Is GABA Involved in Regulating Plant Growth and Development ?

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Abstract

Rapid and large accumulation of GABA (γ-aminobutyric acid) in response to a number of plant stresses has been well documented. But the role(s) of GABA in plants is not well defined. In recent years, the possibility of GABA involvement in regulating plant growth and development has been raised. In the present study, this possibility was examined.

First, to rapidly and accurately determine GABA levels in plant tissues, a spectrometric method for GABA determination was developed based on a commercially available enzyme Gabase. Seventy mM $LaCl_3$ almost completely removed water-soluble pigments from plant tissues which greatly interfere with the absorbance reading at 340nm. Inactivation of GAD (glutamate decarboxylase) by immediately adding methanol to a frozen plant tissue powder was suggested to prevent GABA production during extraction. The recovery of GABA with this method was approximately 100%.

Second, the relationship between GABA levels and hypocotyl elongation in soybean seedlings was analyzed using different approaches to regulate *in vivo* GABA levels and the elongation of hypocotyls. The following major observations were made.

(1) Mechanical stimulation by stroking elevated GABA levels and concurrently induced a rapid and significant reduction in hypocotyl elongation.

(2) External GABA was demonstrated to penetrate into the hypocotyls using ^{14}C-GABA. Application of external GABA elevated *in vivo* GABA levels, but failed to inhibit hypocotyl elongation.

(3) $LaCl_3$ and blue light irradiation caused an inhibition in the elongation of dark-grown hypocotyls, whereas GABA levels were not significantly affected.

(4) Ca^{2+} was suggested to be involved in the signal transduction pathway leading from mechanical stimulation to GABA production, as indicated by the ability of La^{3+} to inhibit GABA production in stimulated hypocotyls.

(5) Bicuculline, saclofen and baclofen (agonists and antagonists of GABA receptors in animals) had no effect on hypocotyl elongation. It might indicate that GABA-binding components which are structurally similar to animal GABA receptors and functionally capable of regulating plant growth may not exist in plants.

Therefore, the conclusion was drawn that GABA alone is not sufficient to inhibit hypocotyl elongation.

Third, chloride influx in isolated *Asparagus* cells was enhanced by 10mM GABA during a 3 hour incubation, but the effect was not specific for GABA. Chloride efflux was not influenced by GABA. Both influx and efflux of chloride were significantly inhibited by NPPB, a chloride channel blocker. These results suggest that GABA does not influence the activity of plant chloride channels.

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Literature Review

Introduction

GABA is a non-protein amino acid with a molecular weight of 103.1 daltons. It appears ubiquitously in living organisms (Bown and Shelp, 1989; 1997). In animals, GABA was discovered in mammalian brains in 1950 by several groups and first demonstrated as a major inhibitory neurotransmitter in the central nervous systems (CNS) in 1956 by Florey's research group at McGill University (Bazemore *et al.*, 1956). It was also found in invertebrates *i.e.* lobster (Kravitz *et al.*, 1965). GABA also occurs in nonneuronal tissues (Okada, *et al.*, 1976). In plants, the first discovery of GABA was documented in 1949 in potato tubers (Steward *et al.*, 1949). Since then, the presence and accumulation of GABA in response to a variety of environmental stresses in a wide range of plants have been well documented. The resting levels of GABA are around 50 nmol per gram fresh weight. With a variety of stresses, there is a large increase of GABA in a period of minutes. For example, in soybean leaves stressed with cold shock (decreasing the temperature from 23°C to 6°C), GABA levels increased from 0.1 to 1.77 μmol/g fresh weight within 5 minutes (Wallace *et al.*, 1984). These stresses include cold shock (Wallace *et al.*, 1984; Cholewa *et al.*, 1997), heat shock (Mayer *et al.*, 1990), darkness (Wallace *et al.*, 1984), mechanical stimulation (Lane and Stiller, 1970; Wallace *et al.*, 1984), mechanical damage (Ramputh and Bown, 1996), anaerobic conditions (Tsushida and Murai, 1987; Reggiani *et al.*, 1988; Aurisano *et al.*, 1995), acidosis (Crawford *et al.*, 1994), water stress (Rhodes *et al.*, 1986; Bolarin *et al.*, 1995), NH_4^+ stress (Kishinami and Ojima, 1980), senescence (Lahdesmaki, 1968; Vandewalle and Olssen,1983), stimulation with phytohormone such as auxin and ABA (Kishinami 1988; Reggiani *et al.*, 1993; Ford *et al.*, 1996), and viral attack (Cooper and Selman, 1974). Recently the mechanisms of rapid GABA production have been elucidated (Bown and Shelp, 1997). However, the role or roles of GABA in plants have not yet been clearly defined though some have been

suggested (Bown and Shelp, 1997). The objective of this study was to investigate the possible role of GABA in plant development. Before presenting the data obtained, several aspects which are related to this study are reviewed below.

Metabolism of GABA in plants

1. *GABA biosynthesis and regulation* As in animals, GABA in plants is produced primarily by the decarboxylation of glutamate (Glu), using the enzyme glutamate decarboxylase (GAD, EC 4.1.1.15), a cytosolic enzyme (Bown and Shelp, 1997):

$$^{-}OOCCH_2CH_2\underset{\displaystyle NH_3^{+}}{\underset{|}{C}}HCOO^{-} + H^{+} \xrightarrow{GAD} {}^{+}H_3NCH_2CH_2CH_2COO^{-} + CO_2$$

L-Glutamate GABA

This reaction is irreversible and occurs in the cytosol (Breitkreuz and Shelp, 1995). There is no evidence that the rapid and large accumulation of GABA induced by stresses is due to *de novo* synthesis of GAD (Bown and Shelp, 1997). This response involves post-translational modification of GAD activity by Ca^{2+}/calmodulin and cytosolic H^{+} levels.

The tobacco or petunia GAD is a multisubunit complex (Fromm, 1997). Potato tuber GAD might be a dimer (Satyanarayan and Nair, 1985). The molecular weight of GAD has been reported to be 43kD in potato tubers (Satyanarayan and Nair, 1985) or 58kD in petunia (Chen *et al.*, 1994). The cDNA sequence of a petunia GAD reveals that unlike GAD in animals (Erlander and Tobin, 1991), plant-derived GAD has a calmodulin (CaM) binding domain (Baum *et al.*, 1993). The existence of this domain has been further proved by *in vitro* activation of GAD from fava bean (Ling *et al.*, 1994) and soybean (Snedden *et al.*, 1995) with Ca^{2+} /CaM. This CaM binding domain is located at the carboxy-terminal of GAD (Baum *et al.*, 1993). Interestingly, the

recombinant petunia GAD could also be activated by an antibody which recognizes the CaM domain (Snedden *et al.*, 1996). Removal of the CaM binding domain by deleting 27 amino acids at the C-terminal of GAD makes this truncated GAD become active in the absence of Ca^{2+}/CaM. (Arazi *et al.*, 1995). These data indicate that the CaM domain in the absence of Ca^{2+}/CaM may lock up the reaction center and/or the substrate binding domain in GAD. However, the binding of Ca^{2+}/CaM or antibody to the CaM binding domain of GAD might induce a conformational alteration of GAD, such that the reaction center or the substrate binding domain could be exposed, and the enzyme activated. Thus, the interaction between the CaM binding domain and the reaction center or the substrate binding domain could be characterized as autoinhibitory (Bown and Shelp, 1997). Furthermore, *in vivo* experiments with transgenic tobacco plants which express the truncated GAD lacking the CaM-binding domain indicate that GAD activity was over-expressed, and GABA was over-produced in these tobacco plants. In addition, the assembly of the GAD-CaM complex is promoted by Ca^{2+} in wild type (Baum *et al.*, 1996). Both *in vitro* and *in vivo* evidence prove that Ca^{2+} is involved in the regulation of GAD activity and GABA biosynthesis in plants. However, this stimulation seems to be pH-dependent. At the optimal pH of 5.8 no significant stimulation of GAD activity by Ca^{2+}/CaM was seen, but at pH 7.0 a 3-fold increase in GAD activity in response to Ca^{2+}/CaM was observed (Snedden *et al.*, 1995). The regulation of GAD by Ca^{2+} may enable unstimulated or resting cells to maintain a low level of GABA, since there is a low level of free Ca^{2+} (30 to 200 nM) in the cytosol of higher plants (Bush, 1993), and $K_{1/2}$ (the dissociation coefficient) of GAD for Ca^{2+} is 800 nM (Snedden *et al.*, 1996).

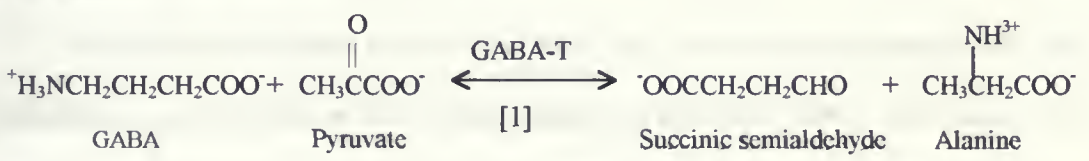
GAD is also regulated by cytosolic pH. Purified potato GAD (Satyanarayan and Nair, 1985) and soybean GAD (Snedden *et al.*, 1995) both have an optimal pH of 5.8, and the active pH range of GAD appears to be very narrow (Snedden *et al.*, 1992, 1995). At pH 7.0, compared to the optimal pH, GAD exhibits 10% of V_{max} in asparagus (Snedden *et al.*, 1992), 12% in soybean (Snedden *et al.*, 1995) and about 25% in potato tubers (Satyanarayan and Nair, 1985). Generally, the pH of the cytosol ranges

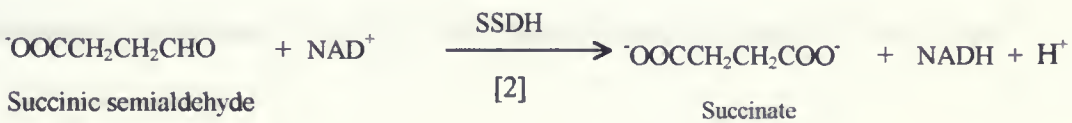
from 7.0 to 7.5 (Kurkdjian and Guern, 1989). Therefore, a small reduction in cytosolic pH from the normal physiological pH level causes a large increase in GAD activity. Crawford *et al.* (1994) observed that acidification of the cytosol by addition of 5mM butyric acid cause a decrease of 0.6 pH unit in the cytosol, which rapidly and greatly stimulates GABA production in isolated asparagus cells. In an independent study, using NMR, Carroll *et al.* (1994) demonstrated that cytosolic acidification from pH7.5 to almost pH7.3 induced by ammonium assimilation in cultured carrot cells resulted in a significant accumulation of GABA.

Taken together, the literature indicates that GABA production through GAD is controlled by Ca^{2+} and/or H^+ concentrations in the cytosol of cells.

In addition to GABA production through GAD, there are three minor pathways of GABA production in plants (Bown and Shelp, 1989). (1) In chestnut fruits, conversion of L-ornithine by L-ornithine-2-oxo-acid aminotransferase directly produces GABA. (2) In spruce plants, ornithine is converted to arginine followed by formation of 4-guanidinobutyric acid, which is further hydrolyzed to produce GABA accompanied with urea. (3) In maize seedlings, ornithine is converted to putrescine, spermidine and pyrroline in sequence. Pyrroline is then oxidized to GABA. Since ornithine is derived from glutamate, glutamate is overall the sole source of GABA production by these four pathways in plants.

2. *GABA catabolism* Two enzymes are responsible for GABA catabolism to succinate in plants. They are GABA : pyruvate transaminase (GABA -T, EC 2.6.1.19) and succinic semialdehyde dehydrogenase (SSDH, EC 1.2.1.16). The two reactions are shown below:





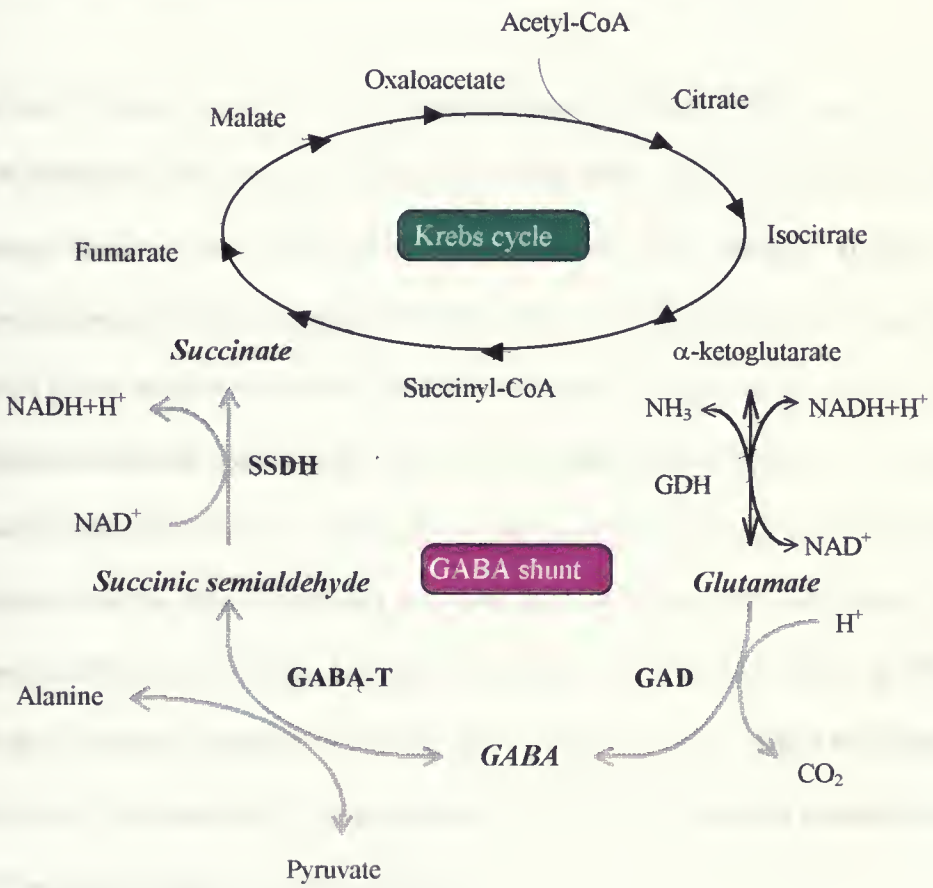
GABA-T catalyzes reaction I, the reversible transamination of GABA producing succinic semialdehyde, whereas SSDH catalyzes the irreversible oxidation of succinic semialdehyde (reaction 2) (Satyanarayan and Nair, 1990). GABA-T is a mitochondrial enzyme that has recently been purified from tobacco (Van Cauwenberghe and Shelp, 1997). It was reported that GABA-T has an alkaline optimum pH in the range of 8.6-9.0 (Shelp *et al.* 1995; Satyanarayan and Nair, 1986). Unlike animal or bacterial GABA-transaminases which are GABA : glutamic transaminases (called GABG-T) and only use α-ketoglutarate as a substrate (Scott and Jakoby, 1959; Schousboe *et al.*, 1973), plant GABA-T prefers pyruvate as its substrate instead of α-ketoglutarate. The K_ms of GABA-T for GABA and pyruvate are 1.5 and 300 μM, respectively, whereas GABA -T can be inhibited by α-ketoglutarate with K_i of 3mM (Van Cauwenberghe and Shelp, 1997). It was also reported that GABA-T from radish (Streeter and Thompson, 1972a) or soybean (Wallace *et al.*, 1984; Shelp *et al.*, 1995) is 10 to 20-fold more active with pyruvate than with α-ketoglutarate. The activity of potato tuber GABA-T with pyruvate is about twice as much as with α-ketoglutarate (Satyanarayan and Nair, 1986).

SSDH (reaction 2) is also a mitochondrial enzyme and has been purified from several plants (Breitkreuz and Shelp, 1995; Shelp *et al.*, 1995). The K_m of potato tuber SSDH was reported to be 4.65 μM for succinate semialdehyde (Satyanaryan and Nair, 1990b). The optimal pH of SSDH is between 9.0 to 10.0 depending on the source of the enzyme. Plant SSDH in certain characteristics somewhat closely resembles that from animals (Satyanarayan and Nair, 1990) and appears to be less controversial in its enzymological properties than GABA-T.

