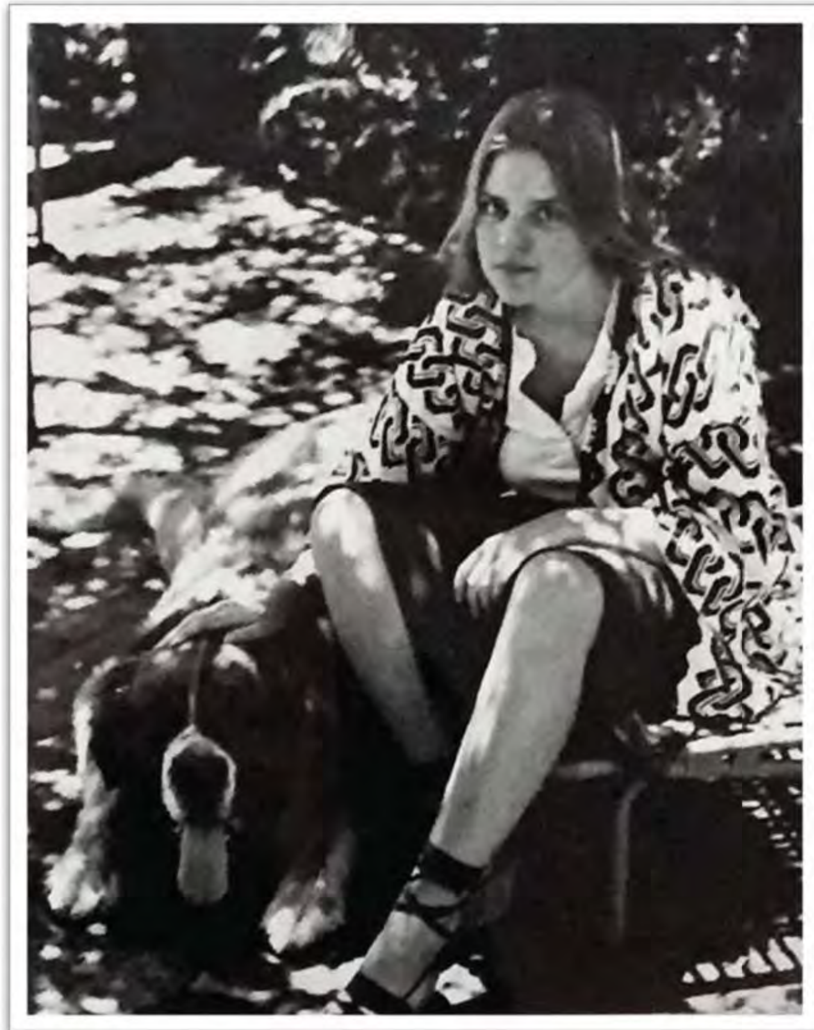


Alexandra Page Clark

September 28, 1961 – August 22, 2017

Curriculum Vitae

Alexandra Page Clark

Born: September 28, 1961, San Francisco, CA.

Education

University of California, Berkeley – BS. 1993

Monterey Peninsula College, Monterey, CA
Horticulture Degree

City College, San Francisco
Culinary Degree

New York University - MS Biology 5/16/1996
NYU Student ID: N19175326
GPA: 3.588/4.0

Relevant Courses

New York University, Dept. of Biology

Course	Grade
Biochemistry I & II	A-/B
Plant Resources I & II	B+/B
Lab in Molecular Biology I & II	A-/A
Lab Research I & II	A/A

Appointments

Assistant Research Scientist 1996 - 2001
New York University- Department of Biology
Lab of Plant Molecular Biology – Mentor, Gloria Coruzzi

Publications*:

Clark, A (1996) NYU Biology MS THESIS: "Further characterization of the genes and mutants of aspartate aminotransferase in Arabidopsis"

Brenner E, Martinez-Barbosa N, **Clark A**, Liang Q, Stevenson DW, and **Coruzzi G** (2000) Arabidopsis mutants resistant to BMAA, a Cycad-derived glutamate receptor agonist. ***Plant Physiology*** 124: 1615-1624. (Cited 87 times in other publications)

Oliveira I, Brears T, Knight T, **Clark, A** & Coruzzi G (2002) Overexpression of cytosolic glutamine synthetase. Relation to Nitrogen, Light and photorespiration. ***Plant Physiology*** 129: 1170-1180. (Cited 185 times in other publications)

*See attached PDFs of papers.

Alex Clark- Researcher, Mentee, Dear Colleague and Friend – by Gloria Coruzzi

I first met Alex when she was a student in my graduate level class at the New York University Biology Department entitled, "Plant Resources". The class covered plant from their molecular biology and DNA to plant biodiversity, and was co-taught by my colleagues at the New York Botanical Garden.

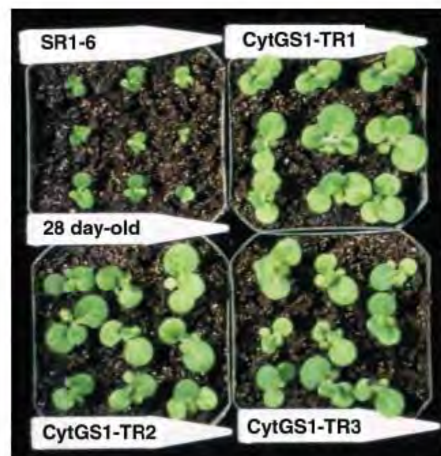
Alex stood out in a class of about 20 MS and Ph.D. students for her maturity and genuine curiosity about plants and their uses as medicines and products. I vividly remember the first day I met Alex outside of class. She was sitting on a bench outside of NYU's Bobst Library. We had what I consider our first "mentor/mentee" conversation about her life and career goals. Alex said she was quite idle and bored in between graduate classes at NYU, so I told her that if she was interested, we could keep her "busy" in our Plant Molecular Biology laboratory – and that was the start of her research career and projects and our life-long friendship.

Alexandra Clark - MS Biology, NYU, May 1996
Mentor: Gloria Coruzzi

In our Plant Molecular Biology Lab at NYU, Alex worked on plant genes – she isolated them from the model plant "Arabidopsis". and also made transgenic plants in which she introduced new genes into tobacco that enabled the plants to grow better than a wild-type strain (SR1-6) (Fig. 3, see Fig. 3, Oliveira, Clark et al 2002). She did this by manipulating expression of a gene encoding the enzyme "glutamine synthetase" which controls the assimilation of inorganic nitrogen from the soil into the amino acid glutamine – a building block of all DNA, proteins and chlorophyll. Alex was meticulous in her experiments – both at the lab bench and in her lab notebooks (see lab notes from Alex's book at the end of this tribute).

Oliveira et al.

Figure 3. Qualitative growth phenotype of soil-grown GS transgenic plants. Control line (SR1-6; A), CytGS1-TR1 (B), CytGS1-TR2 (C), and CytGS1-TR3 (D) were germinated and grown for 28 d in soil as described in "Materials and Methods."



Alex worked with several other members of our Plant Molecular Biology laboratory, notably Rosana Melo, a then Ph.D. student who became her dear, dear life-long friend. Alex also worked with several other members the lab including Maria Shamis, Igor Oliveira, Laurence Lejay, Eric Brenner and Barbara Miesak, amongst others. Alex was part of the Coruzzi lab family at NYU. As you will see from the photos below (courtesy of Rosana Melo & Igor Oliveira), Alex and her colleagues worked hard and played hard together….

Alex was a remarkable person – she had a deep, deep intellect and curiosity about science and how things worked. She was also immensely warm and generous of spirit. She was always willing to help others and put the needs of her family and close friends ahead of herself.

Alex introduced us to Oscar Dunn - her soul mate and husband. Oscar was such a gentleman, he adopted all of us. He went along on lab dinners and was always there to help when he could with career moves of those in the lab that went on to business ventures.

Alex was fiercely smart, and extraordinarily kind and generous of spirit. She was so very loyal to her family and friends. For me, I started out as her professor and mentor, and ended as her dear, dear friend – I will miss many parts of Alex, but especially her insatiable curiosity, her esprit de coeur (spirit of the heart) and her joy for life.

In her final year – I got to know and became in close contact with Alex's dear Aunt Pamela. Aunt Pamela was a tremendous source of comfort and strength for Alex – and also for me and Rosana Melo during her ordeal. We formed a bond that would make Alex proud.

Remarkably, the Sunday before Alex left this earth, she came to me in my dream as real as day. She was standing straight and tall. I feel Alex came to say goodbye. I am so very grateful that my dear friend Natasha Raikhel enabled me to reroute my trip back home from a scientific conference to spend one last day, June 28, 2017 - my birthday - with Alex in San Francisco. What a gift.

Alex was a true, true, friend and a remarkably strong human being - her strength of character especially shone in her last year, months, days.
Alex – may god keep you in the palm of his hand.

With much love – from your loyal mentor and friend

Gloria Coruzzi
Carroll & Milton Petrie Professor
NYU Department of Biology

Alexandra Clark, Assistant Research Scientist
NYU Dept. of Biology – 1997

Alex Clark and Rosana Melo, NYU Biology

For my friend Alex Clark – by Rosana Melo, Research Scientist, Monsanto

I am writing to celebrate Alex's life. I had the privilege of being her friend for over 23 years. I first met Alex at New York University (NYU). Although we went separate ways after NYU, I was happy we continued to stay in touch with each other via phone calls and visits.

I have fond memories of working with her in the same lab at NYU. We often worked together and Alex was very meticulous on planning and conducting experiments. Afternoon coffee runs were memorable, one of us would buy coffee across the street from NYU… and we would take orders from people around the lab. Lunches varied from Pizza to Japanese carryout. Her laughter, kindness, and positive energy were contagious.

I have so many good memories of Alex from all the years we were friends. Alex would always send birthday cards, Christmas cards, or a card just to say "I love you" or "have a great summer". She would always buy 3-4 cards for each occasion because she couldn't make up her mind regarding which card she liked the best. Then, she would send each card separately and a few days apart to insure there was unexpected surprises in the mailbox.

Rosana Melo and Alex Clark, NYU Plant Molecular Biology

Alex lived life intensively. I loved hearing all about her fascinating travels around the world. And, she always had a "lifetime lesson message" to share with friends. Alex had an incredible generous, compassionate, and loving spirit. Alex never stopped trying to find or create opportunities to help people around her. She always put the needs of others first. Her priorities were always to help her family and her friends.

When Alex's learned of her illness, I was going through a stressful time in my life.   She waited for the right moment to tell me about her illness so I wouldn't lose focus. Then one day when I called her, she told me of her illness. I was shocked, speechless, and numb. I went to visit her San Francisco and even though debilitated, Alex was her typical positive, never complaining and ready to fight self! We always kept in touch through text, Facetime, even when she could not speak.

I often would tell her not to forget who she was and all the positive impacts she had on people's lives. Alex was a unique and very special friend that will be missed and always be in my heart.

Alex, I am so proud of all you have accomplished in life.
Kisses, hugs and love from your friend.

Rosana Melo

For my friend Alex Clark – by Igor Oliveira

Alex, you will be missed. Each day working with you was never the same… It was always something different but your smile and upbeat energy was always the same. You made each day unique. Alex, you will be missed forever.

Alex, your "little secret", small, that you kept in such humility: you worked like you were “four” people. You worked because you loved it. Because you loved us. Each one of us. And the lab. That’s your secret Alex, that’s why you were the person that you were. You will be truly missed and will be with us forever, Alex Clark.

It was great writing about Alex, emotional but good. She is such a great person.

L to R: Yanna & undergraduate student, Igor Oliveira, Alex Clark NYU Biology – Lab of Plant Molecular Biology

Aug 22, 2017

Dear Gloria,

Thank you so much for letting me know. (about Alex's passing)

I have been thinking about Alex every day and was anxiously waiting for your return from Ireland.

In the beginning, I did not have the courage to go see her in California. I wanted to always remember her young, healthy, and full of energy and life. And later, it was too late for both of us.

Alex was a very dear friend. She had a tendency to disappear often, but when she reappeared again she always was the same old Alex. I have so many fond memories (and cute little cards) from our 21 years together.

She will remain in my heart forever...

Maria (Shamis)

Maria Shamis
NYU Plant Molecular Biology Lab

A tribute to Alex from Laurence Lejay-Lefebvre, Montpellier, FR

“A friend who dies, it’s something of you who dies” Gustave Flaubert.

I first met Alex when I arrived in Gloria’s Coruzzi’s lab in October 2000. Knowing that Alex was speaking French, Gloria kindly put me in her hands to help me settle down and find my way in the lab and in New York City. More than a colleague, Alex quickly became my first real friend in this new world. The first thing you noticed, when you met Alex, is her energy and her big smile. For what I can remember she was always in a good mood, always ready to carry you on her back and show you the world. And no matter what was happening, she would always stay positive and optimistic. But the crab is cruel and let her no chance. I will forever remember our coffee at O’rens in the morning, the GreenMarket at Union Square on Saturday, and the Oyster bar in Grand Central. When I told you that my husband Ionas was a big fan of Arthur Rimbaud, you took me for a glass of wine at the French Wine bar “Le Bateau Ivre”. I also remember this big painting of Ionas that you hung on the wall of your bedroom. You told me that you could see your life in it. It became like an unbreakable bond between us, even long after I left New York. Art can be such a powerful vessel to take you through time and space. I hope it helped you until the end. Life is so unfair, Alex, but you loved it so much.

Rest in peace my dear friend and say hi to Ionas for me.
I will miss you until we meet again. I love you.

Laurence Lejay-Lefebvre, Montpellier, FR

Alex in the Lab with Plant Molecular Friends and Family Members

Alex and Oscar Dunn at Lab Party

Alex making Pizza!

Alex, Rosana Melo and Tatiana Oliveria in Naples, FL

Alex and Rosana – true friends.

Alex and Aunt Pamela Galy –

A special union…

“Our prayers and love for Alex will keep us all together.”

- **Aunt Pamela Galy –**

“This has been an emotional journey with Alex. We shared those quiet moments of conversation on the sofa together here in the Autumn and Winter along with the hikes in the forest. Alex was always in the lead with Winston.”

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News

Plant Systems Biology @ The Coruzzi Lab

From predictive network modeling to trait evolution

August 2017

In memory and tribute to Alexandra Page Clark: Student, Research Scientist, Friend

See: "people" page, scroll to Alumni

http://coruzzilab.bio.nyu.edu/wordpress/?page_id=5

Alex Clark Tribute

Carlos Cordon-Cardo, MD, PhD
Professor and Chairman
Department of Pathology

Mount Sinai Health System
One Gustave L. Levy Place, Box 1194
New York, NY 10029-6574
T 212-241-8014
F 212-426-5129
carlos.cordon-cardo@mssm.edu

July 6, 2017

Ms. Alexandra Clark
860 U.N. Plaza, Apt. 29B
New York, NY 10017

Dear Ms. Clark,

As the Chairman of the Department of Pathology at the Mount Sinai Health System, I am pleased to inform you that Elliott and Ruth Joseph are making an important donation in honor of you and Dr. Joseph Schein. This support will enable a study in "Immune Regulation and Immune Escape Mechanisms in Brain Diseases" in order to better understand the immunological pathways that activate disease, including the impact of stress on brain diseases. Every human body is made of approximately one trillion cells, one billion of which die and are replaced each day. When this process is in balance, the body can enjoy good health and life. When this process is dysregulated, cancer and other diseases result. Many studies have shown correlations between stress and disease and this will be a new lens from which to view that link.

