The putative glutamate receptor 1.1 (AtGLR1.1) functions as a regulator of carbon and nitrogen metabolism in Arabidopsis thaliana

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The ability to coordinate carbon (C) and nitrogen (N) metabolism enables plants to regulate development and metabolic responses to different environmental conditions. The regulator(s) or sensor(s) that monitor crosstalk between biosynthetic pathways and ultimately control the flow of C or N through them have remained elusive. We used an antisense strategy to demonstrate that the putative glutamate receptor 1.1 (AtGLR1.1) functions as a regulator of C and N metabolism in Arabidopsis. Seeds from AtGLR1.1-deficient Arabidopsis (antiAtGLR1.1) lines did not germinate in the presence of an animal ionotropic glutamate receptor (iGLR) agonist, but germination was restored upon coincubation with an iGLR agonist on the putative ligand glutamate. In antiAtGLR1.1 lines, endogenous acidic abscisic acid (ABA) concentrations increased with iGLR antagonist treatments and decreased with coincubation with an iGLR agonist, suggesting that germination was controlled by ABA. antiAtGLR1.1 seedlings also exhibited sensitivity to increased levels of Ca2+ compared with wild type, and they exhibited a conditional phenotype that was sensitive to the C:N ratio. In the presence of C, specifically sucrose, but not glucose, mannitol, or sorbitol, antiAtGLR1.1 seeds did not germinate, but germination was restored upon coincubation with NO3-, but not NH4+. Immunoblot, isoenzyme, and RT-PCR analyses indicate that AtGLR1.1 regulates the accumulation of distinct C- and N-metabolic enzymes, hexokinase 1 (HXK1) and zeaxanthin epoxidase (ABA1), by transcriptional control. We provide a model to describe the role of AtGLR1.1 in C/N metabolism and ABA biosynthesis, which in turn controls seed germination.

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Abbreviations: Suc, sucrose; Glc, glucose; Gln, glutamine; Glu, glutamate; iGLR, ionotropic Glu receptor; AtGLR, Arabidopsis thaliana Glu receptor; DNOX, 6,7-dinitroquinoxaline-2,3-(1H,4H)-dione; BMAA, S-(+)-β-methyl-α,β-diaminopropionic acid; BMAA, abscisic acid; G5, Gln synthetase; FeD-GOGAT, ferredoxin-dependent Glu synthase; AAT, asparate aminotransferase; GDH, Glu dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; ICDH, NADP-dependent isocitrate dehydrogenase; HXK1, hexokinase 1.
our findings suggest that AtGLR1.1 regulates C and N metabolism and controls seed germination by means of changes in ABA.

Materials and Methods

Vertical Growth Assay and Maintenance of Plants. Seedlings were maintained in the vertical position on square Petri plates on complete Murashige and Skoog (MS) medium or MS minus inorganic nitrogen medium (Caisson Laboratories, Sugar City, ID) supplemented with different C or N compounds, amino acids, or DNOX and/or BMAA and 0.8% (wt/vol) Phytagar (Life Technologies) for 7 days. Growth conditions were maintained at 20–21°C, with 60–70% relative humidity, under cool white fluorescent lights (140 μmol of photons per m² per s) with a 16-h light/8-h dark cycle. Seeds from transgenic line 2 (Fig. 1A) were used in all of the vertical growth experiments. All experiments described below were conducted in duplicate or triplicate.

Antisense Construct. A 654-bp BamHI fragment from the Arabidopsis EST clone 107M14T7 (GenBank accession no. T22862) obtained from The Arabidopsis Biological Resource Center (Ohio State University, Columbus), was cloned into a BamHI-digested modified binary pBI221 vector (CLONTECH), pPV1, in the reverse orientation. Arabidopsis thaliana (L.) Heynh. ecotype Wassilewskija was transformed by vacuum infiltration (11).

Antibody Production. The C terminus, 88 aa, of AtGLR1.1 was expressed in a GST-fusion system. PCR was performed with the gene-specific primers 5’-trnAtGLR1.1oxEco (5’-GCCCAGATTCCGAATGATT-3’) and 3’-trnAtGLR1.1oxEco (5’-GCCCAGATTCATTACCCGCAACTCAG-GAA-3’) and the EST clone as the template. All primers were commercially synthesized (Bio-Synthesis, Lewisville, TX). The amplification reaction, cloning, purification of the truncated AtGLR1.1 protein, and antibody production were performed as described by Turano et al. (12).