GABA synthesis occurs exclusively in the cytosol, whereas the two reactions of GABA catabolism occur exclusively in mitochondria (Breitkreuz and Shelp, 1995). This compartmentation requires specific transport proteins on the mitochondrial membranes to facilitate GABA entry into mitochondria (Breitkreuz *et al.*,1997). Such transport proteins in plants have not yet been identified.

The GABA shunt The three reactions described above, namely, GABA formation mediated by GAD, GABA transamination by GABA-T and the subsequent oxidation of succinic semialdehyde by SSDH, make up the so-called the GABA shunt (Fig.1) (Bown and Shelp, 1989; Satyanarayan and Nair, 1990). The GABA shunt in plants was first postulated by Naylor and Tolbert (1956) and experimentally demonstrated by Dixon and Fowden (1961) by tracing the fate of ^{14}C-GABA in pea. Since then, many reports have proved the occurrence of the GABA shunt (Satyanarayan and Nair, 1990). The GABA shunt provides a pathway for glutamate catabolism by the Krebs cycle. Glutamate is a main component in the free amino acid pool in most plants [3 μmol Glu per gram fresh weight in soybean leaves (Secor and Schrader, 1984)]. The GABA shunt converts GABA to succinate which is an intermediate of the Krebs cycle and produces 3 ATPs *via* NADH formation by SSDH. Glutamate is also able to enter the Krebs cycle through either deamination by glutamate dehydrogenase (GDH, EC 1. 4. 1. 3) or transamination with pyruvate or oxaloacetate to form α-ketoglutarate which is the another intermediate of the Krebs cycle (Bown and Shelp, 1997). The deamination pathway of glutamate catabolism to form succinate produces 6 ATP and 1 GTP. Obviously, the GABA shunt is less efficient in energy conservation. However, both pathways exist in normal metabolism (Tuin and Shelp, 1996). One advantage of the GABA shunt is not to produce toxic NH_3 which is a by-product of deamination. Another advantage of the GABA shunt may be to provide the Krebs cycle with succinate to overcome a shortage of krebs cycle intermediates *i.e.* α-ketoglutarate when GDH is inhibited due to stress. In rape seedlings, water stress caused inhibition of GDH (Srivastava and Singh, 1987), which implies that the production of *a*-ketoglutarate declines and would further negatively affect the Krebs cycle. In fact, it was also reported that the respiration rate did not change significantly when pea seedlings were subjected to a mechanical stress (Goeschl *et al.*, 1966). Thus, these advantages may outweigh the cost of producing less energy. The other potential roles of the GABA shunt will be presented in the section of

Fig. 1. Operation of the GABA shunt



"Roles of GABA in plants" below.

In the GABA shunt, there may be three factors which favor GABA accumulation rather than GABA consumption. (a) The maximal activity of GAD is 20 times more than that of GABA-T (Streeter and Thompson, 1972b); (b) The equilibrium of transamination by GABA-T favors GABA formation (Satyanarayan and Nair, 1990). (c) GABA catabolism requires transport of GABA into mitochondria from the cytosol where it is produced (Breitkreuz and Shelp, 1995).

Transport of GABA between cells When GABA accumulates, does GABA remain inside cells or leave cells? In isolated soybean cells, GABA exhibited a slow efflux (Secor and Schrader, 1984). In isolated asparagus mesophyll cells, on the other hand, most of the newly synthesized GABA was released to the incubation medium (Chung *et al.*, 1992). To answer this question, more data might be needed. Little is known about the mechanism of GABA transport in plant cells. Using the technique of functional complementation of yeast, Breitkreuz *et al.* (1997) have isolated a gene from *Arabidopsis thaliana* genome coding for an amino acid permease (AAP3). This permease is capable of facilitating GABA transport across the plasma membrane in a yeast strain deficient in GABA transport. The transgenic version of this strain with the AAP3 gene inserted is able to grow efficiently on 20 mM GABA. This gene has been sequenced. It was reported that this transport mediates the uptake of basic α-amino acids with a broad specificity besides GABA. Interestingly, in *Xenopus* oocytes expressing AAP3, GABA induces changes in membrane potential.

The roles of GABA in plants

The control of cytosolic pH Cytosolic pH is controlled both by a biophysical pH-stat which is operated by H^+ -ATPases or other H^+ pumps residing on the plasma membranes and/or vacuolar membranes and also by a biochemical pH stat which is operated by metabolic processes. These

metabolic processes produce or consume H^+ as indicated below, *e.g.* a malate-base pH-stat (Davies, 1986).

$$RCOO^- + H^+ \rightleftharpoons RH + CO_2$$

H^+-consumption in decarboxylation and the sharp acidic optimal pH of GAD allow GAD to operate as a component of the biochemical pH-stat. Crawford *et al.* (1994) and Carroll *et al.* (1994) independently correlated *in vivo* production of GABA with changes in cytosolic pH using different approaches. Acidification of either asparagus cell cytosol by applying 5mM butyrate at pH 5.0 to the medium (Crawford *et al.*, 1994) or cultured carrot cell cytosol by assimilation of NH_4^+ (Carroll *et al.*, 1994) resulted in a large accumulation of GABA. Moreover, Carroll *et al.* (1994) demonstrated that the recovery of cytosolic pH recovered to normal physiological level after GABA production. Thus, the causal relationship between GABA production and the cytosolic pH change seems to be clear. A stress induces cytosolic acidification, activates GAD, which initiates H^+-consumption during glutamate decarboxylation and GABA synthesis. Finally the cytosolic pH gradually recovers as decarboxylation proceeds. This GABA-mediated pH homeostasis may provide a way for plants under acid stress to precisely control cytosolic pH.

Temporary store of nitrogen.. It has been suggested that GABA can be viewed as a temporary nitrogen storage (Satyanarayan and Nair, 1990; Bown and Shelp, 1997), especially under stress conditions (Mizusaki *et al.*, 1964). As GDH becomes inhibited under stress conditions(Steward *et al.*, 1980; Srivastava and Singh, 1987), cells may switch the catabolic pathway of glutamate from the deamination by GDH to the GABA shunt. Consequently, GABA may accumulate. In terms of GABA levels, first, in root nodules of *Medicago sativa* and a few other N_2-fixing plants, GABA accounts for up to 20% of the total N content of the organs (Larher *et al.*, 1983), which implies that the assimilated

nitrogen somehow temporarily accumulates in the nodules in the form of GABA. Second, a significant proportion of GABA out of the total free amino acids was found in soybean phloem sap (Servaites *et al.*, 1979) and in asparagus xylem sap (11.4%) (Chung *et al.*, 1992). As to nitrogen turnover in developing seeds, GABA together with glutamate is actively produced during protein storage and mobilization as a means of recycling nitrogen (Bown and Shelp, 1997; Micallef and Shelp, 1989a, b). The evidence seems to support the hypothesis that plants use GABA as a temporary nitrogen store, since it has a relatively high ratio of N/C compared to other amino acids, and it can not be directly utilized by protein synthesis.

Plant defense against pests Mechanical damage induces rapid and substantial synthesis of GABA in plants (Wallace *et al.*, 1984). In soybean leaves, simulation of the mechanical damage caused by the biting action of phytophagous larvae of the oblique-banded leaf rollers (OBLR) (*Choristoneura rosaceana*), elevates GABA levels 10- to 25-fold within 2 minutes to values around 2 μmol per gram fresh weight of leaves (Ramputh and Bown, 1996). The biting action is thought to destroy the vacuolar compartmentation which releases H^+ into the cytosol and in turn enhances GAD activity and GABA production (Ramputh and Bown, 1996). A synthetic insect diet in which total GABA levels were adjusted to about 2 μmol per gram fresh weight inhibits growth, retards development and reduces the survival rates of the OBLR larvae (Ramputh and Bown, 1996). GABA is a major inhibitory neurotransmitter in the CNS, and acts as a chemotropic signal during CNS development (see "The roles of GABA in animals" below on page 13). Thus, insect larvae which lack a blood-brain barrier may experience adverse effects from a high level GABA introduced during feeding. The long-term stimulation of GABA receptors may cause abnormalities in feeding, growth and development of larvae. Thus, GABA may play a role in plant defense against phytophagous insects.

Involvement in plant development There are several findings which may imply that GABA is involved in plant development. First, Fromm's lab at the Weizmann Institute in Israel found that GAD exists in all organs including flowers and all floral parts, leaves, stems, roots, and seeds in petunia plants, and they showed that both GAD activity and expression of its gene are developmentally regulated (Chen *et al.*, 1994).

Second, Fromm's lab also established transgenic tobacco plants expressing a truncated GAD lacking the CaM binding domain, designated GADΔC (Baum *et al.*, 1996). In GADΔC plants, GAD activity is overexpressed, and the steady-state levels of GABA are 3 to 7 times as high as controls (untransformed or transformed with the normal GAD gene). Concomitantly, glutamate levels are 1/18 of those in controls. GADΔC plants exhibit stunted growth with shorter stems, narrower leaves and more branches than those in control plants. Histologically, the stem cortex parenchyma cells in GADΔC plants are 3 times as short in length as those in controls. In addition, GADΔC plants are able to grow continuously for over 2 years. In fact, normal tobacco plants are annual plants. These observations indicate that overexpression of GAD in plants causes abnormal development, though it is hard to establish a clear causal relationship between the morphological abnormality and GABA levels.

Third, Ford *et al.* at Oxford University reported that dedifferentiation of carrot root tissues induced by the hormones α-NAA and kinetin were accompanied by increases in GABA levels. These increases were not due to cytosolic acidification, as indicated by ^{31}P NMR data used for *in vivo* pH measurements. Ca^{2+} is likely to be involved in the process of dedifferentiation (Ford *et al.*, 1996).

Fourth, Reid's group at the University of Calgary observed that GABA added to culture media at 0.25mM stimulated elongation of hypocotyls in *in vitro* sunflower plantlets over 21days (personal communication from A. W. Bown). They also observed that 100 mM GABA stimulated ethylene

production, though the concentration of 100 mM is far beyond physiological levels (Kathiresan *et al.*, 1997).

Fifth, Locy *et al.* (1997) at Auburn University recently observed in *Arabidopsis thaliana* that GABA supplied in the growth medium inhibits growth of roots and hypocotyls and promotes root branching and formation of leaves. Their ongoing investigations with GABA-resistant mutants and GABA-insensitive mutants of *Arabidopsis thaliana* may help to elucidate the role(s) of GABA in plant development.

Finally, Bown's laboratory at Brock University found that GABA rapidly accumulates to high values and growth was inhibited when dark-grown soybean seedlings were stroked (Grognet, 1996). This indicates a possible causal relationship between GABA accumulation and hypocotyl elongation. In addition, most newly synthesized GABA was released into the media surrounding isolated asparagus cells (Chung *et al.*, 1992), which suggests that GABA may act as an intercellular messenger (Bown and Shelp, 1997).

These findings raise the possibility that GABA may be involved in regulating plant development. However convincing evidence is still required, and how GABA functions in plants is unknown. In animals, however, intercellular GABA functions as an inhibitory neurotransmitter in the central nervous system and as a chemotropic signal molecule in neuron migration *via* GABA receptors. The following paragraphs will describe GABA's functions and its receptors in animals.

The roles of GABA in animals

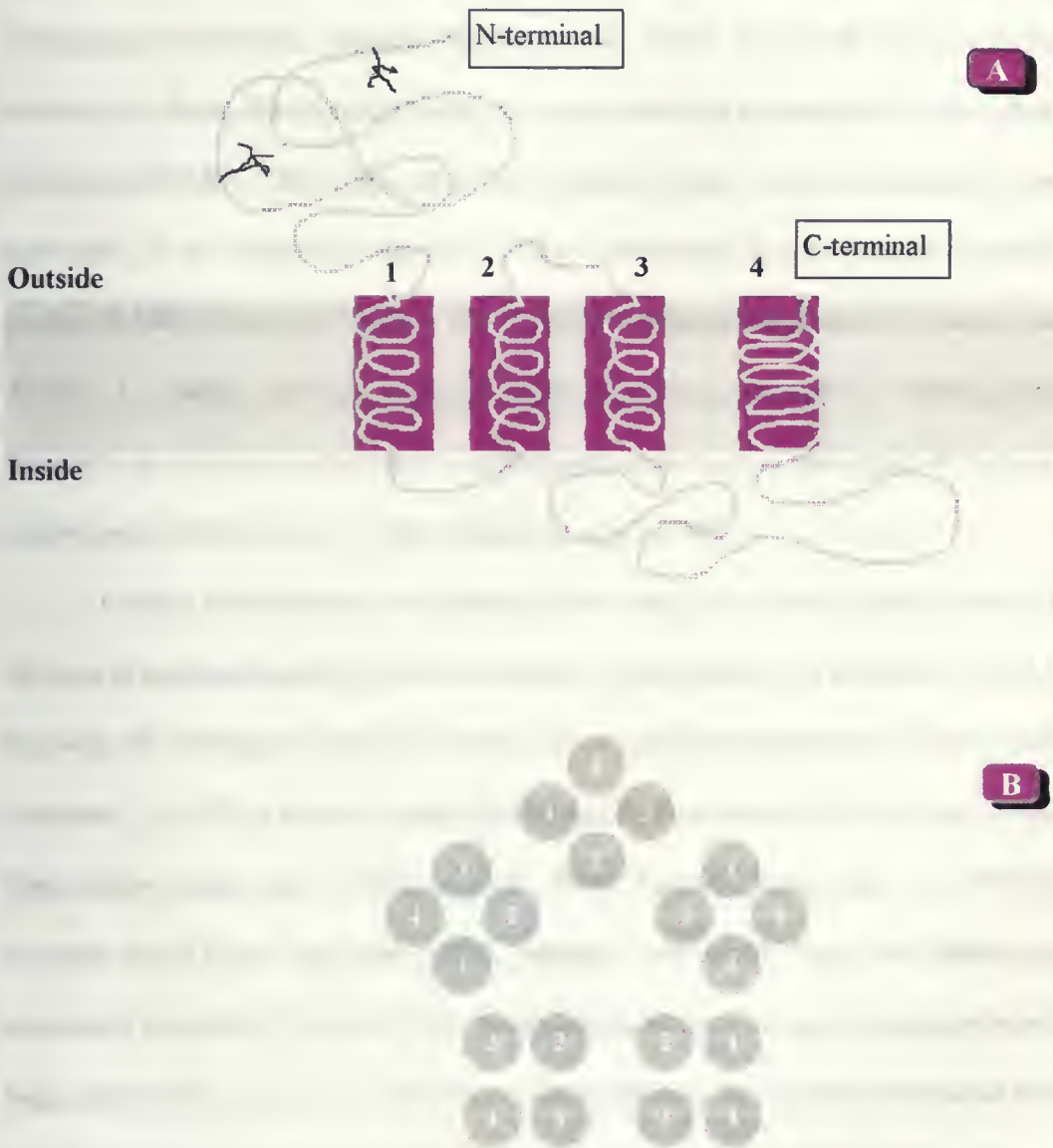
GABA as an inhibitory neurotransmitter and its receptors GABA has been demonstrated as a major inhibitory neurotransmitter in the nervous system. There are three receptors : $GABA_A$, $GABA_B$ and $GABA_C$.

1. $GABA_A$ belongs to the class of ionotropic receptors. It is a plasma membrane located, GABA-gated, fast-acting chloride channel and occurs within mammalian CNS. The presence of $GABA_A$ receptors was demonstrated in the 1970s. It was purified in 1983 from bovine cerebral cortex by Siegl *et al.* (1983). $GABA_A$ is a heterooligomer glycoprotein complex composed of 5 subunits with a molecular weight of about 275kD. Each subunit has its own gene family based on sequence similarity, which causes a large difference in composition of the channel during developmental stages and in varied tissues. These 5 subunits form a quasisymmetric structure around the pore, each subunit contributing to the wall of the pore (Fig. 2A) (Macdonald and Olsen, 1994). The effective pore diameter of the ion channel at the main conductance level of 10 to 30 pS is around 5.6 nm (Bormman *et al.*, 1987). In terms of each subunit: generally, the N-terminal is exposed to the extracellular environment; four α-helix domains span the cell membrane with the C-terminal at the extracellular end of the fourth membrane-spanning domain; a large intracellular loop is located between the third and the fourth α-helices (Fig. 2B) (Schofield *et al.*, 1987).