Specifically, this important work will further our understanding of cells that can escape the surveillance of the immune system, since they do not express histocompatibility proteins. As products in any store, our cells also have their "bar codes" that identify them as part of our own body. Recent discoveries point to a cell with "no bar codes" (or HLA negative cells) that cannot be recognized nor attacked by our immune cells, including T-cells and natural killer cells that are activated in diseases like cancer. Most human cells are HLA positive, but HLA negative cells are found throughout the body in very small percentages and, we believe, can offer insight into the development of cancer and other inflammatory diseases. Using the resources of Mount Sinai's tissue and data banks, we will isolate HLA negative cells and perform in-depth immunologic and genetic profiling of these cells, especially in brain disease. The further characterization of these cells will build our understanding of how these cells function and expand our understanding of the triggers of health and disease.

We are so grateful that the Josephs are creating this wonderful tribute to you and Dr. Schein, and any publications that result from these studies will acknowledge this tribute in your honor. This study would simply not be possible without philanthropic support, and it is the Joseph's vision and generosity that is moving this innovative work forward. I would be delighted to answer any questions you may have. If you or your family would like to be in touch, don't hesitate to call me at 212-241-8014.

My warmest regards,

Carlos Cordon-Cardo, MD, PhD

cc: Elliott and Ruth Joseph
Dr. Joseph Schein

Name:	**Alexandra Page Clark**
Birthdate (MM/DD):	**09/28**
Print Date:	**08/28/2017**
Student ID:	**N19175326**
Institution ID:	**002785**
Page:	**1 of 1**

New York University
Beginning of Graduate Record

Degrees Awarded

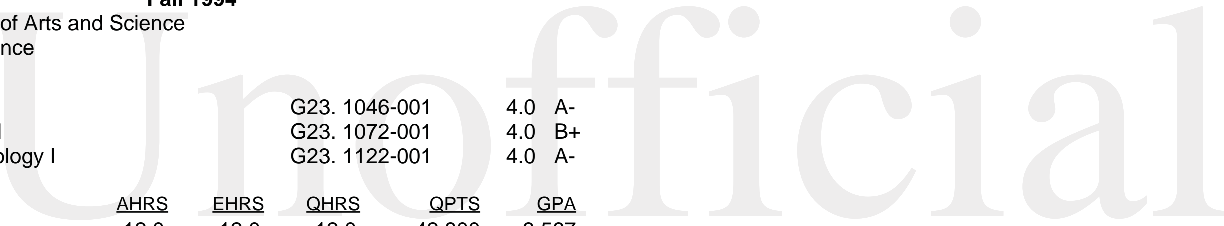
Master of Science 05/16/1996
Graduate School of Arts and Science
Major: Biology

Other Institutions Attended

Univ Calif Berkeley
From: 08/01/1992 To: 12/01/1993

Test Scores

Test ID	Test Component	Test Date	Score
GRE	Biological Sciences	04/01/1994	730.00
GRE	Quantitative	04/01/1994	550.00
GRE	Subscore1	04/01/1994	74.00
GRE	Subscore2	04/01/1994	75.00
GRE	Subscore3	04/01/1994	64.00
GRE	Verbal	04/01/1994	540.00



Fall 1994

Graduate School of Arts and Science
Master of Science
Major: Biology

Biochemistry I	G23. 1046-001	4.0	A-
Plant Resources I	G23. 1072-001	4.0	B+
Lab Molecular Biology I	G23. 1122-001	4.0	A-

	AHRS	EHRS	QHRS	QPTS	GPA
Current	12.0	12.0	12.0	42.800	3.567
Cumulative	12.0	12.0	12.0	42.800	3.567

Spring 1995

Graduate School of Arts and Science
Master of Science
Major: Biology

Biochemistry II	G23. 1047-001	4.0	B
Plant Resources II: Econ Botany & Global Env	G23. 1073-001	4.0	B
Lab Molecular Biology II	G23. 1123-001	4.0	A

	AHRS	EHRS	QHRS	QPTS	GPA
Current	12.0	12.0	12.0	40.000	3.333
Cumulative	24.0	24.0	24.0	82.800	3.450

Summer 1995

Graduate School of Arts and Science
Master of Science
Major: Biology

Research	G23. 3303-001	4.0	A
Research	G23. 3304-001	4.0	A

	AHRS	EHRS	QHRS	QPTS	GPA
Current	8.0	8.0	8.0	32.000	4.000
Cumulative	32.0	32.0	32.0	114.800	3.588

Fall 1995

Graduate School of Arts and Science
Master of Science
Major: Biology

Maintain Matriculation	G47. 4747-001	0.0	***

	AHRS	EHRS	QHRS	QPTS	GPA
Current	0.0	0.0	0.0	0.000	0.000
Cumulative	32.0	32.0	32.0	114.800	3.588

Spring 1996

Graduate School of Arts and Science
Master of Science
Major: Biology

Maintain Matriculation	G47. 4747-001	0.0	***

	AHRS	EHRS	QHRS	QPTS	GPA
Current	0.0	0.0	0.0	0.000	0.000
Cumulative	32.0	32.0	32.0	114.800	3.588

MS THESIS: FURTHER CHARACTERIZATION OF THE GENES AND MUTANTS OF ASPARTATE AMINOTRANSFERASE IN ARABIDOPSIS

End of Graduate Record

Alexandra Clark
NYU Lab Notebook
1997

Experiment n° 452 — 6/26/97

PROTOCOL SET-UP for transgenic tobacco Western Blotting using Bio-Rad Mini-Trans-Blot Cell

I MATERIALS

Ⓐ Electrophoresis
Ⓑ Transfer
Ⓒ Detect

Ⓐ ① 5x Tris-glycine-SDS Running Buffer

5x tris-glycine	100 ml	V_F = 500 ml
20% SDS	2.5 ml	
H_2O	397.5 ml	

② 5x SDS gel-loading buffer (4°C)

10 ml	
Tris	380 mg
glycerol	5 ml
SDS	500 mg
BME	1 ml
B ... blue	pinch
H_2O	4 ml

③ SDS gel (Maniatis 18.52)

	10% SEPARATING GEL	5% STACKING GEL
d H_2O	4 ml	3.4
1.5 M tris HCl pH 8.8	2.5 ml	—
1.0 M tris HCl pH 6.8	—	630 µl
20% SDS	50 µl	25 µl
30% acrylamide	3.3 ml	830 µl
10% APS (0.1 g/ml)	100 µl	50 µl
TEMED	4 µl	5 µl

pour separating gel, overlay w/ H_2O, allow solidification, pour off H_2O, pour stacking, insert comb, allow to solidify, remove comb

Exp nº 452 6/23 p2

rinse wells, prepare 500 ml 1x running buffer and assemble to running chamber

(4) Samples acetone suspension, exp 451
resusp in 10 μl 0.1 M NaOH, 3 μl 1 M tris 8, 10 μl 0.1 M HCl, up to 300 μl H_2O

- Soluble protein extracts from exp SR1 and 23
- Kaleidoscope prestained standards marker

See exp 451

Bradford 10 μl
(1) 0,426
(2) 0,390

→ μg tot μg/μl
(1) 17.64 (1) 1,76
(2) 16.2 (2) 1,62

6S →

BIO-RAD Kaleidoscope Prestained Standards
Catalog 161-0324, Control 79358

Protein	Color	Calibrated MW (daltons)
Myosin	Blue	200,000
ß-galactosidase	Magenta	135,000
Bovine serum albumin	Green	81,000
Carbonic anhydrase	Violet	41,900
Soybean trypsin inhibitor	Orange	31,400
Lysozyme	Red	18,000
Aprotinin	Blue	6,900

CL1610324 REVB

Bio-Rad Laboratories • 2000 Alfred Nobel Drive • Hercules, CA 94547

Concentration	sample	6S enzyme	H_2O	5x loading buffer
20 μg	1	11.3 μl	24.7 μl	4 μl
	3	12.3	23.7	↓
10	1	5.7	30.3	
	3	6.2	33.4	
5	1	2.8	33.2	
	3	3.1	36.5	
2.5	1	1.4	34.6	
	3	1.5	34.5	

Run = 200V, ~40'

Comb → 5 well 0.75mm, can hold VF = 40 μl
70 μl

Exp n° 452

6/24
p3

(B) Transfer

materials

(1) • PVDF membrane (0.2 μm)
(bio-rad 162-0184)
• Whatman 3MM paper
• mini-trans-blot cell bio-rad
• meOH
• transfer buffer/l (fresh)

3.03 g tris base
14.4 g glycine
200 ml meOH

(can be made 10x : use 100 ml)

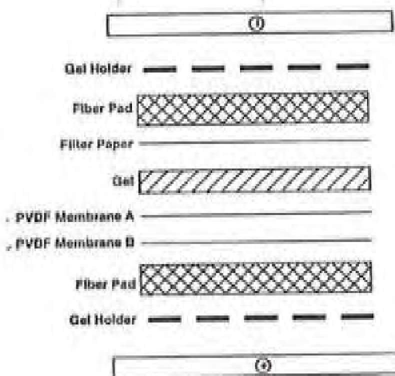
• 10X TBS/l

80 g NaCl
0.2 g KCl
30 g tris
pH 7.4 w/HCl

← 800 ml

(2) apparatus set-up

(a) bio ice cooling units, fill, -20°C
(b) apparatus



clear (b)

whatmann
gel
PVDF
whatman

Black

Exp nº 452

6/24
p4

(C) Detection
materials

Block (1) Blotto (200 ml) — [10 g nonfat dry milk (Alba brand); 20 ml 10X TBS]

Wash (2) TBS-T = 1X TBS + 0.1% Tween-20

1er anti-body (3) Dr. Gloria Coruzzi's frozen stock -80°C of diluted antibodies (used 22 times)

= [tobacco leaf 6S 1:1000
P. vulgaris nod 6S 1:250
dated 8/12/86]

thaw room t° and store at 4°C
(add Na azide 0.02%) 6/25

HRP labelled antibody (4) Anti-rabbit Ig, peroxidase-linked species specific whole antibody (from Donkey)

(Amersham) ECL Western Blotting protocols

(catalogue MA 9-34)

dilute 1:300 (10 mls use 34 µl)
keep 4°C

(5) Detection (kit Amersham RPN 2108 ECL Western blotting analysis system)

detection reagent 1 + 2

(6) Biomax MR film
expose 10" to 10'

II PROCEDURES

(1) prepare gels 6/24

(2) Before loading samples:
heat to 95°C for 5' to denature proteins
pb * (marker as well (did not do)*

* (3) load samples and run gel at
*(samples should have been re-dissolved in Na_2CO_3 / DTT / par Corvuzzi protocol)
200 Volt (constant) for 45' RT
dye front just out of gel

* (4) dissamemble and soak gels in transfer buffer as well as membrane and whatman)
*(should have soaked membrane in MeOH and rinsed in H_2O) then to equilibrate in transfer buffer.

Exp n° 452

(5) Set up transfer apparatus and soak sponges in transfer buffer. — 6/24

assemble and run at

350 mA for 2h at 4°C

check trf by using the blot markers did NOT see the kaleidoscope pattern on the (m) so trf was NOT successful.

(6) Placed the 2 (m) in Blotto (30ml) in petri dish OH, 4°, shaker

(7) Followed kit protocol. — 6/25

Western blotting protocol summary — RPN 2106/8/9

Stage	1 Electrophoresis and blotting	2 Block	3 Wash	4 Primary antibody	5 Wash
Reagent		5% blocking reagent in TBS-T or PBS-T	TBS-T or PBS-T	Diluted in TBS-T or PBS-T	TBS-T or PBS-T
Volume used		10ml	10ml	10ml	10ml
Time	Usual electrophoresis and blotting times	1 hour	1 x 15 mins 2 x 5 mins	1 hour	1 x 15 mins 2 x 5 mins (see over)

6 Biotinylated antibody or HRP labelled antibody	7 Wash - if using HRP labelled antibody omit steps 7 and 8	8 Streptavidin -HRP	9 Wash	10 Detection	11 Exposure
Diluted in TBS-T or PBS-T	TBS-T or PBS-T	Diluted in TBS-T or PBS-T	TBS-T or PBS-T	Mix the two reagents 1:1	Drain excess reagent, cover with SaranWrap™
10ml	10ml	10ml	10ml	0.125ml/cm²	
20mins - 1hour	1 x 15mins 2 x 5mins	20 mins - 1hour	1 x 15mins 4 x 5mins	1 min	Immediately expose to film for 30 seconds - 10 mins

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Amersham International plc, Amersham Place, Little Chalfont, Buckinghamshire, England, HP7 9NA

™SaranWrap is a trademark of the Dow Chemical Co.

5434/5066/95/04

(a) 30 ml 1er antibody (Corozzi mixed)

(b) 500 µl reagent 1, 500µl reagent 2

(c) 5' expose

~~Result~~

REDO

Overexpression of Cytosolic Glutamine Synthetase. Relation to Nitrogen, Light, and Photorespiration[1]

Igor C. Oliveira[2], Timothy Brears[3], Thomas J. Knight, Alexandra Clark, and Gloria M. Coruzzi*

Department of Biology, New York University, 1009 Main Building, 100 Washington Square East, New York, New York 10003 (I.C.O., T.B., A.C., G.M.C.); and Department of Biology, University of Southern Maine, 96 Falmouth Street, Portland, Maine 04103 (T.J.K.)

In plants, ammonium released during photorespiration exceeds primary nitrogen assimilation by as much as 10-fold. Analysis of photorespiratory mutants indicates that photorespiratory ammonium released in mitochondria is reassimilated in the chloroplast by a chloroplastic isoenzyme of glutamine synthetase (GS2), the predominant GS isoform in leaves of Solanaceous species including tobacco (*Nicotiana tabacum*). By contrast, cytosolic GS1 is expressed in the vasculature of several species including tobacco. Here, we report the effects on growth and photorespiration of overexpressing a cytosolic GS1 isoenzyme in leaf mesophyll cells of tobacco. The plants, which ectopically overexpress cytosolic GS1 in leaves, display a light-dependent improved growth phenotype under nitrogen-limiting and nitrogen-non-limiting conditions. Improved growth was evidenced by increases in fresh weight, dry weight, and leaf soluble protein. Because the improved growth phenotype was dependent on light, this suggested that the ectopic expression of cytosolic GS1 in leaves may act via photosynthetic/photorespiratory process. The ectopic overexpression of cytosolic GS1 in tobacco leaves resulted in a 6- to 7-fold decrease in levels of free ammonium in leaves. Thus, the overexpression of cytosolic GS1 in leaf mesophyll cells seems to provide an alternate route to chloroplastic GS2 for the assimilation of photorespiratory ammonium. The cytosolic GS1 transgenic plants also exhibit an increase in the CO_2 photorespiratory burst and an increase in levels of photorespiratory intermediates, suggesting changes in photorespiration. Because the GS1 transgenic plants have an unaltered CO_2 compensation point, this may reflect an accompanying increase in photosynthetic capacity. Together, these results provide new insights into the possible mechanisms responsible for the improved growth phenotype of cytosolic GS1 overexpressing plants. Our studies provide further support for the notion that the ectopic overexpression of genes for cytosolic GS1 can potentially be used to affect increases in nitrogen use efficiency in transgenic crop plants.

