

ASR1 Mediates Glucose-Hormone Cross Talk by Affecting Sugar Trafficking in Tobacco Plants^{1[C][W][OA]}

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Asr (for *ABA*, *stress*, *ripening*) genes are exclusively found in the genomes of higher plants, and the encoded proteins have been found localized both to the nucleus and cytoplasm. However, before the mechanisms underlying the activity of ASR proteins can be determined, the role of these proteins in planta should be deciphered. Results from this study suggest that ASR is positioned within the signaling cascade of interactions among glucose, abscisic acid, and gibberellins. Tobacco (*Nicotiana tabacum*) transgenic lines with reduced levels of ASR protein showed impaired glucose metabolism and altered abscisic acid and gibberellin levels. These changes were associated with dwarfism, reduced carbon dioxide assimilation, and accelerated leaf senescence as a consequence of a fine regulation exerted by ASR to the glucose metabolism. This regulation resulted in an impact on glucose signaling mediated by *Hexokinase1* and *Snf1-related kinase*, which would subsequently have been responsible for photosynthesis, leaf senescence, and hormone level alterations. It thus can be postulated that ASR is not only involved in the control of hexose uptake in heterotrophic organs, as we have previously reported, but also in the control of carbon fixation by the leaves mediated by a similar mechanism.

Sugars supply necessary carbon and energy for the vast majority of metabolic processes in plants. Moreover, they act as signaling molecules in a wide number of physiological processes, such as growth, development, and senescence (Rolland et al., 2006; Baena-González and Sheen, 2008; Hanson and Smeekens, 2009; Eveland and Jackson, 2012). To date, the most studied sugar signaling factors include HEXOKINASE1, SNF1-RELATED KINASE (SnRK1), TOR KINASE, and C/S1 bZip factors (Baena-González and

Sheen, 2008; Hanson and Smeekens, 2009; Eveland and Jackson, 2012). Sugar signaling pathways interact extensively with hormone pathways, and these interactions are partly responsible for the control of basic physiological processes (Rolland et al., 2006; Eveland and Jackson, 2012). Studying the nature of these interactions and the mechanisms of control involved in them will lead to a better understanding of these physiological processes. In this vein, awareness of the cross talk between sugars and hormones has arisen from studies with mutants of the hormonal pathways and/or sugar responses. For instance, a key link between sugars and abscisic acid (ABA) is exemplified by ABA INSENSITIVE4, which encodes an Apetala2 domain-containing transcription factor that binds a COUPLING ELEMENT1-like element present in many ABA and sugar-regulated promoters and is required in the Glc-mediated developmental arrest during vegetative morphogenesis (Arenas-Huertero et al., 2000; Dekkers et al., 2008). Despite this, there is relatively scarce information available on transcription factors regulating sugar-hormone cross talk.

The *Asr* (for *ABA*, *stress*, *ripening*) gene family is widely distributed in higher plants (Carrari et al., 2004; Frankel et al., 2006; Fischer et al., 2011) and constitutes group 7 of LATE EMBRYOGENESIS ABUNDANT proteins (Battaglia et al., 2008). It has been demonstrated that the ASR1 protein is present in the nucleus (Çakir et al., 2003; Wang et al., 2003; Kalifa et al., 2004a) and possesses a zinc-dependent DNA-binding

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activity (Kalifa et al., 2004a; Rom et al., 2006). In addition, fractionation assays and functional analyses suggest that the protein also localizes to the cytosol, where it has chaperone-like activity (Kalifa et al., 2004a; Konrad and Bar-Zvi, 2008; Urtasun et al., 2010; Dai et al., 2011; Hsu et al., 2011; Ricardi et al., 2012). Although the involvement of *Asr* genes in various abiotic and biotic stresses has been described (Amitai-Zeigersona et al., 1995; Jeanneau et al., 2002; Kalifa et al., 2004b; Yang et al., 2005; Liu et al., 2010; Dai et al., 2011; Henry et al., 2011; Hsu et al., 2011; Virilouvet et al., 2011; Jha et al., 2012; Arenhart et al., 2013), the exact molecular mechanism underlying their function remains unknown. Nevertheless, it has been demonstrated that the grape (*Vitis vinifera*) ASR VvMSA (for MATURATION-, STRESS-, ABA-INDUCED PROTEIN) protein interacts with a DROUGHT RESPONSE ELEMENT BINDING transcription factor in the nucleus (Saumonneau et al., 2008).

Several *Asr* orthologous and paralogous genes have been found to be transcriptionally regulated by ABA and sugars (Amitai-Zeigersona et al., 1995; Rossi et al., 1998; Çakir et al., 2003; Kalifa et al., 2004a; Liu et al., 2010; Chen et al., 2011; Henry et al., 2011; Virilouvet et al., 2011). Furthermore, a model for VvMSA transcriptional regulation at the convergence of Glc and ABA signaling cascades through HEXOKINASE1 and SnRK1 has recently been proposed (Saumonneau et al., 2012). Although the physiological role of *Asr* genes has proven elusive, *VvMsa* has been clearly demonstrated to recognize specific binding sites in the promoter/enhancer region of the hexose transporter *VvHt1* (Çakir et al., 2003). In tubers of transgenic potato (*Solanum tuberosum*) plants, the levels of expression of *Asr1* negatively correlated with tuber Glc content and mRNA levels of the hexose transporter *Ht2* (Frankel et al., 2007). Recently, it has been reported that the overexpression of ASR1 in maize (*Zea mays*) has a large impact on vegetative biomass and that this phenotype correlates with changes in the branched-chain amino acid biosynthesis (Virilouvet et al., 2011). The molecular studies mentioned above suggest that ASR1 could play a regulatory role in the cross talk between primary carbon metabolism and hormone signaling. To gain insight into the physiological role of ASR1, we generated and comprehensively evaluated transgenic tobacco (*Nicotiana tabacum*) plants with altered expression levels of *Asr1*. Our results indicate that ASR1 regulates leaf Glc levels and carbon partitioning. Moreover, both hormone levels and sensitivity analyses suggest that this protein is not only involved in Glc signaling but also in Glc-ABA and Glc-GA cross talk in photosynthetically active leaves. These combined data are discussed in the context of current models of sugar and hormone sensing and their influence on whole-plant carbon partitioning.

RESULTS

Generation and Selection of Tobacco Transgenic Lines

Fifty and thirty-four independent shoots of T0 antisense and sense lines, respectively, were obtained after

selection in kanamycin-containing medium and transferred to the greenhouse. These lines were screened on the basis of the levels of *Asr1* expression by means of northern blot (data not shown). For further analyses, three lines from each construct were selected (namely, overexpressors S14, S22, and S31 and silenced lines AS2, AS26, and AS42) and propagated *in vitro* in order to obtain six plants per line, which were subsequently analyzed by western (Fig. 1A) or northern (Fig. 1B) blots of fully expanded leaves. Progeny tests of the six transgenic lines showed segregation ratios of 3:1 (resistance:susceptibility to kanamycin), suggesting a single-copy insertion of the transgene. In addition, two transgenic lines (one overexpressor, S 22, and one silenced, AS 2) were selfed to T4 homozygous generation; two siblings per line in T1 generation were selected through progeny tests for further experiments in order to avoid possible maternal effects. Thus, siblings S 22 2 and S 22 3 derive from S 22, and siblings AS 2 2 and AS 2 4 derive from AS 2. When these lines were analyzed by quantitative real-time PCR (qRT-PCR), significant reductions and increments in *Asr1* mRNA levels were confirmed in fully expanded leaves of antisense and sense plants, respectively, compared with wild-type controls (Fig. 1C).

Phenotypic Characterization of *Asr1* Transgenic Lines

Selected transgenic lines were grown alongside wild-type controls and characterized in detail

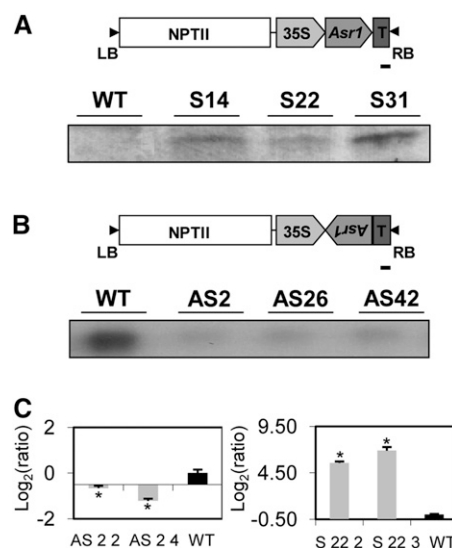


Figure 1. *Asr1* expression in tobacco transgenic lines. Western- (A) and northern-blot (B) analyses of three overexpressing (S) and three silenced (AS) transgenic lines (T0). Schemes of the constructs used for plant transformation are shown in the top side of each figure. Bars = 100 bp. C, qRT-PCR expression analyses in source leaves from T4 homozygous lines (left, antisense plants; right, overexpressing plants). Asterisks indicate statistically significant differences by the permutation test ($P < 0.05$). $n = 3$ to 5. WT, Wild type.

phenotypically. The three silenced lines showed a severe stunted phenotype (Fig. 2A), which was already evident at plantlet stage (data not shown). This phenotype is reflected in a significant decrease in stem fresh matter accumulation after 6 weeks of growth (Fig. 2C). In homozygous silenced lines, average internode lengths were shorter; consequently, plant height and stem matter accumulation were again significantly reduced (Fig. 2, E, F, and H). Also, these plants showed a dramatic reduction in fresh matter

accumulation in leaves and roots (Fig. 2H), which was not observed in T0 silenced lines (Fig. 2, B and D), and an extended flowering time (Fig. 2G). By contrast, overexpressing lines showed no differences in any of the measured traits (Fig. 2).