Once GABA binds to $GABA_A$, the channel opens and then closes rapidly (Macdonald and Olsen, 1994). During the open state, chloride ions enter the cells and induce hyperpolarization which inhibits initiation of the action potential.

$GABA_A$ receptors are activated by GABA and its structural analogues, such as muscimol; but are inhibited competitively by bicuculline and noncompetitively by picrotoxin (Macdonald and Olsen, 1994).

Fig. 2. The $GABA_A$ receptor.



A: The topological structure of a subunit protein of $GABA_A$ receptors.

B: Model of the $GABA_A$ receptor- chloride ion channel protein complex.

These two models are based on (Macdonald and Olsen, 1994).

2. $GABA_B$ belongs to metabotropic receptors which are slow-acting. The $GABA_B$ receptor works quite differently from the $GABA_A$ receptor. It links to a GTP-binding protein (G-protein) to produce several divergent effects: inhibition of adenylate cyclase, closure of voltage-gated Ca^{2+} channels and opening of adjacent K^+ channels (Nakayasu *et al.*, 1993). As a result of this signal transduction network, the effects of activating $GABA_B$ are slower and more diverse than the effects that follow the activation of $GABA_A$. Stimulation of $GABA_B$ produces a slow and prolonged inhibition in post-synaptic nerve cells. It also inhibits the release of GABA by autoinhibition in presynaptic terminals of axons that produce GABA (Bowery and Brown, 1997). $GABA_B$ receptors are involved in various neurological diseases, *e.g.* multiple sclerosis and spinal injury (Kaupmann *et al.*, 1997). $GABA_B$ receptors are also thought to be involved in complicated cerebral mechanisms such as some types of learning and memory, and behavior (Nakayasu *et al.*, 1993; Kaupmann *et al.*, 1997).

$GABA_B$ receptors were first identified by Bowery *et al.* (1980) at the University of Birmingham. Because of its physiological and clinical importance, many attempts to characterize $GABA_B$ receptors at the molecular level have since been made, but only recently was the molecular structure of this receptor discovered. In 1993, a Japanese group purified and characterized an 80kD protein $GABA_B$ receptor from bovine cerebral cortex (Nakayasu *et al.*, 1993). Recently, Kaupmann *et al.* (1997) reported that the genes of two forms of rat brain $GABA_B$ receptors were cloned. These two $GABA_B$ receptors are monomeric proteins of 100 and 130kD. Each $GABA_B$ receptor has seven transmembrane helices, a large extracellular domain at the N-terminal, and a relatively short C-terminal situated inside the cells. The gene sequence shows that the GABA binding site is positioned at the N-terminal region and a possible G-protein binding domain is postponed between transmembrane helix 2 and helix 3 (Fig. 3). The connecting domain to adenylate cyclase has not yet been defined (Kaupmann *et al.*, 1997).

One GABA molecule binds to the extracellular domain and activates $GABA_B$. The activated $GABA_B$ couples to the α-subunit of associated G-proteins (G_i), and then opens a K^+ channel (probably

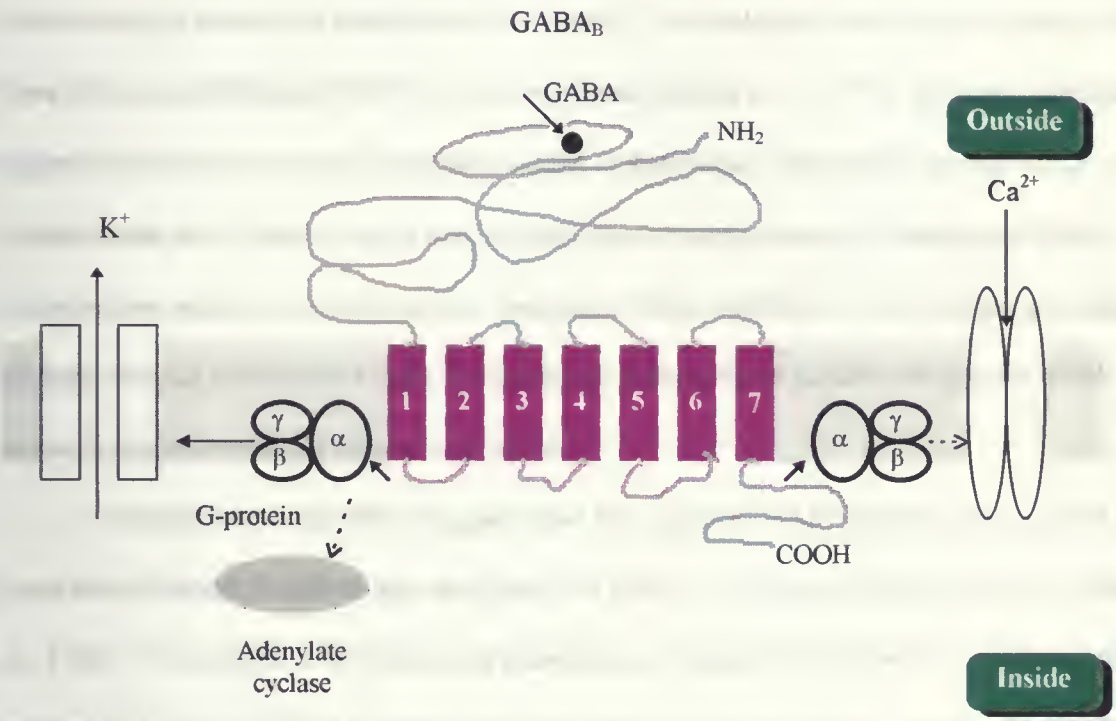
through the βγ subunit of the G-protein). Another type of G-protein (G_o) inhibits a voltage-gated Ca^{2+} channel (probably through its βγ subunit) (Bowery and Brown, 1997). Kaupmann *et al.* (1997) also reported that activated $GABA_B$ inhibited cAMP production, which indicates that $GABA_B$ negatively modulates adenylate cyclase activity.

$GABA_B$ receptors are activated by GABA and its agonist baclofen, , but are strongly inhibited by its antagonist, 2-hydroxysaclofen. Unlike $GABA_A$ receptors, $GABA_B$ receptors are bicuculline- and picrotoxin-insensitive (Behar *et al.*, 1996).

3. $GABA_C$ belongs to the class of ionotropic receptors. It is also a fast-acting GABA-gated chloride channel. Because of its unique pharmacology (see below), it is classed as the third type of the GABA receptors. However, some people describe it as a subgroup of the $GABA_A$ receptor class, since $GABA_C$ receptors structurally resemble $GABA_A$ receptors (Bowery and Brown, 1997). The $GABA_C$ receptor was identified in 1993 by two research groups (Feigenspan *et al.*, 1993; Qian and Dowling, 1993). It occurs in the visual nervous system, *e.g.* rod cells and bipolar cells in the retina of vertebrate animals (Feigenspan *et al.*, 1993: Qian and Dowling, 1993) and in the nervous system of invertebrates (Jackel *et al.*, 1994).

Pharmacologically, $GABA_C$ receptors are picrotoxin-sensitive, but are inhibited neither by bicuculline (a $GABA_A$ antagonist), nor by saclofen (a $GABA_B$ antagonist), and fail to be activated by baclofen (a $GABA_B$ agonist) (Behar *et al.*, 1996).

Fig. 3. The $GABA_B$ receptor



One molecule of GABA binds to the large extracellular domain of the monomeric $GABA_B$ receptor, which has seven transmembrane helices. The activated receptor then couples to the α-subunit of associated G-proteins. One of these (G_i) inhibits adenylate cyclase and opens a potassium channel. Another G-protein (G_o) retards the opening of a calcium channel (Bowery and Brown, 1997).

GABA as a chemoattractant for neuron cell migration During the development of the mammalian cerebral cortex, neuronal progenitors proliferate within ventricular regions and then migrate to the cortical plate where they differentiate and organize into layers. Thus, nerve cells undergo a migratory phase. Molecules that are possible candidates for chemoattractants should be presented in appropriate locations and be released by cells (Behar *et al.*, 1994). Two molecules that have been shown to meet these criteria are GABA and NGF (nerve growth factor) (Behar *et al.*, 1994). As a chemoattractant in triggering and guiding neuronal movement, GABA at femtomolar (fM) and micromolar (μM) concentrations were found to induce two different types of neuron motility. Femtomolar GABA concentrations primarily stimulate neuron chemotaxis which is defined as migration along a chemical gradient; whereas micromolar GABA concentrations predominantly initiate chemokinesis which increases random movement (Behar *et al.*, 1996).

Investigations using different agonists and antagonists of GABA receptors indicate that GABA exerts these functions in neuron migration likely *via* $GABA_A$, $GABA_B$ and $GABA_C$ receptors (Behar *et al.*, 1996). Furthermore, it was found that chemotaxis in response to femtomolar GABA concentrations involves all 3 types of GABA receptors, whereas chemokinesis in response to micromolar GABA levels involves $GABA_B$ and $GABA_C$ receptors. In general, submicromolar to micromolar of GABA concentrations are required to open chloride channels associated with $GABA_A$ receptors. Thus femtomolar GABA levels are not enough to activate the chloride channel activity of $GABA_A$ receptors. However, both femtomolar and micromolar GABA elevate intracellular Ca^{2+} levels. Moreover, cells loaded with a Ca^{2+} -chelator, BAPTA-AM (Bis(2-aminophenox) ethane-N,N,N',N'-tetra-acetic acid), are not able to migrate in response to both concentrations of GABA. Therefore, Ca^{2+} mediates chemotaxis and chemokinesis induced by GABA (Behar *et al.*, 1996).

Summary As a ubiquitous molecule and a prominent component of the free amino acid pool in plants, GABA has been given considerable attention. Many aspects regarding GABA in plants have been revealed, *e.g.* demonstration of GABA accumulation in response to a number of stresses, elucidation of the GABA shunt, the enzymology of GABA metabolism, the mechanism of regulating GABA synthesis, and the molecular biology of GAD. The roles of GABA in plants, however, are still not well defined, although GABA has been suggested to play a role in pH regulation, plant defense against phytophagous insects, nitrogen metabolism and plant development. In contrast to animals, in plants the equivalent of a $GABA_A$, $GABA_B$ or $GABA_C$ receptor has not been found, and neither is there conclusive evidence that GABA is involved in plant development. Therefore, it is of a great interest to determine whether GABA is involved in plant growth and development. We hypothesized that GABA is involved in inhibition of soybean hypocotyl elongation trigged by mechanical stimulation. The primary questions addressed in this study were :

1. Does GABA accumulate during growth inhibition induced by mechanical stimulation ?
2. What is the temporal relationship between GABA accumulation and growth inhibition induced by mechanical stimulation ?
3. Is GABA capable of inhibiting growth?
4. Does GABA accumulate during growth inhibition caused by lanthanum or blue light?
5. Does GABA regulate chloride channels in plant cells.

In addition, part of this study was intended to develop a method for the rapid, inexpensive and convenient measurement of GABA in plants.

Materials and Methods

A. Chemicals

Name	Source
ACS (Counting scintillant)	Amersham
Aminobutyric acid (GABA)	Sigma
(U)^{14}C- Aminobutyric acid (^{14}C-GABA)	Sigma
Baclofen	RBI
Bicuculline	RBI
Gabaculine	RBI
Gabase	Sigma
Glutamate	Sigma
Glutamic acid assay kit	Boehringer
Glycerol	BDH
α- Ketoglutarate (α- KG)	Sigma
Lanthanum chloride ($LaCl_3$)	Sigma
β-Mercaptoethanol (CH_3CH_2SH)	Kodak Eastman
Methanol (CH_3OH)	BDH
$Na^{36}Cl$	Amersham
Nicotinamine adenine dinucleotide phosphate ($NADP^+$)	Sigma
5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB)	RBI
Saclofen	RBI
Sudan 3	BDH
Tetrapotassium pyrophosphate ($K_4P_2O_7$)	Sigma

B. Plant Materials

Soybean (*Glycine max* [L]. Merr. cv Corsoy 79) seeds were supplied by Harrowvinton Seeds, Ontario. They were germinated and grown in vermiculite. (1) For GABA determination experiments, fully expanded leaves were harvested from 50-day-old soybean plants grown in the university greenhouse under normal growth conditions, with weekly fertilization with a fertilizer Plant-Prod 20-20-20 purchased from Plant Products Company Limited, Ontario, Canada. (2) For hypocotyl and stem elongation determination in light-grown seedlings, plants were grown under the conditions described above and watered only on the vermiculite to avoid mechanical stresses by watering. Seven-day-old seedlings were used. (3) For experiments dealing with hypocotyl elongation in etiolated seedlings, the seeds were grown for 3 days in the dark in a growth chamber at 25°C. All manipulations were carried out in green light.

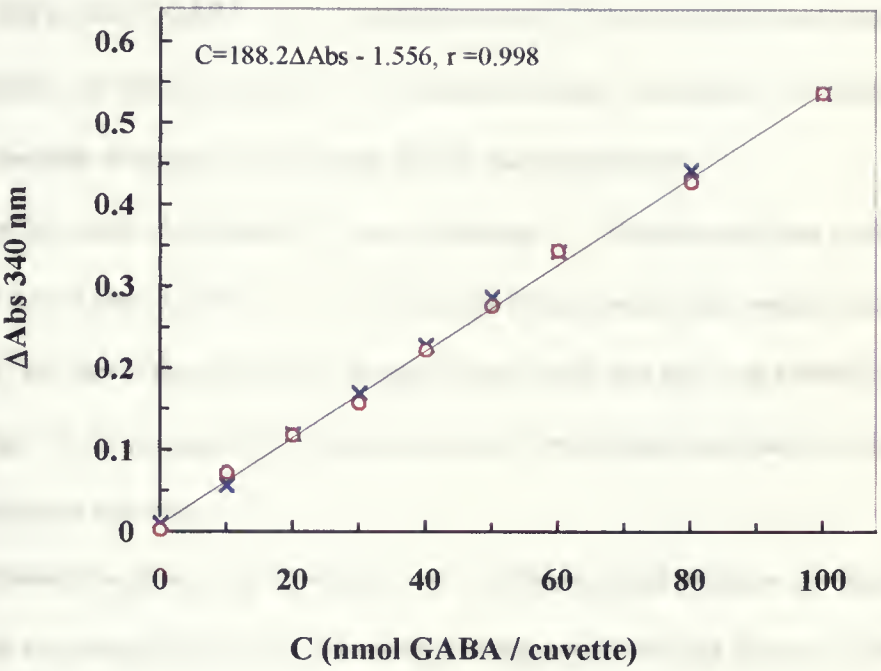
Asparagus (*Asparagus sprengeri* Regel) plants were grown under the same conditions as light-grown soybean plants described in (1) above.

C. Methods

Extraction of GABA from soybean leaves Leaves after detachment from the plants were immediately placed into liquid nitrogen, and then ground in liquid nitrogen to a fine powder using a mortar and pestle. About 0.1 g of the frozen powder was transferred into a pre-weighed Eppendorf tube containing 400 µl methanol, then the Eppendorf tube was weighed again in order to obtain the precise weight of the homogenate transferred. After the sample was dried at 70°C, 1.0 ml of 70 mM lanthanum chloride ($LaCl_3$) or a specified concentration of it was added. The homogenate was shaken intermittently for 10 - 15 minutes and then centrifuged at 13,600 g for 5 minutes. The pellet was retained for chlorophyll determination. Eight hundred µl of the supernatant was transferred into another Eppendorf tube containing 160µl of 1N KOH or an amount equivalent to the La^{3+} added. Following 5 minutes of shaking, centrifugation was performed as before. The resulting supernatant was used for GABA determination and the pellet consisting of lanthanum hydroxyl and yellow pigments was discarded.