Nitrogen is a costly and rate-limiting element in plant growth. Nitrogenous fertilizer accounts for 40% of costs associated with crops such as corn (*Zea mays*) and wheat (*Triticum aestivum*; Sheldrick, 1987). Increasing the efficiency of nitrogen use would be cost-effective and would minimize problems of ground water contamination by excess nitrate application (Sheldrick, 1987). Attempts to select crop plants with enhanced nitrogen use by conventional breeding strategies have been largely unsuccessful because of problems associated with screening large populations for a trait that is difficult to assess under field conditions. Plants do not seem to be limited in their ability to uptake or convert nitrate to ammonium (Crawford et al., 1986), although it does seem that some crop plants may be limited in their ability to incorporate inorganic nitrogen into protein. Gln synthetase (GS; E.C.6.3.1.2) catalyzes the conversion of inorganic nitrogen (ammonium) into organic form (Gln) and, for this reason, is a good candidate to be a critical and possibly rate-limiting enzyme in ammonium assimilation.

Biochemical studies have shown that distinct isoenzymes of GS are located in the chloroplast (GS2) and cytosol (GS1) of numerous plant species (Hirel and Gadal, 1980). In all higher plants examined to date, there is a single nuclear gene for chloroplastic GS2 and multiple homologous but distinct genes for cytosolic GS1 (Tingey and Coruzzi, 1987; Tingey et al., 1987; Sakamoto et al., 1990; Cock et al., 1991; Peterman and Goodman, 1991; Sakakibara et al., 1992; Li et al., 1993; Oliveira et al., 1997; Oliveira and Coruzzi, 1999). The chloroplastic and cytosolic GS isoenzymes seem to serve distinct roles, based on their organ- and cell-specific expression patterns (Edwards et al., 1990; Carvalho et al., 1992; Kamachi et al., 1992). Chloroplastic GS2 is expressed abundantly in leaf mesophyll cells, whereas expression of cytosolic GS1 is detected at low levels in leaves, and it is normally restricted to the phloem (Edwards et al., 1990; Carvalho et al., 1992; Kamachi et al., 1992).

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The high-level expression of chloroplastic GS2 in leaf mesophyll cells underscores its role in the reassimilation of photorespiratory ammonium, which is supported by biochemical, genetic, and more recent molecular evidence (Keys et al., 1978; Wallsgrove et al., 1987; Edwards and Coruzzi, 1989; Lea and Forde, 1994; Kozaki and Takeba, 1996; Migge et al., 2000). Reassimilation of photorespiratory ammonium by chloroplast GS2 is crucial to plant growth, as levels of ammonium released during photorespiration may exceed primary nitrogen assimilation by 10-fold (Keys et al., 1978). Barley (*Hordeum vulgare*) mutants defective in chloroplastic GS2 are unable to reassimilate photorespiratory ammonium and die when grown in air, indicating that chloroplastic GS2 plays a major role in the reassimilation of photorespiratory ammonium in leaf mesophyll cells. It was surprising that these barley mutants in chloroplastic GS2 died when grown under photorespiratory conditions (air), even though leaves contain low levels of cytosolic GS1 (Wallsgrove et al., 1987; Lea and Forde, 1994). The nonoverlapping and cell-specific expression patterns of chloroplastic and cytosolic GS isoenzymes may explain why cytosolic GS1 cannot compensate for the loss of chloroplastic GS2 in leaf mesophyll cells of these barley photorespiratory mutants.

The barley GS mutant studies cited above suggest that there is a subcellular trafficking of photorespiratory ammonium out of the mitochondria and into the chloroplast for reassimilation by chloroplastic GS2. We, therefore, reasoned that the ectopic overexpression of a cytosolic GS1 isoenzyme in the leaf mesophyll cells, where it is not normally expressed at high levels, could potentially provide an additional and/or alternate route to native chloroplastic GS2 in the reassimilation of photorespiratory ammonium. This type of metabolic engineering of cytosolic GS1 could potentially result in an increase in the efficiency of reassimilation of photorespiratory ammonium, leading to increases in nitrogen use efficiency and plant growth. Previous studies showed that overexpression of a gene for chloroplast GS2 from rice in transgenic tobacco (*Nicotiana tabacum*) increased photorespiratory capacity and resistance to photooxidation, although in this case no effect on growth has been reported (Kozaki and Takeba, 1996).

Several groups have attempted to improve nitrogen assimilation by the overexpression of GS genes with mixed results (Eckes et al., 1989; Hemon et al., 1990; Hirel et al., 1992; Temple et al., 1993; Vincent et al., 1997; Gallardo et al., 1999; Migge et al., 2000; Ortega et al., 2001). For instance, Hirel and coworkers observed accelerated growth rate in transgenic *Lotus corniculatus* plants, which overexpress a soybean (*Glycine max*) GS isoenzyme (Vincent et al., 1997). Growth improvements have been reported more recently for poplar (*Populus* spp.) trees and tobacco plants overexpressing distinct isoforms of GS (Gallardo et al., 1999; Migge et al., 2000; Fuentes et al., 2001). Experimental data available to date have provided evidence that overexpression of GS may affect the modulation/maintenance of photosynthetic rates (Kozaki and Takeba, 1996; Fuentes et al., 2001), and it is a possible mechanism by which GS can improve/accelerate growth in these GS transgenic plants (Fuentes et al., 2001).

Herein, we report that transgenic tobacco plants that ectopically overexpress a cytosolic GS1 isoenzyme in leaves have alterations in the photorespiratory pathway. This is evidenced by lower levels of free ammonium, by higher levels of photorespiratory intermediates, and by an increase in the CO_2 photorespiratory burst measurements. These GS1 transgenic plants also display an enhanced growth phenotype as quantified by increases in fresh weight, dry weight, and leaf soluble protein. Moreover, these increases are paralleled by corresponding increases in GS activity. These studies provide insights into the mechanism by which overexpression of a cytosolic GS1 isoenzyme may lead to changes in growth and suggest that it may be possible to increase nitrogen use efficiency by the manipulation of genes for specific GS isoenzymes in transgenic crop plants.

RESULTS

Characterization of GS Transgenic Plants

Transgenic lines of tobacco were generated in which a 35S cauliflower mosaic virus promoter was used to drive the ectopic overexpression of pea (*Pisum sativum*) cDNAs encoding either chloroplastic GS2 or cytosolic GS1 isoenzymes. Two homologous but distinct GS cDNAs encoding cytosolic isoenzymes of GS (80% nucleotide homology and 86% amino acid homology within the coding region) were used; cytosolic GS1 (CytGS1-TR) or cytosolic GS3A (CytGS3A-TR; Tingey et al., 1988). Transgenic lines containing the pea chloroplastic GS2 cDNA were also generated (ChlGS2-TR; Tingey et al., 1988). Controls used in these studies were tobacco plants transformed with an insertless vector (SR1–6). For each construct, multiple independent lines were generated. The results reported below are representative of four CytGS1-TR (three shown below), two CytGS3A-TR (not shown), and nine ChlGS2-TR (one shown below) independent GS transgenic lines, respectively.

GS expression was examined in transgenic plants at the level of GS mRNA, GS protein, GS holoenzyme, and total GS activity (Figs. 1 and 2). The growth phenotype of two individuals of representative transgenic and control lines are shown side-by-side in Figure 2A. Leaves of CytGS1-TR plants accumulated high levels of mRNA for cytosolic GS1 transgene (Fig. 1A, lanes 2 and 3) and cytosolic GS1 protein (Fig. 1B, lanes 2 and 3). The ectopically expressed pea cytosolic GS1 protein also assembled into a native cytosolic GS1 holoenzyme in leaves (Fig.

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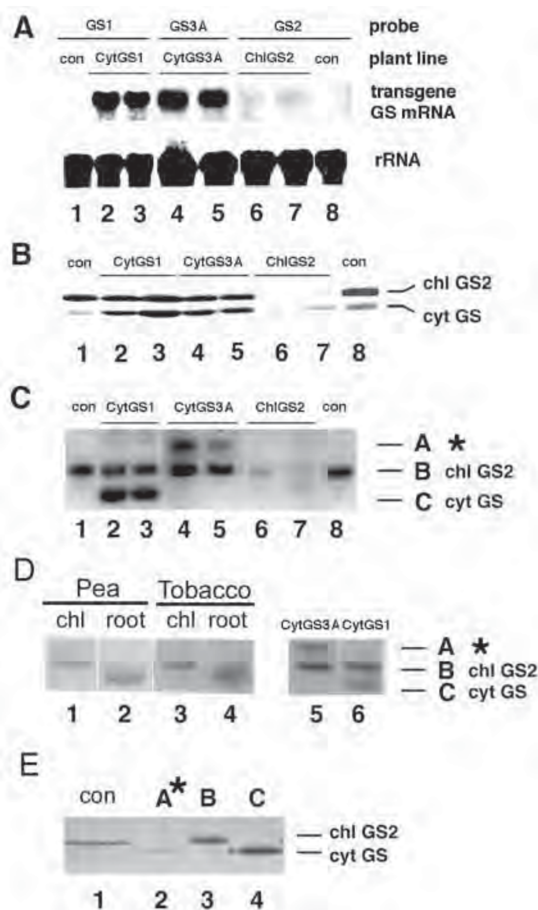

Figure 1. GS expression profiles in leaves of 35S-GS transgenic tobacco plants. A, GS mRNA was detected by hybridization with full-length cDNA probes for pea cytosolic GS1 (lanes 1–3), pea cytosolic GS3A (lanes 4 and 5), and pea chloroplastic GS2 (lanes 6–8). B, Western-blot analysis with a mixture of antibodies to bean (*Phaseolus vulgaris*) cytosolic GS1 and tobacco chloroplastic GS2 (Hirel et al., 1984; Lara et al., 1984; Tingey et al., 1988). C, Non-denaturing gel and GS activity stain showing GS holoenzymes A, B, and C. GS holoenzyme A (*) is a nonnative GS isoenzyme detected only in CytGS3A-TR plants. CytGS1-TR and CytGS3A-TR lines contain normal levels of native chloroplastic GS2 (band B). D, Non-denaturing gel and GS activity stain showing a side-by-side comparison between CytGS3A-TR (lane 6) and CytGS1-TR (lane 5) leaf extracts. The cytosolic GS1 holoenzyme (band C), which is detected in leaves of CytGS1-TR plants but not in the control plants, corresponds to the native root-specific tobacco cytosolic GS1 holoenzyme (lanes 4 and 6). Controls: lanes 1 and 2, pea chloroplast and root extracts; lanes 3 and 4, tobacco chloroplast and root extracts. E, Subunit composition of GS holoenzymes. GS holoenzymes A*, B, and C, respectively, were excised from preparative native gels, denatured, separated by PAGE, and detected by western-blot analysis. Crude leaf extract of untransformed tobacco (lane 1), GS holoenzyme A* from CytGS3A-TR (lane 2), GS holoenzyme band B isolated from isolated chloroplasts from untransformed tobacco (lane 3), and GS holoenzyme C from CytGS1-TR (lane 4).

1C, lanes 2 and 3, band C). This cytosolic GS1 holoenzyme is normally only detected at significant levels in roots of tobacco (Fig. 1D, lane 4) but not in leaves (Fig. 1C, lane 1). It is noteworthy that the levels of cytosolic GS1 protein present in leaves of control plants detected by western blot (Fig. 1B, lanes 1 and 8) are too low to produce a detectable GS1 holoenzyme band when assayed by enzyme activity staining of extracts run on non-denaturing PAGE (Fig. 1C, lanes 1 and 8). These differences in detection of low levels of native cytosolic GS1 in leaves of tobacco are most likely due to different sensitivities between the two techniques. The increased level of the cytosolic GS1 holoenzyme in leaves of CytGS1-TR plants, resulted in significant increases in levels of total GS activity when compared with controls (Fig. 2, B–D, black circles).

Plants overexpressing a distinct pea cytosolic GS isoenzyme named GS3A (CytGS3A-TR) showed increases in levels of GS3A mRNA (Fig. 1A, lanes 4 and 5) and GS3A protein (Fig. 1B, lanes 4 and 5). However, the GS3A protein assembled into a nonnative-sized GS holoenzyme (Fig. 1C, lanes 4 and 5 and band A*), as demonstrated by its anomalous migration pattern when compared with either pea or tobacco native isoforms from chloroplasts and roots (Fig. 1D). To determine the subunit composition of the GS holoenzymes in the CytGS3A-TR plants, bands A*, B, and C were excised from preparative gels, and the GS subunit peptides were detected by western-blot analysis (Fig. 1E). GS activity bands A* and C are composed exclusively of GS polypeptides (Fig. 1E, lanes 2 and 4). This discounted the possibility that the larger GS activity band A* was the result of the assembly of GS3A subunits expressed ectopically in leaf mesophyll cells with endogenous prechloroplastic GS2 subunits containing an unprocessed chloroplastic transit peptide. Therefore, because the anomalous migrating GS3A holoenzyme was shown to be composed of normal-sized cytosolic GS3A polypeptides (Fig. 1E), one formal possibility is that the larger GS activity band A* in the CytGS3A-TR plants could result from a post-translation modification by the association of this GS holoenzyme with another uncharacterized protein. Evidence for the association of cytosolic GS with other associated proteins has previously been suggested by other studies (Temple et al., 1993; Ortega et al., 2001). Therefore, the unusual migration of the cytosolic GS3A holoenzyme in the CytGS3A-TR plants may reflect changes in conformation and/or additional GS-associated proteins. These CytGS3A-TR plants, which had the anomalous GS holoenzyme, exhibited only modest changes in total GS enzyme activity and growth when compared with controls (not shown). These results with the CytGS3A-TR lines are reminiscent of previous reports in which posttranslational modification of a transgenic cytosolic GS protein was suggested to be associated with the lack of increase in GS enzyme activity and/or ameliorated plant growth in the transgenic GS lines (Eckes et al., 1989; Hemon et al., 1990; Hirel et al., 1992; Temple et al., 1993; Vincent et al., 1997).