Fully expanded leaves from silenced lines were also characterized by chlorotic and necrotic spots that appeared early during leaf expansion (Fig. 2A). When they were assayed for the oxidation of diaminobenzidine (DAB) by the hydrogen peroxide (H₂O₂) content of the

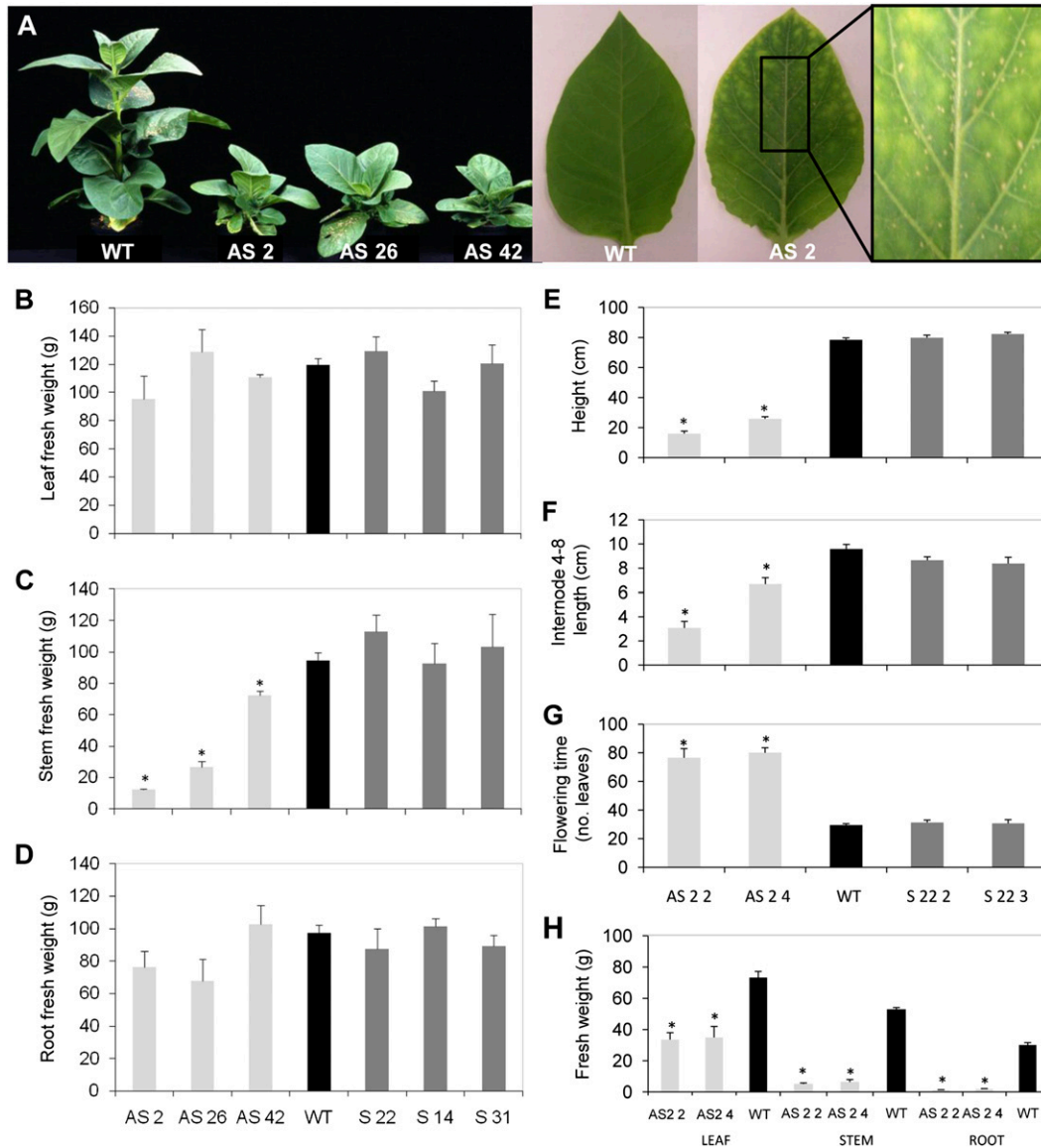


Figure 2. Phenotypic characterization of the *Asr1* transgenic lines. A, Visual phenotype of 6-week-old plants of three independent T0 silenced transgenic lines (left). Expanded seventh leaves from a wild-type (WT) plant and a T4 homozygous silenced plant (AS 2 4; right). Fresh matter accumulated in leaves (B), stems (C), and roots (D) of independent T0 transgenic lines. Phenotypic characterization of T4 homozygous lines: plant height (E), internode 4 to 8 length (F), flowering time measured as the number of total leaves of the plant at the time of the setting of flower buds (G), and matter accumulation in leaves, stems, and roots (H). Data represent the means \pm SE of measurements from five to eight plants per line. Asterisks indicate statistically significant differences by Student's *t* test ($P < 0.05$). AS, Silenced plants; S, overexpressing plants. [See online article for color version of this figure.]

cells, the typical insoluble brownish precipitate was stronger in silenced lines (Supplemental Fig. S1). Since this phenotype could be related to leaf senescence, ASR1 protein levels were assessed by western blot in leaves of wild-type plants. Older leaves, whose senescence is more advanced, accumulated higher levels of the ASR1 protein (data not shown).

Photosynthetic Parameters Are Altered in *Asr1*-Silenced Lines

We reasoned that the dramatic reduction in fresh matter accumulation along with the early chlorosis in expanded leaves observed in the silenced *Asr1* lines (Fig. 2) could be related to a reduction in CO₂ assimilation. Indeed, this parameter was significantly reduced in homozygous silenced plants at all tested photosynthetically active radiations (PARs; Fig. 3A). However, transpiration rate and leaf conductance were reduced only at high irradiances (Fig. 3, B and C). These results correlated with weaker intensities of the bands corresponding to the large and small subunits of Rubisco in leaves from these plants, which also seem to have a different protein pattern (Supplemental Fig. S2). On the other hand, homozygous overexpressing lines were invariant compared with wild-type plants in all measured parameters (Fig. 3).

Changes in Leaf Carbohydrate Contents

Since *Asr1*-silenced plants showed a reduction in CO₂ assimilation and *Asr* genes have been related to carbon transport and metabolism in sink tissues, sugar contents were measured in leaves and phloem sap. Silenced plants showed significantly higher levels of leaf Glc during the middle of the light (1 PM) and dark (5 AM) periods, while Fru was only increased during the light period (Fig. 4A). In the experiment performed with homozygous lines, the diurnal curve of leaf sugars showed that silenced plant leaves accumulated between 3 and 10 times more Glc than wild-type and overexpressing lines (Fig. 4B). However, no other variations in sugar contents were observed either in the silenced or overexpressing lines (Fig. 4, A and B).

A significant reduction in Suc level was found in the phloem sap of homozygous silenced plants (Supplemental Fig. S3). Remarkably, a strong deterioration was observed in leaves of silenced plants after the 17 h ex vivo, whereas wild-type and overexpressing leaves exhibited no visible changes (Supplemental Fig. S3). On the other hand, a decrease of Suc was detected in the phloem sap of the S 22 3-overexpressing line. Hexoses were detected in phloem sap (data not shown). However, this would suggest that there was some contamination from the vacuolar compartment of compromised, non-phloem cells during sampling, as detailed by Turgeon and Wolf (2009) and Liu et al. (2012), since tobaccos are Suc apoplasmic loaders (von Schaewen et al., 1990; Sonnewald et al., 1991).