Immunoblot Analysis and Enzyme Activity Stains. Immunoblot and isoenzyme stains were performed on crude protein preparations from leaves of 30-day-old plants. Immunoblot analysis of Gln synthetase (GS) and ferredoxin-dependent Glu synthase (FD-GOGAT, glutamate–2-oxoglutarate aminotransferase) were performed with antibodies and protocols described by Turano and Muhitch (13). Antibodies for aspartate aminotransferase (AAT)2 (14) and AAT3 (15) were obtained from Ben Matthews (U.S. Department of Agriculture/Agricultural Research Service, Beltsville, MD). Antiserum to NADP(H)-dependent glutamate dehydrogenase (GDH) was prepared to a portion of the deduced amino acid sequence (RDIKSQQRSLRDYSKTYARAKYFDELKP-MERTEENYHWNWER) by following the protocols developed by Animal Pharm Services (Healdsburg, CA). Enzyme stains for NAD-dependent GDH were conducted as described by Turano et al. (16), and the other stains were conducted as described by Vallejos (17) except that Dl-isocitrate was added to a final concentration of 40 mM. Samples were standardized by equal protein loading (20 μg per lane) on the MiniPROTEAN 3 system (Bio-Rad) and 3 μg per lane on the 8–25 gradient PhastGel System (Amersham Pharmacia Biosciences) for 6-phosphogluconate dehydrogenase (6PGDH) activity. Immunoblots and stained gels were photographed and densitometrically analyzed with the Kodak Digital Science 1D Image Analysis Software package (version 3.0).

RT-PCR Analysis. Total RNA was extracted from leaves of 30-day-old plants with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Total RNA (1 μg) served as the template for RT-PCR using the RT-PCR beads System (Amersham Pharmacia Biosciences) with 25 pmol of each transcript-specific primer. All primers were commercially synthesized (Bio-Synthesis). The RT-PCR was conducted as follows: 42°C for 15 min, 95°C for 5 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, and a 72°C extension reaction for 2 min. The primers used and the corresponding accession numbers are available on request. RNA integrity and concentration, PCR controls and fragment identifications were conducted and analyzed as previously described (12).
ABA Determinations. To quantify the ABA content of antiAtGLR1.1 and WT seeds and seedlings, 0.1 g of frozen homogenized tissue was suspended in 1.5 ml of extraction buffer (100 mg/liter butylated hydroxytoluene/0.5 g/liter citric acid monohydrate in 80% methanol). The suspension was rotated overnight at 4°C and centrifuged at 1,000 × g for 20 min. The supernatant was collected and transferred to a new tube and vacuum dried. The dried residue was dissolved with 100 μl of methanol plus TBS (50 mM Tris/0.1 mM MgCl₂/0.15 M NaCl, pH 7.8), and ABA was quantified with the PhytoDtek-ABA-Kit as described by the manufacturer (Idetek, Sunnyvale, CA).