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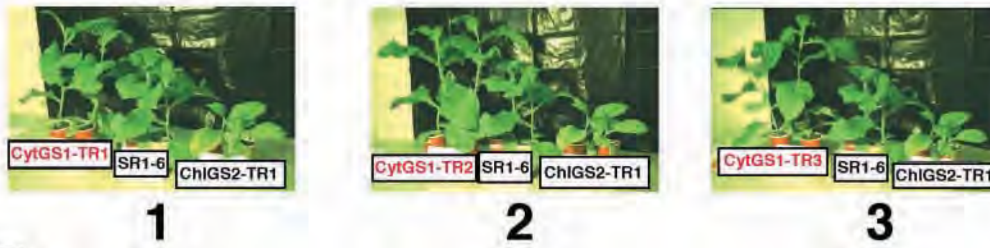

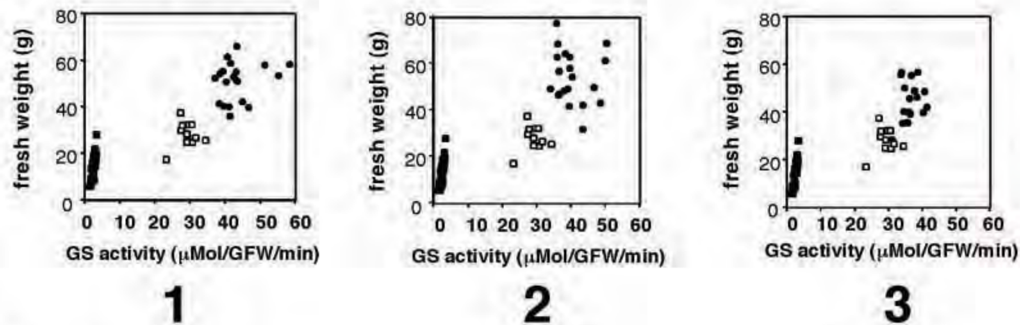

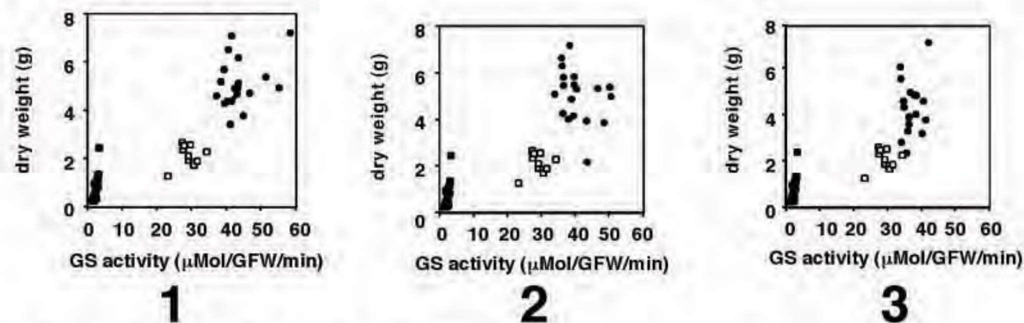

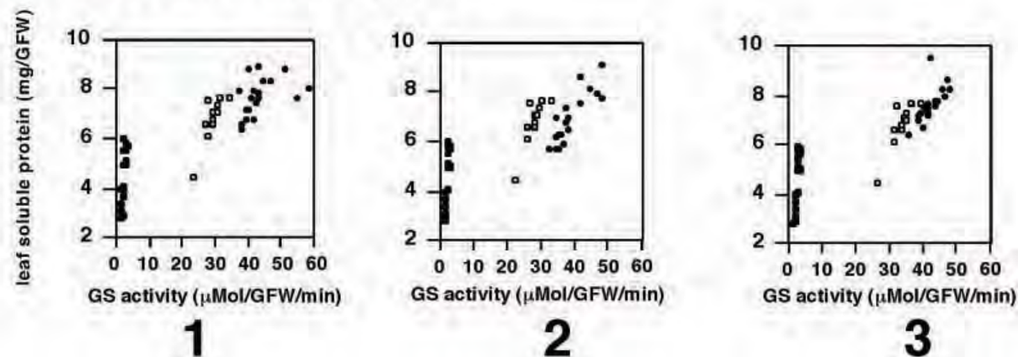

Figure 2. Qualitative and quantitative growth phenotype of GS transgenic plants. A, Plants from the control line (SR1–6) and the cosuppressed chloroplastic GS2 (ChlGS2-TR) line are shown next to three independent lines of cytosolic GS1 overexpressors: CytGS1-TR1 (1), CytGS1-TR2 (2), and CytGS1-TR3 (3). The same ameliorated growth phenotype was also observed in another independent CytGS1-TR line, CytGS1-TR4 (not shown). B through D, Growth analysis of cytosolic GS1 overexpressor lines (●) CytGS1-TR1 (1), CytGS1-TR2 (2), and CytGS1-TR3 (3). Also represented are the control tobacco line

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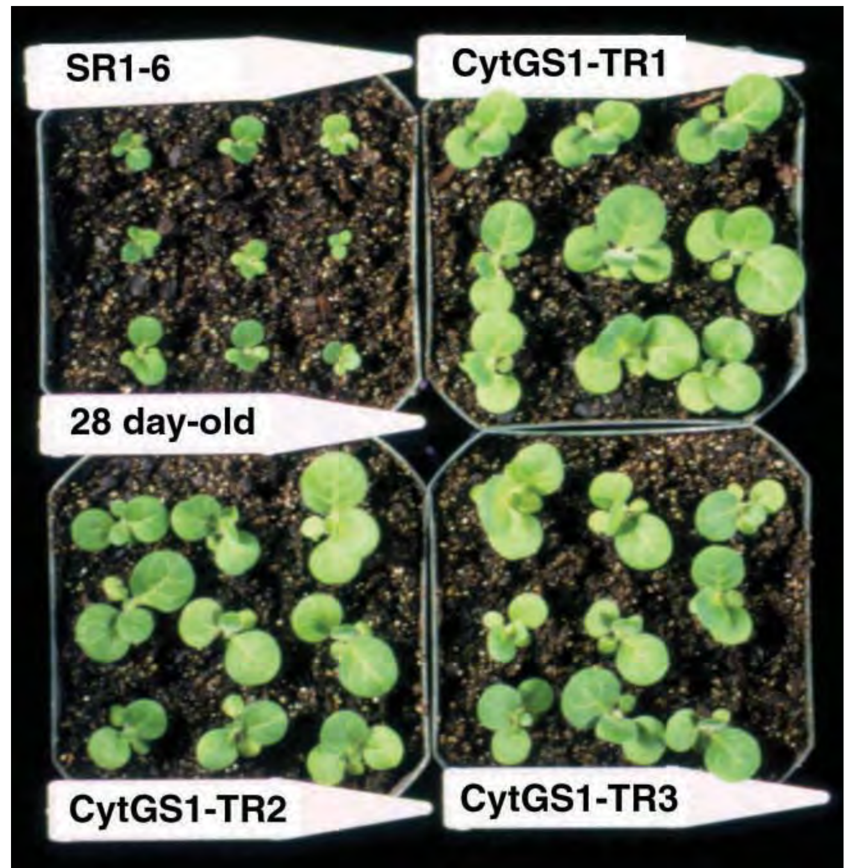

Figure 3. Qualitative growth phenotype of soil-grown GS transgenic plants. Control line (SR1–6; A), CytGS1-TR1 (B), CytGS1-TR2 (C), and CytGS1-TR3 (D) were germinated and grown for 28 d in soil as described in "Materials and Methods."

All transgenic lines engineered to overexpress pea chloroplastic GS2 (ChlGS2-TR) showed a cosuppressed phenotype. Cosuppression was manifested by no expression of transgene GS2 mRNA (Fig. 1A, lanes 6 and 7) and by a dramatic reduction in levels of native tobacco GS protein and holoenzyme for chloroplastic GS2 and cytosolic GS1 (Fig. 1, B and C, lanes 6 and 7). The cosuppression effect on ChlGS2-TR was very consistent and was observed in 23 independent transformants using two different constructs (not shown). It is noteworthy that the pea GS2 transgene was able to suppress expression of genes for chloroplastic GS2 and cytosolic GS1 of tobacco. This is consistent with the relatively high identity between the GS genes of these two plant species (76%–88% amino acid homology; Tingey and Coruzzi, 1987). There are other examples where one member of a gene family can cause cosuppression of other gene family members with significant homology (e.g. ACC synthase; Que et al., 1998). Because the actual mechanism(s) underlying the phenomenon of cosuppression in plants is not totally understood (Vaucheret et al., 1998), the cause for the observed cosuppression of both GS isoenzymes in the ChlGS2-TR plants can only be conjectured.

Transgenic GS Lines Show a Correlation between GS Activity and Fresh Weight, Dry Weight, and Leaf Soluble Protein

We monitored the above transgenic GS lines for growth phenotypes (Fig. 2A, 1–3), and observed a correlation between the levels of GS enzyme activity and plant fresh weight, dry weight, and leaf soluble protein (Fig. 2, B–D). Analysis of at least three independent lines for each construct consistently showed that the transgenic lines transformed with the pea cytosolic GS1 cDNA (CytGS1-TR1, CytGS1-TR2, and CytGS1-TR3; black circles), showed the highest levels of GS activity and the highest increases in plant fresh weight, dry weight, and leaf soluble protein compared with controls (open squares; Fig. 2, B–D). These increases in fresh weight, dry weight, and leaf soluble protein exhibited by the CytGS1-TR plants were most pronounced at early stages of develop-

Figure 2. (*Legend continued from facing page.*)
(SR1–6, □) and the cosuppressed chloroplastic GS2 line (ChlGS2-TR, ■). The growth assays were performed in 19 plants for the CytGS1-TR or ChlGS2-TR lines and 10 plants for the SR1–6 line. All plants were analyzed individually for total plant fresh weight (B), dry weight (C), and soluble protein (D) as a function of total leaf GS specific activity (Shapiro and Stadtman, 1971). The plants were grown and assayed as described in "Materials and Methods."

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ment (Figs. 3 and 4), but also persisted in older plants (Fig. 2A) and in flowering plants (50–60 d old; not shown). The improved growth phenotype of transgenic lines transformed with the pea cytosolic GS1 cDNA was observed in soil-germinated seedlings (Fig. 3) and in plants cultured in media, before transfer to soil (Fig. 2A). Lines transformed with the gene encoding a distinct cytosolic GS gene (CytGS3A) showed only modest increases in GS activity and correspondingly modest increases in fresh weight, dry weight, and leaf soluble protein when compared with the control (not shown). All lines containing the chloroplast GS2 gene (ChlGS2-TR lines) were cosuppressed, and the growth of these lines was characterized by extensive leaf chlorosis (Fig. 2A, 1–3) and by reductions in growth, fresh weight and dry weight (Fig. 2, B–D). The chlorotic phenotype of the cosuppressed ChlGS2-TR plants was relieved when plants were grown in an atmosphere of elevated CO_2 (0.8%–1.2%) to suppress photorespiration or when plants were supplemented with exogenous Gln (not shown). As such, these GS cosuppressed transformants resembled the GS2-deficient photorespiratory mutants of barley (Wallsgrove et al., 1987; Lea and Forde, 1994). Previous studies showed that the barley GS2 mutants could also survive if photorespiration was suppressed (by 1% [v/v] CO_2) or if supplemented with Gln (Blackwell et al., 1987). These results indicate that chloroplastic GS2 mutants (and the cosuppressed GS transgenic plants described herein) die from the depletion of amino donors from the pool of organic nitrogen, caused by their inability to reassimilate photorespiratory ammonium (Blackwell et al., 1987).

Transgenic Plants That Ectopically Overexpress Cytosolic GS1 Display a Light-Dependent, Improved Growth Phenotype under Nitrogen-Limiting and Nitrogen-Non-Limiting Conditions

To determine the possible mechanisms underlying the enhanced growth of transgenic plants overexpressing cytosolic GS1 (CytGS1-TR), we examined whether this improved growth was related to the concentration of exogenously supplied inorganic nitrogen or by light (Fig. 4). The CytGS1-TR plants showed increases in fresh weight under nitrogen-limiting and nitrogen-non-limiting conditions when compared with plants grown at lower PFDs (Fig. 4, A and B). The effects of light and inorganic nitrogen were additive, because the growth of CytGS1-TR plants was maximal under conditions of high inorganic nitrogen (40 mM nitrate and 20 mM ammonium) and "moderate light" (moderate PFD, 200 μmol cm^{-2} s^{-1}). It is noteworthy that even under conditions of no exogenous nitrogen application (0× nitrogen), the CytGS1-TR plants still show a growth advantage compared with control plants (Fig. 4). This suggests that the observed growth advantage of the GS transgenics may relate to increased efficiencies in use of internal stores of nitrogen such as the reassimilation of "recycled" ammonium released during photorespiration (see below).

Transgenic Plants That Ectopically Overexpress Cytosolic GS1 Display Increased Photorespiratory CO_2 Burst

Because primary nitrogen assimilation, photorespiration, and the reassimilation of photorespiratory

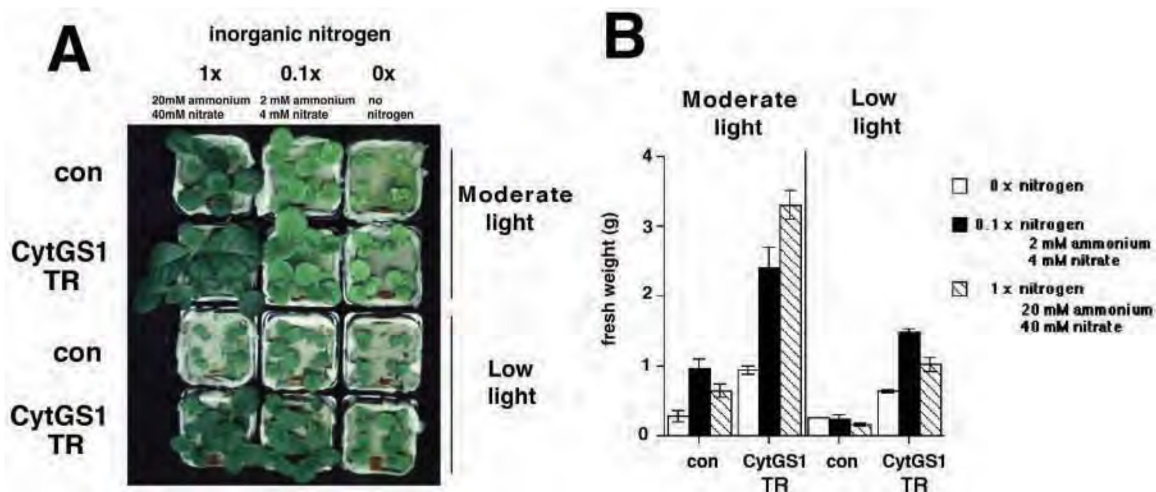

Figure 4. Effect of light on growth of GS transgenic plants grown under different nitrogen regimes. Plants were incubated in a normal day/night cycle either under high light (moderate PFD, 200 μmol cm^{-2} s^{-1}) or low light (low PFD, 50 μmol cm^{-2} s^{-1}) and subirrigated with ammonium-free/nitrate-free liquid Murashige and Skoog medium containing 0× nitrogen (no nitrogen supplementation), 0.1× nitrogen (4 mM nitrate/2 mM ammonium), or 1× nitrogen (40 mM nitrate/20 mM ammonium). A, Qualitative growth phenotype. B, Fresh weight (n = 4, mean ± SE) from plants in A. The plants for this experiment were grown as described in "Materials and Methods."

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ammonium are all light-dependent processes (Blackwell et al., 1987; Wallsgrove et al., 1987; Lea and Forde, 1994; Kozaki and Takeba, 1996), we next tested whether photorespiration was affected in the CytGS1-TR transgenic plants by measuring the post-illumination photorespiratory CO_2 burst. Several independent lines of evidence suggest a direct correlation between increased levels of cytosolic GS1 overexpression in the CytGS1-TR plants and increased rates of photorespiration. First, gas exchange experiments revealed that postillumination photorespiratory CO_2 evolution was increased in the overexpressing CytGS1-TR and decreased in the ChlGS2-TR-cosuppressed plants when compared with the controls (Fig. 5; Table I). Second, levels of amino acids known to be involved in the photorespiratory cycle were elevated in the leaves of CytGS1-TR transgenic plants. CytGS1-TR plants showed a 3.5-fold increase in the Ser/Gly ratios (669.0 ± 86.6 Ser/71.3 ± 4.6 Gly) when compared with the SR1–6 controls (465.7 ± 4.4 Ser/175.7 ± 3.2 Gly) and a 2-fold increase in Glu levels (719.5 ± 27.8) when compared with the SR1–6 controls (338.8 ± 3.7), as measured in picomoles per milligram fresh weight (±SE, n = 3 individual plants). Third, the increased photorespiratory rates in the CytGS1-TR plants correlated with a 6.3- to 7-fold reduction in the total levels of free ammonium when compared with the SR1–6 controls (Fig. 6). This reduction in levels of ammonium was related to the level of GS expression, because transgenic plants that are cosuppressed for GS activity display the opposite phenotype (i.e. 44-fold increase in the levels ammonium; Fig. 6). These results collectively provide three independent measures suggesting that the CytGS1-TR plants have changes associated with photorespiration: (a) increased post-illumination CO_2 evolution, (b) increased levels of photorespiratory amino acids, and (c) decreases in free ammonium. These correlated changes support the notion that ectopic overexpression of cytosolic GS1 in the cytoplasm of leaf mesophyll cells leads to increases in the levels of photorespiration in the transgenic GS1 plants. Although these measures indicate increased photorespiratory rate, the CO_2 compensation point in the GS1-TR plants was unchanged from wild type (not shown). Because the CO_2 compensation point is the point at which CO_2 consumption by photosynthesis equals the rate of CO_2 evolution by photorespiration, this suggests that the

Table I. *Determination of CO_2 evolution in detached leaves of tobacco plants*

Results shown are a representative one from measurements done in two individual plants from each transgenic line analyzed.