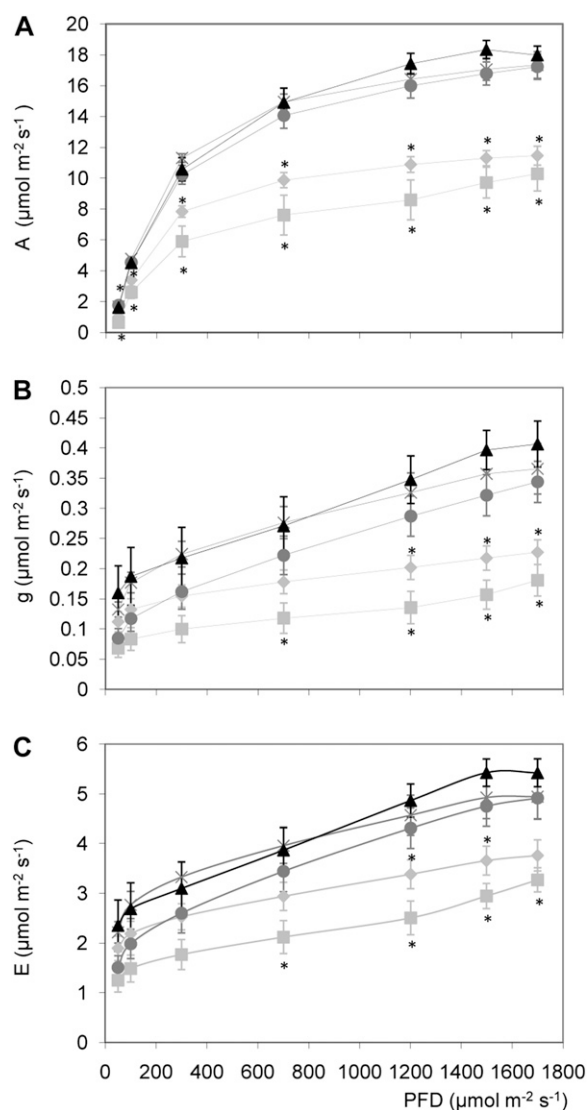


Figure 3. Effect of altered *Asr1* expression on photosynthetic parameters of T4 plants. A, CO₂ assimilation rate or A; B, stomatal conductance or G; and C, transpiration rate or E, measured at the indicated photon flux density (PFD) in fully expanded leaves of T4 plants at vegetative stage. Light-gray squares, AS 2 2; light-gray rhombuses, AS 2 4; black triangles, wild type; dark-gray circles, S 22 2; dark-gray crosses, S 22 3. The data represent the mean \pm se of measurements from four to six plants per line. Asterisks indicate statistically significant differences by Student's *t* test ($P < 0.05$).

[U-¹⁴C]Glc Assimilation in Leaves

In order to study whether Glc assimilation in leaves could be affected by *Asr1*, we incubated leaf discs of transgenics and wild-type plants with [¹⁴C]Glc in the dark (Fig. 5). The total [¹⁴C]Glc uptake was invariant in silenced and overexpressing lines (Fig. 5). However, distribution of radioactivity between soluble and insoluble metabolite fractions differed significantly after 225 min of incubation (and also after 60 and 120 min; data not shown), with percentages of soluble ¹⁴C being

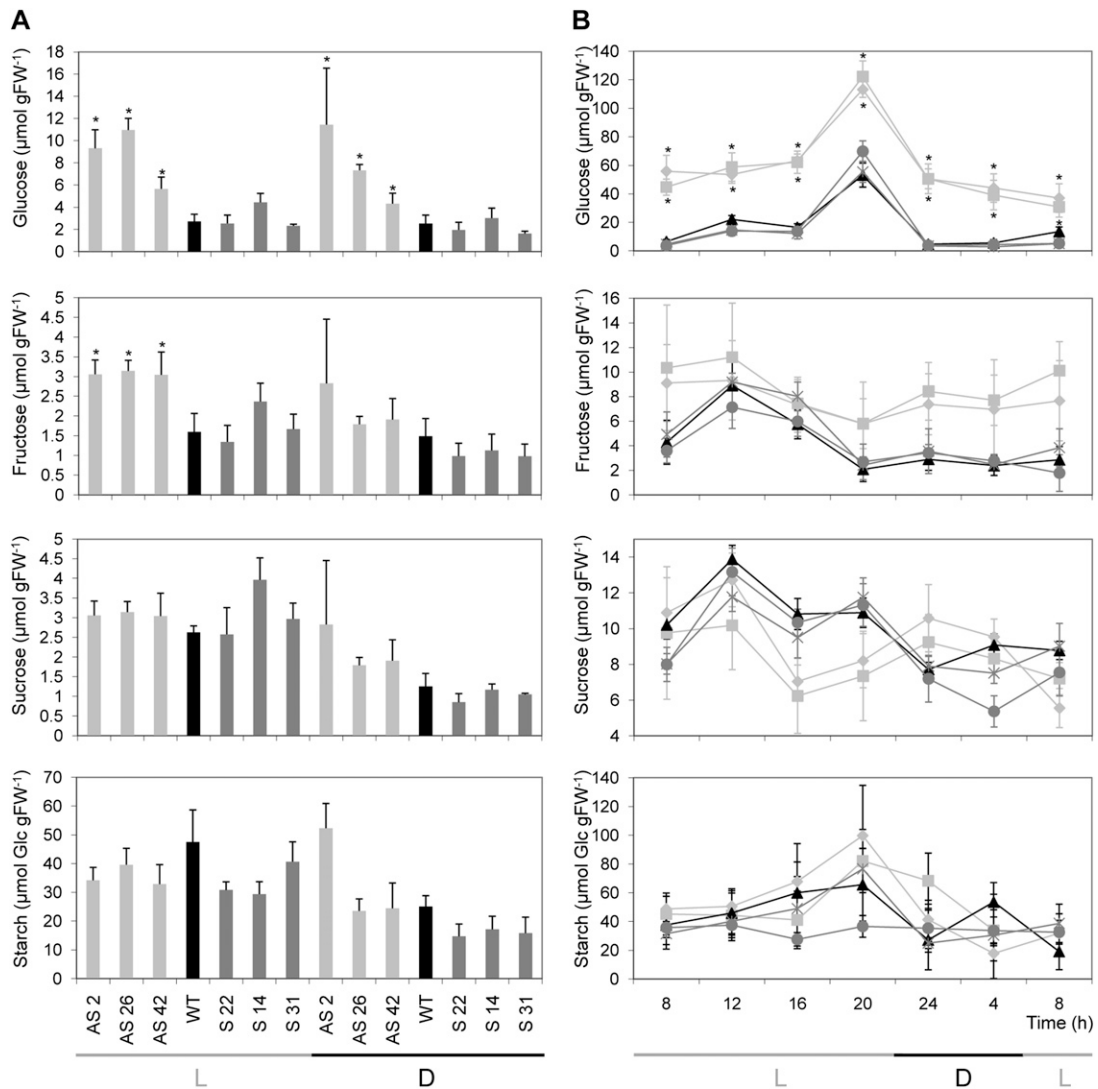


Figure 4. Leaf carbohydrate contents in *Asr1* transgenic lines. A, Carbohydrate contents in source leaves of T0 plants at a light time point (1 PM) and a dark time point (5 AM). B, Diurnal changes in carbohydrate contents in leaves from T4 homozygous plants. At each time point, samples were taken from mature source leaves and data represent the means \pm SE of measurements from four to six plants per line. L, Light period; D, dark period; FW, fresh weight. Light-gray squares, AS 2 2; light-gray rhombuses, AS 2 4; black triangles, wild type (WT); dark-gray circles, S 22 2; dark-gray crosses, S 22 3. Asterisks indicate statistically significant differences relative to wild-type controls by Student's *t* test ($P < 0.05$).

significantly higher in discs from silenced leaves (Fig. 5). The insoluble fraction, which is mainly composed of starch, membrane components, and proteins, revealed a minor, albeit nonsignificant, decrease in the silenced plants (Fig. 5). In the overexpressing lines, the opposite effects were observed (Fig. 5). Within the soluble fraction, the neutral fraction (mostly soluble sugars) was also significantly increased in silenced plants (Fig. 5). Moreover, the combined acid and basic fractions, which together are composed by major components of the tricarboxylic acid cycle, were decreased in the three silenced plants compared with wild-type controls (Fig. 5). In addition, neutral fractions were separated in thin-layer chromatography

(TLC) plates alongside authentic radioactive sugar standards. This analysis revealed up to 11-fold increases in Glc-to-Suc ratios in silenced plants, whereas these ratios were unaltered or marginally reduced in the overexpressing lines (Fig. 5). When taken together, these results thus suggest that Glc metabolism is impaired in silenced plants.