Results and Discussion
AtGLR1.1 Has iGLR-Like Characteristics. To determine the mechanistic action of AtGLR1.1, we tested antiAtGLR1.1 lines for altered sensitivity to the iGLR antagonist DNQX and/or agonist BMAA (Fig. 1A). Previous pharmacological studies in plants determined the bioactive concentrations of these chemicals to be 200 μM DNQX, which resulted in the inhibition of hypocotyl shortening and leaf greening (5), and 50 μM BMAA, which increased hypocotyl elongation and decreased cotyledon opening (6). antiAtGLR1.1 seeds maintained on MS medium with 3% Suc supplemented with low concentrations (100 μM) of DNQX (Fig. 1A) or without DNQX (data not shown) germinated and seedlings developed similarly to WT. However, the germination of antiAtGLR1.1 seeds was inhibited (91 ± 0.3%, n = 40) in the presence of 200 μM DNQX. PCR analysis confirmed the existence of the antiAtGLR1.1 construct, and decreased accumulation of the AtGLR1.1 peptide was confirmed by immunoblot analysis (Fig. 1B). This screen was used to identify three representative transgenic lines with a detectable reduction in the accumulation of AtGLR1.1 peptide compared with that of WT. Our results show an inverse relationship between the accumulation of the AtGLR1.1 peptide and antagonist sensitivity. DNQX hypersensitivity in antiAtGLR1.1 lines is consistent with the hypothesis that these lines have functional GLRs, but the GLRs contain fewer AtGLR1.1 subunits and are thus more readily saturated by the inhibitory effects of the antagonist compared with WT. Conversely, in WT plants there are more AtGLR1.1-containing receptors, and at the same concentration of DNQX that adversely affects the antiAtGLR1.1 lines the antagonist is not saturating and is therefore not inhibitory. Seed germination and seedling development were similar among antiAtGLR1.1 and WT lines treated with 50 μM BMAA (Fig. 1A). The DNQX-mediated inhibition of germination and development of antiAtGLR1.1 seedlings was reversed by simultaneous incubation with 50 μM BMAA or 10 mM Glu. The 50 μM BMAA treatment was more effective and less variable for the reversal of the inhibitory effects of DNQX than 10 mM Glu. BMAA and Glu reversal of DNQX-mediated inhibition of germination is consistent with presumption that these compounds compete for similar binding sites and act as an antagonist or agonist to AtGLR1.1 in a manner similar to the iGLRs. At 10 mM, Glu reversed the DNQX-mediated inhibition of seed germination (Fig. 1A). To test the specificity of Glu, the ability of other amino acids (Gln and Asp) to reverse the DNQX-mediated inhibition of seed germination was tested (Fig. 1C). Neither Asp nor Gln was as effective as Glu (85%) in the restoration of germination to DNQX-treated antiAtGLR1.1 lines. Interestingly, at 10 mM, Gln did increase germination to 50%; this increase may be due to the rapid conversion of Gln to Glu, the putative ligand. Similar observations and explanations for a Glu response have been reported for WT lines co-inoculated with an iGLR agonist (8). In animals, iGLRs function as an inward-rectifying K⁺, Na⁺, or Ca²⁺ ligand-gated ion channel. If AtGLR1.1 functions in a manner similar to the iGLRs, then antiAtGLR1.1 lines would be expected to have altered sensitivity to at least one of these ions. Results from vertical growth assays revealed that antiAtGLR1.1 lines were more sensitive to increased levels of supplemented Ca²⁺ than WT. These findings suggest a relationship between altered levels of AtGLR1.1 and Ca²⁺ sensitivity. Genetic and physiological studies of another AtGLR (AtGLR3.2) also observed changes in sensitivity to Ca²⁺, and not to K⁺ or Na⁺ (9). The results from both physiological studies are supported by extensive phylogenetic analysis of the pore-forming region (ion-permeability region) of the AtGLRs and corresponding regions of other ligand-gated ion channels and K⁺ channels that demonstrated the AtGLRs are not similar to the K⁺ channels (18).

Table 1. ABA content in 3-day-old WT and antiAtGLR1.1 seedlings maintained on MS plus 3% Suc medium supplemented with an iGLR agonist or antagonist

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ABA, μg/g</th>
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<tbody>
<tr>
<td>Control (MS + 3% Suc)</td>
<td>0.174 ± 0.002</td>
</tr>
<tr>
<td>200 μM DNQX</td>
<td>0.159 ± 0.004</td>
</tr>
<tr>
<td>200 μM DNQX + 50 μM BMAA</td>
<td>0.739 ± 0.083</td>
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Results are expressed as μg of ABA per g of frozen tissue. Data are means ± SD (n = 3).