Time	ChlGS2-TR	SR1-6	CytGS1-TR
s		*μmol m⁻² s⁻¹*	
0	−2.895	−9.300	−8.932
12	0	9.300	10.586
24	5.308	8.636	16.242
36	4.504	5.646	12.570
48	4.182	3.820	7.675
60	4.182	3.155	6.881
72	4.343	3.023	6.318
90	4.504	2.989	6.285
102	4.665	2.989	6.285
114	4.826	3.023	6.285
126	4.826	3.155	6.451
138	4.890	3.255	−8.435
150	−1.930	3.355	−9295
162	−2.413	−8.636	−8.932
174	−2.574	−8.636	−8.435

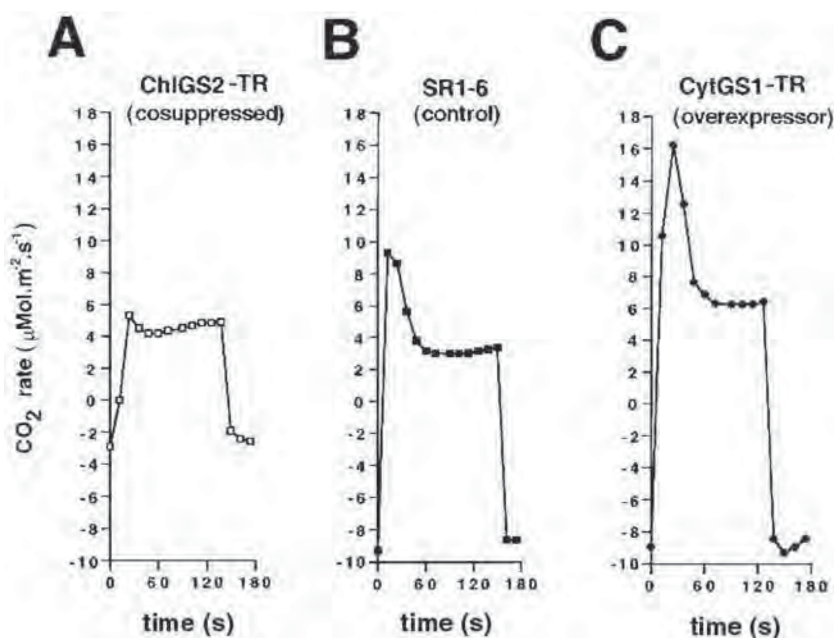

Figure 5. Levels of photorespiration correlate with GS expression in transgenic plants. Detached leaves of the cosuppressed chloroplastic GS2 line (ChlGS2-TR1, □), the control tobacco line (SR1–6, ■), and a cytosolic GS1 overexpressor line (CytGS1-TR1, ●) were initially illuminated (1,000 μmol cm^{-2} s^{-1}) for 1 h and subsequently exposed to dark by blocking the light source for a period of 2 min. The composition of the gas entering the chamber was 79 μL CO_2 L^{-1} (PPM), 21% (v/v) O_2, and balanced nitrogen. Total gas flow was approximately 1 L min^{-1}. The temperature was kept at 28°C to 29°C for dark and light conditions. The rate of CO_2 exchange was measured at 12-s intervals. The measurements were done in two individual plants from each transgenic line analyzed. A representative result is shown.

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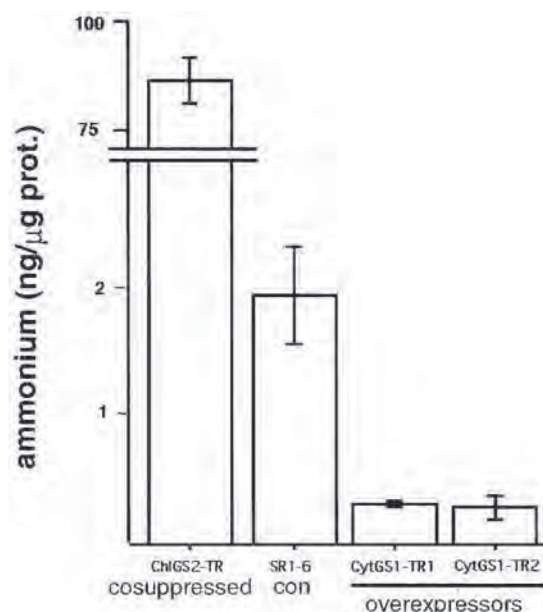

Figure 6. Correlation between the levels of ammonium and expression of GS in tobacco transgenic plants. The plants were incubated under moderate light (moderate PFD, 200 μmol cm^{-2} s^{-1}) subirrigated with 0.5× Hoagland for 20 to 30 d. Ammonium was determined from leaf extracts of the tobacco transgenic lines as indicated. Results are in nanograms of NH_4^+ per microgram of protein ± SE, n = 3 individual plants.

changes in photorespiration were most likely accompanied by commensurate changes in photosynthesis.

DISCUSSION

Here, we report the positive effects on plant growth related to the ectopic overexpression of a pea gene for cytosolic GS1 in transgenic tobacco (CytGS1-TR lines). To begin to uncover the mechanisms by which the ectopic expression of cytosolic GS1 leads to improved growth, we examined the effects of light, nitrogen, and photorespiration in the GS transgenic plants. Analysis of GS levels in transgenic and control lines indicated a correlation between levels of GS activity and fresh weight, dry weight, and leaf soluble protein. The CytGS1-TR plants display increases in photorespiration, as judged by three measurements: (a) an increased pool size of photorespiratory metabolites, (b) an increased CO_2 burst, and (c) an accompanying decrease in the levels of free ammonium. These results suggest that cytosolic GS1 is not solely operating to suppress the negative effects of photorespiration (e.g. nitrogen drain). Instead, because photorespiratory rates are actually elevated in the CytGS1-TR lines, these results suggest that GS may be a critical enzyme linking photosynthesis with photorespiration (via ammonium assimilation). This role for GS is supported by previous studies on the overexpression of chloroplastic GS2 in tobacco (Kozaki and Takeba, 1996), where increased levels of chloroplastic GS2 in tobacco led to increased photorespiration and resistance to photooxidation. In our studies, overexpression of cytosolic GS1 isoenzyme in tobacco leaves in the CytGS1-TR lines led to increased photorespiratory rates as indicated by an increased CO_2 burst and by increased levels of photorespiratory intermediates. However, the CO_2 compensation point (71) of the CytGS1-TR is unchanged compared with control plants (data not shown). The CO_2 compensation point is the concentration of CO_2 at which the rate of CO_2 evolution from photorespiration equals the rate of CO_2 assimilated via photosynthesis at a given O_2 level (Tolbert, 1997). The fact that the CO_2 compensation point is unchanged in the CytGS1-TR lines suggests that the increase in photorespiration in CytGS1-TR plants is most likely accompanied by a concomitant increase in photosynthesis. This conclusion is supported by the recent findings of Fuentes et al. (2001). The Fuentes et al. (2001) study has demonstrated that tobacco plants overexpressing the alfalfa GS_1 gene under control of the 35S promoter display growth advantage when compared with the controls. The authors concluded that this was due to the ability of these transgenic plants to maintain normal photosynthetic rates even under nitrogen limiting conditions. The observations of these authors are complementary with our own observations that the growth phenotype of the CytGS1-TR plants is positively affected by light and is accompanied by changes in photorespiration.

We cannot rule out the possibility that the observed increase in growth and photosynthesis/photorespiration may be an indirect effect of the increase in leaf soluble protein observed in the CytGS1-TR plants. However, other genetic and biochemical evidence support a direct correlation between changes in GS expression, levels of photorespiration, and photosynthetic rates (Blackwell et al., 1987; Wallsgrove et al., 1987; Edwards and Coruzzi, 1989; Hausler et al., 1994a, 1994b). Photorespiratory mutants in GS2 display a decline in photorespiration and in photosynthetic carbon fixation (Blackwell et al., 1987; Wallsgrove et al., 1987; Edwards and Coruzzi, 1989; Hausler et al., 1994b). These photorespiratory GS mutants also show a 2- to 50-fold increase in ammonium accumulation (Wallsgrove et al., 1987; Hausler et al., 1994a). In the barley GS2 mutants, the failure to reassimilate photorespiratory ammonium into Gln also resulted in a 5-fold decrease in the Ser/Gly ratio (Hausler et al., 1994a) and a reduction of photorespiratory amino acids (Blackwell et al., 1987; Hausler et al., 1994a). The CytGS1-TR tobacco plants that ectopically overexpress cytosolic GS1 described herein, show the exact opposite phenotypes compared with the barley GS2 mutants, deficient in GS activity. The CytGS1-TR plants display enhanced photorespiration, an increase in the Ser/Gly ratio (3.5-fold), and a dramatic reduction in the levels of free ammonium.

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In addition, the levels of Glu (the product of photorespiratory ammonium assimilation) were also increased in CytGS1-TR plants when compared with controls (2-fold; not shown).

Our growth assays suggest that enhanced photorespiratory rates combined with increased reassimilation of photorespiratory ammonium in the CytGS1-TR plants (7-fold reduction) have beneficial effects on plant growth. Migge et al. (2000) have overexpressed a plastidic form of Gln synthetase (GS2) in leaves of tobacco, which led to a 3.7-fold reduction in the leaf ammonium pool with parallel effects on growth. The increased ammonium assimilation observed in our study (7-fold) may be due to the fact that the CytGS1-TR plants ectopically overexpress a cytosolic GS1 isoform in tobacco leaf mesophyll cells, where it is normally not expressed at high levels. The ectopic expression of cytosolic GS1 in leaf mesophyll cells may provide a complementary and/or alternative route to chloroplastic GS2 for the reassimilation of photorespiratory ammonium. Because nitrogen flux through the photorespiratory pathway is 10-fold higher than primary *N*-assimilation, the enhanced reassimilation of photorespiratory ammonium could lead to enhanced nitrogen use efficiency. Mechanistically, the improved growth phenotype observed in the CytGS1-TR plants may be a consequence of increased photosynthesis/photorespiration, combined with enhanced nitrogen efficiency. These findings for cytosolic GS1 seem to be generally applicable to other C3 plants, because preliminary results from our laboratory indicate that a similar improved growth phenotype also occurs in Arabidopsis plants overexpressing the pea cytosolic GS1 gene (not shown).

The overexpression of GS genes has been attempted before by several groups with mixed results (Eckes et al., 1989; Hemon et al., 1990; Hirel et al., 1992; Temple et al., 1993; Vincent et al., 1997; Ortega et al., 2001). For instance, Hirel and co-workers have observed accelerated growth rate in transgenic *L. corniculatus* plants that overexpress a soybean cytosolic GS isoenzyme. Those plants also displayed increases in some amino acids. However, that report does not indicate a correlation between plant dry/fresh weight and GS activity (Vincent et al., 1997). Previous studies also showed that overexpression of a gene for chloroplast GS2 from rice in transgenic tobacco led to increased levels of photorespiration and resistance to photooxidation, although no accompanying increase in growth or yield was reported (Kozaki and Takeba, 1996). It is unlikely that photoprotection plays a major role in the improved growth phenotype in the CytGS1-TR plants reported herein, because of the moderate PFD used in our experiments (200 μmol cm^{-2} s^{-1}). In more recent reports, overexpression of distinct GS isoenzymes has been associated with improvement of plant growth in two other species, including poplar, supporting the generality of this approach. However, in those studies, no studies were performed to gain insight into the mechanisms underlying such growth improvement (Gallardo et al., 1999; Migge et al., 2000). The recent study by Fuentes et al. (2001) has demonstrated that tobacco plants overexpressing the alfalfa GS_1 gene under control of the 35S promoter display growth advantage when compared with the controls, and they cite increases in photosynthetic rate as a possible mechanism. The improvement in plant growth for the CytGS1-TR plants reported herein most likely results from a combination of factors including: (a) ectopic overexpression of a cytosolic GS1 isoenzyme in leaf mesophyll cells of a species where it is normally expressed at low levels (e.g. Solanaceous species); (b) a threshold level of transgene expression; (c) a cytosolic GS1 isoenzyme that assembles into a native holoenzyme in the host plant system; and (d) an appropriate plant background (e.g. plants with low levels of native cytosolic GS1 in leaves or C_3 plants).

The CytGS1-TR plants described herein exhibit increases in biomass (dry weight) at all stages of growth tested, up to flowering (50–60 d old; not shown). This increase may reflect an accelerated growth rate (Vincent et al., 1997) and/or an increase in total biomass. Either trait could have important agronomic applications. The physiological parameters relevant to seed yield and seed-nitrogen content include not only the efficiency of nitrogen assimilation or reassimilation in vegetative tissues, but also the remobilization of nitrogen reserves at the onset of bolting and flowering. Whether the increases in dry weight and soluble protein observed in transgenic lines overexpressing cytosolic GS1 will also lead to a significant improvement in seed yield or seed quality is an important question that remains to be answered in future studies of these and other transgenic lines currently under investigation in our laboratory.

MATERIALS AND METHODS

Plasmids and Plant Transformation

The plant expression vector and the cDNAs corresponding to the pea (*Pisum sativum*) genes GS1, GS2, and GS3A have been described elsewhere (Tingey and Coruzzi, 1987; Tingey et al., 1988; Brears et al., 1993). Transfer of constructs to the *Agrobacterium tumefaciens* strain LBA4404 and tobacco (*Nicotiana tabacum* line SR1) transformation was as described (Bevan, 1984; Horsch et al., 1985; Brears et al., 1993). All experiments described below were performed with T_3 and T_4 generation transgenic plants.

Plant Growth Conditions. Growth Assays for Plants Germinated on Medium

Plants were germinated on Murashige and Skoog/kanamycin medium under a light irradiance of 90 μmol cm^{-2} s^{-1} generated by a mixture of fluorescent, incandescent, high-pressure sodium, and metal halide lights. After 14 to 18 d, kanamycin-resistant seedlings were transferred to white sand. The plants were further grown for 20 to 42 d (depending on the experiment) and subirrigated with 0.5× Hoagland (0.6 mM ammonium and 7 mM nitrate) in a 16-h-light/8-h-dark cycle. Fresh weight and dry weight determinations were from the whole plant. Dry weight was determined after incubation of the plant at 65°C for 72 h. Soluble protein was calculated

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by measuring the total soluble protein from approximately 1 g of leaf tissue (Bradford, 1976). All protein measurements were conducted either in fresh harvested tissue ground immediately after excision or from leaves quickly deep-frozen in liquid nitrogen and kept at −80°C until the assay. Material for all biochemical determinations (including protein measurement) was collected from plants in mid-light cycle. The transgenic lines used in these experiments have not been analyzed for transgene copy number or homozygosity. Therefore, to compensate for possible variations within individuals of each line, a large number of individuals were analyzed (19 individuals/line).