Determination of GABA This was performed according to the methods described by the Sigma Chemical Company with the modification of 150 µl instead of 50 µl of 4 mM $NADP^+$ (Crawford *et al.*, 1994). One ml of the GABA assay mixture was composed of 550 µl of the sample, 200 µl of 0.5 M potassium pyrophosphate buffer (pH8.6), 150 µl of 4 mM $NADP^+$, 50 µl of 2 units Gabase/ml, and 50 µl of 20 mM α-ketoglutarate. Before adding α-ketoglutarate, the initial absorbance of the sample was measured at 340 nm. The final absorbance reading was made 60 minutes after adding α-ketoglutarate and mixing. By subtracting the initial A340 nm from the final A340 nm, ΔA340 nm was obtained and used to calculate the amount of GABA according to a calibration curve in the range of 0 - 100 nmoles standard GABA per cuvette (Fig. 4).

Fig.4 Calibration curve for GABA determination



One ml of GABA assay mixture was composed of 0 - 100 µl of 1.0 mM standard GABA, 550 - 450 µl H_2O, 200 µl of 0.5 M tetrapotassium pyrophosphate buffer (pH8.6), 150 µl of 4 mM $NADP^+$, 50 µl of 2 units Gabase / ml and 50 µl of 20 mM α-ketoglutarate. Before adding α-ketoglutarate, the initial absorbance at 340nm (A340nm) was measured. After adding α-ketoglutarate and incubation for 60 minutes at 25°C, the final absorbance was measured. By subtracting the initial A340nm from the final A340nm, ΔA340nm was obtained.

Assay of Gabase activity The method described by the Sigma Chemical Company was employed for the assay of Gabase activity. The 1 ml assay system consisted of 200 µl of 0.5 M tetrapotassium pyrophosphate buffer (pH8.6), 6 µl β-mercaptoethanol, 250 µl of 20 mM α-ketoglutarate, 125 µl of 10 mM $NADP^+$, 100 µl 60mM GABA, 10 µl 2unit Gabase /ml, 0-150 µl of 200mM $LaCl_3$ and 310 -160 µl H_2O. The reaction was started by adding 10 µl of Gabase solution. Absorbances at 340nm were recorded as a function of time using a Beckman DU-50 spectrophotometer.

Localization of hypocotyl elongation in soybean seedlings The hypocotyl was marked with Sudan 3, a red dye, at intervals of 5 mm. Then the length of each section was measured after 12 hours. The segment 17 mm below the apical hook elongated most rapidly and was most sensitive to mechanical stimulation (Fig. 14). This segment was chosen as the site of mechanical stimulation or chemical treatments throughout this study.

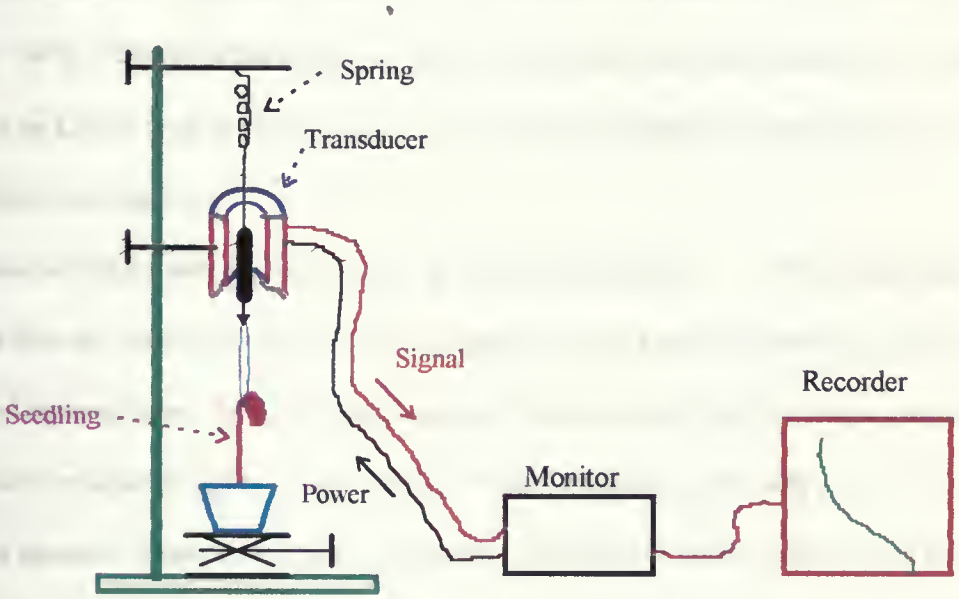
Mechanical stimulation of soybean hypocotyls Twenty strokes between the tips of a thumb and a forefinger were done within 20 seconds along the hypocotyl (Jones and Mitchell, 1989). Latex gloves were worn to prevent any chemical effect from the fingers.

Application of chemicals to soybean hypocotyls Ten µl of each specified chemical in 1mM MES (pH5.7) in 6 to 8 droplets was applied along the segment of a hypocotyl with a 10 µl Hamilton syringe (Jones and Mitchell, 1989).

Measurement of hypocotyl elongation in soybean seedlings For the hypocotyl growth increment over 24 hours, the heights of seedlings were measured using a ruler before and 12 or 24 hours after treatment.

To monitor the elongation rate of a seedling, an electronic transducer was employed. This apparatus is indicated in Fig. 5. Briefly, the apical hook of a seedling was caught in the loop of a thread which at the other end links to the transducer and is tightened by a small spring (1.5 x 15 mm). This

Fig.5 Scheme of the transducer system for growth measurement.



spring was stretched about 2 mm at the beginning of an experiment, which provides about 2 grams of stretch force to the seedling. Fifteen minutes after manipulation, the rate of hypocotyl elongation measurement was started. Mechanical stimulation, blue light irradiation, or chemical application was administered after a stable growth rate was seen and recording continued.

Blue light radiation Etiolated seedlings were irradiated with blue light (340 -480nm, 350 - 450 $\mu mol \cdot s^{-1} \cdot m^{-2}$). Changes in the elongation rates were recorded using the transducer. To investigate any influence on GABA level, the irradiated seedlings were removed from the pots and then were dropped immediately into liquid N_2.

Estimation of GABA and glutamate levels in soybean hypocotyls Whole seedlings were removed from the vermiculite medium and immediately placed into liquid nitrogen. About 2-cm-long hypocotyl segment below the apical hook, mentioned above was cut from the frozen seedling, and placed immediately in liquid N_2 again. It was weighed within 10 seconds, and finally ground in liquid N_2. The segments remained frozen during these procedures. The resulting frozen tissue powder was then transferred completely to an Eppendorf tube using 4 or 5 aliquots of 300 µl of methanol. After the methanol was evaporated at 68 ° C, the sample was resuspended in 700 µl of distilled water and shaken intermittently for 1 hour. After centrifugation at 13,600 g for 5 minutes, the resulting supernatant was used for further experiments.

To measure Glu, a Glu assay kit provided by Boehringer was used. One hundred µl of the supernatant above was placed in a cuvette which contained 200 µl of triethanolamine phosphate buffer (pH 8.6), 606 µl of H_2O, 65 µl of NAD^+/diaphorase solution, and 19 µl of int.(iodonitrotetrazolium chloride) solution. The initial absorbance readings at 492 nm were taken. The reaction was initiated by adding 10 µl of glutamate dehydrogenase (GDH) solution and was continued at 25°C in the dark for 45 minutes. Then the final absorbance readings were made. Both the initial readings and final readings at

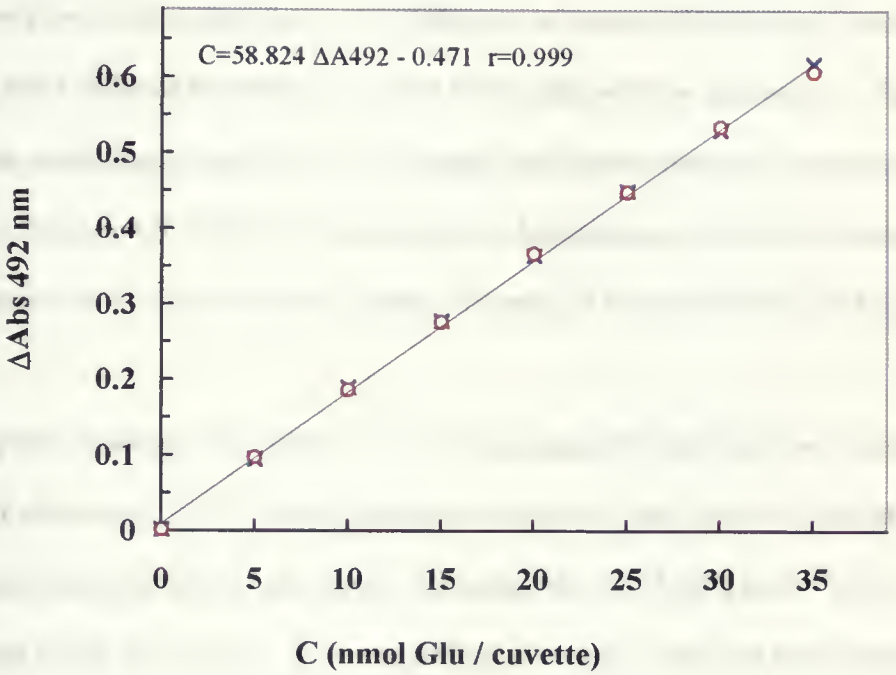
492 nm were measured with a Beckman DU-50 spectrometer. The ΔA492 nm obtained by subtracting the initial A492 nm from the final A492 nm was used to calculate Glu using a calibration curve ranging from 0-100 nmol Glu per cuvette (Fig. 6). One hundred µl of distilled water instead of the tissue extract was used for control.

The remaining sample (600 µl) was used for GABA determination with the procedures described above. However, 150 µl of 350 mM $LaCl_3$ was added into the suspension to give a 70 mM final concentration of $LaCl_3$. After centrifugation, 600 µl of the supernatant was placed in a new Eppendorf tube and 120 µl of 1N KOH was added. After centrifugation, 550 µl of the supernatant fluid was used for GABA measurement following the procedures described above.

Absorption of GABA and ^{14}C- GABA by soybean seedlings Ten µl of 10 mM GABA solution (in 2 mM MES buffer, pH 5.7) was applied to the hypocotyl segments of dark-grown seedlings. In controls, seedlings were treated with the buffer solution only. Two or 4 hours after application, the hypocotyls were rinsed gently with large volume of distilled water, and then placed in liquid N_2 quickly. A previous experiment had demonstrated that 15 ml of water could completely rinse off 10 µl cold GABA from the hypocotyl. *In vivo* GABA levels in the segments were measured using the above method.

Ten µl of 2.2×10^4 DPM ^{14}C-GABA / µl (238 mCi/mmol) in 2mM MES buffer (pH5.7) was applied the same way as cold GABA to hypocotyls. Four hours after application, the hypocotyl was rinsed with 15 ml of H_2O to remove extemal radioactivity and then placed in liquid N_2. The cotyledons were cut off from the frozen seedlings, and the hypocotyls were further excised into 3 sections 2 cm long. They were then individually ground in liquid N_2 to a fine powder. Five ml of 90% methanol was added to the cotyledon powder and 1.5 ml of this homogenate was transferred to an Eppendorf tube. All the powder from a hypocotyl section was transferred to an Eppendorf tube with 4 or 5 aliquots of 300 µl 90% methanol. After drying, 500 µl of 70mM $LaCl_3$ was added to the sample to remove pigments which

Fig.6 Calibration curve for glutamate determination



One ml of glutamate assay mixture was composed of 100 µl of standard glutamate ranging from 0 - 0.35 mM, 200 µl triethanollamine phosphate buffer (pH8.6), 606µl H_2O, 65 µl NAD^+/diaphorase, 19µl Int. (iodo-nitrotetrazolium chloride) and10 µl of 900 units / ml glutamate dehydrogenase. Before adding glutamate dehydrogenase, the initial absorbance at 492nm (A492nm) was measured. After adding glutamate dehydrogenase and incubating for 45 minutes at 25°C, the final absorbance was made. By subtracting the initial A492nm from the final A492nm, ΔA492nm was obtained.

quench scintillation counting. After 1 hour of intermittently shaking, the samples were spun for 5 minutes at 13,600g and 300 µl of the fluid was transferred to another Eppendorf tube containing 60 µl of 1N KOH. The resulting $La(OH)_3$ pellet was removed by centrifugation as before, and 200 µl supernatant was pipetted into a scintillation vial. Fifteen ml of the wash fluid derived from rinsing a hypocotyl was dried, followed by addition of 2 ml of H_2O to redissolve the radioactivity. Finally 200 µl of this fluid was pipetted into a scintillation vial. Liquid scintillation counting was performed with 10 ml of ACS using a Beckman LS-4800 scintillation counter at Agriculture and Agri-food Canada--Vineland Station. The radioactivity recovery and the counting efficiency of the samples were 99.4% and 91.2%, respectively.

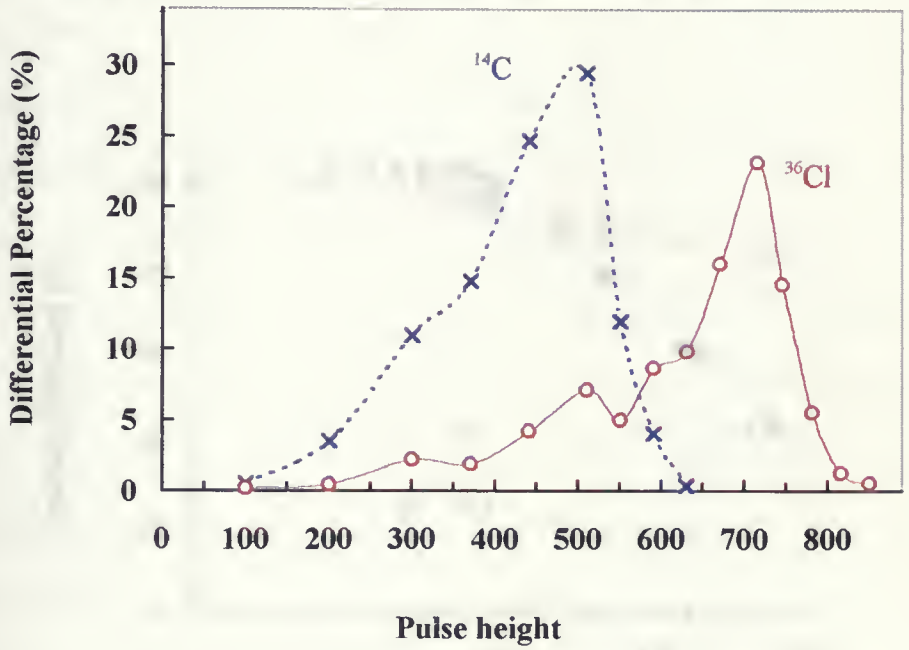
Cell isolation from Asparagus cladophylls Fresh asparagus mesophyll cells were mechanically isolated daily (Colman *et al.*, 1979). Briefly, asparagus cladophylls were collected, washed with tap water, sliced into 2 mm long sections and vacuum infiltrated with 1 mM MES buffer (pH 6.0) containing 1 mM $CaSO_4$ for 5 minutes to remove air from the tissues. Then, the sliced cladophylls were placed in a mortar containing the above buffer and were hit gently using a pestle. The resulting green cell suspension was filtered with 4 layers of cheesecloth, collected and spun for 3 minutes at 100 × g with a desktop medical centrifuge to pellet cells. The cell pellet was washed using 50 ml of 1 mM $CaSO_4$ (pH 6.0) by centrifugation as above. Finally, the cell pellet was resuspended and cell density was adjusted to 3-5 × 10^7 cells/ml with the fresh buffer. These cells were used for further experiments.

