRK3 (Halford and Hey, 2009). For instance, in Arabidopsis, these three subfamilies comprise 3, 10 and 25 members, respectively (Hrabak et al., 2003; Halford and Hey, 2009). At present, it is unclear how these kinases coordinate the regulation of stress and energy signaling in plants.
Members of the SnRK1 subfamily, based on sequence similarity, are the closest homologues of the yeast Snf1 and the mammalian AMPK. The findings that rye and tobacco genes can complement the \textit{snf1} mutation in yeast predict a functional similarity of plant SnRK1 genes to \textit{SNF1}. Accordingly, SnRK1 has been implicated in carbohydrate and starch metabolism in light of its roles in the regulation of sucrose synthase expression in potato (Purcell et al., 1998), post-translational redox activation of ADP-glucose pyrophosphorylase, a key regulatory step in starch biosynthesis (Tiessen et al., 2003), and starch synthesis in pollen grains of barley (Zhang et al., 2001). In addition, the SnRK1 complex appears to be involved in carbon partitioning as it promotes the allocation of carbon to root upon herbivore attack, thereby enabling better tolerance in the annual \textit{Nicotiana attenuate} (Schwachtje et al., 2006). Recently, \textit{Arabidopsis} SnRK1 kinases have been implicated as central integrators of transcription networks in response to stress and energy signaling (Baena-González et al., 2007; Baena-González and Sheen, 2008). This was further supported by the work of Jossier \textit{et al.} that highlights the involvement of SnRK1 in sugar and ABA signaling (Jossier et al., 2009). Collectively, the SnRK1 kinases appear to share crucial roles with the orthologous yeast Snf1 and mammalian AMPK in controlling energy and metabolic homeostasis through regulating fundamental metabolic pathways in response to nutritional and environmental stresses (Baena-González and Sheen, 2008).

In contrast to SnRK1, no counterparts of SnRK2 and SnRK3 are found in animals and fungi due to high divergence of their regulatory domains at the C-terminus that are thought to function in protein-protein interactions or regulate kinase activity (Hrabak et al., 2003). It has been shown that plant SnRK3 kinases, also termed as CIPKs (CBL-interacting protein kinases), can interact with a family of calcium sensors, called calcineurin B-like proteins (CBLs) (Kudla et al., 1999; Shi et al., 1999; Kim et al., 2000; Luan et al., 2002; Batistic and Kudla, 2004; Kolukisaoglu et al., 2004; Batistic and Kudla, 2009; Luan, 2009). Under stress conditions, calcium signatures change and decode specific interaction between different CBL and SnRK3 (CIPK) members, leading to altered expression of the downstream genes followed by specific physiological responses (Luan, 2009). Noticeably, the CBL-SnRK3 (CIPK) network largely interacts
with the plant hormone abscisic acid (ABA), which is referred to as the stress hormone because of its pivotal roles in stress responses. The evidence supporting this mechanism includes: (i) under stress-like conditions, ABA can induce the expression of CBL1 and CIPK3 genes (Cheong et al., 2003; Kim et al., 2003); (ii) cbl9 and cipk3 mutants are hypersensitive to ABA (Kim et al., 2003; Pandey et al., 2004); and (iii) overexpression of PKS18 (At5g45820), a member of SnRK3 group, in Arabidopsis conferred hypersensitivity to ABA during seed germination, whereas silencing of the gene resulted in ABA-insensitivity (Gong et al., 2003).

The SnRK2 subfamily is implicated in osmotic signaling in light of the finding that all the SnRK2 kinases except SnRK2.9 can be activated by hyperosmotic and NaCl stress in Arabidopsis (Boudsocq et al., 2004). Because of low sequence similarity in the C-terminal domains, however, the Arabidopsis SnRK2 kinases can be further divided into two subgroups, namely SnRK2a and SnRK2b (Boudsocq et al., 2004; Umezawa et al., 2004). SnRK2a consists of the five members 2, 3, 6, 7 and 8, while the other five members 1, 4, 5, 9 and 10 belong to SnRK2b (Umezawa et al., 2004). Strikingly, ABA can only activate the SnRK2a but not SnRK2b kinases (Boudsocq et al., 2004), suggesting that each group fulfills distinct functions. Moreover, individual kinases even within the same subgroup may function differently. For instance, SnRK2.2 and SnRK2.3 are required for the control of responses to ABA during seed germination, dormancy and seedling growth but have very minor roles in stomatal control (Fujii et al., 2007), thereby distinguishing their function from that of SnRK2.6 (also known as OST1/At4g33950) whose inactivation impairs stomatal closure leading to severe leaf water loss (Mustilli et al., 2002; Yoshida et al., 2002; Assmann, 2003).

Despite the functional significance of the SnRK2 and SnRK3 subfamilies in stress and ABA signaling, little is known thus far about their roles in modulating fundamental metabolic pathways that govern carbon and energy supply for various biological processes, such as plant growth and reserve deposition in seeds. We were interested in understanding if these kinases mediate the regulation of the most prominent ability of higher plants which is to reduce carbon dioxide in the presence of water and light to
sugars via photosynthesis and translocate the assimilated carbon to sink tissues (Sturm, 1999; Lunn and MacRae, 2003; Roitsch and González, 2004). In most species, sucrose is the main product of photosynthesis exported from the leaves to furnish the rest of the plant with the carbon and energy needed for growth and reproduction as well as the synthesis of the storage reserves such as oil, starch and protein (Sturm, 1999; Lunn and MacRae, 2003; Koch, 2004; Roitsch and González, 2004). Given that sugars are essential both as a building block and as an energy source, the demand for sugars generally increases during active plant growth and seed production and in response to stress. Thus, the signaling components involved in sensing sugar levels and determining the capacity of carbon assimilation and translocation are expected to have crucial roles in regulating the cellular and whole-body energy levels and enhancing plant production and stress tolerance.

To unravel the roles of the SnRK2 and SnRK3 subfamilies in the regulation of carbohydrate and energy metabolism, we initially aimed to identify those kinases involved in seed oil production in Arabidopsis. This is because in oilseed plants such as Arabidopsis and Brassica, the vast majority of photoassimilate is translocated to seeds for triacylglycerol synthesis. Triacylglycerol serves as a carbon and energy storage reservoir, and high energy load and fatty acid supply are thought to favor triacylglycerol synthesis. The high and low carbon level relative to nitrogen accelerates and decelerates oil synthetic rate, respectively (Saravitz and Raper Jr, 1995; Pipolo et al., 2004). This implies that alteration in seed oil synthesis may signify, to large extent, the status of sugar and energy supply from the source tissue in oilseed plants.