Growth Assays for Plants Germinated on Soil

Plants were germinated on soil under a light irradiance of 60 to 90 μmol cm^{-2} s^{-1} generated by a mixture of fluorescent and incandescent lights. The plants were grown for 28 d subirrigated with 0.5× Hoagland (0.6 mM ammonium and 7 mM nitrate) in a 16-h-light/8-h-dark cycle.

Light and Inorganic Nitrogen Dependence

Plants were germinated on Murashige and Skoog/kanamycin medium under a light irradiance of 60 μmol cm^{-2} s^{-1} provided by incandescent and hi-gro fluorescent lights. After 14 to 18 d, the kanamycin-resistant seedlings were transferred to white sand. Plants were subirrigated with ammonium-free/nitrate-free Murashige and Skoog liquid medium containing 0× nitrogen (no nitrogen supplementation), 0.1× nitrogen (4 mM nitrate/2 mM ammonium), or 1× nitrogen (40 mM nitrate/20 mM ammonium), further subdivided into two sets, incubated under moderate light (moderate PFD, 200 μmol cm^{-2} s^{-1}) or low light (low PFD, 50 μmol cm^{-2} s^{-1}), and further grown for 20 to 30 d in a 16-h-light/8-h-dark cycle.

Ammonium Determination

Plants were germinated in Murashige and Skoog/kanamycin medium, transferred to white sand, and subirrigated with 0.5× Hoagland as above. Thereafter, the plants were incubated under moderate light (moderate PFD, 200 μmol cm^{-2} s^{-1}) for 20 to 30 d.

Postillumination Photorespiratory CO_2 Evolution Experiments

Plants were germinated and grown as above except that in this case the plants were transferred to soil and subirrigated with 0.5× Hoagland. Thereafter plants were grown in a greenhouse and subirrigated with 0.5× Hoagland for 20 to 25 d.

Measurement of Photorespiration

The levels of photorespiration were estimated by postillumination photorespiratory CO_2 evolution (Decker, 1955; Peterson, 1983) using an infrared CO_2 gas analyzer (LI-COR, Lincoln, NE).

HPLC Analysis of Free Amino Acids

HPLC analysis was performed as previously described (Brears et al., 1993) with minor modifications. In brief, leaf samples were harvested and quickly frozen in liquid nitrogen until the moment of the assay. Thereafter, the leaf samples were frozen-ground and mixed in an ice-cold buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM imidazole, and 0.5% (w/v) β-mercaptoethanol quickly followed by extraction with 200 μL of methanol:chloroform (6:2.5, v/v). HPLC analysis of amino acid was performed using a supelcosil LC-18 reversed-phase analytical column (25-cm × 4.6-mm i.d., particle size 5 μm; Supelco Inc., Bellefonte, PA). The mobile phase consisted of a gradient of 26 mM phosphate buffer, pH 7.5 (buffer A), with increasing concentrations of 72% (v/v) methanol in water (buffer B). The column eluate was read by a LS30 luminescence spectrometer (PerkinElmer, South Plainfield, NJ) and recorded in a ChromJet integrator (ThermoSeparations, Bergenfield, NJ). The amino acid analog nor-Val was used as an internal standard.

Determination of Gln Synthetase Activity and Free Ammonium Levels

GS enzyme activity analysis was essentially as described (Shapiro and Stadtman, 1971). Ammonium was extracted by grinding liquid nitrogen-frozen plant tissue samples with a mortar in cold GS assay buffer (50 mM Tris-HCl, pH 8.0, 10 mM imidazole, and 0.5% [w/v] β-mercaptoethanol). Samples were kept on ice until assay that was performed immediately after grinding with a kit (Boehringer Mannheim, Mannheim, Germany) following instructions from the manufacturer. Material for all determinations was collected from plants in mid-light cycle.

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Arabidopsis Mutants Resistant to S(+)-β-Methyl-α, β-Diaminopropionic Acid, a Cycad-Derived Glutamate Receptor Agonist[1]

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Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that are the predominant neuroreceptors in the mammalian brain. Genes with high sequence similarity to animal iGluRs have been identified in Arabidopsis. To understand the role of Arabidopsis glutamate receptor-like (AtGLR) genes in plants, we have taken a pharmacological approach by examining the effects of BMAA [S(+)-β-methyl-α, β-diaminopropionic acid], a cycad-derived iGluR agonist, on Arabidopsis morphogenesis. When applied to Arabidopsis seedlings, BMAA caused a 2- to 3-fold increase in hypocotyl elongation and inhibited cotyledon opening during early seedling development. The effect of BMAA on hypocotyl elongation is light specific. Furthermore, BMAA effects on early morphogenesis of Arabidopsis can be reversed by the simultaneous application of glutamate, the native iGluR agonist in animals. To determine the targets of BMAA action in Arabidopsis, a genetic screen was devised to isolate Arabidopsis mutants with a BMAA insensitive morphology (*bim*). When grown in the light on BMAA, *bim* mutants exhibited short hypocotyls compared with wild type. *bim* mutants were grouped into three classes based on their morphology when grown in the dark in the absence of BMAA. Class-I *bim* mutants have a normal, etiolated morphology, similar to wild-type plants. Class-II *bim* mutants have shorter hypocotyls and closed cotyledons when grown in the dark. Class-III *bim* mutants have short hypocotyls and open cotyledons when grown in the dark, resembling the previously characterized constitutively photomorphogenic mutants (*cop, det, fus,* and *shy*). Further analysis of the *bim* mutants should help define whether plant-derived iGluR agonists target glutamate receptor signaling pathways in plants.

Glu is the predominant neurotransmitter in the brain. As a neurotransmitter, it activates Glu receptors at the post-synaptic membrane, which are involved in sensing environmental cues and in memory function (Nowak et al., 1984; Isquierdo and Medina, 1995; Tsien et al., 1996). Improper ionotropic Glu receptor (iGluR) function has been implicated in a variety of human diseases including Alzheimers and Parkinsons dementia (Ikonomidou and Turski, 1996; Forsythe and Barnes-Davies, 1997). One subgroup of Glu receptors is comprised of the iGluRs, which function as Glu-gated ion channels that convey rapid synaptic transmission. iGluRs are pharmacologically classified into subgroups based on agonist response. The two main iGluR subfamilies in animals are *N*-methyl-D-Asp (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate (KA) (non-NMDA) activated iGluRs (Ikonomidou and Turski, 1996; Forsythe and Barnes-Davies, 1997).

In plants, it appears that Glu may also act as a signaling molecule. Glu supplied to plant growth media has been shown to alter the expression of genes encoding enzymes involved in amino acid metabolism (Lam et al., 1994, 1998b; Oliveira and Coruzzi, 1999). Despite the evidence that amino acids may act as signals in higher plants, the mechanism of amino acid sensing and signaling is poorly understood. Genes for putative amino acid sensors have been uncovered in Arabidopsis that have high sequence similarity to ionotropic Glu receptors of animals (Lam et al., 1998a; Chiu et al., 1999). Arabidopsis GLRs have all the signature features of animal iGLRs, including a plasma membrane signaling peptide, two putative ligand-binding domains, and a "three-plus-one" transmembrane region (Lam et al., 1998a; Chiu et al., 1999).

To assess the function of putative Glu receptor genes in plants, Arabidopsis seedlings were treated with the iGluR antagonist 6,7 dinotropuinoxaline 2,3(1H, 4H) dione (DNQX), known to block AMPA/KA iGLRs in animals (Muller et al., 1989). It was shown that DNQX inhibits two key aspects of seedling photomorphogenesis in Arabidopsis: light-induced hypocotyl shortening and light-induced greening (Lam et al., 1998a). To further explore the targets of AtGLR function in plants, we tested whether other compounds known to block iGluR function in animals could also block aspects of Ara-

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bidopsis growth and development. Several of these iGluR agonists are plant-derived products: kainate (KA; Monaghan et al., 1989; Bettler and Mulle, 1995) made by seaweed (*Digenea simplex*); β-*N*-oxalylamino-L-alanine (Ross et al., 1989) made by chickpeas (*Lathyrus* sp.), and BMAA (Copani et al., 1991) made by cycads. BMAA [S(+)-β-methyl-α, β-diaminopropionic acid] has been detected in members of the family Cycadaceae or "cycads" (Pan et al., 1997). Cycads are believed to be the most primitive of gymnosperms, whose remnant surviving members descended from the Mesozoic and the Paleozoic when cycads predominated the vegetation (Chamberlain, 1919). BMAA was first isolated as the suspected cause of Parkinsonians dementia complex and amyotrophic lateral sclerosis in Guam's Chamorro human population, where consumption of *Cycas circinalis* L., a local food source, was prevalent (Whiting, 1963; Spencer et al., 1987). Subsequent to its detection and purification, BMAA has been shown to cause neural degeneration in primates when supplemented in their food (Spencer et al., 1987). Because BMAA is a natural plant product that blocks iGluR function in animals, we decided to test whether it would have any effect on plant GLRs using Arabidopsis as a model. In this study, we show that BMAA promotes hypocotyl elongation and inhibits cotyledon opening when applied to light-grown Arabidopsis seedlings. To identify the targets of BMAA action in plants, we used BMAA as a pharmacological tool to screen for Arabidopsis mutants resistant to BMAA-induced changes in photomorphogenesis. The isolation and preliminary characterization of these mutants is described below.

RESULTS

BMAA, a Cycad-Derived Glu Receptor Agonist, Causes a Long Hypocotyl Phenotype in Arabidopsis

To probe the putative function of AtGLR genes in plants, we sought to determine whether the iGluR agonist, BMAA, caused any observable phenotypic effects on plant growth when supplied to Arabidopsis seedlings. Arabidopsis seedlings were germinated and cultivated on Murashige and Skoog media in the presence or absence of BMAA. BMAA-treated seedlings were evaluated for phenotypic alterations in treated plants compared with untreated control plants. At 8 d post-germination, the hypocotyls of seedlings grown in the light on Murashige and Skoog media containing 50 μM BMAA, displayed elongated hypocotyls (Fig. 1A), compared with control untreated plants (Fig. 1B). The effect of BMAA on hypocotyl elongation was quantified. A dose-dependent response was observed at increasing concentrations of BMAA (Fig. 2A). In light-grown plants, a concentration of 20 μM BMAA caused an increase in length of approximately 30%, and 50 μM BMAA caused approximately a 100% increase in hypocotyl length when compared with untreated plants (Fig. 2A). The effect on hypocotyl elongation is weaker at 100 μM BMAA, and at greater concentrations (200 μM) BMAA becomes inhibitory to growth (data not shown). In contrast, BMAA does not induce hypocotyl elongation in dark-grown plants (Fig. 2B). Instead, BMAA has an increasingly negative effect on hypocotyl length in dark-grown plants at concentrations of 50 μM or greater (Fig. 2B). BMAA was also inhibitory to root growth (Fig. 1A). BMAA also inhibits cotyledon opening in the light (Fig. 1A). The arc of cotyledon opening is reduced to 50° in BMAA-treated plants (Fig. 1A) when compared with 120° in control plants (Fig. 1B).

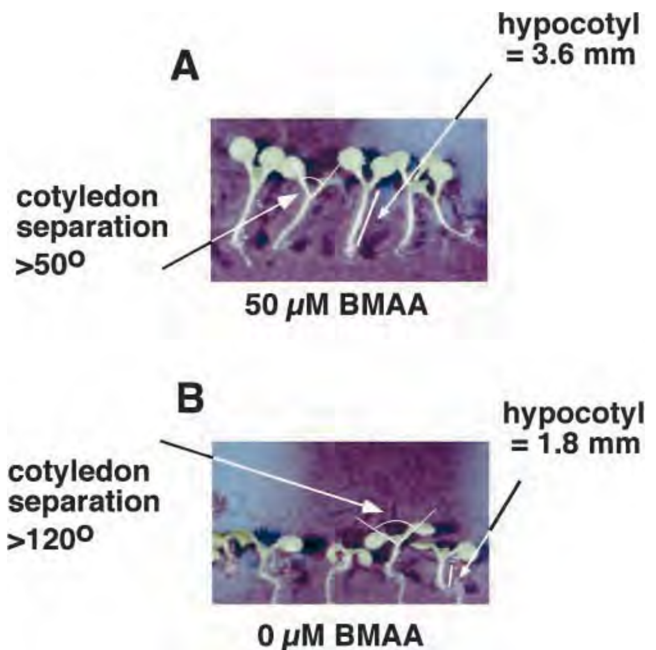

Figure 1. BMAA causes hypocotyl elongation and inhibits cotyledon unfolding in light-grown Arabidopsis. Arabidopsis plants were germinated and cultivated on Murashige and Skoog media and 0.7% (w/v) agar containing 50 μM BMAA (A) or no BMAA (B) for 8 d in the light. The average hypocotyl length for each treatment, as well as the arc-angle of opening for the cotyledons, is shown (n = 30).

To determine whether the effects of BMAA (a Glu analog) could be reversed by Glu (the native agonist of iGluRs in animals), plants treated with 25 μM BMAA were grown on media containing increasing amounts of L-Glu (Fig. 3). The BMAA-induced effects on hypocotyl elongation and inhibition of cotyledon opening can be partially reversed by the simultaneous addition of L-Glu to the growth media (Fig. 3, A and B). The L-Glu reversal of the BMAA effects occurs in a dose-dependent manner. BMAA-induced hypocotyl elongation is reversed by approximately 50% with 1 mM L-Glu and by approximately 100% with 10 mM L-Glu (Fig. 3A). BMAA-induced inhibition of cotyledon opening is reversed by 20% with 1

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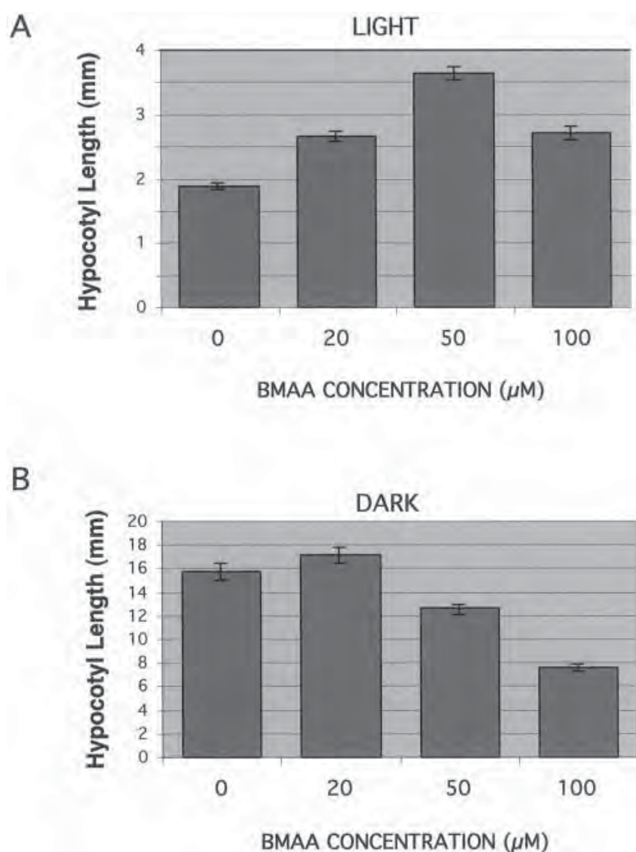

Figure 2. Quantification of BMAA-induced hypocotyl elongation in light-grown and etiolated plants. Arabidopsis plants were grown on increasing concentrations of BMAA (0–100 μM), in the light (A) or in continuous dark (B) for 5 d. The average hypocotyl length for each treatment is shown. Error bars show the SE of the mean (n = 30).

mM L-Glu, and by nearly 50% with 10 mM L-Glu (Fig. 3B). To determine whether the reversal of BMAA effects on morphogenesis was specific to the amino acid L-Glu, we tested whether two other amino acids could similarly counteract the effects of BMAA. L-Asp could not reverse the effects of BMAA, however L-Gln could also reverse the effects of BMAA (data not shown).