ABA and GA Metabolisms Are Altered in *Asr1* Plants

Regulation of the *Asr* gene family at the mRNA levels by ABA is well documented in tomato (*Solanum lycopersicum*; Amitai-Zeigersona et al., 1995; Rossi

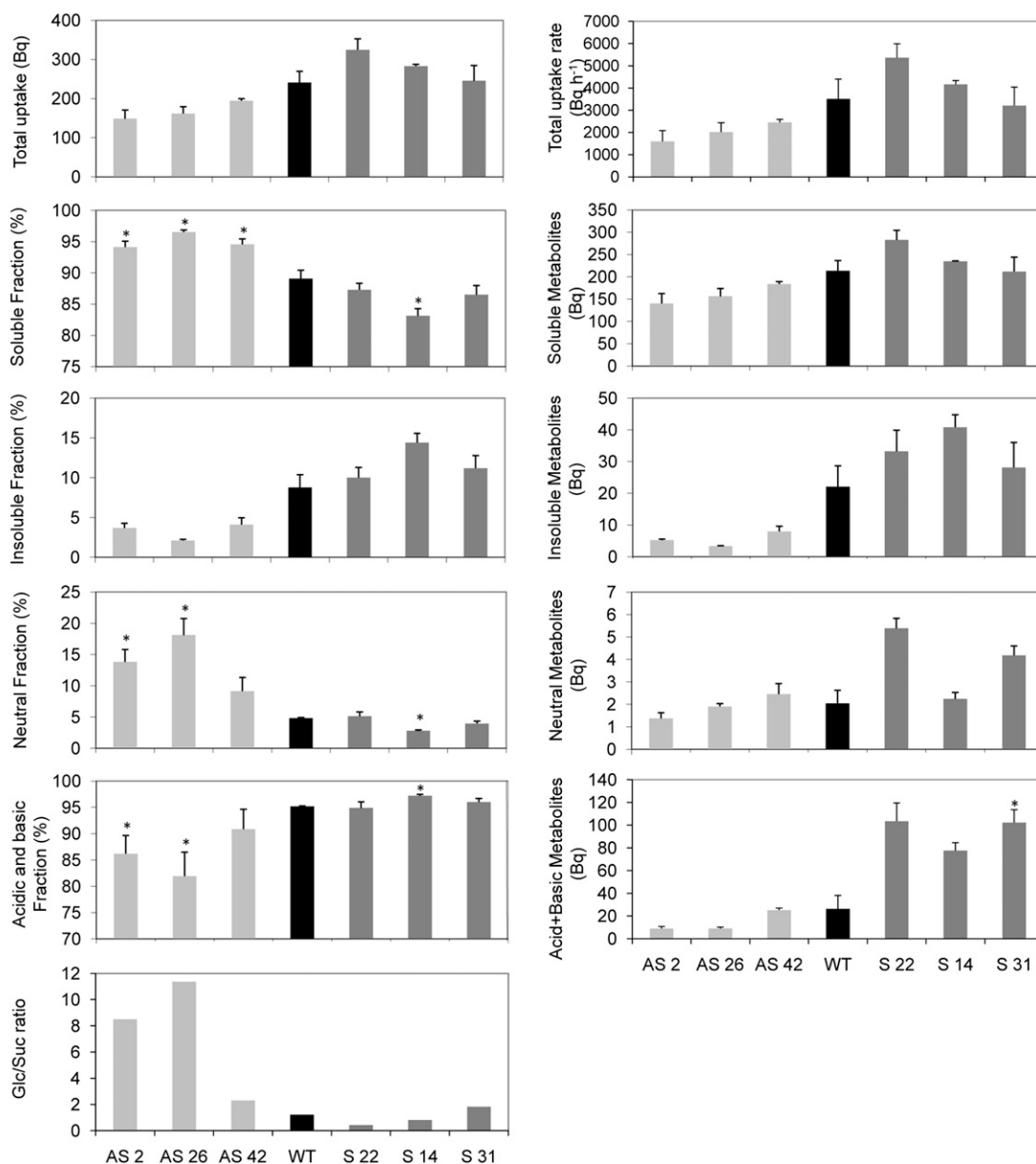


Figure 5. Glc assimilation in *Asr1* transgenic leaves (T0). Leaf discs were cut from 6-week-old plants at the onset of the light period and incubated with [U-¹⁴C]Glc. Soluble and insoluble fractions were measured in a scintillation counter. Soluble fractions were subsequently separated by ion-exchange chromatography. Total [U-¹⁴C]Glc uptake and soluble, insoluble, neutral, acid, and basic fractions are shown on the left. Total uptake rate, absolute soluble, insoluble, neutral, acid, and basic metabolites are shown on the right. Sugars from neutral fractions were separated on TLC plates, and Glc-to-Suc ratios are presented at bottom left. Data represent the mean \pm SE of measurements from three plants per line. Asterisks indicate statistically significant differences by Student's *t* test ($P < 0.05$). WT, Wild type.

et al., 1998), grape (Çakir et al., 2003), and maize (Virlouvet et al., 2011). It is also known that Glc modulates genes involved in ABA biosynthesis and signaling (Rolland et al., 2006). Therefore, we next measured ABA contents by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) in source leaves. In homozygous silenced plants, ABA levels were significantly reduced (more than or equal to 3 times; Fig. 6A). Conversely, overexpressing plants

showed a marginal increase in the levels of this hormone; however, this was only statistically significant in line S 22 3 (Fig. 6A).

We also reasoned that the stunted phenotype of the silenced lines could be due to altered GA levels and decided to quantify these hormones in expanded leaves. Out of the 20 GA standards ($GA_{8/29/23/3/1/6/5/19/13/20/44/34/53/51/7/4/24/15/9/12}$) used, only $GA_{8/29/23/1/19/20/53}$ could be reliably identified and quantified in

our samples (Fig. 6B). GA₃₄ was convincingly detected, but the level was below the reliable level of quantification. A reconstruction of the most probable pathway of GA metabolism in tobacco leaves was carried out based on the pathways published by Yamaguchi (2008) and MacMillan (1997) (Fig. 6B). GA₁ was the only growth-active GA that could be detected and quantified (Fig. 6B). Surprisingly, silenced homozygous plants did not show alterations in the levels of this GA (Fig. 6B). However, both its immediate precursor GA₂₀ and its degradation product GA₈ turned out to be significantly reduced and increased, respectively, in silenced plants (Fig. 6B). GA₂₉, which derives from GA₂₀, is also reduced in silenced plants (Fig. 6B). In contrast, overexpressing plants showed a marginal, though significant, decrease in GA₁ (Fig. 6B). Except for GA₂₃, which showed increased and decreased levels in the antisense line AS 2 4 and in the overexpressing line S 22 2, respectively, there were no changes in the levels of other active GA precursors (GA₅₃ and GA₁₉; Fig. 6B). For a more comprehensive analysis, product-to-substrate ratio for each oxidation step was calculated. Silenced lines exhibited significantly different values; while C20 oxidation steps GA₁₉>GA₂₀ and GA₂₃>GA₁ (catalyzed by the GA 20-oxidase) were lower than in wild-type controls, the C3 oxidation reactions GA₂₀>GA₁ and GA₁₉>GA₂₃ (catalyzed by the GA 3-oxidase) displayed 5-fold increased ratios (Supplemental Table S1). These results suggest that both GA 20-oxidase and GA 3-oxidase activities are altered in silenced plants.

Sensitivity to GA₃

Given that GA₁, the only growth-active GA that was detected, is unaltered in the stunted silenced plants and that the levels of ASR1 protein were increased in leaves of wild-type plants following a GA₃ treatment (data not shown), we decided to investigate if the shorter internode lengths of the silenced plants were a consequence of an alteration in GA sensitivity.

For this purpose, the fold change in stem growth curve was used as a measure of the response to GA₃. Although clear phenotypic differences were observed after 53 d of GA₃ treatment between the silenced and wild-type plants (Fig. 6C), slopes of GA₃-treated silenced plants were similar to those of the wild-type controls, thus suggesting similar extent of GA₃ sensitivity between the genotypes (Fig. 6D).

Soluble sugars of treated wild-type plants were altered in comparison to untreated wild-type plants (Supplemental Table S2). However, silenced lines did not show any difference between treatments (Supplemental Table S2). Interestingly, similar results were observed for biomass accumulation: silenced treated plants showing no differences but wild-type plants accumulating higher levels of biomass (Supplemental Table S2). In addition, GA₃ caused a delay in flowering time of the wild-type plants (Supplemental Table S2). However,

in the silenced treated plants, this trait could not be investigated due to death of the treated plants before flowering.