Fig. 2. Effect of C and/or N on germination. (A) Seeds from WT or antiAtGLR1.1 lines were sown on NH₄NO₃-free MS medium containing 0.8% (wt/vol) Phytagar supplemented with no C or inorganic N (−C/−N), with C (3.0% Suc) and no inorganic N (−C/+N), or with C (3.0% Suc) and inorganic N (5 mM NH₄ and 10 mM NO₃) (+C/+N). (B) WT or antiAtGLR1.1 seeds were sown on NH₄NO₃-free MS medium supplemented with 25 mM Suc, Glc, mannitol (Man), or sorbitol ( Sor). (C) Seeds from WT or antiAtGLR1.1 lines were sown on NH₄NO₃-free MS medium supplemented with 87 mM (3%) Suc and 5 mM NH₄ or NO₃.
ABA Concentrations in the DNQX- or BMAA-Treated Plants. It has been established that ABA plays an important role in seed germination (19). As shown in Fig. 1A, the germination of seeds from antiAtGLR1.1 lines was inhibited on MS medium with 3% Suc supplemented with 200 μM DNQX. We hypothesized that decreased germination of the antiAtGLR1.1 seeds by DNQX was due to elevated ABA titers. To test this hypothesis, endogenous ABA concentrations were determined in 3-day-old WT and antiAtGLR1.1 seedlings incubated in liquid culture containing MS medium with 3% Suc, in the absence or presence of 200 μM DNQX and/or 50 μM BMAA (Table 1). In the absence of either agonist or antagonist, the antiAtGLR1.1 seedlings contained 8 times more ABA than did WT. When the medium was supplemented with 200 μM DNQX the ABA content in WT seedlings was similar to that of the nontreated WT controls. Coincubation of WT seedlings with 200 μM DNQX and 50 μM BMAA resulted in a 425% or 460% increase in ABA content compared with nontreated or DNQX-treated WT seedlings, respectively. When the medium was supplemented with 200 μM DNQX, the ABA content in antiAtGLR1.1 seedlings increased 160% to that of the nontreated antiAtGLR1.1 controls. Coincubation of antiAtGLR1.1 seedlings with 200 μM DNQX and 50 μM BMAA resulted in a 37% decrease in ABA compared with the DNQX-treated antiAtGLR1.1 seedlings. However, the ABA content in antiAtGLR1.1 seedlings treated with 200 μM DNQX and 50 μM BMAA was nearly 80% higher than that of the antiAtGLR1.1 seedlings control. Compared with WT seedlings treated with 200 μM DNQX and 50 μM BMAA, similarly treated antiAtGLR1.1 seedlings had a 240% increase in ABA. Combined, these findings suggest that decreased AtGLR1.1 “activity,” caused either by less protein (antisense) or by inhibition of the GLRs by the antagonist, results in increased ABA. The inhibitory effects of the two approaches are cumulative, because the antiAtGLR1.1 seedlings were more sensitive to effects of the antagonist, which resulted in the highest levels of ABA observed in these experiments. Furthermore, these findings strongly suggest that endogenous ABA levels must reach a threshold to inhibit seed germination.

AtGLR1.1 Alters C/N Sensitivity. Because Glu is a potential ligand, and Glu metabolism and plant development are affected by different C:N ratios, we subjected plants to different C:N regimes to determine the effect on seedling development. AntiAtGLR1.1 plants exhibited a conditional phenotype that was sensitive to different C:N treatments (Fig. 2A). Seeds from antiAtGLR1.1 and WT lines germinated and seedlings developed similarly on MS nutrient plates in the absence of inorganic N or supplemental C (−C/−N). When supplemental C (3% Suc) was provided to the medium, germination of the antiAtGLR1.1 lines was inhibited (87 ± 0.5%, n = 44) compared with WT (+C/−N). However, the addition of N (5 mM NH4NO3 and 5 mM KNO3) to the C-containing medium restored the antiAtGLR1.1 seeds and seedlings to WT germination rates and growth, respectively (+C/+N). These results suggest that differences in C and N availability alter germination and development, and high C:N ratios inhibit germination.

To test the specificity of Suc as a C source involved in the inhibition of germination of antiAtGLR1.1 seeds, antiAtGLR1.1 and WT seeds were germinated on MS plates minus inorganic N in the presence of different C (25 mM) sources (Fig. 2B). Previously we determined that 25 mM Suc was the lowest effective concentration to inhibit germination of the antiAtGLR1.1 seeds (data not shown). Germination of antiAtGLR1.1 seeds was inhibited by 25 mM Suc, whereas under the same conditions WT seeds germinated and seedlings developed normally. The antiAtGLR1.1 and WT seeds germinated and seedlings developed normally when germinated in MS minus inorganic nitrogen supplemented with 25 mM Glc, mannitol, or sorbitol. High levels of C (300 mM Suc or Glc) arrest a variety of plant developmental processes (1), through the elevation of ABA (20). Our observations suggest that (i) AtGLR1.1 may be associated with Suc-sensing or -mediated regulation; (ii) Suc has a negative or inhibitory effect on AtGLR1.1, because Suc in the
absence of N, like the DNQX treatment, results in the inhibition of seed germination; (iii) AtGLR1.1 also effects Glc sensing or Glc-mediated regulation because the antiAtGLR1.1 lines are hypersensitive to Suc and not Glc; and (iv) AtGLR1.1 alters ABA biosynthesis because Suc-treatment antiAtGLR1.1 seeds did not germinate.