Selection of Arabidopsis Mutants Resistant to the Effects of BMAA on Morphogenesis

To determine the targets and mode of action of BMAA in Arabidopsis, a screen was devised to isolate mutants insensitive to the BMAA-induced effects on seedling morphology. For this screen, mutagenized (M2) Arabidopsis seeds were plated and grown in the light on Murashige and Skoog media containing 50 μM BMAA (Fig. 4). On this BMAA-containing media, wild-type plants exhibit elongated hypocotyls and partially closed cotyledons. M2 plants that exhibited short hypocotyls and open cotyledons when grown on BMAA were identified as "BMAA insensitive morphology" (*bim*) mutants (Fig. 4A). A total of 18,000 ethyl methanesulfonate (EMS) M2 seedlings were screened in the light on 50 μM BMAA, and 10 *bim* mutants were isolated (*bim 18*, *26*, *40*, *50*, *59*, *77*, *131*, *136*, *167*, and *175*). A representative *bim* mutant seedling, *bim 26*, identified in the M2 screen is shown in Figure 4B.

M3 progeny from M2 *bim* plants were tested for genetic inheritance of resistance to the effects of BMAA. M3 progeny of two *bim* mutants are shown in Figure 5. When treated with 50 μM BMAA and grown in the light, wild-type plants have elongated hypocotyls (Fig. 5A). In contrast, two representative mutants, *bim 131* and *bim 26*, each have visibly shorter hypocotyls when grown in the light on 50 μM BMAA (Fig. 5A). When grown in the light minus BMAA, *bim 131* and *bim 26* are indistinguishable from wild type as young seedlings (Fig. 5B). BMAA treatment impairs root growth in wild-type plants, and in the majority of *bim* mutants. However, one mutant, *bim 50*, showed partial resistance to BMAA-mediated inhibition of root growth when compared with wild type (data not shown).

The effect of BMAA on hypocotyl length of light-grown plants was quantified for all *bim* mutants and wild-type plants (Fig. 6A) and was compared with untreated plants (Fig. 6B). When grown in the light plus 50 μM BMAA, wild type has a 2- to 3-fold increase in hypocotyl length, compared with *bim* mutants (Fig. 6A). In contrast, when grown in the light minus BMAA, the majority of *bim* mutants are indistinguishable from wild type with regard to hypocotyl length (Fig. 6B). Only *bim 131* and *bim 167* have obviously shorter hypocotyls than wild type, when grown in the absence of BMAA (Fig. 6B).

A Subset of *bim* Mutants Exhibit Constitutive Photomorphogenesis in the Dark

The *bim* mutants have been grouped into three classes based on their dark morphology when grown in the absence of BMAA (Fig. 7). Hypocotyl length of the etiolated plants is quantified in Figure 8. Class I *bim* mutants have a normal etiolated morphology (elongated hypocotyls and closed cotyledons) when cultivated in the dark in the absence of BMAA (Fig. 7A). This is shown for a representative *bim* mutant (*bim 131*) (Figs. 7A and 8A). Class-II and class-III *bim* mutants each have short hypocotyls when grown in the dark minus BMAA (Figs. 7, B and C, and 8A). Class-II *bim* mutants (*bim 18*, *40*, *77*, *136*, *59*, *167*) have short hypocotyls in the dark, but their cotyledons remain closed (Fig. 7B). Class-III *bim* mutants (*bim 26* and *50*) have short hypocotyls but also display open cotyledons in the dark (Figs. 7C and 8A), similar to the *cop/det/fus* mutants. The effects of BMAA on a representative *cop* mutant (*cop1-6*), is shown in Figure 6 and 8. BMAA has no effect on hypocotyl elongation of the *cop 6-1* mutation in the light (Fig. 6B versus 6A) or in the dark (Fig. 8B versus 8A). By contrast, BMAA

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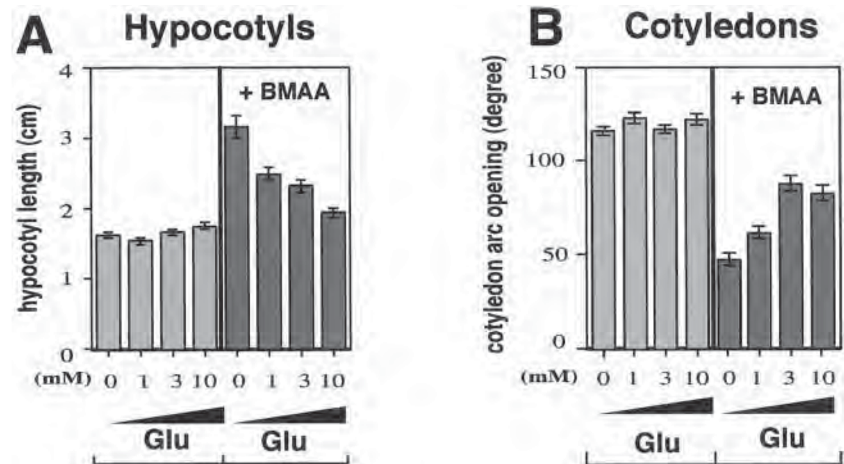

Figure 3. BMAA-induced hypocotyl elongation, as well as inhibition of cotyledon opening is reversed in a dose-dependent manner by Glu. Arabidospsis seedlings were cultivated in the light on Murashige and Skoog media containing 1, 3, or 10 mM Glu in the absence (left, A and B) or presence (right, A and B) of 25 μM BMAA. A, The average hypocotyl length for each treatment. B, The arc of cotyledon opening. Error bars show the SE of the mean (n = 30)

causes slight elongation of *bim* mutants grown in the light (Fig. 6A). Thus, it appears that the mutation conferring BMAA-resistance in the *bim* mutants affects aspects of skotomorphogenesis.

DISCUSSION

We have shown that BMAA, a plant-derived agonist that blocks Glu receptor function in animals, appears to alter early morphogenesis of light-grown Arabidopsis seedlings. BMAA promotes hypocotyl elongation and inhibits cotyledon opening in the light. As such, BMAA-induced effects on Arabidopsis seedlings phenocopy the long hypocotyl or "*hy*" mutants, defective in perceiving light and/or transmitting light signals in Arabidopsis (von Arnim and Deng, 1996; Fankhauser and Chory, 1997).

We reported previously that DNQX, an antagonist of AMPA/KA receptors in animals, also causes a "*hy*"-like phenotype when supplemented in the culture media of Arabidopsis seedlings (Lam et al., 1998a). The fact that two different iGluR interacting compounds (DNQX and BMAA) each induce hypocotyl elongation in light-grown seedlings provides support for the hypothesis that endogenous AtGLR genes in plants may be involved in photomorphogenic development in Arabidopsis. DNQX (an iGluR antagonist) could potentially antagonize Arabidopsis GLRs, which may be involved in light-mediated inhibition of hypocotyl growth. BMAA (an iGluR agonist) might inhibit AtGLR function but via a different mechanism. In animal systems, non-native agonists such as BMAA, can impair iGluR function because often iGluRs remain sensitized to these non-native

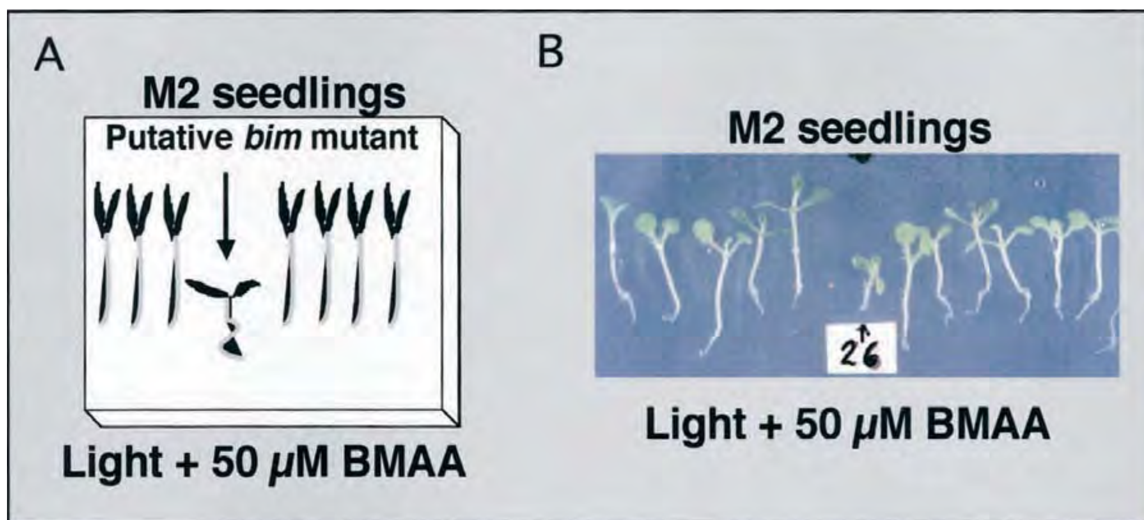

Figure 4. A genetic screen to isolate BMAA insensitive morphology (*bim*) mutants. A, Strategy to isolate Arabidopsis mutants resistant to the effects of BMAA is shown. Individual EMS mutagenized M2 seedlings are grown for up to 2 weeks on Murashige and Skoog media containing 50 μM BMAA in the light. M2 individuals with short hypocotyls, compared with neighboring plants, are recovered from the BMAA-containing media and allowed to produce seed for analysis in the M3 generation. B, Representative M2 *bim* plant (*bim26*) is shown as detected in the primary screen.

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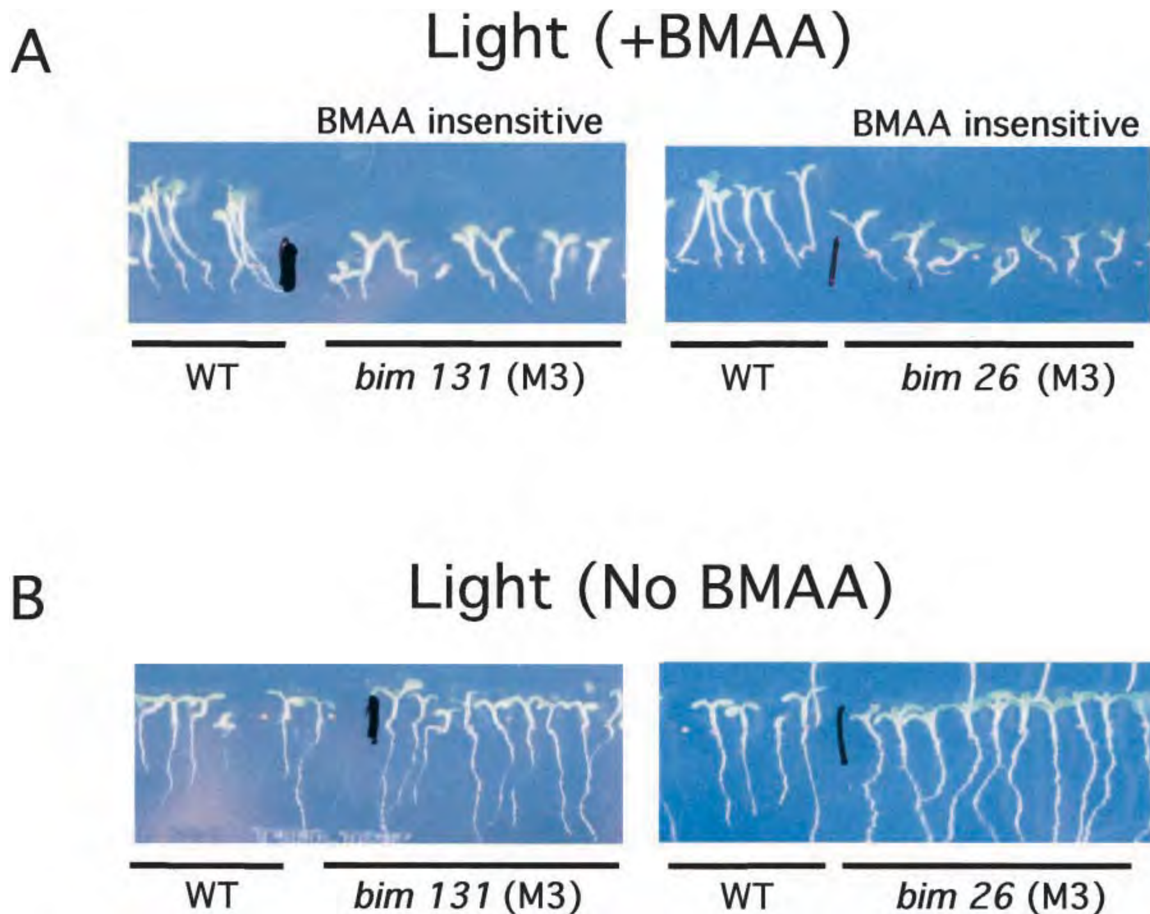

Figure 5. *bim* mutants are insensitive to BMAA-induced hypocotyl elongation. Two representative M3 *bim* mutants (*bim 131* and *bim 26)* were cultivated for 5 d in the light on Murashige and Skoog media in the presence of 50 μM BMAA (A) or in the absence of BMAA (B). A, Both *bim 131* and *bim 26* have a short hypocotyl phenotype (right) when compared with wild type (left), which has an elongated hypocotyl in the presence of BMAA. B, Plants grown in the absence of BMAA where *bim 131* and *bim 26* appear similar to wild type at the early seedling stage.

ligands (Ross et al., 1989). In contrast, animal iGluRs become desensitized to the native agonist, Glu. Desensitization is necessary for ion channel closure and proper iGluR function (Geoffroy et al., 1991; Sprengel and Seeburg, 1995). The fact that the BMAA-induced effects on Arabidopsis morphogenesis are reversed by the addition of Glu (the native iGluR ligand), is consistent with the hypothesis that BMAA may act by blocking plant AtGLR signaling in Arabidopsis. In this scenario, increasing levels of Glu would compete with BMAA at the ligand-binding site and restore normal AtGLR desensitization and function. Alternatively, BMAA could act as an agonist to activate and open iGluR channels in plants, potentially regulating ion flow necessary for hypocotyl elongation. Hypocotyl expansion is largely due to increases in cell size, since most cells in the hypocotyl are formed during embryogenesis (Gendreau et al., 1997). Previous work has already detected the activation of chloride channels during hypocotyl elongation (Cho and Spalding, 1996). In this scenario, we hypothesize that BMAA-induced hypocotyl elongation may be caused by activation of Arabidopsis GLRs important for cell expansion during hypocotyl elongation.

Among other amino acids tested, we have found that Gln could also reverse the effects of BMAA on Arabidopsis growth, whereas Asp could not. Thus, Gln may also act similar to Glu as a potential agonist at a BMAA responsive site. Gln and Glu both trigger ion transport of Glu receptors from the cyanobacteria, *Synechocystis* (Chen et al., 1999), when expressed in a heterologous system. Glu receptors from cyanobacteria show the strongest sequence similarity to Arabidopsis Glu receptors. Another possibility is that exogenously supplied Gln is assimilated and metabolized to Glu, which is then able to reverse the effects of BMAA on Arabidopsis. In fact, HPLC analysis has shown that exogenously added Gln leads to significantly higher levels of endogenous Glu in Arabidopsis (Oliveira and Coruzzi, 1999).