Asr1 Cross Talks at Sugar Transport and Signaling by Regulating mRNA Levels

It has been described that a grape ASR protein binds to conserved motifs in the promoter/enhancer region of the *VvHt1* monosaccharide transporter (Çakir et al., 2003). In order to test a possible connection between *Asr1* and sugar transporters in tobacco, we evaluated the mRNA levels of four putative sugar transporters, namely, *Ht1* (ortholog to *VvHt1*), *Sut2* (for *Suc transporter2*; Meyer et al., 2000; Hackel et al., 2006), *Tmt3* (for *Tonoplast monosaccharide transporter3*; Wormit et al., 2006), and *Vgt* (for *Vacuolar Glc transporter*, although it seems to be localized to the chloroplast membrane; Büttner, 2007). Silenced homozygous plants showed lower mRNA levels of *Ht1* and *Sut2* than wild-type plants (Fig. 7, A and B). However, no differences were observed in the mRNA levels of *Tmt3* and *Vgt* in these plants (Fig. 7, C and D). To gain further insight into the Glc signaling pathway, we also measured the mRNA levels of *Hexokinase1* and *SnRk1.1* (the catalytic subunit of *SnRK1*), two key genes in the Glc signaling pathway. Silenced homozygous plants had higher mRNA levels of *Hexokinase1* and lower mRNA levels of *SnRk1.1* (Fig. 7, E and F). By contrast, overexpressing plants were invariant with regard to the mRNA levels of any of these genes (Fig. 7).

DISCUSSION

Silencing *Asr1* in tobacco resulted in dramatic alterations in all measured physiological parameters. By contrast, *Asr1* overexpression did not generate significant changes under controlled growth conditions. Previous studies have shown that the grape ASR ortholog, VvMSA, regulates the expression of the hexose transporter *ht1* gene (Çakir et al., 2003). Interestingly, the *Asr1* transcript is expressed in the companion cells of the phloem of tomato leaves (Maskin et al., 2008). Likewise, *ht1* is expressed in the phloem region of vascular bundles of grape leaves, petioles, and fruits (Vignault et al., 2005). Intriguingly, *Asr1* silencing led to reduced mRNA levels of the tobacco *ht1* ortholog and high leaf Glc levels (Figs. 4 and 7A). We also found that Glc metabolism is impaired in the silenced plants (Fig. 5). According to the proposed role of transporters such as HT1 (Lalonde et al., 2003; Vignault et al., 2005; Slewinski, 2011), the decreased expression of *ht1* could explain the increased levels in Glc, since a failure of phloem cells to retrieve hexoses would result in sugar accumulation in the apoplast. It is conceivable that Glc could spread to other cell types, such as parenchyma and mesophyll cells. This hypothesis is in agreement with the phenotypic

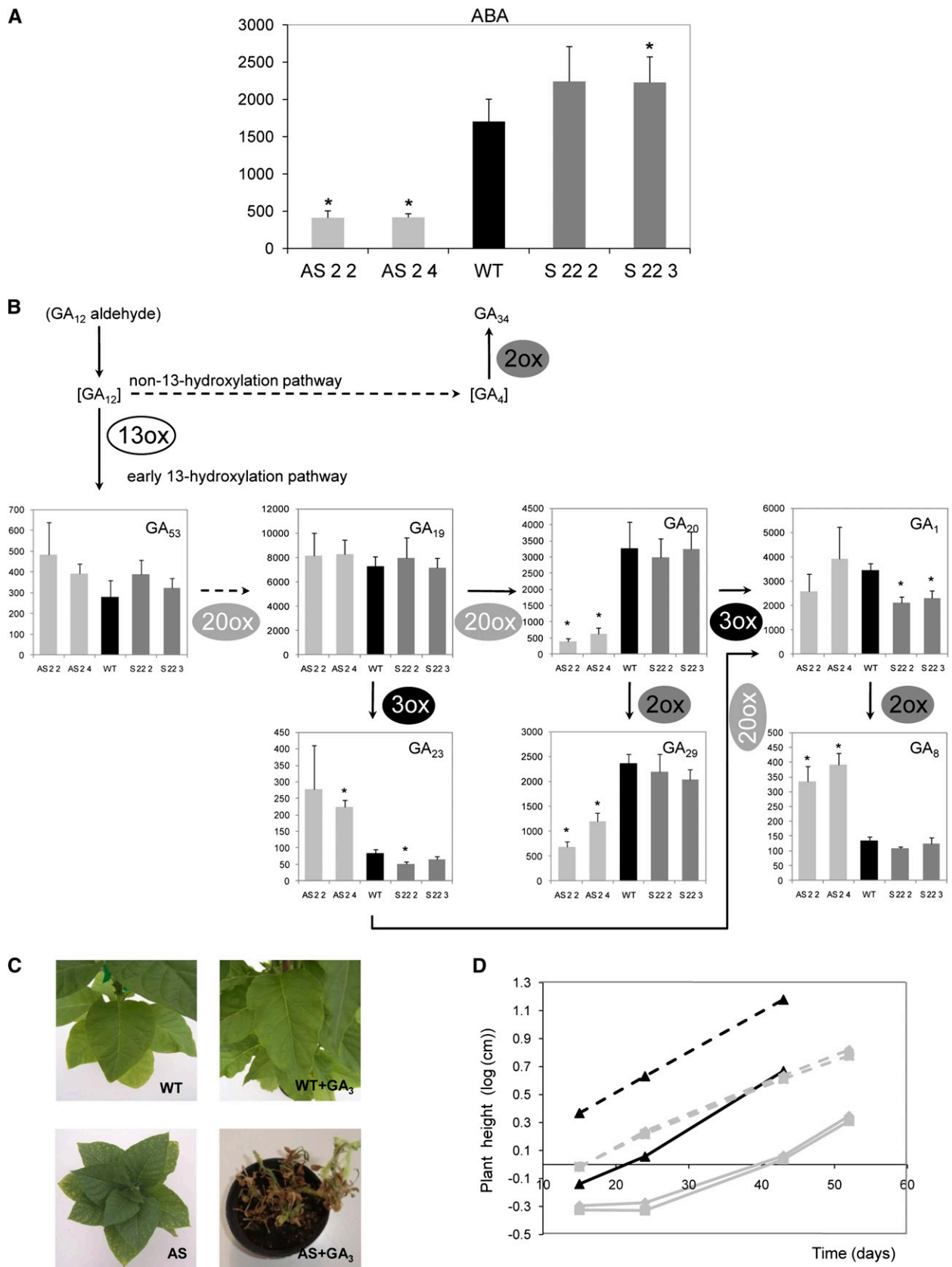


Figure 6. ABA and GA metabolism in *Asr1* tobacco plants and GA₃ sensitivity of *Asr1*-silenced plants. ABA (A) and GA (B) levels were measured by LC-MS/MS in source leaves from 6-week-old plants. ABA and GA data are expressed in ng g dry weight⁻¹ and pg g dry weight⁻¹, respectively, and represent the means \pm SE of measurements from five independent plants per