Influx of $^{36}Cl^-$ The ^{36}Cl loading medium was composed of 2 µCi $Na^{36}Cl$ /ml (0.5 mCi/mmol Cl^-), 5 mM K_2SO_4, 5% DMSO, 5mM HEPES (pH 7.5) or MES (pH 6.5 or 5.5), and 3-5 × 10^7 asparagus cells/ml. At 1, 2 or 3 hours after incubation with intermittently gently shaking, 100 µl of the cell suspension was collected on a Millipore filter (HA type, 0.65 µm) and washed with 1×1.5 ml followed by 4×3 ml of washing solution (°C) which consisted of 2 mM HEPES or MES buffer, 100 mM

cold NaCl, 5 mM K_2SO_4, and 5% DMSO. The filters with cells were dried at 90°C and then placed into Eppendorf tubes. After 600 µl of 0.2 M KOH was added to the tubes, the tubes were sealed and heated at 90 °C for about 1 hour and vortexed twice during incubation to break down both cells and filters. Subsequently, 120 µl of 350 mM $LaCl_3$ was added to remove pigments which quench liquid scintillation counting. After centrifugation, clear extraction fluids were obtained. Four hundred µl of these fluids was pipetted into a scintillation vial. Finally, radioactivity of the sample was determined in 10 ml of ACS using a Beckman LS-1800 scintillation counter (at Brock university) at 610 to 780 pulse height (Fig. 7) of channel 3. The counting efficiency and the radioactivity recovery of this procedure were estimated. The H number which reflects the quench effect of a sample on pulse height distribution of Compton electrons was between 80 to 90, which represents at least 95% counting efficiency (Fig. 8). It was also shown that 94.5±1.5% of radioactivity was recovered with this procedure. To determine the background radioactivity of this procedure, initial loading media containing cells and $^{36}Cl^-$ were transferred directly onto the filters within 10 seconds of addition of $^{36}Cl^-$, washed immediately, and then treated as above.

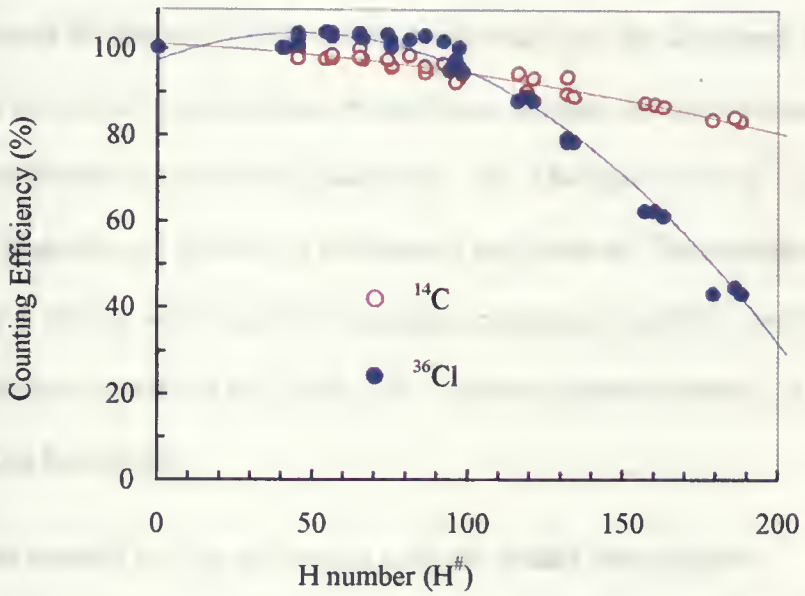
Efflux of $^{36}Cl^-$ Cells were loaded with $^{36}Cl^-$ using the same procedure as the $^{36}Cl^-$ influx experiment. Washed cells were resuspended in the $^{36}Cl^-$ -free loading buffer described above. To obtain the time course of $^{36}Cl^-$ efflux, 300 µl of cell suspension was pipetted into an Eppendorf tube at the beginning, 20, 60 or 120 minutes after resuspension, and centrifuged immediately for 10 seconds at 13,600 g. Two hundred and fifty µl of the clear supernatant was placed into a scintillation vial and the radioactivity was counted in 10 ml ACS as above.

Fig.7 Pulse height distribution of ^{14}C and ^{36}Cl for liquid scintillation counting of radioactivity



Twenty μl of $Na^{36}Cl$ ($4.4x10^5$DPM/ml) or 10 μl of ^{14}C-GABA (3.8 x 10^6DPM/ml) was added to a scintillation vial which containing 10 ml aqueous counting solvent (ACS). The CPM integral was measured between 0 and a set pulse height shown in the figure using a Beckman LS-1800. The CPM differential was obtained by taking away the previous CPM integral from the present CPM integral. Differential percentage was computed by dividing a CPM differential by the maxium CPM integral.

Fig. 8 Quench curves of liquid scintillation counting for ^{14}C and ^{36}Cl



Quench (H number, $H^{\#}$) was manipulated by addition of various volumes (μl) of CCl_4 to 10 ml ACS scintillation solvent. For ^{14}C, a channel which counts pulses from 0 to 670 was set. For ^{36}Cl, a channel which counts pulses from 610 to 780 was set. To each vial 50 μl of radioactivity was added to give a calculated DPM of 11,000. This calculation was based on the specific activity data provided by the manufacture. A Beckman LS1800 was employed.

Results

A. Development of a method for the rapid Determination of GABA *

Elimination of water-soluble pigments from leaf extract using $LaCl_3$ Leaf extracts of soybeans contain large amounts of pigments which absorb light below 450 nm (Fig. 9). At 340 nm, the working wavelength for detecting NADPH in GABA determination, the absorbance due to pigments led to values which prevented the measurement of absorbance increases due to the presence of GABA (Fig. 10). $LaCl_3$ significantly removed these pigments (Fig. 10). After removal of La^{3+} by precipitation and centrifugation (Materials and Methods), a clear extract was obtained. The absorbances of the aqueous GABA extracts at 340 nm were reduced by increasing concentrations of $LaCl_3$, and dropped to values around 10^{-2} absorbance at 60 mM $LaCl_3$ (Fig. 10). To ensure pigment-removal, 1.0 ml of 70 mM $LaCl_3$ was added to 0.1g leaf sample.

The influence of residual La^{3+} on the enzyme assay for GABA determination The influence, if any, of residual La^{3+} on the GABA assay was investigated. First, the inhibition of Gabase activity by La^{3+} was investigated. More than 90% of Gabase activity remained if the concentration of La^{3+} in the cuvette was below 20mM (Table 1). Second, the concentration of La^{3+} remaining in the reaction medium was estimated by precipitation tests. It was noticed that when the reaction medium initially contained standard $LaCl_3$ between 1 and 30 mM, a transient white colloid of lanthanum tetrapyrophosphate appeared when pyrophosphate buffer was added to the cuvette. The colloid disappeared quickly after shaking for a few seconds. However, no such colloid was seen in the cuvette when a tissue extract was added to the cuvette. This suggested that La^{3+} remaining in the reaction medium was less than 1mM. Furthermore, two reaction time courses with 100 nmoles standard GABA per cuvette which have been treated with or without (control) this procedure were compared. No significant difference between control and the La^{3+}-treated sample was

* Some data from this section have been published (Zhang and Bown, 1997).

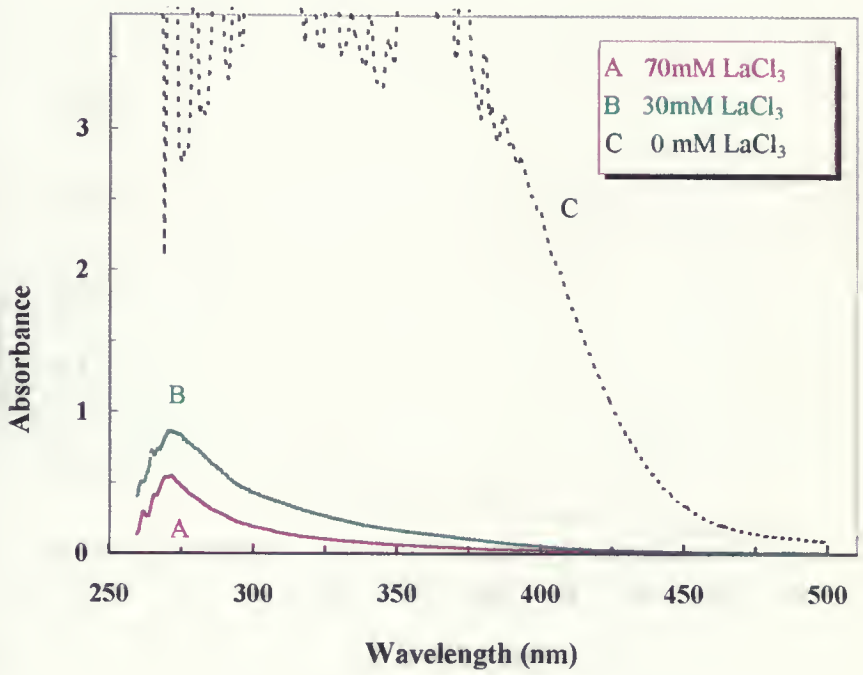
observed (Fig. 11). Therefore, the concentration of $LaCl_3$ in the reaction medium introduced from the tissue extracts did not markedly inhibit Gabase activity or interfere with the assay.

The necessity for methanol treatment of the samples To ensure that GABA synthesis does not occur during sampling and homogenization, which can cause increases in the apparent *in vivo* GABA levels, frozen leaf powders were immediately treated with methanol, 30 mM HCl (60°C) or 70 mM $LaCl_3$ (60°C) for 10 minutes. Subsequent GABA determination indicated that there were much higher GABA levels (19 or 41-fold) in the samples treated with HCl and hot $LaCl_3$ compared with methanol-treated samples (Fig. 12). This suggests that it is necessary for methanol treatment of the samples before further extraction into aqueous solution to prevent GABA production.

The influence of leaf powder temperature on GABA values It is not clear that liquid N_2 treatment and grinding prevents GABA accumulation in defrozen leaf powders. Frozen leaf powders were left on ice or at room temperature (25°C). Methanol was then added at the time indicated in Fig.13. At the beginning, the GABA level was 7 nmol/gfw. Five minutes later, it dramatically increased 4.9 -fold at 0 °C and 303-fold at 25 °C. As the storage duration increased, more GABA was produced in the homogenates (Fig. 13). Thus, immediate addition of methanol to frozen leaf powders is required to inhibit GABA synthesis.

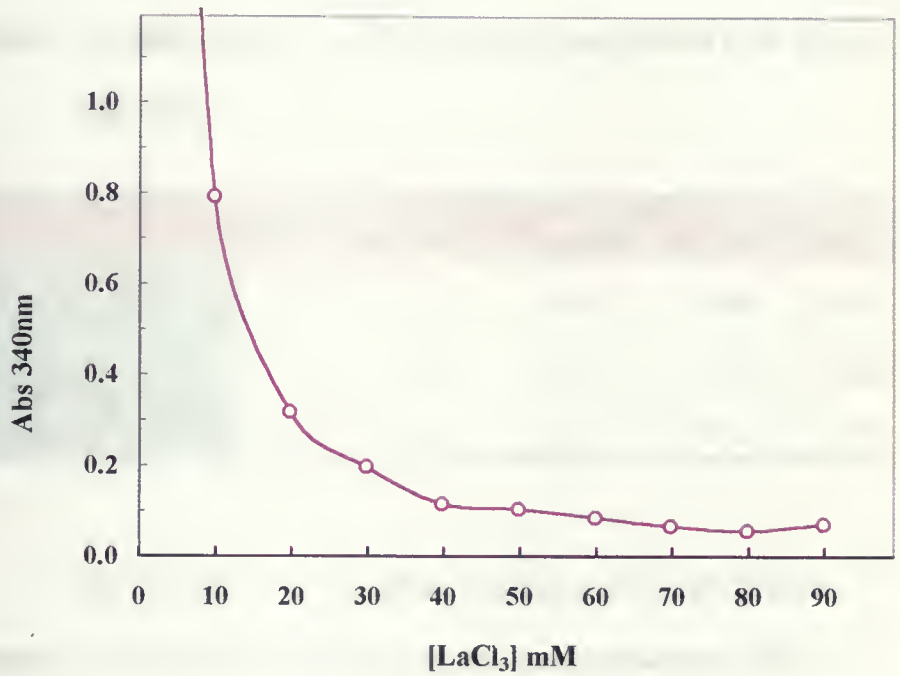
GABA recovery It was seen that mixing of $LaCl_3$ and GABA at the concentrations employed did not form any precipitate in the aqueous solution, so GABA remained in the aqueous phase when $LaCl_3$ was introduced into this procedure. To estimate the recovery of GABA with this method, standard GABA was added into tissue samples. The difference in the amount of GABA between control (without GABA addition) and experimental groups indicated $100.2 \pm 2.5\%$ recovery of GABA (Table 2). Thus, this method did not result in a significant loss of GABA.

Fig.9 Removal of pigments by $LaCl_3$ from tissue extracts prior to GABA determination.



0.1g of frozen soybean leaf powder was transferred into 400 µl methanol. The resulting homogenate was dried and resuspended in 1ml $LaCl_3$ solution at the indicated concentrations. After centrifugation for 5 minutes at 13,600g, 800 µl of the supernatant was taken and mixed with 160 µl KOH (with molarity adjusted to give a stoichiometric amount to La^{3+} employed). The resulting pellet was removed by centrifugation as above, and 550 µl of the supernatant was added into the GABA assay mixture for scanning from 260 to 500nm using a Beckman DU-7400.

Fig.10 Absorbances at 340 nm of tissue extracts treated with various concentrations of $LaCl_3$.



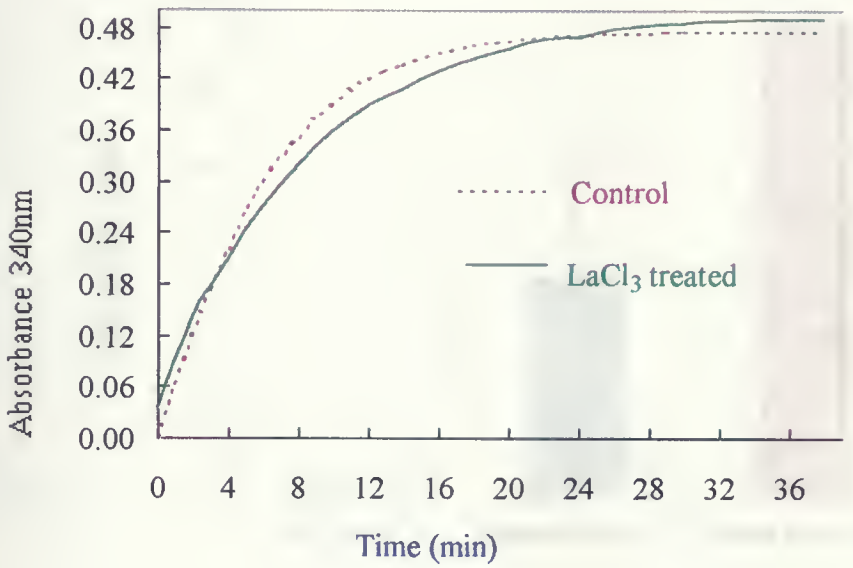
0.1g of frozen soybean leaf powder was transferred into 400 µl methanol. The resulting homogenate was dried and then resuspended in 1ml $LaCl_3$ solution at the indicated concentrations. After centrifugation for 5 minutes at 13,600g, 800 µl of the supernatant was taken and mixed with 160 µl KOH (with molarity adjusted to give a stoichiometric amount to La^{3+}). The resulting pellet was removed by centrifugation as above, and 550 µl of the supernatant was added into the GABA assay mixture for absorbances measurements at 340nm using a Beckman DU-7400.

Table 1. The effect of Lanthanum Chloride on Gabase activities in the GABA assay system.