Our reverse genetic study uncovered the role of the SnRK2 subfamily member SnRK2.6 as a positive regulator in seed oil production in Arabidopsis. To further elucidate its function, we overexpressed this kinase in Arabidopsis and demonstrated its roles in enhancing sucrose synthesis and fatty acid desaturation in the leaves. Moreover, the metabolic alterations in the SnRK2.6 overexpressors were accompanied by amelioration of those physiological processes that require high levels of carbon and energy input, such as plant growth and seed production. Our study demonstrates for the first time at the
metabolic level that SnRK2.6 is implicated in the regulation of carbon and energy supply. The mechanisms underlying the roles of SnRK2.6 are discussed.

RESULTS

Inactivation of SnRK2.6 Reduced Seed Oil Content

To elucidate the roles of the SnRK2 and SnRK3 kinases in the regulation of plant metabolism and energy levels, we initially investigated if inactivation of individual kinase genes affects seed oil production. A reverse genetic approach was employed based on screening of SALK lines carrying T-DNA insertion into the kinase genes and determining the impact of the gene knockout on seed oil content. Our results showed that disruption of several SnRK kinase genes by T-DNA insertion could cause alteration in seed oil content, one of which is the \textit{SnRK2.6} gene (At4g33950). This gene, previously known as \textit{OST1} (\textit{OPEN STOMATA 1}), is a member of \textit{SnRK2a} subgroup encoding a protein that possesses serine/threonine kinase activity and mediates the regulation of stomatal aperture by ABA and reactive oxygen species production (Mustilli et al., 2002). For convenience, the \textit{Arabidopsis} plant homozygous for T-DNA insertion into \textit{SnRK2.6} was designated \textit{snrk2.6}. Repeated experiments over various planting times showed that inactivation of this gene caused 7% to 25% decrease in seed oil content as compared to null segregants. In addition, more than 24% reduction in seed yield under dehydration conditions was detected. In comparison, disruption of \textit{SnRK2.4}, a member of \textit{SnRK2b} subgroup, did not result in a significant change in seed oil content, suggesting that SnRK2.6 and SnRK2.4 have distinct functionality. Collectively, the above results indicate that SnRK2.6 acts as a positive regulator for seed oil production in \textit{Arabidopsis}.

SnRK2.6 Protein Has a Broad Expression Pattern

To delineate the functionality of SnRK2.6, it is necessary to comprehend its expression profile. Previous studies with the GUS reporter driven by the native \textit{SnRK2.6} promoter
suggested specific expression of this kinase in stomata and vascular tissues in *Arabidopsis* (Mustilli et al., 2002; Fujii et al., 2007). To establish a closer relationship between the function and expression of this kinase, its protein levels in different tissues were monitored by Western blot.

We assumed that antibodies against a conserved domain shared within the SnRK2 subfamily may be useful as a reference to estimate the total amount of SnRK2 kinases, but for accurate determination of endogenous SnRK2.6 protein, antibodies against a specific domain in SnRK2.6 are required. Thus, two different kinds of polyclonal antibodies, designated PAB-K and PAB-R, were generated against two different peptides located in the N-terminal conserved kinase domain (K) and the C-terminal divergent regulatory domain (R), respectively. Although a specific N-terminal extension exists in SnRK2.6, it was not chosen for preparation of antibodies specific to this kinase because of the lack of information about intactness of this extension sequence in the mature form of SnRK2.6 protein.

The utility and specificity of the antibodies were assessed through Western blot analysis on the proteins isolated from the wild type and snrk2.6 mutant *Arabidopsis* rosettes. Using PAB-K, a protein band at the position of SnRK2.6 was observed in both the *snrk2.6* and wild type samples (data not shown), suggesting that PAB-K may be capable of binding to different SnRK2 kinases. In contrast, in the assay with PAB-R, the SnRK2.6 protein was readily detected in the wild type but at undetectable levels in the *snrk2.6* plant (data not shown), indicating that PAB-R possesses relatively high specificity toward SnRK2.6 and is useful for detecting this protein.

We thus utilized PAB-R to examine the levels of SnRK2.6 in different tissues of wild type *Arabidopsis*. The results showed that the protein accumulated at high and low levels in the stem and leaf, respectively (Fig. 1). This seemed to be in accordance with the previous observation that the GUS reporter gene under the control of the SnRK2.6 promoter was highly expressed in vascular tissue and guard cells but not in other cells of leaf tissue (Mustilli et al., 2002). However, we cannot exclude the possibility that
SnRK2.6 protein is present at low levels in mesophyll cells. In addition, our study showed that while low in the root, the protein levels in the flower/bud and silique were as high as that of the stem. The reliability of the above results was validated by using the snrk2.6 mutant as a negative control in which no proteins similar in size to SnRK2.6 (42 kDa) were detected (Fig. 1). Collectively, our results indicate that this kinase has a broad protein expression pattern, suggesting that it may possess other functionalities in addition to its role in the regulation of stomatal aperture.

Despite the low protein expression of SnRK2.6 in the leaf tissue, we attempted to examine the effect of its inactivation on leaf sucrose synthesis given that sucrose synthesized in the leaves is the primary form of photosynthate translocated to the seed for the synthesis of seed oils in Arabidopsis. However, no significant difference in soluble sugar or starch content was found between the snrk2.6 mutant and its null sibling grown under well-watered conditions (Supplemental Fig. S1). Although one explanation for this phenomenon could be provided based on the previous finding regarding the restricted expression of the SnRK2.6 gene in guard cells (Mustilli et al., 2002) that have little, if any, effect on overall photoassimilate production in the leaves, the precise roles of this kinase in carbon supply remain to be defined.

Generation and Characterization of Transgenic Arabidopsis Events Harboring CsVMV-Driven SnRK2.6 Gene

Considering the possibility that gene redundancy may hide the effect of SnRK2.6 deficiency, we overexpressed this gene in Arabidopsis under the control of the constitutive Cassava vein mosaic virus (CsVMV) promoter (Verdaguer et al., 1996, 1998) to dissect its functionality. More than 40 independent transgenic events were generated, among which six events had a single copy of the transgene based on genetic segregation analysis. RT-PCR analysis showed a noticeable increase in SnRK2.6 transcript accumulating in the rosettes of these simple events at T3 generation relative to wild type (data not shown). To determine if the constitutive expression of SnRK2.6 increases the protein level, root and leaf tissues were chosen for Western blot analysis because of the
low endogenous SnRK2.6 protein levels in these tissues. It is evident that the kinase protein is present at higher levels in the roots and leaves of individual transgenic events relative to wild type (Fig. 2). Together with the previous findings that CsVMV drives gene expression in a constitutive manner (Verdaguer et al., 1996, 1998), our results indicate that the SnRK2.6 transgene is expressed in Arabidopsis.