To test these different hypotheses and to determine the targets of BMAA action in plants, we have undertaken a mutant screen in Arabidopsis using BMAA as a pharmacological tool. This molecular-

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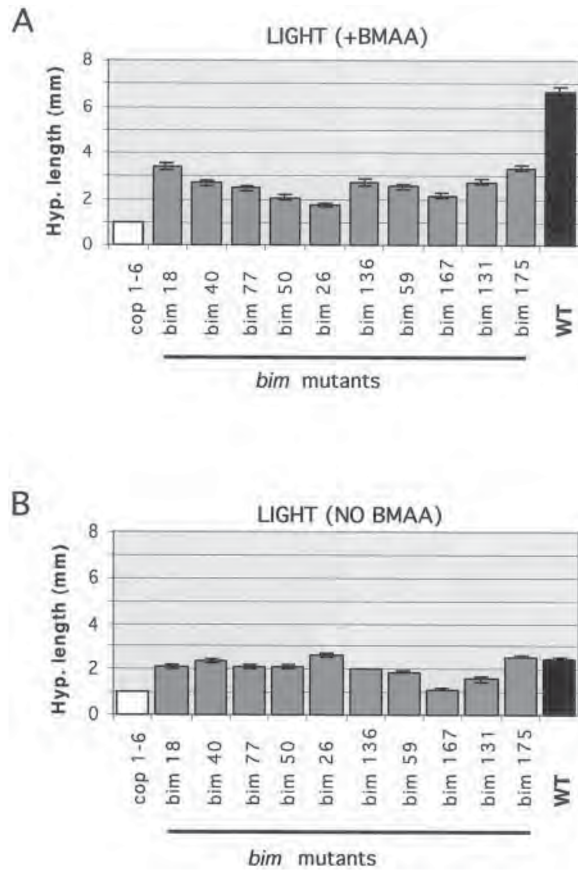

Figure 6. Quantification of hypocotyl lengths of *bim* mutants grown in the light in the presence or absence of BMAA. *bim 18*, *40*, *77*, *50*, *26*, *136*, *59*, *131*, *167*, and *175* were grown for 5 d in the light in the presence of 50 μM BMAA (A) or with no BMAA (B). Hypocotyl lengths of *bim* seedlings and wild type were measured and quantified. Wild type is indicated with a black bar on the far right of each graph. *cop1-6* mutant is indicated with a white bar on the far left of each graph. The average hypocotyl length for each treatment is shown (n = 30). Error bars show the SE of the mean.

genetic approach should enable us to understand how BMAA might induce hypocotyl elongation and block cotyledon separation in light-grown Arabidopsis. We have isolated Arabidopsis mutants insensitive to the effects of BMAA on early morphogenesis in the light. BMAA insensitive morphology (*bim*) mutants have short hypocotyls when grown in the light in the presence of BMAA (Figs. 4–6). In contrast, wild-type plants display elongated hypocotyls under these conditions. The *bim* mutants were further separated into three classes based on their morphology in the dark (Fig. 7). The first class of *bim* mutants has a normal etiolated morphology in the dark. We have identified two *bim* mutants in this class (*bim 131* and *bim 175)*. The second class of *bim* mutant (*bim 18*, *40*, *59*, *77*, *136*, *167*), has short hypocotyls in the dark and closed cotyledons. This phenotype is similar to the *proscute* (Desnos et al., 1996) and *korrigan* (Nicol et al., 1998) mutations, which affect cell wall formation during development. This phenotype is also similar to a number of hormone mutants deficient in growth including *gai* (Gendreau et al., 1999) and *ctr1* (Kieber et al., 1993). The third class of *bim* mutants (*bim 26* and *50)* has short hypocotyls and open cotyledons. These two characteristics are analogous to the photomorphogenic mutants *cop* (Hou et al., 1993), *det* (Chory et al., 1989, 1991b), *fus* (Miséra et al., 1994; Kwok et al., 1996), and *shy* (Reed et al., 1994; Tian and Reed, 1999) mutants, which share these phenotypes. We tested the effects of BMAA on one constitutively

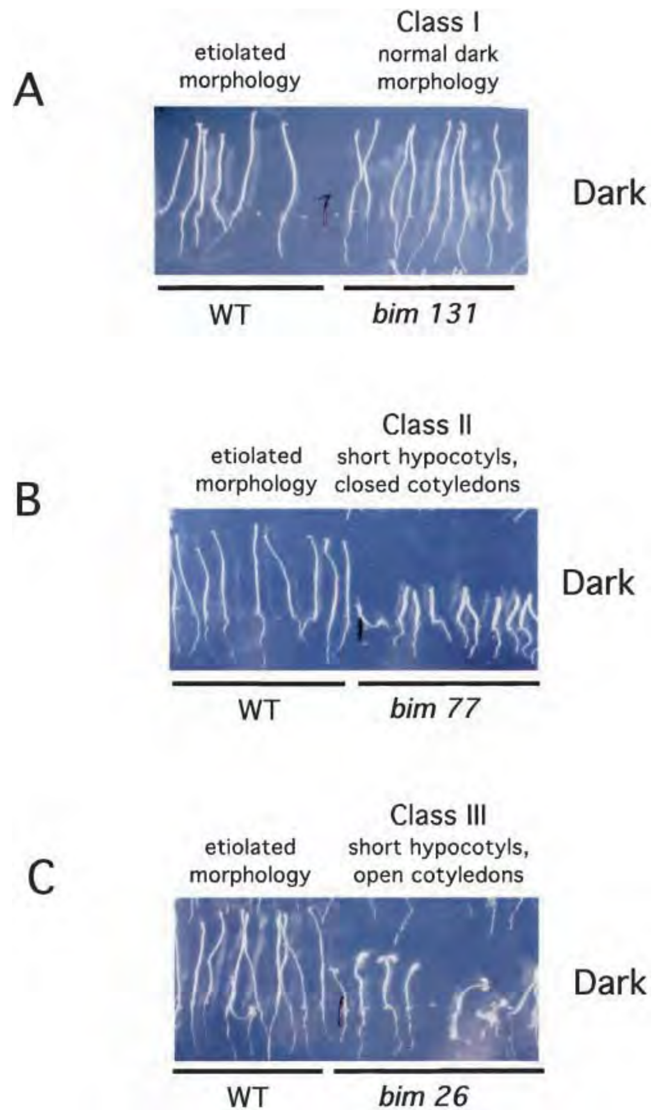

Figure 7. *bim* mutants are subgrouped into three separate classes based on their dark-grown morphology. *bim* mutants were cultivated on Murashige and Skoog media in the absence of BMAA and grown in the dark for 5 d. Class-I *bim* mutants exhibit a normal etiolated phenotype (Fig. 7A, right) when grown in the dark compared with wild type (Fig. 7A, left). Class-II *bim* mutants have a short hypocotyl and closed cotyledons (Fig. 7B, right) when compared with wild type (Fig. 7B, left). Class-III *bim* mutants have a short hypocotyl and open cotyledons (Fig. 7C, left) when compared with wild type (Fig. 7C, right).

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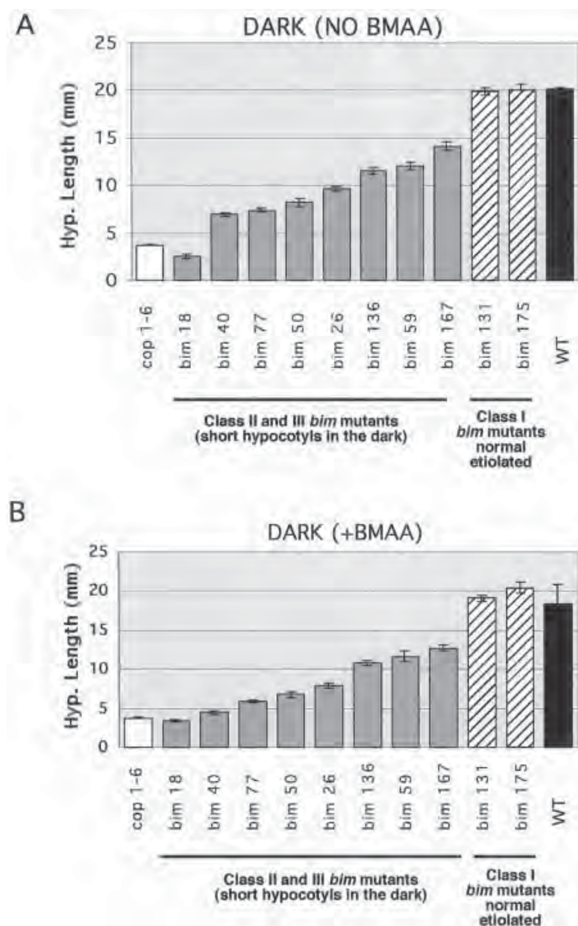

Figure 8. Quantification of hypocotyl lengths of *bim* mutants grown in the dark in the absence or presence of BMAA. *bim 18*, *40*, *77*, *50*, *26*, *136*, *59*, *131*, *167*, and *175* and wild type were grown for 5 d in the dark in the absence (Fig. 8A) or presence of 50 μM BMAA (Fig. 8B). Hypocotyl lengths were quantified. Wild type is indicated with black bars. Class-I *bim* mutants (*bim 131* and *bim 175*) have wild-type length hypocotyls and are indicated with hatched bars. Class-II and -III *bim* mutants, which have short hypocotyls in the dark, are marked with gray shaded bars. *cop1-6* is shown in the far left side of the graph (white bar). The average hypocotyl length for each treatment is shown (n = 30). Error bars show the SE of the mean.

photomorphogenic mutant, *cop1-6* (Kendrick and Nagatani, 1991; Deng and Quail, 1992) (Figs. 6 and 8). BMAA does not induce elongation of the *cop1-6* hypocotyl. However, interpretation of these results must await molecular analysis of lesions in the *bim* mutants. It is important to test whether class II and III *bim* mutants are allelic to these previously characterized *cop*, *det*, *fus*, and *shy* mutants or whether they represent new loci. It is also important to test for allelism between the different *bim* mutants and to map the *bim* mutants to determine whether they are genetically linked to any AtGLR genes in Arabidopsis.

An important aspect of development in seedlings involves the complicated interplay of light and various phytohormones. Auxin (Jensen et al., 1998; Kim et al., 1998), gibberellin (Jacobsen and Olszewski, 1993; Steber et al., 1998), and brassinolide (Fujioka et al., 1997; Azpiroz et al., 1998) all act as positive regulators of hypocotyl elongation in Arabidopsis. Ethylene (Kieber et al., 1993) and cytokinins (Chory et al., 1991a), conversely, are believed to act as inhibitors of hypocotyl elongation. Thus, the BMAA-mediated effects on hypocotyl length in light-grown plants may also involve the interaction of one or more of these phytohormones. It is also possible that BMAA blocks transduction of light signals, which inhibit hypocotyl elongation. Blue light (Liscum and Hangarter, 1991; Ahmad and Cashmore, 1993; Lasceve, et al., 1999), red light (Somers et al., 1991; Nagatani et al., 1993), and far-red light (Dehesh et al., 1993) are the major, incident wavelengths perceived by plants that repress hypocotyl elongation in Arabidopsis. In future studies, it will be important to determine whether BMAA effects are specific to one or more of these wavelengths in Arabidopsis.

One interesting result from our studies is that BMAA induces hypocotyl elongation in Arabidopsis specifically in the light at low concentrations (20–50 μM BMAA) (Fig. 2A). Because BMAA exerts its effects at low (μM) concentrations, this suggests that BMAA could act as a signaling compound in plants. In species of the Cycadaceae, BMAA is detected at high levels (milligram BMAA/gram of tissue) (Vega and Bell, 1967; Duncan et al., 1989; Pan et al., 1997). The presence of high levels of BMAA in such tissues has led some researchers to suggest that BMAA may act as a toxin against predators (Ladd et al., 1993). This theory of herbivore deterrence may explain why neurotoxins, such as BMAA are synthesized at high levels in plants. Our phylogenetic studies on GLR genes in plants and animals suggests that iGluRs are derived from a primitive signaling mechanism that existed before plants and animals diverged (Chen et al., 1999; Chiu et al., 1999). Those studies, plus the ones described herein, suggest the intriguing possibility that iGluR agonists made by plants may serve not only as herbivore deterrents, but may also act as signaling molecules affecting developmental processes in plants. We postulate, for example that BMAA, which appears to affect photomorphogenesis in Arabidopsis, may also alter light signaling in cycads. Whether BMAA plays a signaling role in cycads, or is even present at low levels in other species of higher plants are open questions that remain to be answered. Using the Arabidopsis *bim* mutants to understand the mode of action of a cycad-derived iGluR agonist in plants may help to address these questions. Furthermore, using *bim* mutants to understand how BMAA mediates its effects in Arabidopsis could potentially lead to new therapeutic treatments of iGluR-related neurological disorders in humans.

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MATERIALS AND METHODS

Culture of Arabidopsis Plants

Arabidopsis ecotype Columbia seeds were plated on Murashige and Skoog media (Murashige and Skoog, 1962), 0.1% (w/v) MES [2-(*N*-morpholino)ethanesulfonic acid] (Sigma M-2933) containing 0.5% (w/v) Suc and 0.7% (w/v) agar. Arabidopsis seeds were placed for 2 d at 4°C on the growth media. Plants were grown in square (100 × 15 mm) plates in a vertical position. Light grown plants were grown at 22°C during a cycle of 16-h light/8-h dark under cool-white fluorescent bulbs (General Electric, Fairfield, CT). Plants received a fluence level ranging from 40 to 60 μE. For dark grown seedlings, plants were incubated for an initial 4 h in the light to stimulate germination. After the light pretreatment, dark-grown plants were wrapped in two layers of foil and grown in the dark for 5 d at 22°C.

BMAA (L-BMAA hydrochloride) was purchased from RBI. L-Glu (Sigma G-1501), L-Asp (Sigma A-6558), and L-Gln (Sigma G-3126) stocks were dissolved in water and the pH was adjusted to 5.7.

cop 6-1 seeds used as a control were a gift from Dr. Kameda, Hokkaido University (Hokkaido, Japan).

Measurement of Hypocotyl Length and Cotyledon Opening

Plants were grown in the light for 5 to 8 d. Hypocotyl length was measured under the view of a dissecting scope. The top of the hypocotyl was defined as the point where the petioles of the cotyledons are attached to the axis. The bottom of the hypocotyl was determined as the root-shoot junction (with the root being defined as the point where root hairs are initially apparent). Cotyledon separation was measured by projecting the slide image of the seedlings onto a screen. Two lines were then drawn along the petioles over the image of the cotyledons and the angle was measured where these two lines intersected (at the shoot apex of the seedling).

Screen for *bim* Mutants in EMS Mutagenized M2 Lines

EMS mutagenized Columbia Co-3, glabrous seedlings (Lehle Seeds, Round Rock, TX) were cultivated as described above on Murashige and Skoog media (with 0.1% [w/v] MES, pH 5.7; 0.5% [w/v] Suc) plates containing 50 μM BMAA. Mutants with a *BMAA insensitive morphology* (*bim*) were screened after 8 to 12 d of growth, at which time they were transferred to Murashige and Skoog media lacking BMAA. After 1 to 2 weeks, the plants were transplanted to soil and allowed to set seed.

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