$[La^{3+}]$ (mM)		0	1.0	10.0	20.0	30.0
Gabase activity	Δabs/min	0.109	0.103	0.101	0.099	0.079
	SE	0.005	0.003	0.004	0.004	0.004
	%	100	94.5	92.7	90.8	72.5

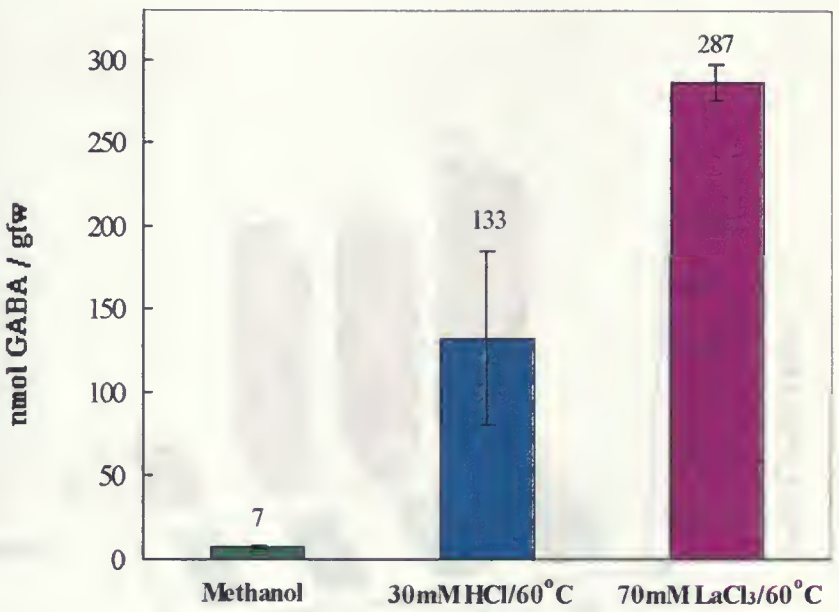
The 1.0 ml Gabase assay system consisted of 200 µl of 0.5M tetra-potassium pyrophosphate buffer (pH 8.6), 6µl β-mercaptoethanol, 250µl of 20mM α-ketoglutarate, 125µl of 10mM $NADP^+$, 100µl of 60mM GABA, 10 µl of 2 units Gabase /ml, 0 - 150µl of 200mM $LaCl_3$ and 310 - 160 µl H_2O according to the volume of $LaCl_3$ employed. The reaction was started by adding 10 µl of Gabase solution. Absorbances at 340 nm were recorded as a function of time using a Beckman DU-50 spectrophotometer.

Fig. 11 Time courses of the Gabase- mediated reaction for GABA determination in samples treated with or without 70mM $LaCl_3$.



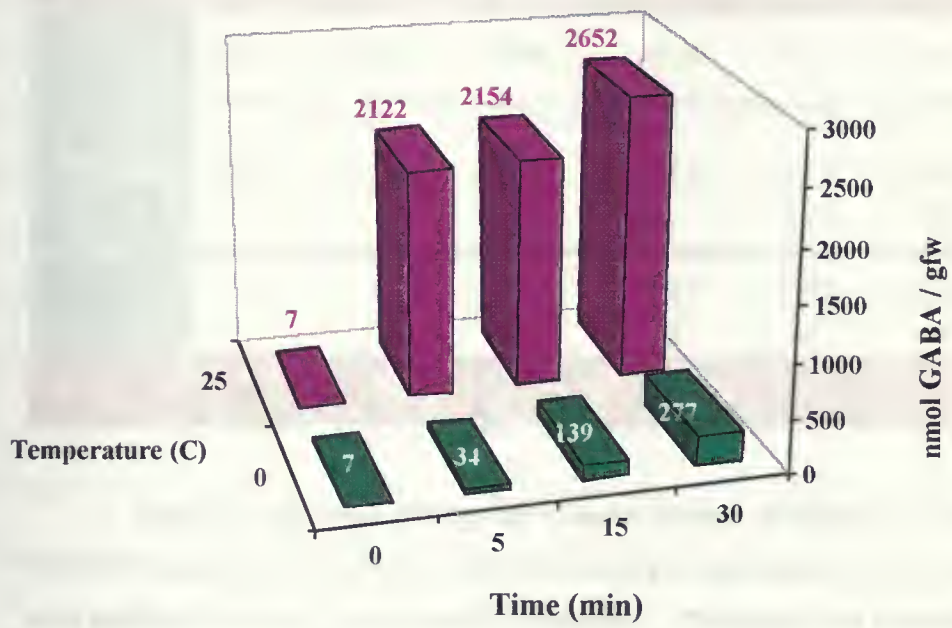
For $LaCl_3$ -treated samples, 520 µl of 1 mM standard GABA solution was mixed with 280 µl of 200 mM $LaCl_3$ to give a 70mM $LaCl_3$ solution. Then 160 µl of 1M KOH was added and the samples were shaken for 5 minutes. After centrifugation, 185 µl of the resulting supernatant containing 100 nmoles GABA and 358 µl of H_2O were added to a cuvette containing the GABA assay mixture (Methods). Absorbances at 340 nm were recorded as a function of time using a Beckman DU-50 spectrophotometer.

Fig. 12 Inactivation of GAD prior to GABA determination.



Frozen soybean leaf powders obtained by grinding in liquid nitrogen were treated immediately with methanol, 30 mM HCl (60°C) or 70 mM $LaCl_3$ (60°C) for 10 minutes. Methanol treated samples were dried and then resuspended in 1.0ml of 70 mM $LaCl_3$ (25 °C). GABA was then determined for all 3 treatments (Methods). The data indicated the means of 3 repeats with standard errors.

Fig. 13 The influence of storage duration and temperature on GABA levels in leaf powder.



Frozen soybean leaf powders were placed on ice or at room temperature (25°C). Methanol was added to inactivate GAD at the times indicated. Samples were then dried. GABA was determined following the procedures (Methods). The data indicate the means of 3 repeats.

Table 2. Recovery of GABA added to frozen leaf powder prior to GABA determination using the $LaCl_3$ method.

	Control			Experimental		
	A	B	C	A	B	C
Chl (μg)	256.3	281.6	345.4	231.4	294.5	264.7
GABA(nmol)						
Measured	70.3	81.6	107.4	191.4	246.5	192.7
Calculated				67.4	85.7	77.0
Difference				124.0	160.8	115.8
Added				120	160	120
Recovery (%)				103.4	100.5	96.5

Standard GABA was added to experimental samples of soybean leaf powder. No GABA was added to controls. GABA extraction and determination in these samples were performed with procedures described (Methods). Chlorophyll was extracted with 1.0 ml of 80% acetone from the green pellet resulting from the first centrifugation of the tissue homogenate in 70 mM $LaCl_3$. The average value of GABA in leaves was 0.29 nmol per mg chlorophyll calculated from the data in control group. This value was then used to calculate the *in vivo* amount of GABA (Calculated) in the experimental samples. The differences between the measured amount and the calculated *in vivo* amount of GABA in the experimental samples were used to calculate the percentages of recovery.

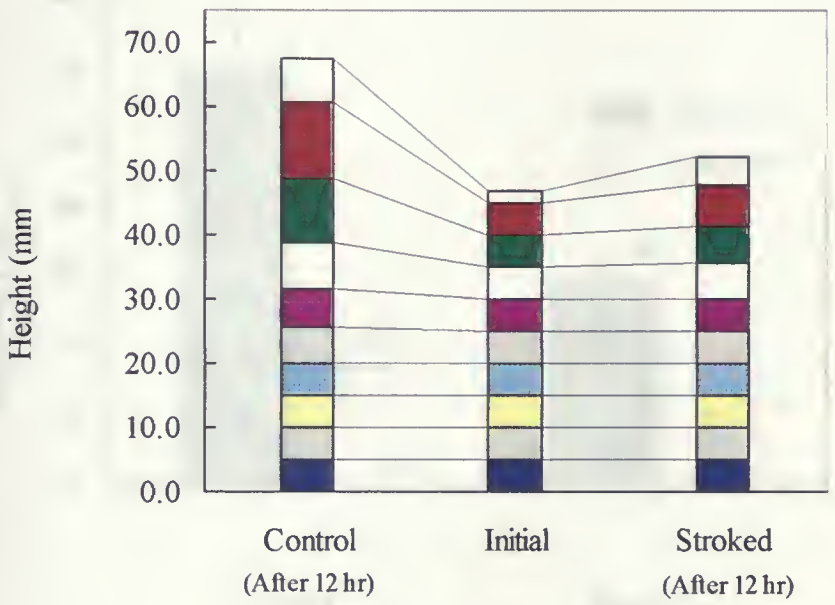
B. The relationship between GABA levels and hypocotyl elongation in soybean seedlings.

Localization of hypocotyl elongation in soybean seedlings To determine which part of a hypocotyl should be chosen as the site for application of mechanical stimulus or chemicals, hypocotyl elongation was investigated in etiolated seedlings. The top 17 mm of a 47-mm-high hypocotyl contributed 92.7% (19.1mm) of the overall increment of 20.6 mm over 12 hours in unstroked seedlings (control). In stroked hypocotyls, however, this segment elongated only 5.2mm compared to the 19.1 mm increment in control (Fig. 14). The data indicate that this segment elongated rapidly and was sensitive to mechanical stimulation. Thus, all treatments in further experiments were performed on the 2cm segment below the apical hook, and this segment was used for GABA and glutamate analysis.

Inhibition of hypocotyl elongation induced by mechanical stimulation To establish an experimental approach for investigating the relationships between growth and *in vivo* GABA levels, the hypocotyls were given 20 strokes at the segment mentioned above. In both dark-grown and light-grown seedlings, the mechanical stimulation of stroking caused a 50% and 60% inhibition of elongation over 24 hours, respectively (Fig. 15). It was also observed that elongation rates declined within 1 minute following stroking from 1.18 to 0.26 mm/h in dark-grown hypocotyls and from 0.23 to 0.082 mm/h in light-grown hypocotyls (Fig. 16). The inhibition lasted at least 26 hours in dark-grown hypocotyls (Fig. 17). These results demonstrated that mechanical stimulation rapidly and significantly inhibited hypocotyl elongation in soybean seedlings.

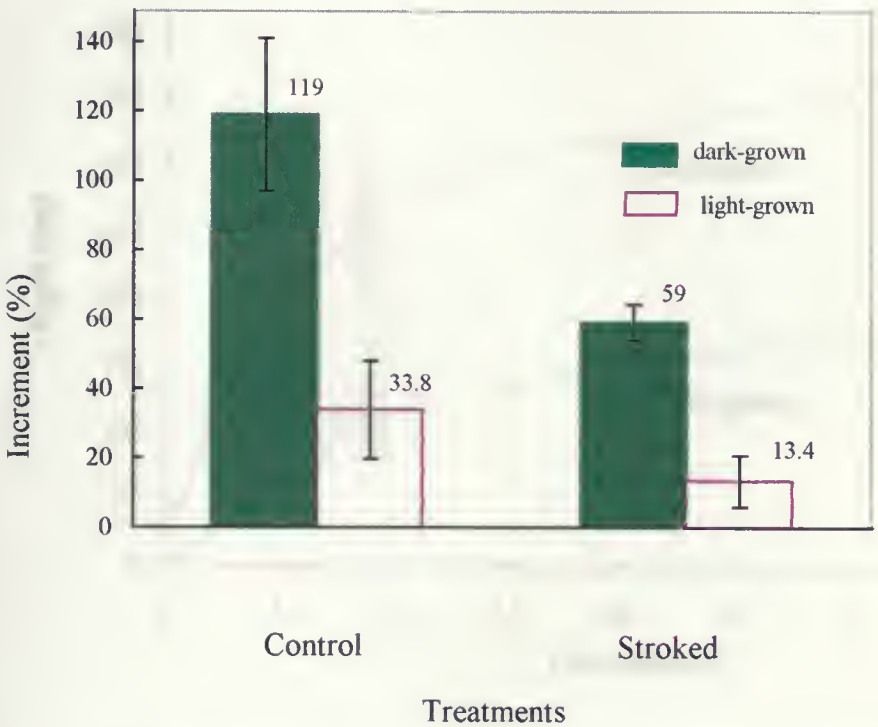
Elevation of *in vivo* GABA levels triggered by mechanical stimulation To analyze changes in *in vivo* GABA levels in elongation-inhibited seedlings induced by mechanical stimulation, time courses of *in vivo* GABA levels in stroked hypocotyls were investigated. In both dark-grown and light-grown seedlings, stroking raised *in vivo* GABA levels within 4 minutes. Eight minutes after stroking, GABA levels reached maxim of 367 and 732 nmol/gfw which are 4.3-fold and 10.2-fold greater than those observed before stroking in dark-grown and light-grown seedlings, respectively (Fig. 18). These data demonstrated that the mechanical stimulation of stroking triggered a rapid and large accumulation of GABA in soybean seedlings, while it also induced an inhibition of hypocotyl elongation.

Fig. 14 Localization of elongation inhibition by stroking in the hypocotyls of dark-grown soybean seedlings



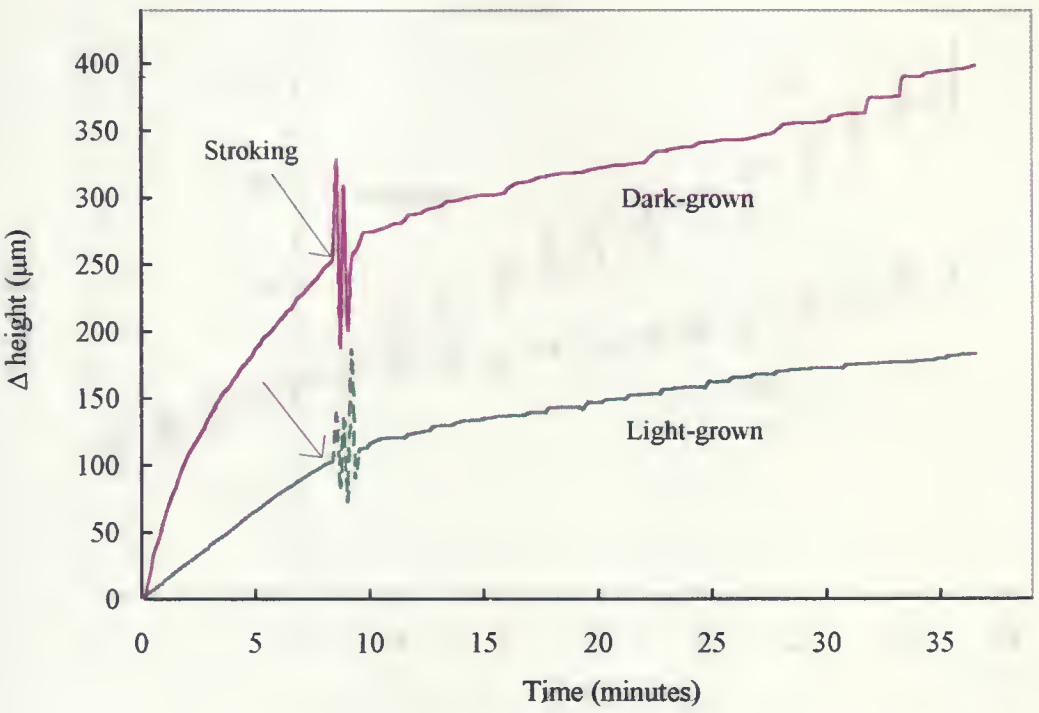
Dark-grown hypocotyls with an average height of 47mm were given 20 strokes over the 2 cm below the apical hook. Then control and stroked hypocotyls were marked with Sudan 3 dye using a water color brush. The first mark from the apical hook was made 2 mm below, and the following marks were made at the intervals of 5 mm from the first one. 12 hours after stroking, the length of each segment was measured. All manipulations were performed in green light. The mean values of elongation were obtained from 4 seedlings.

Fig. 15 Inhibition of soybean hypocotyl elongation by mechanical stimulation.