SnRK2.6 Is Involved in the Control of Plant Growth and Seed production

Phenotypic characterization of the transgenic plants uncovered a role of SnRK2.6 in plant growth. Overexpression of SnRK2.6 led to marked increases in leaf expansion, aboveground biomass and lateral branching under well-watered conditions (Fig. 3, Fig. 4). Increasing leaf growth at the vegetative stage was evident across different generations of transgenic plants. In addition, four or more lateral branches typically appeared in the kinase overexpressors, while only three were seen for wild type and knockout plants in most cases. Moreover, SnRK2.6 overexpression led to a reduction in water loss from the excised aerial parts of the transgenic plants compared to the wild-type. In contrast, knockout of this gene accelerated water loss (Supplemental Fig. S2), as in the previous study with the ost1 mutants (Mustilli et al., 2002).

A reduction in time to flowering was also observed in the SnRK2.6 overexpressors, which is in contrast to the role of SnRK1 kinases in delaying flowering (Baena-González et al., 2007). Early flowering is a desirable trait in most plant breeding programs because it is often associated with yield advantage. Typically, the transgenic plants started flowering one day earlier than wild type. Accordingly, senescence of the mature leaves at the late reproductive stage (approximately 30 days after planting) was accelerated, as evidenced by an earlier onset of anthocyanin accumulation in the old transgenic leaves. There is a prevailing view that leaf senescence in the old leaves plays a vital role in nutrient recycling, especially in the remobilization of nitrogen, although it reduces photosynthetic carbon fixation (Pourtau et al., 2004, 2006; Wingler et al., 2006, 2008). A high availability of photoassimilates relative to nitrogen in the mature leaves can trigger leaf senescence, and such a process is favorable to sustaining photosynthetic activity in the
young leaves and ultimately reproductive success (Pourtau et al., 2004, 2006; Wingler et al., 2006, 2008).

In addition to the profound effects of the SnRK2.6 transgene on the physiological and developmental processes described above, constitutive expression of SnRK2.6 also caused an increase in seed yield due to increased seed number. As compared to wild type plants, the transgenic plants showed 24%, 16% and 35% increases in seed yield, respectively, under normal, mild drought, and severe drought conditions (Fig. 5). Taken together, our results suggest that SnRK2.6 is implicated in the control of plant growth and development in addition to the previously identified role in the control of stomatal aperture.

**SnRK2.6 Overexpression Increased Leaf Sucrose Synthesis**

The increased plant growth and seed production in the transgenic plants suggest that carbon sinks are enlarged by the SnRK2.6 transgene. In light of an intimate relationship between sink strength and the rate of sugar synthesis in the source leaves (Rocher et al., 1989; Farrar, 1996; Paul et al., 2001; Baxter et al., 2003), we determined if sugar metabolism is affected in the SnRK2.6 overexpressors. LC-MS/MS was used to analyze leaf soluble sugars at three stages of plant development, 22, 24 and 28 days after planting (DAP). The results showed that sucrose constituted the vast majority of soluble sugars in both the transgenic and wild type leaves in these three stages. However, it was present at higher levels in the transgenic leaves (Fig. 6A). Accordingly, the total soluble sugar content in the transgenic leaves was 34.7% higher than that of wild type at 22 DAP. Sugar content of the transgenic leaves continued to be maintained at higher levels at 24 and 28 DAP. Although sugar contents in the leaves of wild type increased to a greater degree at 28 DAP relative to 22 and 24 DAP, they were not above the levels of the transgenic leaves (Fig. 6B). To determine if the increased soluble sugar levels result from a decrease in starch synthesis, starch was analyzed in the same leaf samples used for soluble sugar analyses. Relative to the wild type, no decrease in starch content was found in the transgenic leaves (Fig. 6C), indicating that increasing synthesis of soluble sugars
by the transgene was not at the expense of starch. These results indicate that the availability of carbon in the transgenic leaves is higher than in wild type in the period of 22 to 28 DAF.

To corroborate the effect of SnRK2.6 overexpression on carbohydrate metabolism, a separate experiment was conducted using the leaves of 30-day-old Arabidopsis plants grown under well-watered conditions. Consistent with the previous results from sugar analysis of 22-, 24- and 28-day-old plants, the average total amount of soluble sugars in the leaves of five independent transgenic events was twice that of the wild-type (Fig. 7A). Noticeably, glucose and fructose, as opposed to sucrose, were the main soluble sugars in both the transgenic and wild type leaves, suggesting that sucrose cleavage was very active at 30 DAP. But the amount of glucose and fructose accumulating in the transgenic leaves was twice and three times greater than that of wild type, respectively (Fig. 7B). Despite the greater increase in both hexoses, sucrose in the transgenic leaves was still more than twice that of wild type. These findings demonstrate that the SnRK2.6 transgene also is capable of increasing soluble sugars at 30 DAP and along with our previous observation that constitutive expression of SnRK2.6 caused an earlier onset of leaf senescence, strongly support the notion that high hexose supply triggers leaf senescence (Wingler et al., 2006). Furthermore, a positive, instead of inverse, correlation occurred between soluble sugar level and starch accumulation in the SnRK2.6 transgenic plants at 30 DAP. The average content of starch in the transgenic leaves was 156% of the wild type level (Fig. 7C).

Collectively, the above results indicate that the SnRK2.6 transgene altered carbohydrate metabolism and increased the pool sizes of soluble sugars in the source leaves. This may constitute a metabolic basis for the enhanced growth and seed production in the SnRK2.6 overexpressors.

**SnRK2.6 Overexpression Increased Leaf Fatty Acid Desaturation**
The increased sugar availability prompted us to examine if lipid metabolism is affected in the leaves of the SnRK2.6 overexpressors. GC analysis of leaf fatty acid composition revealed that the two trienoic fatty acids 16:3 and 18:3 in the transgenic leaves increased by 81% and 26%, respectively, relative to wild type (Fig. 8A, B). This result indicates that polyunsaturation of chloroplast membrane is elevated by the transgene considering that 16:3 is mainly present in thylakoid lipids, especially monogalactosyldiacylglycerol (McConn and Browse, 1998). Evidence has been accumulating that trienoic fatty acids of thylakoid membrane lipids are required to maintain photosynthetic competence, particularly at low temperature (McConn and Browse, 1998; Routaboul et al., 2000; Vijayan and Browse, 2002; Barkan et al., 2006). In addition, membrane polyunsaturation has been shown to be correlated with drought tolerance as well as with cold and freezing tolerance (Im et al., 2002; Zhang et al., 2005; Mène-Saffrané et al., 2009; Torres-Franklin et al., 2009). Reduction of 18:3 fatty acid in chloroplast membrane by silencing a plastidial ω-3 desaturase (FAD7) in tobacco plants causes a decrease in drought/salt tolerance (Im et al., 2002). Conversely, overexpression of either extraplastidial FAD3 or plastidial FAD8 ω-3 desaturase increases tolerance to drought in tobacco plants (Zhang et al., 2005). Taken together, we propose that SnRK2.6-mediated membrane polyunsaturation may be associated with the increased carbon assimilation.