To test the specificity of different forms of inorganic N necessary for the reversal of Suc-mediated inhibition of germination of antiAtGLR1.1 seeds, seeds from antiAtGLR1.1 and WT lines were maintained on MS plates plus 3% Suc in the presence of 5 mM NH₄ or NO₃ (Fig. 2C). The Suc-mediated inhibition of germination of the antiAtGLR1.1 seeds was reversed by NO₃ but not NH₄. Our observations suggest that (i) AtGLR1.1 may be associated with NO₃-sensing or -mediated regulation, and (ii) NO₃ has a positive or stimulatory effect on AtGLR1.1, because NO₃ reverses Suc-mediated inhibition of seed germination. This reversal is analogous to the BMAA reversal of the DNQX treatment.

**AtGLR1.1 Regulates the Accumulation of C- and N-Metabolic Enzymes.**

To elucidate the effects of antiAtGLR1.1 on C/N metabolism, we tested three independent transgenic lines by immunoblot analysis or specific enzyme activity stains for changes in the accumulation of isoenzymes involved in N or C metabolism (Fig. 3). In antiAtGLR1.1 plants, the levels of the cytosolic isoforms of GS (GS1) (21) and AAT (AAT2) (22) were 90% and 93% less, respectively, than those levels observed in WT plants (Fig. 3A and C). The corresponding chloroplastic isoenzymes, GS2 and AAT3, remained unchanged compared with WT plants. The accumulation of other C- and N-metabolic enzymes, including the chloroplastic isoenzymes (23, 24) Fd-GOGAT and NADP(H)-GDH and the mitochondrial (16) NAD-GDH, were unaltered compared with WT plants as determined by immunoblot analysis or enzyme-specific activity gel stain (Fig. 3C). Enzyme-specific activity stains for the cytosolic C-metabolic isoenzymes (25, 26) 6PGDH and NADP-dependent isocitrate dehydrogenase (ICDH) showed decreased levels (80% and 78%, respectively, of both isoforms in antiAtGLR1.1 plants (Fig. 3B) compared with WT. Collectively, these results suggest that the AtGLR1.1 gene product is involved in the maintenance of steady-state levels of specific N- and C-metabolic enzymes.

**AtGLR1.1 Regulates the Accumulation of Transcripts Involved in C and N Metabolism, Glc Sensing, and ABA Biosynthesis.**

To determine whether the C- and N-metabolic isoenzymes were transcriptionally or translationally controlled, we used RT-PCR (Fig. 4). For all of the transcripts that we previously tested by immunoblotting or isoenzyme analysis, i.e., GS1, AAT2, and 6PDGH, except one ICDH, there was a parallel decrease in the peptide or isoenzyme activity or translationally controlled, we used RT-PCR (Fig. 4). For all of the transcripts that we previously tested by immunoblotting or isoenzyme analysis, i.e., GS1, AAT2, and 6PDGH, except one ICDH, there was a parallel decrease in the peptide or isoenzyme 30% decrease) in antiAtGLR1.1 plants compared with WT. Because the antiAtGLR1.1 seedlings were more sensitive to Suc than to Glc (Fig. 2B), we hypothesized that the antiAtGLR1.1 plants would have less HXK1, the Glc sensor, than WT plants. Indeed, there was less accumulation (50% decrease) of the HXK1 transcript in antiAtGLR1.1 than in the WT plants, thus the decreased accumulation of the HXK1 transcript in antiAtGLR1.1 lines may explain why these lines are more sensitive to Suc as opposed to Glc (Fig. 2B). This notion is supported by earlier findings with antisense HXK1 plants, which were shown to be less sensitive to Glc than WT (3). Interestingly, the transcript for ABA1, a gene involved in the regulation of ABA biosynthesis, was more abundant (300% increase) in antiAtGLR1.1 plants compared with WT. This observation is consistent with the notion that a decrease in functional AtGLRs results in elevated ABA biosynthesis.