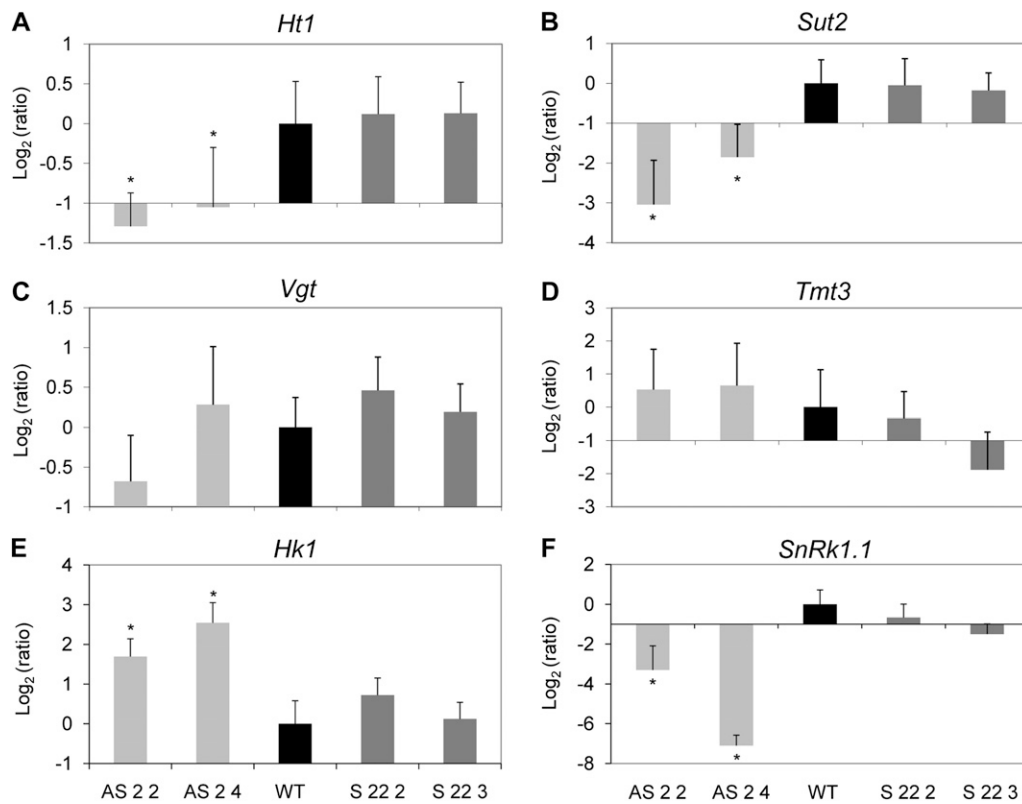


Figure 7. Expression analyses of sugar sensors and transporters in *Asr1* tobacco lines. mRNA levels of sugar transporters *Ht1* (A), *Sut2* (B), *Vgt* (C), and *Tmt3* (D) and sugar sensors *Hexokinase1* (E) and *SnRk1.1* (F) were quantified by qRT-PCR in leaves of T4 lines relative to wild-type (WT) plants. Asterisks indicate statistically significant differences by the permutation test ($P < 0.05$). $n = 3$ to 5. Ratio = $(E_{\text{target}})^{\Delta\text{Cp target}/(\text{MEAN control} - \text{MEAN sample})} / ((E_{\text{target}})^{\Delta\text{Cp target}/(\text{MEAN control} - \text{MEAN sample})})^{-1}$ (Pfaffl 2001).

alterations observed in silenced plants, which are associated with high levels of Glc in mesophyll cells (see below). Expression of the *Sut2* gene was also down-regulated in silenced plants (Fig. 7B). *Sut2* is a Suc transporter localized to the sieve elements of the phloem whose expression is regulated by Suc and whose function in leaf tissues has not been elucidated (Barker et al., 2000; Meyer et al., 2004; Hackel et al., 2006; Kühn, 2011). *Sut2* down-regulation could be due to a direct regulation by ASR1, considering that proteins of sieve elements are synthesized in companion cells (Liesche et al., 2011). Even though carbon assimilation is lower in silenced plants, total leaf Suc levels are unaltered (Figs. 3 and 4). Hence, the low levels of Suc in phloem sap could be explained by the decrease in Suc loading into the phloem in order to maintain normal leaf Suc levels.

The effect of Glc as signal molecule is partly mediated by HEXOKINASE1, which among other functions, is involved in the inhibition of photosynthetic gene transcription (Moore et al., 2003) and the development of senescence (Dai et al., 1999). Another pivotal protein in sugar sensing and signal transduction is SnRK1, a global regulator of plant metabolism that controls energy-conserving processes and the remobilization of alternative energy sources (Chillebert et al., 2011). *SnRK1* regulation seems to rely on the overall energy status of the cell, independently of specific sugar molecules, and both Suc and Glc have a repressive effect on SnRK1 target genes (Baena-González and Sheen, 2008). It seems reasonable to assume that SnRK1 and HEXOKINASE1 pathways are connected. However, precise details concerning the nature of this connection are presently unknown (Cho et al., 2012).

Figure 6. (Continued.)

line. Asterisks indicate statistically significant differences compared with wild-type (WT) controls by Student's *t* test ($P < 0.05$). Species in brackets were not detected. GA₃₄ was convincingly detected, but levels were below the reliable level of quantification. Reconstruction of GA pathway was done based by Yamaguchi (2008) and MacMillan (1997). Continuous and dashed lines indicate single and multiple reactions, respectively. 13ox, 13-Oxidase; 20ox, 20-oxidase; 3ox, 3-oxidase; 2ox, 2-oxidase. Exogenous GA₃ (10 μM) was applied to young seedling as described in "Materials and Methods." Pictures of GA₃-treated (right) and untreated (left) silenced transgenics (T4) and control plants (C). Plant height along growth (D). Gray squares, AS 2 2; gray triangles, AS 2 4; black triangles, wild type; continuous lines, untreated plants; dashed lines, treated plants. [See online article for color version of this figure.]

Extensive interactions between Glc and hormone pathways have been reported (Rolland et al., 2006; Eveland and Jackson, 2012). However, the knowledge of the mechanisms underlying these interactions remains incomplete. It is known that in some cases, HEXOKINASE1 regulates the response to certain hormones, such as cytokinins and auxins (Moore et al., 2003). Our experiments showed that silenced plants have decreased ABA levels and high Glc (Figs. 4 and 6A), which is unexpected considering the literature to date (Rolland et al., 2006; Eveland and Jackson, 2012). However, most of the studies showing the effect of Glc on ABA have been conducted with seedlings rather than with adult plants. Our results suggest that ABA catabolism could be enhanced in the leaves of the silenced plants; the higher Glc and *Hexokinase1* transcript levels (Figs. 4 and 7E) might result in ABA glycosylation generating inactive ABA-glucosyl esters (Xu et al., 2002; Bolouri-Moghaddam et al., 2010).

GA metabolic pathway also considerably differed in these lines (Fig. 6B); ratios of a given GA and its precursor were altered (Supplemental Table S1), probably as a consequence of changes in the activities of GA 20- and GA 3-oxidases. Also, treatment of wild-type plants with GA₃ increases and/or stabilizes the level of ASR1 protein (data not shown). This is in agreement with the increased transcript levels of rice (*Oryza sativa*) ASR5 occurring upon treatment with GA₃ (Takasaki et al., 2008). There is a precedent that interactions between GAs and sugars occur in the leaves, as it has been described that GAs regulate photosynthesis and source-sink relationships via the regulation of SUCROSE PHOSPHATE SYNTHASE and invertases (Biemelt et al., 2004; Huerta et al., 2008; Iqbal et al., 2011). As for other hormones, the regulation of the bioactivity of GAs can also be achieved by the glycosylation of its species (Bolouri-Moghaddam et al., 2010). Regulation of ASR1 by ABA, GA, and sugars thereby provides a novel link between hormonal and sugar pathways.

A remarkable aspect of the phenotype of the silenced plants is the occurrence of necrosis and chlorosis of the leaves (Fig. 2A). Along with the decrease in CO₂ assimilation (Fig. 3A), the degradation of the large and the small subunits of Rubisco (Supplemental Fig. S3), the accelerated deterioration of the leaves after a period of incubation (Supplemental Fig. S2), and the increased reactive oxygen species (ROS) levels in leaves (Supplemental Fig. S1) indicate that these plants undergo a process of accelerated senescence (Yoshida et al., 2002; Rolland et al., 2006; Gregersen et al., 2008; Breeze et al., 2011). It has been proposed that developmental senescence is led by increasing levels of Glc in mesophyll cells and that the sugar signaling during senescence is dependent on HEXOKINASE1 (Dai et al., 1999; Rolland et al., 2006; Slewinski, 2011; Wingler et al., 2012). It is thus conceivable that the increments in Glc and *Hexokinase1* transcript levels in silenced plants (Figs. 4 and 7E) could trigger leaf senescence. Furthermore, high Glc levels inhibit photosynthesis

by down-regulating transcription of photosynthetic genes through HEXOKINASE1 (Sheen, 1990; Moore et al., 2003). In turn, photosynthesis inhibition can also lead to senescence (Dai et al., 1999). Moreover, silenced plants also show high levels of H₂O₂, a ROS (Supplemental Fig. S1). This molecule could also contribute to the establishment of senescence, since ROS can act as signaling molecules that activate senescence genes (Jongebloed et al., 2004; Bolouri-Moghaddam et al., 2010). Additionally, it has been proposed that a drastic overshoot of the ROS levels can serve as a trigger of senescence after the release of HEXOKINASE1 from the mitochondrion (or the chloroplast) membrane (Bolouri-Moghaddam et al., 2010). SnRK1 activity is also involved in the development of senescence. Strong evidence suggests that low SnRK1 activity accelerates senescence and the opposite occurs with high SnRK1 activity at different stages of plant development (Rolland et al., 2006; Cho et al., 2012; Wingler et al., 2012). Silenced tobacco plants displayed decreased *SnRk1.1* mRNA levels (Fig. 7F), a feature that could be influencing the early onset of senescence. In brief, our results suggest that the altered levels of Glc, *Hexokinase1*, *SnRK1.1*, and ROS all contribute to the early onset of senescence observed in the silenced plants.

At the hormonal level, ABA is not necessary for the induction of senescence associated with sugars (Pourtau et al., 2004). GAs, on the other hand, delay senescence (Gan, 2010). Although the silenced plants revealed no significant differences in GA₁ content (Fig. 6B), we cannot exclude that other GA species and/or other hormones may be influencing the observed phenotype. The role of ASR in senescence is also supported by the fact that levels of this protein increase as leaf age progresses (data not shown) and by the suggestion of the involvement of the maize *Asr1* ortholog in stress-induced senescence (Jeanneau et al., 2002). Surprisingly, GA₃ treatment provoked a remarkable deterioration in the silenced plants (Fig. 6C). This is opposite to the known effects of GAs on plant senescence (Gan, 2010). Therefore, we suppose that the observed phenotype is likely the result of a complex interaction of changes caused by the lack of ASR1 and the treatment with GA₃.