To understand if the increased leaf sugar synthesis affects seed oil content, we carried out seed oil analysis of five independent transgenic lines. The results showed that constitutive expression of SnRK2.6 did not significantly increase seed oil content (data not shown). Nevertheless, overall seed oil production in the SnRK2.6 overexpressors increased due to the increased seed yield (Fig. 5) and the preservation of the wild type level of seed oil content in the transgenic seeds. One possibility for the neutral effect of the transgene on seed oil content is that the increased sugar supply from the leaves may increase nitrogen assimilation, especially in the nitrogen-rich greenhouse conditions, thereby maintaining the balance between oil and protein levels in the seeds.

SnRK2.6 Increases ABA Sensitivity during Seed Germination and Seedling Growth
To decipher the mechanisms governing the roles of SnRK2.6 in sugar metabolism, plant growth and development, we attempted to determine the implications of this kinase in ABA and ethylene signaling during seed germination and seedling growth. Seeds of the SnRK2.6 overexpressor, snrk2.6 mutant and wild type were germinated at various concentrations of ABA and 1-aminocyclopropane-1-carboxylic acid (ACC), an immediate precursor of ethylene. Germination was scored based on radicle emergence at 64 h after sowing. At low ABA concentrations (not above 0.5 μM), the rates of germination in all the genotypes were nearly 100%. However, the rates of the overexpressors dropped from nearly 100% to less than 25% at 1 μM of ABA, while no obvious decline occurred for the wild type and snrk2.6 mutant. At 2 μM of ABA, a further reduction in germination rate was evident in the overexpressors, and meanwhile, the rates of the wild type fell below 23% as opposed to 52% for the mutant (Fig. 9A). In contrast to ABA, treatment with ACC at various concentrations from 2.5 to 25 μM did not affect seed germination of any of the genotypes. These results revealed a role for SnRK2.6 in increasing ABA sensitivity in the process of seed germination.

As in the case of seed germination, ACC treatment did not result in apparent difference in seedling growth between different genotypes. However, they showed distinct responses to ABA when grown in the dark (Fig. 9B, C). Compared to wild type, the snrk2.6 seedlings showed much less reduction in hypocotyl length at 0.25 or 0.5 μM ABA (Fig. 9B). This is unlikely to be due to alteration of the sensitivity of radicle emergence to inhibition by ABA because no significant difference in root growth at these two ABA concentrations was observed between the two genotypes (Fig. 9C). Root growth of the mutant seedlings was enhanced and inhibited by 0.25 and 0.5 μM ABA, respectively, both to the same degree as for the wild type (Fig. 9C), consistent with the results from previous Western blot analysis revealing very weak expression of SnRK2.6 in roots of wild type plants (Fig. 1). The present results are reconciled with the notion that ABA at low and high concentrations has growth-promoting and -inhibitory roles in root growth, respectively (León and Sheen, 2003; Fujii et al., 2007). Strikingly, SnRK2.6 overexpression resulted in marked decreases in both hypocotyl and root length even at 0.25 μM ABA (Fig. 9B, C). As ABA concentration increased to 1 μM, its inhibitory
effect appeared to be more profound in three independent SnRK2.6 overexpressors compared to the wild type and snrk2.6 mutant (Fig. 9D).

It is concluded that SnRK2.6 confers ABA hypersensitivity during seedling growth. This is consistent with its role as a positive regulator of ABA signaling in guard cells (Mustilli et al., 2002; Assmann 2003). On the contrary, the impact of SnRK2.6 on ACC response was not evident in this experiment. However, given that the actions of plant hormones are dependent on the concentration, tissue, developmental stage as well as on internal and external signals (León and Sheen, 2003; Pierik et al., 2006), we cannot exclude the role of SnRK2.6 in the regulation of ethylene response in other tissues or under other conditions.

Transcriptional Regulation by SnRK2.6

To understand the mechanism of action of SnRK2.6 in these diverse processes, Agilent microarray analysis with 21,000 A. thaliana probes was conducted using RNA derived from leaves of the SnRK2.6 overexpressor and wild type plants. Our experiment with two independent transgenic events identifies 96 up-regulated and 96 down-regulated genes in the transgenic leaves. Among them, 27 and 45 genes showed more than two-fold increase and reduction, respectively, in each respective transcript. These genes were further analyzed for their responses to ABA using Arabidopsis eFP Browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Eight of the 27 SnRK2.6-inducible genes were induced by ABA in seedlings and/or mesophyll and/or guard cells, whereas 13 of the 45 SnRK2.6-repressible genes were repressed by ABA. This result indicates that a significant portion (~29%) of SnRK2.6-responsive genes overlap with the genes responsive to ABA, consistent with the role of SnRK2.6 as a positive regulator of ABA signaling (Mustilli et al., 2002; Fujii and Zhu, 2009; Nakashima et al., 2009). However, the finding that many SnRK2.6-regulated genes are not responsive to ABA, suggests that this kinase also functions independently of ABA.
Of particular interest is a gene (At5g43450) encoding 1-aminocyclopropane-1-carboxylate oxidase (ACO), whose transcript showed a three-fold increase in the transgenic leaves compared to the wild type. Because ACO catalyzes the last step of ethylene synthesis, an increased rate of ethylene synthesis is expected in the transgenic leaves. In addition, several genes coding for enzymes involved in primary metabolism were up-regulated. Transcripts of the genes encoding glucose-6-phosphate 1-dehydrogenase (At1g09420), starch synthase (At1g32900) and SPS (At4g10120) in the transgenic leaves were 2.8, 1.6 and 1.5 times higher than those for the wild type, respectively. These results support the roles of SnRK2.6 in sugar metabolism.

DISCUSSION

Despite that the SnRK2 and SnRK3 subfamilies are related to the evolutionarily conserved metabolic and energy sensors Snf1/AMPK/SnRK1 kinases, little is known thus far about their metabolic roles. In this study, we present evidence that SnRK2.6 mediates the regulation of carbon supply for plant growth and seed production.