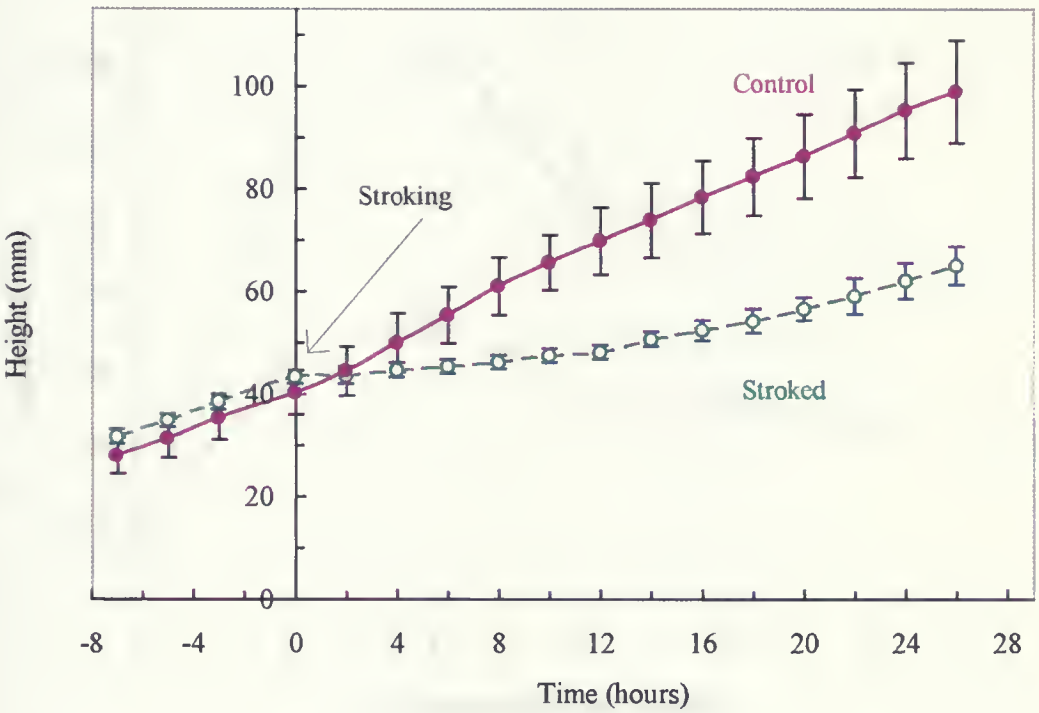
Dark-grown or light-grown hypocotyls were given 20 strokes over the 2 cm below the apical hook. No stroke was given to control seedlings. Before and 24 hours after stroking, the height of each seedling was measured. All manipulations were performed in green light. The mean values and standard errors (SE) are indicated for dark-grown plants (n=9) and light-grown plants (n=14).

Fig. 16 Rapid inhibition of hypocotyl elongation induced by mechanical stimulation of dark-grown and light-grown soybean seedlings.



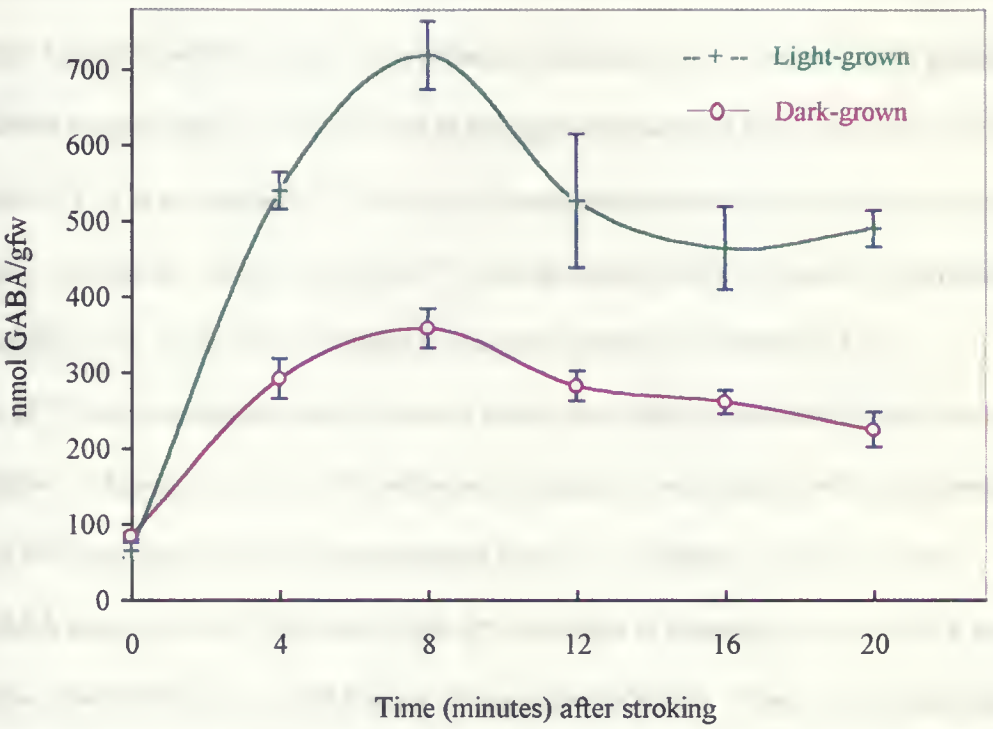
Dark-grown or light-grown hypocotyls were given 20 strokes within 20 seconds over the 2 cm below the apical hook at the time indicated. Elongation was monitored by a transducer as described (Methods).

Fig. 17 Inhibition of hypocotyl elongation induced by mechanical stimulation of dark-grown soybean seedlings.



The heights of dark-grown soybean seedlings were measured every 2 hours before and after stroking. The stroked hypocotyls received 20 strokes within 20 seconds over the 2 cm below the apical hook. No stroke was given to the hypocotyls of control seedlings. All manipulations were performed in green light. The average heights with SE shown derives from 3 seedlings

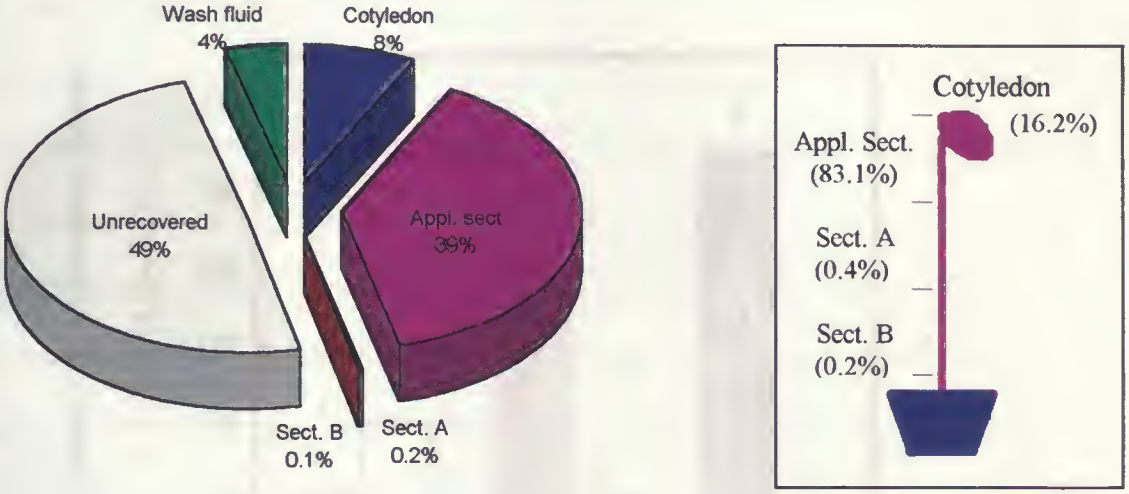
Fig. 18 GABA accumulation in light-grown and dark-grown soybean hypocotyls induced by mechanical stimulation.



Twenty strokes (Methods) were given within 20 seconds over the 2 cm segment below the apical hook. The seedlings were taken at the time indicated and immediately placed in liquid nitrogen. The 2 cm long segments below the apical hook were excised from the frozen seedlings for GABA assay. 3 and 8 repeats were done in light-grown and dark-grown seedlings, respectively. The error bars represent standard errors (SE).

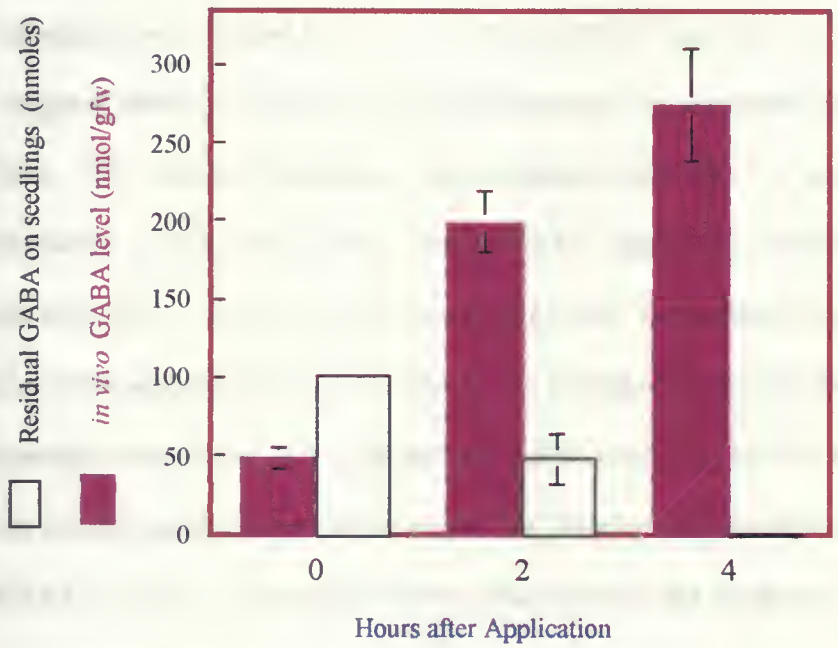
The effect of exogenously applied GABA on *in vivo* GABA levels and hypocotyl elongation To examine the hypothesis that GABA accumulation may cause the inhibition of hypocotyl elongation associated with mechanical stimulation, exogenous GABA was applied to soybean hypocotyls. Ten μl of 10.0 mM GABA did not significantly affect hypocotyl elongation in light-grown and dark-grown seedlings within 2 hours or within 24 hours of application (data not shown). Neither did the application of 10 μl of GABA ranging from 0.25 to 10.0 mM to dark-grown hypocotyls (data not shown). When 10 mM ^{14}C-GABA (0.1 μCi) was applied, 95.7% of applied radioactivity was absorbed by the hypocotyls within 4 hours of application. Half of the applied ^{14}C was recovered from the hypocotyls, cotyledons and wash fluid (Fig. 19). Another half was not recovered due possibly to respiratory loss or incorporation of ^{14}C into proteins and other molecules which were either precipitated by $LaCl_3$ or were not water-soluble. In hypocotyls, 83% of the recovered radioactivity was retained within the treated segments, and 16% was transported into the cotyledons (Fig. 19). In addition, 10 μl of 10 mM exogenous GABA raised *in vivo* GABA levels from 48.2 nmol/gfw in untreated tissues to 199.8 and 274.3 nmol/gfw in treated tissues at 2 and 4 hours of application (Fig. 20). These data demonstrate that exogenous GABA entered the hypocotyl tissues and elevated *in vivo* GABA levels, but failed to inhibit elongation of the hypocotyls.

Fig. 19 Absorption and distribution of ^{14}C-GABA in dark-grown soybean seedlings



Ten µl of 2.2 $\times 10^4$ DPM ^{14}C-GABA /µl was applied in 6 - 8 droplets to the 2 cm long segment below the apical hook. Four hours later, no droplet on the hypocotyl surface was seen. The seedlings were washed gently with 15ml of distilled water, then wrapped with foil and placed immediately in liquid nitrogen. Each frozen hypocotyl was cut into three sections of 2cm long. Radioactivity in the cotyledon, each section of hypocotyls and in the wash fluid was measured (Methods). The data in the pie graph represent the percentage of radioactivity in each portion to the total applied radioactivity. The data in the inset represent the distribution percentage of radioactivity in each section to the recovered radioactivity within the hypocotyls. The mean values were obtained from three seedlings.

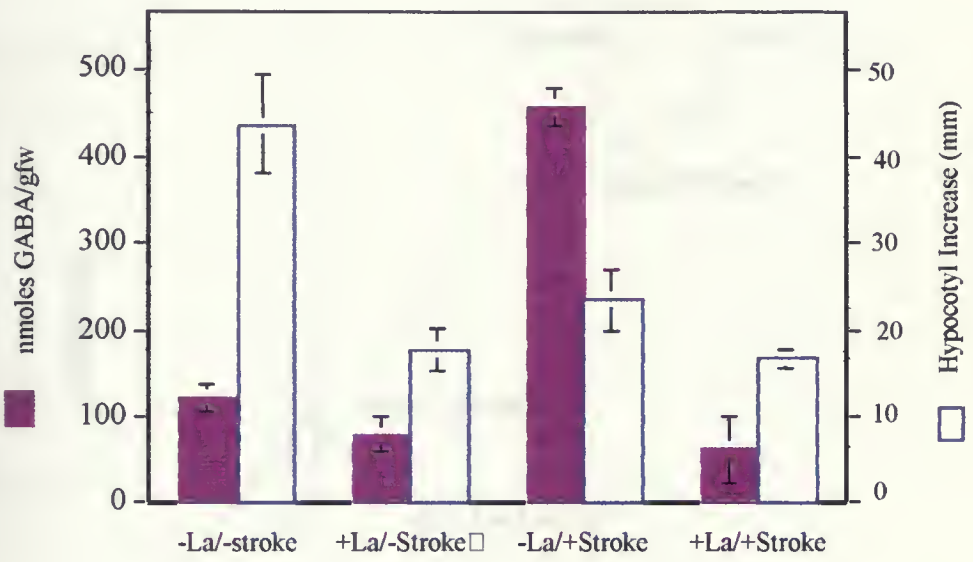
Fig.20 Exogenously applied GABA raised *in vivo* GABA levels in dark-grown soybean seedlings



Six to 8 droplets of 10µl of 10mM GABA solution (100nmoles) were applied to the 1 cm long segment below the apical hook of 3-day old dark-grown soybean seedlings. At the times indicated, the treated hypocotyls were rinsed gently with 20ml of distilled water and wiped with a tissue. Then the seedlings were immediately placed in liquid nitrogen for GABA extraction and measurement (Methods). GABA concentrations in the 20ml rinsing fluid were also assayed. The mean GABA levels with standard errors were obtained from 3 seedlings.

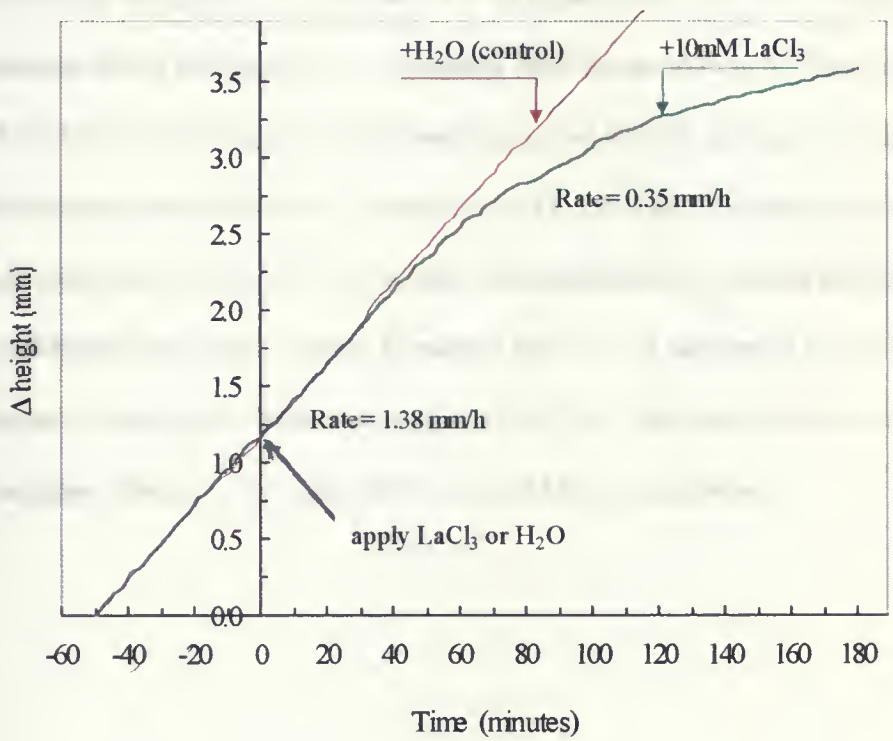
The effect of La^{3+} on *in vivo* GABA levels and hypocotyl elongation La^{3+} is a blocker of plasma membrane Ca^{2+} channels in both animal cells and plant cells. We therefore investigated the influence of La^{3+} on GABA production and hypocotyl elongation in response to the mechanical stimulation of stroking. Ten µl of 10 mM $LaCl_3$ was applied to dark-grown soybean seedlings. Hypocotyl elongation was inhibited 59.5% within 24 hours of La^{3+} application (Fig. 21). To determine when La^{3+} triggered inhibition, elongation rates of the hypocotyls were recorded using the transducer. It was found that a 75% decline in the elongation rate occurred 2 hours after La^{3+} application (Fig. 22). In further experiments, stroking was perfomed 2 hours after La^{3+} application. Hypocotyls were harvested 8 minutes after stroking for measurement of *in vivo* GABA levels. In unstroked hypocotyls, La^{3+} inhibited elongation by 59.5% and slightly reduced GABA levels. In stroked hypocotyls, however, La^{3+} further reduced hypocotyl elongation by 28.6% and inhibited GABA production by 86.4% as well (Fig 21). These results strongly suggest that in stroked tissues, La^{3+} blocked Ca^{2+} channels and then down-regulated GABA production. Associated with this reduction in GABA levels was a reduction in elongation. Obviously, a reduction in elongation is not associated with an increase in GABA levels and *vice versa*. These data do not support the hypothesis being tested.