In addition to accelerated senescence, silenced plants showed other phenotypic changes: dwarfism, delayed flowering, and reduced biomass (Fig. 2). These traits are complex and likely regulated at different levels. Some of the most likely causes of these changes are the lower rates of photosynthesis (Fig. 3) and the concomitant reduction in Suc export, which implies a decrease in carbon flow to sink organs (Supplemental Fig. S2), and the hormonal alterations (Fig. 6) probably caused by the increased Glc levels in the leaves. Additionally, there could be a change in the uptake of sugars by sink organs. Regarding the latter, although ASR1 is probably not directly involved in phloem unloading, as this process is symplasmic in developing tobacco leaves (Turgeon, 1987; Ding et al., 1988), it

may play a role via HT1 in retrieval of the hexoses as was mentioned above. Interestingly, when sink activity decreases, carbohydrates accumulate in leaves and photosynthesis becomes inhibited by feedback control mechanisms (Paul and Foyer, 2001; Lalonde et al., 2003; Ainsworth and Bush, 2011). As such, we cannot formally exclude that both events (hexose leakage in source and sink tissues) are taking place simultaneously. To test this hypothesis, experiments with *Asr1*-silenced tomato plants, whose source-sink relationships are stronger, are being performed.

Dwarfism is a typical phenotype associated with a deficit of active GAs. Although there were no changes in the levels of GA₁, the reduction in GA₂₀ in mature leaves of silenced plants could potentially explain its phenotype (Fig. 6B), since it has recently been proposed that maturing leaves of tobacco shoots produce GA₁ or GA₂₀ as signal molecules necessary to stimulate the growth of stem cells (Dayan et al., 2012). On the other hand, silenced plants were able to elongate the internodes in response to GA₃, confirming that GA₃ sensitivity was not modified (Fig. 6D). It is noteworthy that our observations are in agreement with a previous work where manipulation of GA₂₀ activity by means of *GA 20-oxidase* silencing in tobacco resulted in a dwarf phenotype and conserved sensitivity to GA₃ (Biemelt et al., 2004). We also observed an increase in the biomass of wild-type plants upon GA₃ treatment (Supplemental Table S2), suggesting that there is a change in the whole-plant CO₂ assimilation, an effect previously described for GAs (Biemelt et al., 2004). However, silenced plants did not exhibit altered biomass (Supplemental Table S2). The observation that wild-type plants had lower levels of Suc and higher levels of Glc and Fru in the leaves (Supplemental Table S2) was probably due to the promotion of invertase activity by GA₃, as was previously described (Iqbal et al., 2011). Silenced plants did not show either change in hexose or Suc levels (Supplemental Table S2), possibly owing to inhibition of invertase activity by the high Glc content.

Floral meristem development is controlled by sugar contents and their signals (Francis and Halford, 2006). There are many examples that describe that disruptions in hexose availability generate delays in flowering time (Slewinski, 2011; Eveland and Jackson, 2012). This suggests that flowering time in silenced plants may be delayed (Fig. 2) owing to impaired sugar availability. With regard to hormonal effects, GAs are known to be closely related to flowering. Particularly, in tobacco, it has previously been suggested that the active GAs in apical shoots are not imported from other organs but are locally synthesized (Gallego-Giraldo et al., 2007). Thus, it cannot be excluded that silenced plants display an altered local meristematic synthesis of GAs affecting flowering. However, we also cannot currently rule out that other hormonal changes influence the flowering phenotype of these transgenic lines.

CONCLUSION

Although a number of transcription factors have been positioned within the signaling cascade of interactions between sugars and hormones, most of these factors have been identified in *Arabidopsis thaliana*; for review, see Rolland et al., 2006). In this study, we provide compelling evidence that ASR1, a transcription factor not present in *Arabidopsis* (Carrari et al., 2004), connects sugar, ABA, and GAs. Results presented in this article, together with evidence showing that ASR proteins have transactivation activity in grape fruits (Çakir et al., 2003) and also function as a repressor of transcription of a plasma membrane hexose transporter in potato tubers (Frankel et al., 2007), lead us to propose a role for ASR1 in photosynthetic tissues. The fact that large phenotypic changes were notable in the silenced lines but not in the overexpressing lines suggests that ASR1 levels in wild-type plants are saturating with regard to their role in Glc metabolism. While further studies will clearly add more evidence about the exact mechanisms underlying these interactions, this study unequivocally associates ASR1 with the modulation of Glc levels in photosynthetic tissues through the regulation of Glc metabolism. This regulation also has an impact on Glc signaling probably through *Hexokinase1* and *SnRK1*, which would subsequently affect photosynthesis, leaf senescence, and hormone levels.

MATERIALS AND METHODS

Generation of the Transgenic Lines

The 348-bp coding region of the tomato (*Solanum lycopersicum*) *Asr1* gene (GenBank U86130.1) was cloned in sense or antisense orientation into the multiple cloning sites of the vector pBINAR (Liu et al., 1990) between the *Cauliflower mosaic virus* 35S promoter and the *octopine synthase* terminator. The constructs were transferred into tobacco (*Nicotiana tabacum* 'Xanthi-nc') cotyledons by an *Agrobacterium tumefaciens*-mediated protocol. Emerging shoots were excised and selected on Murashige and Skoog media containing kanamycin (200 mg/L). Once plants had taken root, they were transferred to soil in the greenhouse for their subsequent selection.

Selection of the Transgenic Lines

We screened 34 sense and 50 antisense T0 plants on the basis of *Asr1* expression levels by northern blot as described by Frankel et al. (2007). Sense plants were further tested for ASR1 levels by western blot (for methodological details, see below). Finally, three overexpressing lines (S 22, S 14, and S 31) and three silenced lines (AS 2, AS 26, and AS 42) were selected, propagated, and transferred to the greenhouse for further characterization. Lines S 22 and AS 2 were taken to the T4 generation in order to select for homozygous seeds. *Asr1* expression levels of these lines were checked by qRT-PCR. Total RNA was extracted by TRIzol (Invitrogen) according to the manufacturer's specifications. RNA samples were treated with DNase I (Invitrogen). Complementary DNA was done using reverse transcriptase (M-MVL; Invitrogen), RNase inhibitor (RNaseOUT; Invitrogen), and random primers (Invitrogen) according to the manufacturer's specifications. qRT-PCR was performed with a master mix (QuantiTect SYBR Green PCR kit; Qiagen) in a thermocycler (ABI 7500; Applied Biosystems). The primer sequences used for the detection of *Asr1* levels were as follows: forward (FW) 5'-CCTGTTCCACCACAAGGACAA-3' and reverse (RV) 5'-CGATTGCTCGAGATGTTATGG-3'. The reference gene was *Elongation Factor1α* (*EF1α*; as suggested in Schmidt and Delaney, 2010) and the primer sequences used for the detection of its levels were as

follows: FW 5'-GATTGGTGGTATTGGTACTGTC-3' and RV 5'-AGCTTCGTG-GTGCATCTC-3'. Cycle threshold values and efficiencies values of PCRs were obtained with the LinRegPCR software (Ruijter et al., 2009). Ratios were obtained according to Pfaffl (2001). The statistical analysis of the data was performed by the permutation test of the Infostat software (Di Rienzo, 2009).

Growth and Measurement Conditions

Between six and eight replicates of each genotype were grown in a greenhouse under controlled conditions (80% relative humidity, 200 $\mu\text{mol PAR s}^{-1} \text{m}^{-2}$, 16 h of light/8 h of darkness) at each performed experiment. All measurements (see next sections) were performed on the third or fourth expanded leaf during the vegetative phase of the plants, when they had eight to 10 expanded leaves in total.

Phenotypic Characterization

In T0 plants, fresh weight of leaves, stems, and roots was measured. In the homozygous plants, height, stem length between nodes four and eight, fresh weight of leaves, stems, and roots, and flowering time were measured. Flowering time was recorded as the number of total leaves at the time of flower bud setting.

Photosynthetic Parameters

Light-response curves of photosynthetic parameters for each homozygous line and the wild-type plants were obtained from eight replicates per genotype at increasing photon irradiances (0, 50, 100, 300, 700, 1,200, 1,500, and 1,700 $\mu\text{mol s}^{-1} \text{m}^{-2}$) using a portable gas exchange system (LI-COR 6400).