SnRK2.6 Imparts Carbon Source and Sink Strength through Regulating Sucrose Metabolism

Our reverse genetic study uncovered the role of the SnRK2 subfamily member SnRK2.6 as a positive regulator in seed oil production in Arabidopsis. Knockout of this gene led to a significant decrease in seed oil content. It is reasonable to assume that one mechanism underlying such an effect may be directly linked to the role of this kinase in ABA signaling in Arabidopsis seeds based on the following facts: (i) this kinase acts as a positive regulator of ABA signaling (Fujii and Zhu, 2009; Nakashima et al., 2009) and is expressed in Arabidopsis developing seeds, albeit at a low level based on Western blot analysis (data not shown); and (ii) ABA behaves as a positive regulator for triacylglycerol synthesis during embryo development (Pacheco-Moises et al., 1997; Phillips et al., 1997). However, other mechanisms may also contribute to the distinct role
of SnRK2.6 in seed oil synthesis because this kinase is broadly expressed in the maternal plant tissues (Fig. 1) that determine the availability of various assimilates for seed growth and storage compound deposition.

Considering that high and low carbon supply relative to nitrogen accelerates and decelerates oil synthetic rate, respectively (Saravitz and Raper Jr, 1995; Pipolo et al., 2004), we assumed that the significant decline in seed oil content resulting from the inactivation of SnRK2.6 might be partly attributable to the role of this kinase in the control of either carbon assimilation in the source leaves or carbon translocation into the sink tissue or both. To provide evidence for the role of SnRK2.6 in carbon assimilation, we investigated if inactivation of SnRK2.6 could alter the steady-state levels of leaf soluble sugars and starch under well-watered conditions. But no significant difference was detected between the snrk2.6 mutant and its null sibling, suggesting one possibility that SnRK2.6 might be dispensable for carbon assimilation under our growth conditions due to the low expression levels in leaf tissue (Fig. 1). This result seemed to be in accordance with the previous observation based on the promoter-GUS reporter assay that SnRK2.6/OST1 appeared to be highly expressed in guard cells but not in mesophyll cells where the majority of photoassimilates are synthesized (Mustilli et al., 2002). Alternatively, the mutant plants may use a compensatory mechanism to enhance the expression or activities of other kinases to compensate for the loss of function exerted by SnRK2.6 in the leaves. However, another possibility may exist that knockout of this gene concurrently reduces carbon assimilation and translocation so that the steady-state levels of sugar and starch in leaf tissue maintain at the wild type level. The high-level expression of SnRK2.6 in the vascular tissue provides a molecular basis for the role in carbon translocation.

Gene redundancy appeared to complicate dissection of SnRK2.6 functionality using reverse genetics. We thus examined the impact of SnRK2.6 overexpression on leaf sugar metabolism in Arabidopsis. Our results showed that constitutive expression of SnRK2.6 could drastically boost sucrose and total soluble sugar levels in the leaves at various developmental stages while maintaining starch at the wild type level or even higher levels
(Fig. 6, Fig. 7). Because the capacity for sucrose synthesis in the cytosol limits the maximal rates of photosynthesis by restricting the recycling rate of inorganic phosphate to support electron transport and carbon fixation in the chloroplast (Stitt, 1986), increasing sucrose synthesis has a positive impact on photosynthetic rate (Ho and Thorley, 1978; Stitt, 1986; Battistelli et al., 1991; Baxter et al., 2003). In addition, it has been shown that the rate of sucrose synthesis is also positively correlated with the rate of sucrose export from leaves (Ho and Thorley, 1978; Rocher et al., 1989; Baxter et al., 2003; Park et al., 2007). Hence, the increased sucrose levels in the source leaves resulting from constitutive expression of SnRK2.6 are attributable to an increase in photoassimilation rather than a reduction in sucrose export because the carbon sink is also enhanced by the transgene, as exemplified by increased leaf growth, lateral branching and seed production. We conclude that SnRK2.6 has a role in influencing photosynthetic capacity and carbon sink strength through the regulation of sucrose metabolism.

SPS activity has been shown to be a determinant of the synthetic rate of sucrose in leaves (Battistelli et al., 1991; Winter and Huber, 2000; Baxter et al., 2003; Haigler et al., 2007). Interestingly, two lines of evidence from our study suggest a possible role of SnRK2.6 in increasing SPS activity. First, our microarray analysis revealed that SnRK2.6 overexpression caused increased expression of At4g10120 encoding SPS. Second, the transgenic leaves displayed a higher ratio of sucrose to UDP-glucose than the wild type (Supplemental Figure S3). This might partly reflect increased formation of sucrose-6-phosphate from fructose-6-phosphate and UDP-glucose by SPS provided that the resultant sucrose-6-phosphate can be immediately hydrolyzed by sucrose phosphate phosphatase to yield sucrose due to the close association of the two enzymes (Echeverria et al., 1997; Lunn and MacRae, 2003). The above results provide a clue for future work on determining SnRK2.6 effects on each of four Arabidopsis SPS enzymes and delineating the mechanism underlying the role of SnRK2.6 in carbon assimilation. Given the role of SnRK1 in phosphorylating and inhibiting SPS activity (Halford et al., 2003), it would also be valuable to investigate if SnRK2.6 antagonizes the action of SnRK1.
Potential Involvement of SnRK2.6 in Multiple Signaling Pathways

A prevailing view is that SnRK2.6 is a major positive regulator of ABA signaling (Mustilli et al., 2002; Hiroaki and Zhu, 2009; Nakashima et al., 2009). In agreement, our study showed that knockout and overexpression of this gene decreased and increased sensitivity to ABA, respectively, for arresting seedling growth (Fig. 9). Meanwhile, evidence has been accumulating that ABA plays a role in growth promotion (Barrero et al., 2005; Hiroaki and Zhu, 2009), which is in marked contrast to its role as stress hormone in enhancing stress tolerance because stress tolerance often comes at the expense of plant growth. For example, the aba1 mutants deficient in ABA displayed a stunted phenotype even under well-watered conditions, whereas low concentrations of exogenous ABA (e.g. 10 or 50 nM) could increase the fresh weight of mutant and wild type plants (Barrero et al., 2005). Hence, one possibility exists that the phenotype of enhanced leaf growth in the SnRK2.6 overexpressors might be due to an adequate increase in ABA response associated with increased SnRK2.6 protein levels.