**Implications for the Role and Function of AtGLR1.1.** C and N metabolism are eminently integrated because C skeletons are required for N assimilation. Numerous studies have demonstrated that changes in the availability of different C compounds significantly affects the transcriptional or translational control of certain N-metabolic enzymes and vice versa (1, 2). Therefore, perturbations in the activity or accumulation of any C-metabolic isoenzyme might be expected to alter the activity of a particular N-metabolic isoenzyme. Likewise, similar reciprocating effects might be expected of a C-
Glu was not as effective as BMAA in reversing the inhibitory effects of DNQX on seed germination by Glu, and by published reports of the true ligand? The most likely candidate is Glu. This hypothesis results with the iGLR agonist and antagonist (Fig. 1) are consistent withGLR1.1 may function as a sensor cannot be dismissed. Our results are consistent with the hypothesis that AtGLR1.1 is a regulator of C and N metabolism, but the possibility that AtGLR1.1 may function as a sensor cannot be dismissed. Our results with the iGLR agonist and antagonist (Fig. 1) are consistent with the hypothesis that AtGLR1.1 also functions as a sensor or receptor. If AtGLR1.1 is a sensor, the question still remains: what is the true ligand? The most likely candidate is Glu. This hypothesis is supported by our results that show the reversal of the inhibitory effects of DNQX on seed germination by Glu, and by published reports of a Glu-induced voltage potential in Arabidopsis roots (10). Glu was not as effective as BMAA in reversing the inhibitory effect on germination. Probable explanations for this finding include the possibilities that (i) Glu uptake and/or distribution is limited in the antiAtGLR1.1 plants, or (ii) Glu is not the natural ligand and perhaps a BMAA-like molecule is the endogenous AtGLR1.1 ligand. The latter explanation is credible because BMAA is a plant-derived compound, found mainly in the cicas (28). Results from another laboratory demonstrated that the effects of an iGLR agonist are reversed only with high concentrations (10 mM) of Glu (8).

Collectively our data indicate that AtGLR1.1 functions as a C/N regulator, and/or sensor, that regulates C/N metabolism and distinct physiological processes such as germination through the control of ABA biosynthesis. We have demonstrated that the reduction of functional AtGLRs, either through antisense or by treatment with an iGLR antagonist, DNQX, results in elevated ABA contents (Table 1) or the accumulation of the ABA1 transcript (Fig. 4). Both findings are consistent with the notion that the inhibition of germination that we observed in DNQX and Suc treatments was a result of elevated ABA biosynthesis. ABA biosynthesis has been shown to alter physiological events such as seed germination (19) and lateral root formation (29).

Several models that describe the relationship between C or N sensing, ABA biosynthesis, and the control of physiological events have been recently published (29, 30). We present a model that incorporates portions of the above-mentioned models to explain the potential role of AtGLR1.1 in the regulation of the relationship between Suc, N, and ABA biosynthesis to control seed germination (Fig. 5). In their explanation of C-mediated control of ABA, Cheng et al. (30) mention a HXK-independent ABA biosynthetic pathway. Our data suggest that AtGLR1.1 is a key component of the HXK1-independent ABA pathway. Furthermore, we have shown that AtGLR1.1 is differentially affected by Suc and/or N availability in a manner similarly described by Zhang and Forde (29) for an unnamed C/N regulator/sensor in one branch of their dual pathway model regulation of lateral root formation by NO3. Activation or stimulation of AtGLR1.1, through either increased NO3 availability or reduced nitrogen in the form of amino acids, namely Glu or Gln, (i) maintains C- and N-metabolic pathways and (ii) decreases ABA biosynthesis. Inhibition of AtGLR1.1 by Suc results in decreased expression of distinct genes in C- and N-metabolic pathways, decreased HXK1, and elevated ABA biosynthesis, which results in physiological changes such as inhibition of germination. Because ABA has been shown to play an important role in drought tolerance, seed maturation, lateral root formation, and the regulation of vegetative growth (30), investigations into the link between AtGLR1.1 and these processes will prove to be an interesting and exciting area of research in the near future.

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