Leaf Carbohydrate Levels

Leaf sugar levels of the T0 plants were measured at a light time point (13 PM) and a dark time point (5 AM). Diurnal changes in leaf sugar levels were measured in homozygous plants. From each plant, we sampled 30 mg of leaf fresh weight and immediately froze the samples in liquid nitrogen. Samples were ground in liquid nitrogen, and soluble sugars were successively extracted with 250 μL of 80% (v/v) ethanol, 250 μL of 80% (v/v) ethanol, and 150 μL of 50% (v/v) ethanol. Samples were heated at 80°C during 20 min and centrifuged 5 min at 14,000 rpm after each addition of ethanol. Supernatants of each extraction were combined and the mixture was used for soluble sugar determinations. Glc, Fru, and Suc were measured by the enzymatic method (Fernie et al., 2001). The pellet obtained from the soluble sugars extraction was used for starch measurement. It was resuspended in 400 μL of 0.2 N KOH (Sigma-Aldrich), and it was incubated during 1 h at 95°C. Then, 70 μL of 1 N acetic acid (Merck) was added, and the mixture was centrifuged for 10 min at 13,900 rpm. The supernatant was separated and diluted 1:3. A starch assay kit was used for the determinations (Sigma-Aldrich).

Feeding Leaf Discs with [^{14}C]Glc

Leaf discs were cut from T0 plants at the onset of the light period and washed three times with 10 mM MES-KOH (BioChemika; pH 6.5). They were incubated (18 discs in a volume of 4 mL, shaken at 90 rpm) for 1, 2, and 3.75 h in 2 mM Glc, including 1.4 kilobecquerel mmol^{-1} [^{14}C]Glc (Amersham-Buchler). The discs were harvested, washed three times in buffer, and frozen in liquid nitrogen. Fractionation of ^{14}C -labeled leaf discs and separation of sugars from neutral fractions on TLC plates were performed as described by Fernie et al. (2001). The value of each result for each line is the average of the label of two discs per plant from three plants. In every case, the normalization of the data was done by leaf area.

Sugar Content in Phloem Sap

Third or fourth expanded leaves were detached from the T0 plants by cutting the base of petioles under water. One leaf per plant and five plants per line were sampled. Leaves were weighed and fed through their petioles with 5 mM EDTA (pH 6.0; Sigma-Aldrich) for 1 h, then transferred to a fresh solution and incubated for further 16 h. The incubations were done in a growing chamber under controlled conditions (relative humidity 60%, temperature 23°C, 200 $\mu\text{mol PAR s}^{-1} \text{m}^{-2}$). Sap was lyophilized and resuspended in 1 mL

of water. Soluble sugar contents were determined enzymatically as described previously. Results of the sugar contents from the first and the 16th incubation hours are presented together. The protocol was adapted from Leggewie et al. (2003).

In Situ Localization of H_2O_2 in Leaves

H_2O_2 was detected in leaves of homozygous plants and wild-type plants using DAB dye (Sigma-Aldrich). Leaves were infiltrated under vacuum for 5 min with 0.1% (w/v) DAB in buffer Tris-HCl, pH 6.5 (Tris Base; CalBioChem). They were incubated 16 h in darkness at room temperature. Afterward, they were illuminated until brownish spots appeared. They were bleached with 100% (v/v) ethanol for better visualization of the spots. The spots were quantified by the number of pixels using Photoshop 6.0 software. The results were expressed as the percentage of spots per total leaf area.

Total Protein Profile of Leaves

Total proteins of leaves of homozygous and wild-type plants were extracted. They were quantified utilizing the Bradford method (Bio-Rad protein assay). Equal amounts of protein were run in a 15% SDS-PAGE and stained with Brilliant Blue R dye (Sigma-Aldrich). SDS-PAGE protocol was based on Sambrook et al. (1989).

ASR1 Levels in Leaves of Different Ages

Two or three leaves of different ages (ranging between leaf no. 5 and leaf no. 20) were sampled from three wild-type plants. Total proteins were extracted using the following buffer: 10% (v/v) glycerol (Merck), 25 mM Tris, pH 7.5, 1 mM EDTA (Sigma-Aldrich), 150 mM NaCl (Alcor), 10 mM dithiothreitol (Sigma-Aldrich), 2% (w/v) polyvinylpyrrolidone (Sigma-Aldrich), and 2 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich). Quantification of total proteins was performed utilizing the Bradford method (Bio-Rad protein assay). Protein quality of the extracts was checked by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane (Amersham Hybond ECL; GE Healthcare), and transference was checked by Ponceau S dye (Sigma-Aldrich). Membrane was incubated with anti-ASR1 serum (Maskin et al., 2007), and, after washing, it was incubated with anti-rabbit IgG serum (Sigma-Aldrich). SDS-PAGE and western-blot protocols were based on Sambrook et al. (1989).

Hormone Measurements

ABA and GAs were measured in leaves of homozygous plants. Leaves were harvested at midday, as previously described, and lyophilized. The analysis method for extraction and detection of ABA is similar to a previous report (Chen et al., 2009). For quantification, the data were normalized based on the internal standard [6-D]ABA. The analysis method for extraction and detection of GAs is similar to a previous report (Zhang et al., 2011). The analysis included the following 20 targeted GAs: 8/29/23/3/1/6/5/19/13/20/44/34/53/51/7/4/24/15/9/12. The LC-MS/MS system used is composed of a Shimadzu LC system with a LEAP CTC PAL autosampler coupled to an Applied Biosystems 4000 QTRAP mass spectrometer equipped with a Turbolon Spray electrospray ion source.

Sensitivity to Exogenous GA_3

Silenced homozygous plants and wild-type plants were foliar sprayed with 10 μM GA_3 (Sigma-Aldrich) twice a week, while another identical group of plants was foliar sprayed with 0.002% (v/v) ethanol (GA_3 solvent) at the same time. The treatment was started at seedling emergence and was finished when the plants reached the reproductive stage in a greenhouse. A phenotypic analysis was performed as above, except for height, which was measured along time. Glc, Fru, Suc, and starch contents of the leaves were measured at 12 AM (for sugar measurement details, see above). Also, ASR1 protein was detected with a western blot in the leaves of wild-type plants with or without GA_3 treatment (for western-blot details, see above).

qRT-PCR Analyses of Sugar Transporters and Sensors

mRNA levels of sugar transporters and sensors in the leaves of homozygous plants were measured by qRT-PCR. RNA extractions, complementary DNA

synthesis, and real-time PCRs were performed as described before. mRNA levels were measured for *Ht1* (SGN-U446865 tobacco [*Nicotiana tabacum*], ortholog of GenBank, AY608701.1), *Sut2* (SGN-U423251 tobacco, ortholog of The Arabidopsis Information Resource [TAIR], At2G02860), *Tmt3* (SGN-E129731 tomato, ortholog of TAIR, At3g51490), *Vgt* (SGN-U430963 tobacco, ortholog of TAIR, At5g59250), *Hexokinase1* (SGN-E750217 tobacco, ortholog of SGN, E1203588 *Nicotiana benthamiana*), and *SnRK1.1* (SGN-E750838 tobacco, ortholog of TAIR, At3G01090). The primer sequences used were as follows: *NtHt1* FW, 5'-CCTGGGATTGCATCAGTTT-3'; *NtHt1* RV, 5'-TGGCGGAAGCT-GAAATTAAT-3'; *NtSut2* FW, 5'-GAGGTGGCACAGATGGTTT-3'; *NtSut2* RV, 5'-AGCGATTGGTTGCTTGAGT-3'; *SlTmt3* FW, 5'-CTGCCCCACCAGAAA-TAAGA-3'; *SlTmt3* RV, 5'-AGCCTCTTCATTCGACCTT-3'; *NtVgt* FW, 5'-GGC-TCAGACTGAAGTTTCG-3'; *NtVgt* RV, 5'-CCACAAGACCAAGCTGAACA-3'; *NtHK1* FW, 5'-GAGGCATCAATTCCTCCAAA-3'; *NtHK1* RV, 5'-GCCCTTTGT-CCACCTCATAA-3'; *NtSnRk1.1* FW, 5'-AGATTGTAATGCACCCTTGG-3'; *NtSnRk1.1* RV, 5'-ACGCCACAGTACCCTCATT-3'. The reference gene *EFla* was used as described before.

Statistical Analyses

Statistical analyses of the data were performed by the Student's *t* test, except when otherwise indicated.

Supplemental Data

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

Supplemental Figure S1. Oxidative burst and hypersensitive reaction in *Asr1*-silenced leaves.

Supplemental Figure S2. SDS-PAGE of total proteins extracted from wild-type and transgenic lines (T4) leaves.

Supplemental Figure S3. Suc contents in leaf phloem sap.

Supplemental Table S1. Gibberellin conversion in *Asr1* tobacco transgenic lines.

Supplemental Table S2. Sensitivity of *Asr1*-silenced plants to exogenous GA₃.

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