However, the increased lateral branch formation resulting from SnRK2.6 overexpression under well-watered conditions (Fig. 4) is contradictory with the role of SnRK2.6 as a positive regulator of ABA signaling. This is because ABA moderately represses lateral bud outgrowth (Cline and Oh, 2006), whereas ethylene and cytokinins stimulate lateral bud formation (Van Dijck et al., 1988). In addition, SnRK2.6 overexpression resulted in a greater petiole upward movement, and such hyponastic response was augmented in response to attack by the powdery mildew pathogen (data not shown). This unexpected result also markedly contrasts with the positive regulatory role of SnRK2.6 in ABA signaling in that ABA is a negative regulator in the process of ethylene-induced petiole hyponastity (Benschop et al., 2007). These phenomena, along with our finding that many SnRK2.6-regulated genes are not responsive to ABA, prompt us to propose that SnRK2.6 may be implicated in multiple signaling pathways. Supportive of this view is our finding that constitutive expression of SnRK2.6 caused a 3-fold increase in the expression of an ACO gene (At5g43450) in the leaves under well-watered conditions. It has been shown that enhanced expression of this ACO gene coincides with increased ethylene production
and petiole upward movement in *Arabidopsis* (Vandenbussche et al., 2003). These findings suggest a possible, either direct or indirect, role for SnRK2.6 in ethylene signaling pathway.

Our view regarding the potential involvement of SnRK2.6 in multiple signaling pathways could more plausibly explain the observed phenotype in the *SnRK2.6* overexpressors than viewing this kinase as playing a sole role in ABA signaling. This may also be the case with the stimulating effect of *SnRK2.6* overexpression on carbon assimilation. It has been shown that in contrast to the inhibitory role for ABA (Armstrong et al., 1995), ethylene promotes stomatal opening (Merritt et al., 2001; Tanaka et al., 2005), indicating that coordinated regulation of ABA and ethylene signaling in coping with environmental changes is required for fine-tuning of stomatal conductance that allows for optimizing gas exchange for photosynthesis while limiting water loss through transpiration. In addition, ethylene can suppress the sensitivity to endogenous glucose, which has a positive consequence for photosynthetic capacity, whereas ABA exerts an opposite effect (Tholen et al., 2007). Based on these previous findings, it is hardly conceivable that the increased carbon assimilation and seed production rather than yield penalty as the result of *SnRK2.6* overexpression is due to a sole role of this kinase in ABA signaling.

The mechanism behind the potential involvement of SnRK2.6 in multiple signaling pathways could be partly ascribed to the physical and functional interaction of this kinase with type 2C protein phosphatases. Recent studies illustrate that type 2C protein phosphatases including ABI1 regulate the activation of SnRK2.6 by ABA (Yoshida et al., 2006; Umezawa et al., 2009; Vlad et al., 2009). ABI1 acts as a negative regulator of ABA signaling (Leung et al., 1994; Meyer et al., 1994; Gosti et al., 1999; Saez et al., 2004; Ma et al., 2009). It inactivates ABA-activated SnRK2.6 kinase via dephosphorylation of Ser/Thr residues in the activation loop, whereas this process is repressed by the RCAR/PYR ABA receptors in response to ABA. But a gain-of-function mutation (*abi1*) of ABI1 appears to constitutively inhibit SnRK2.6 kinase activity due to the inability to respond to the receptors (Umezawa et al., 2009). Strikingly, while conferring ABA insensitivity, *abi1* enhances hyponastic response of *Arabidopsis* petiole (Benschop et al.,
which is characteristic of enhanced ethylene response. Concertedly, when expressed in gray poplar, *abi1* elevated ethylene production (Arend et al., 2009). Likewise, ethylene can induce the expression of ABI1 (Benschop et al., 2007). These findings present a linkage between SnRK2.6 dephosphorylation and enhanced ethylene response as well as a close connection between SnRK2.6 phosphorylation and ABA signaling and also suggest that dephosphorylation of SnRK2.6 kinase by ABI1 may represent an important means by which ethylene antagonizes ABA action. Therefore, it seems plausible that SnRK2.6 overexpression may create a potential to boost ethylene signaling through increasing the amount of the dephosphorylated form of this kinase in the transgenic leaves under well-watered conditions where ABA is presumably low. Future experimental work toward determining if each form of SnRK2.6, dephosphorylated or phosphorylated, possesses a distinct role in hormone signaling should significantly extend our knowledge on cross-talk between hormone signaling pathways.

In summary, the present study suggests that SnRK2.6, which is previously known for its role as a major positive regulator of ABA signaling mediating the control of stomatal aperture (Mustilli et al., 2002), has a role in increasing carbon supply. Such a role may constitute a basis for the enhanced plant growth and seed production in the SnRK2.6 overexpressors. Along with its metabolic role, its potential involvement in multiple signaling pathways opens clues for future studies on the regulation of stomatal closing and opening. Because adequate ethylene response enables plants to increase photosynthetic capacity (Tholen et al., 2007) and thus positively influence crop production, the potential role of SnRK2.6 in ethylene signaling merits further investigation.

MATERIALS AND METHODS

*Arabidopsis Growth Conditions*
Arabidopsis plants were grown in 4-inch pots filled with Sunshine mix LP5 soil in a greenhouse under a 16/8 h light/dark photoperiod with a light intensity of 120~150 µmol m^{-2} s^{-1} at 22°C in the day and 19°C at night. Plants were watered with Hoagland's solution three times in the entire life cycle.

**Identification of T-DNA Insertion into the Snf1-related protein kinase genes by PCR**

SALK T-DNA insertion lines corresponding to individual kinase genes were purchased from the Arabidopsis Biological Resource Center (ABRC). Arabidopsis plants homozygous, heterozygous and null for T-DNA insertion into each gene were screened by PCR with genomic DNA isolated from the leaves. For an example, GSP108 (5’-GCA GTG AGT GGT CCA ATG GAT T-3’) and LBa1 (5’- TGG TTC ACG TAG TGG GCC ATC G-3’) primers were used to screen for plants carrying T-DNA insertion into SnRK2.6 (At4g33950) in SALK_008068. On the other hand, the two gene-specific primers GSP108 and GSP124 (5’-CCGCTACTGTCGATGTCAAGA-3’) were used to determine if a wild-type copy of the gene is present in the segregants of SALK_008068. For convenience, the progeny of SALK_008068 homozygous for T-DNA insertion into SnRK2.6 was designated snrk2.6. To establish the function for each kinase in seed oil production, seed oil profiles of plants carrying T-DNA insertion into individual kinase genes were determined by gas chromatography, with null sibilings from the same lines as control.

**Lipid Analysis by Gas Chromatograph Flame Ionization Detection (GC-FID)**

Seed and leaf lipids were analyzed using GC-FID as described in Supplemental Materials and Methods.

**Total Starch and LC-MS/MS Metabolite Analysis**

Arabidopsis leaves were ground to a very fine powder in liquid nitrogen. For starch analysis, the samples were de-sugared with 80% ethanol. Digestion of starch was conducted with α-amylase and amyloglucosidase, respectively. The released glucose was detected by glucose oxidase- and peroxidase-based enzyme assay. Starch content was calculated based on the released glucose with adjustment of free glucose to starch.
LC-MS/MS analysis of soluble sugars in plants was carried out essentially as described previously (Nagai et al., 2009) except that 100 mg of fresh leaf tissue per sample was used. The analyzed sugars include sucrose, fructose, glucose (Glc), ADP-Glc, GDP-Glc, UDP-Glc, Glc 1-P, and Glc 6-P. Each biological sample was assayed in triplicate.

**Construction of SnRK2.6 Expression Vector for Arabidopsis Transformation**

*Arabidopsis* SnRK2.6 gene was amplified by RT-PCR. Total RNA isolated from the leaves was used for single strand cDNA synthesis using Oligo dT20 as primer (Invitrogen SuperScriptIII RT kit). The sscDNA was amplified using a pair of primers, 5’- TAA TTT CCA TGG ATC GAC CAG CAG TGA GT-3’ and 5’- TTT TTT CCA TGG ATC ACA TTG CGT ACA CAA TCT CT-3’, both of which include a Nco I site (underlined) for subsequent cloning. The amplified *SnRK2.6* gene was digested with Nco I and inserted into the gateway entry vector pDAB3731. The insert orientation was determined by sequencing. The resulting plant transcription unit (PTU) comprising *CsVMV*, SnRK2.6 and AtuORF24 3’-UTR was cloned into the binary gateway destination vector pDAB3725 using gateway LR reaction. The resulting plasmid, designated pDAB4504, was used for *Agrobacterium* transformation.

**Agrobacterium and Arabidopsis Transformation**

The expression vector pDAB4504 was transformed into *Agrobacterium tumefaciens* (strain Z707S) cells using a protocol of electrotransformation as described by Weigel and Glazebrook (2002). *Arabidopsis* transformation with the resultant *Agrobacterium* cells harboring *SnRK2.6* gene was performed using the floral dip method (Clough and Bent, 1998). For selection of T1 seeds, seedlings of T1 plants were sprayed twice at different days with a 0.20% glufosinate herbicide (Liberty) solution in a spray volume of 10 ml per tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g/ha glufosinate per application. At four to seven days after the second spray, herbicide resistant plants were identified, transplanted into pots, and grown in the above mentioned growth conditions.
Molecular Characterization of SnRK2.6 Transgenic Arabidopsis

To screen transgenic Arabidopsis plants carrying the transcription unit of SnRK2.6, PCR was conducted with a pair of primers, 5’- TGA GGT CTA CAG GCC AAA TTC GCT CTT AGC-3’ and 5’- ATC ACA TTG CGT ACA CAA TCT CT-3’, designed according to the sequence near T-DNA left border and 3’-end sequence of SnRK2.6, respectively. Genetic segregation of T2 transgenic plants was determined using PAT invader assay and herbicide spraying. Only those transgenic events carrying single insertion associated with 3:1 genetic segregation were selected for further biochemical and physiological studies.

Preparation of Polyclonal Antibodies

The two peptides “CHRDLKLENTLLDGSPAPRLKICDFGYSKS” and “MNDNTMTQFDESQPGQSIEE”, which are located, respectively, in the conserved kinase domain (K) and divergent regulatory domain (R) of SnRK2.6 protein, were chosen for preparation of polyclonal antibodies (PAB). After synthesis, these two polypeptides were conjugated to keyhole limpet hemocyanin (KLH) as carrier protein, and the resulting two conjugates were used for rabbit immunization to generate two different kinds of polyclonal antibodies, designated PAB-K and PAB-R, respectively. To purify the antibodies, the peptides were conjugated to bovine serum albumin (BSA), and the conjugates used for affinity chromatography.

Western Blot Analysis

Arabidopsis tissues were ground to a very fine powder in liquid nitrogen. Approximately 200 mg of each sample was used for protein extraction with 1 mL of 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, 5 mM DTT, 0.05% Triton X-100. The protein concentrations of the extracts were measured by Bio-Rad protein assay. Then equal amounts of the proteins from each sample were loaded and separated by SDS-PAGE using a Nupage 10% Bis-Tris gel (Invitrogen), and transferred onto nitrocellulose membrane (Bio-Rad). The membrane was firstly probed with the rabbit anti-SnRK2.6 peptide polyclonal antibodies followed by the goat anti-rabbit IgG-horseradish peroxidase conjugate. For development, the membrane was treated with freshly prepared ECL detection reagent (PIERCE, detection reagent 1–peroxide solution and detection
reagent 2–luminol enhancer solution). Chemiluminescence film (CL-Xposure Film, PIERCE) was exposed to the treated membrane and developed with the Konica SR-X film developer.

**Microarray Analysis**

mRNA was isolated from leaves of 22-day-old *SnRK2.6* transgenic and wild type plants using the FastTrack kit (Invitrogen). An aliquot of 100 ng of the isolated mRNA was amplified using Low RNA Input Linear Amplification kit from Agilent and the resulting cRNA was precipitated with ethanol. cRNA (2.4 μg) was added to an 85 μL reverse transcription reaction containing 30 nmol of dNTP and 2 nmol of Cy3- or Cy5-labeled dUTP. The reaction was directed by random 9-mer primer. Agilent Arabidopsis 3 Oligo Microarray slides were used for hybridization. Each of the 60 mer oligo arrays represents 21,000 probes twice. Hybridization was performed according to the manufacturer’s recommendation except that the incubation of the slides was conducted at 42°C for 16 h with ArrayBooster (Advalytix DE). After washing and drying, slides were scanned with ScanArray (PerkinElmer) and quantified with ImaGene (Biodiscovery). Data from eight repeated slides were analyzed using GeneSight (Biodiscovery).

**Effects of Hormones on Seed Germination and Seedling Growth**

Seeds were surface sterilized and incubated in 0.1% agar at 4°C for 3 days to allow for stratification to promote uniform germination. To determine the effects of ABA and ACC on seed germination, the stratified seeds were transferred to agar plates containing half MS, 1% sucrose, 0.8% agar, pH 5.7, in the absence or presence of various ABA or ACC concentrations. The plates were sealed with Parafilm and incubated at 23°C under a 16/8 h light/dark photoperiod in a growth chamber. At 64 h after sowing, germination was scored based on radicle emergence.

For analysis of ABA and ACC effects on seedling growth in the dark as well as of ACC effects under the standard long day condition, the stratified seeds were directly plated onto half MS medium containing different concentrations of the respective growth regulators. The plates were sealed with Parafilm and incubated at 23°C either in the dark
or under a 16/8 h light/dark photoperiod unless otherwise indicated. Phenotype of seedling growth was monitored at different days as indicated in the Results.

To inspect ABA effects on the primary root growth under a 16/8 h light/dark photoperiod, the stratified seeds were first germinated on half MS medium (half MS, 1% sucrose, 0.8% agar, pH 5.7). Four days later, the resultant seedlings were transferred to the same medium except that various concentrations of ABA were included. No noticeable disparity in the elongation of the primary roots in response to ABA (0.25, 0.5, 1 and 2 μM) occurred among the wild type, SnRK2.6 transgenic and snrk2.6 mutant.

**Supplemental Figures and Materials and Methods**

The following materials are available in the online version of this article.

**Supplemental Figure S1** Soluble sugar and starch contents in the leaves of snrk2.6 mutant and null sibling plants.

**Supplemental Figure S2.** Rates of water loss from detached aerial portions of wild type, SnRK2.6 transgenic and snrk2.6 mutant Arabidopsis plants.

**Supplemental Figure S3.** Effect of SnRK2.6 overexpression on the ratio of sucrose to UDP-glucose at three different developmental stages.

**Supplemental materials and methods:** Lipid analysis by gas chromatograph flame ionization detection (GC-FID)

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FIGURE LEGEND

Figure 1. Western blot analysis of SnRK2.6 protein expression in different Arabidopsis tissues.

An equal amount of total protein (40 μg per lane) was loaded in each lane. The snrk2.6 mutant is a negative control. As the primary antibody in the assay, PAB-R could bind to
several unknown peptides whose sizes are larger than that of SnRK2.6 (42 kD) in both wild type (WT) and snrk2.6 mutant plants. However, this antibody appeared to distinguish SnRK2.6 from other SnRK2 kinases whose sizes are close to 42 kD.

**Figure 2.** Enhanced expression of SnRK2.6 protein in the SnRK2.6 overexpressors. An equal amount of total protein from roots (40 μg; A) or leaves (67 μg; B) of different genotypes was analyzed by Western blot. SnRK2.6 was found to be very unstable, which could cause the discrepancy in the amount of this protein between different experiments and among different tissues of the same transgenic event. Nevertheless, our repeated experiments showed that this kinase was expressed at higher levels in the five independent transgenic events 340293, 340318, 340367, 340378, and 340397 than in the wild type.

**Figure 3.** SnRK2.6 overexpression promoted Arabidopsis leaf growth. (A) Photographs of 22-day-old wild type plants (WT) and two independent transgenic events (340293 and 340318) of T3 generation. Other transgenic events (340367, 340378 and 340397) that displayed the same phenotype are not shown. (B) Aboveground biomass of the transgenic and wild type plants at different days after planting (DAP). For fresh weight determination, five plants were weighted together and then averaged. Values are means ± SD (n = 3).

**Figure 4.** SnRK2.6 overexpression promoted lateral branching in Arabidopsis. (A) The number of lateral branches in wild type (WT) Arabidopsis, five independent transgenic events (340293, 340318, 340367, 340378 and 340397), snrk2.6 mutant and its null sibling (Null). The recorded lateral branches were defined as any elongated branches of at least 4 cm from cauline nodes on the primary inflorescence of 34-day-old Arabidopsis plants. Values are means ± SD (n≥15, significantly different from wild type plants at \(P<0.01**\), t-test). (B) Four lateral branches in each of the two independent events as opposed to three in the wild type or mutant plant were depicted.
Figure 5. Effects of SnRK2.6 overexpression on seed yield under well-watered and mild drought conditions.

A matched pairs design was employed to reduce the environmental effects on seed yield. To eliminate the positional effects of transgene insertion, seed yield under each growth condition was averaged from five independent transgenic events (340293, 340318, 340367, 340378 and 340397), with six plants per event. Well-watered condition: watering twice a week during the active plant growth period. Mild and severe drought conditions: withholding irrigation for six days during the flowering stage or for 16 days starting at the vegetative stage, respectively. Values are means ± SD (n=5).

Figure 6. Effects of SnRK2.6 overexpression on leaf soluble sugar and starch contents at three different developmental stages.

For soluble sugar (A, B) and starch (C) analysis, leaves of wild type (WT) and transgenic plants were sampled at 22, 24 and 28 days after planting (DAP). The total soluble sugar content (B) was obtained from the sum of sucrose (A), glucose, glucose-6-phosphate, glucose-1-phosphate, GDP-glucose, and UDP-glucose. ADP-glucose and fructose were at undetectable levels. Data were averaged from three independent transgenic events (340367, 340378 and 340397). Values are means ± SD (n=3).

Figure 7. Soluble sugar and starch contents in the leaves of 30-day-old wild type and transgenic Arabidopsis plants.

The total soluble sugar content (A) was obtained from the sum of sucrose (B), glucose (B), fructose (B), glucose-6-phosphate and UDP-glucose. Glucose-1-phosphate, ADP-glucose and GDP-glucose were at undetectable levels. Data were averaged from five independent transgenic events (340293, 340318, 340367, 340378 and 340397), with two biological replicates per event. Values are means ± SD (n=10; significantly different from wild type plants at $P<0.01^{**}/0.05^*$, t-test).

Figure 8. Effect of SnRK2.6 overexpression on leaf fatty acid composition.

Leaves of 30-day-old wild type and transgenic plants were used in the assays on fatty acid composition (A) and trienoic fatty acid content (B). Data were averaged from five
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**Figure 9.** Effects of ABA on seed germination and seedling growth of the wild type, *snrk2.6* mutant and *SnRK2.6* overexpressors.

(A) Germination rate (mean ± SD, n= 3) of each genotype at 64 h after the end of stratification. (B, C) The hypocotyl (B) and root (C) length of the seedlings grown in the dark at various ABA concentrations for 8 days. It is noted that the roots and hypocotyls were too short to be accurately measured at 1 and 2 μM of ABA. Values are means ± SD (n= 25; significantly different from wild type seedlings at $P<0.01^{**}$, $t$-test). (D) Phenotype of the seedlings grown at 1 μM of ABA in the dark for 20 days and then in the light for two days. The transgenic event 340335, which did not show a significant increase in soluble sugar content relative to wild type, was used as a control in this experiment. As predicted, no significant difference in ABA sensitivity between this event and wild type was observed.
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