Fig. 21 La^{3+} inhibited growth, and blocked GABA accumulation induced by mechanical stimulation in dark-grown soybean seedlings.



Ten µl of 10mM $LaCl_3$ (+La) or distilled water (-La)was applied over the 2 cm of hypocotyl below the apical hook. 2 hours later, 20 strokes were given to this site(+stroke). The (-stroke) plants were not stroked. Seedlings were placed in liquid nitrogen 8 minutes after stroking. The 2cm long hypocotyl segments below the apical hook were excised from the frozen seedlings and used for GABA assay (n=3). Hypocotyl elongation was measured 24 hours subsequent to stroking(n=7). The error bars represent standard errors.

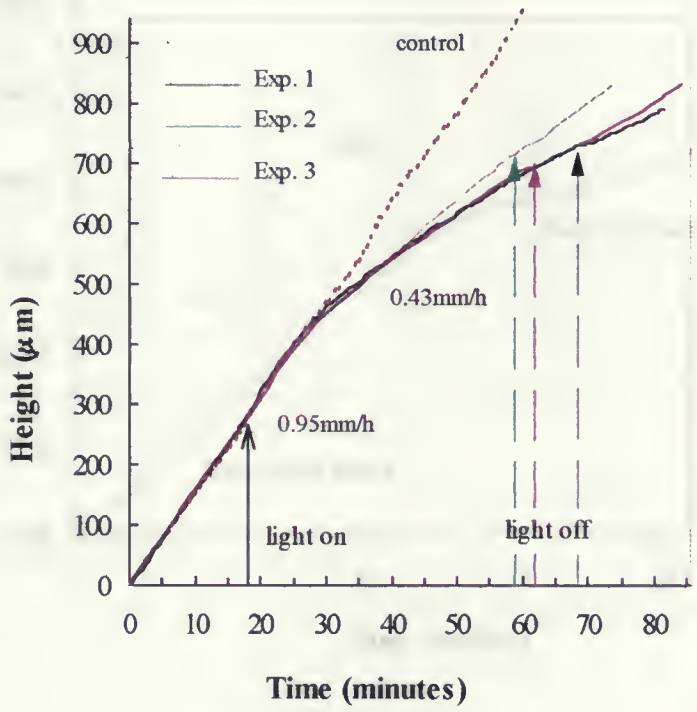
Fig. 22 Inhibition of hypocotyl elongation by $LaCl_3$ in a dark-grown soybean seedling.



A dark-grown hypocotyl was given 10 µl of 10 mM $LaCl_3$ or H_2O (control). Elongation was monitored by a transducer (Methods). The experiment was conducted in green light.

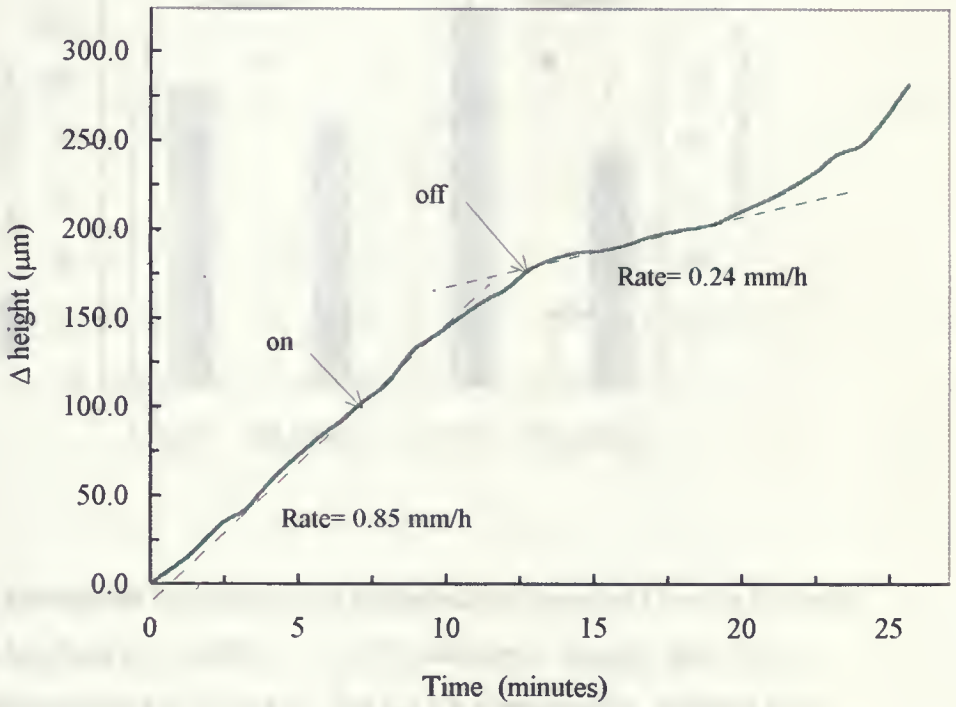
The influence of blue light on hypocotyl elongation and *in vivo* GABA levels In a further attempt to investigate the relationship between *in vivo* GABA levels and the inhibition of hypocotyl elongation, blue light irradiation of dark-grown hypocotyls was employed. Two experiments with continuous irradiation or a pulse of irradiation were carried out. (1) With continuous irradiation, an inhibition of hypocotyl elongation was seen about 5 to 10 minutes after blue light irradiation began. The inhibition continued during the illumination. Elongation rates decreased from 0.95 mm/hr to 0.46 mm/hr (Fig 23). GABA levels in irradiated hypocotyls were monitored after 30 minutes of irradiation and showed a 13% decline compared to control levels (Fig 25.A). (2) With a 5 minute pulse of blue light irradiation, hypocotyl elongation inhibition was observed approximately 5 minutes after the pulse started. The inhibition lasted approximately 10 minutes (Fig. 24). A decrease in *in vivo* GABA levels was also observed 5 minutes after irradiation stopped (Fig.25.B). These data indicate that inhibition of hypocotyl elongation induced by blue light did not cause GABA accumulation.

Fig. 23. Inhibition of hypocotyl elongation induced by continuous irradiation of blue light in dark-grown soybean seedlings.



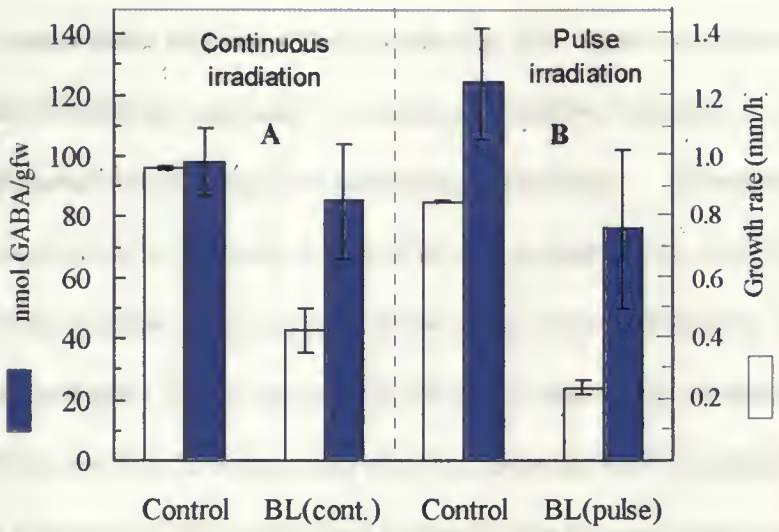
Three dark-grown hypocotyls were irradiated continuously with blue light (340 - 480 nm, 350-450 μmol/s/m^2) after the recording trace showed a constant elongation rate. Elongation was monitored by a transducer (Methods). Control seedlings were not irradiated with blue light. The experiment was conducted in green light.

Fig. 24. Inhibition of hypocotyl elongation induced by a pulse irradiation of blue light in dark-grown soybean seedlings.



Three dark-grown hypocotyls were irradiated for 5 minutes with blue light (340 - 480 nm, 350-450 μmol/s/m^2) after the recording trace showed a constant elongation rate. Elongation was monitored by a transducer (Methods). The experiment was conducted in green light.

Fig. 25. Effects of blue light irradiation on GABA levels and hypocotyl elongation rates in dark-grown soybean seedlings.

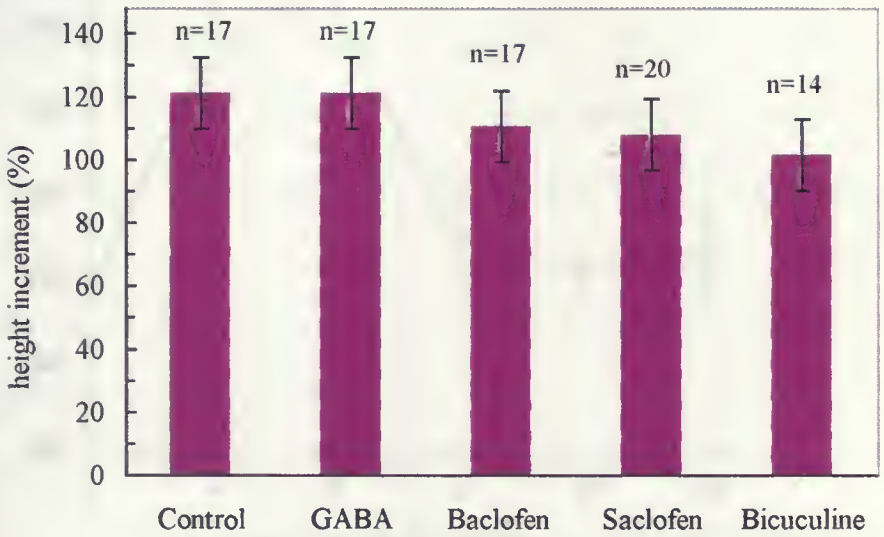


Dark-grown hypocotyls were irradiated continuously (A) or for 5 minute (B) with blue light (340 - 480 nm, 350-450 µmol/s/m^2). Growth rates of the hypocotyls are from Fig. 23 and 24. For GABA measurements, seedlings were harvested and placed into liquid nitrogen after 30 minutes of illumination in the continuous irradiation group or 5 minutes after illumination stopped in the pulse irradiation group, respectively. The mean values with standard errors were obtained from 3 replicates.

Effects of the agonists and an antagonist of GABA on hypocotyl elongation Ten µl of 10 mM of two GABA agonists (Baclofen, Bicuculine) and an antagonist (Saclofen) were applied to investigate their effect on soybean hypocotyl elongation. No significant difference was found between control and a treated tissue with any of these agents (Fig. 26). These experiments do not support the existence of GABA binding components in plant tissues capable of regulating growth.

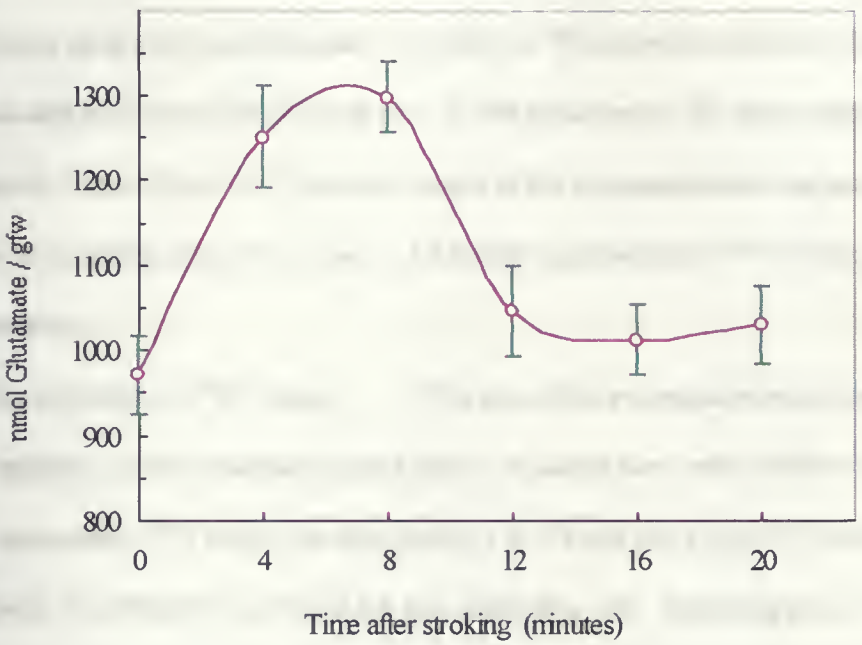
Changes in glutamate levels induced by mechanical stimulation Elongation inhibition in the stroked hypocotyls could be due to the decline of *in vivo* glutamate levels when GABA accumulates, since GABA is derived from glutamate by decarboxylation (Literature review). The changes in glutamate levels within the first 20 minutes (Fig.27) and 24 hours (data not shown) after stroking were monitored. Within the first 20 minutes after stroking, glutamate levels increased from 1276 nmol/gfw and reached a maximum of 1945 nmol/gfw at 8 minutes, then decreased close to the initial level by 12 minutes (Fig. 27). In addition, application of 10 µl of 10 mM glutamate to stroked hypocotyls did not restore elongation and did not enhance the elongation either in unstroked hypocotyls (data not shown). These results indicate that glutamate was not responsible for the inhibition of elongation induced by mechanical stimulation.

Fig. 26. Effects of agonists and an antagonist of GABA on soybean hypocotyl elongation.



Ten μl of each tested drug was applied to the 2 cm long segment below the hook of a seedling (3-day old). The initial height of a seedling and its final height 24 hours after application were measured.

Fig. 27 Changes in glutamate levels in dark-grown soybean hypocotyls induced by mechanical stimulation.



Twenty strokes were given over the 2 cm below the apical hook within 20 seconds (Methods). The seedlings were taken at the times indicated and immediately placed in liquid nitrogen. The 2 cm long segments below the apical hook were excised from frozen seedlings for glutamate assay. The mean values are derived from 6 seedlings. The error bars represent standard errors (SE).

C. The influence of GABA on chloride flux in isolated asparagus cells

1. Influx of chloride

pH and time courses of $^{36}Cl^-$ influx To optimize the influx of $^{36}Cl^-$, the effect of pH on $^{36}Cl^-$ influx was investigated at pH5.5, 6.5 and 7.5. After 1.0, 2.0 and 3.0 hours of incubation, radioactivity of $^{36}Cl^-$ taken up by cells was measured. The influx of $^{36}Cl^-$ increased within the first 2 hours at the three pH values and then leveled off except at pH 5.5. No difference in $^{36}Cl^-$ influx was seen among the three pH values at 3 hours (Fig. 28). Therefore, the pH of the incubation media was set to pH 7.5, and the duration of incubation was set to 3 hours. All further experiments for $^{36}Cl^-$ influx were performed under these conditions.

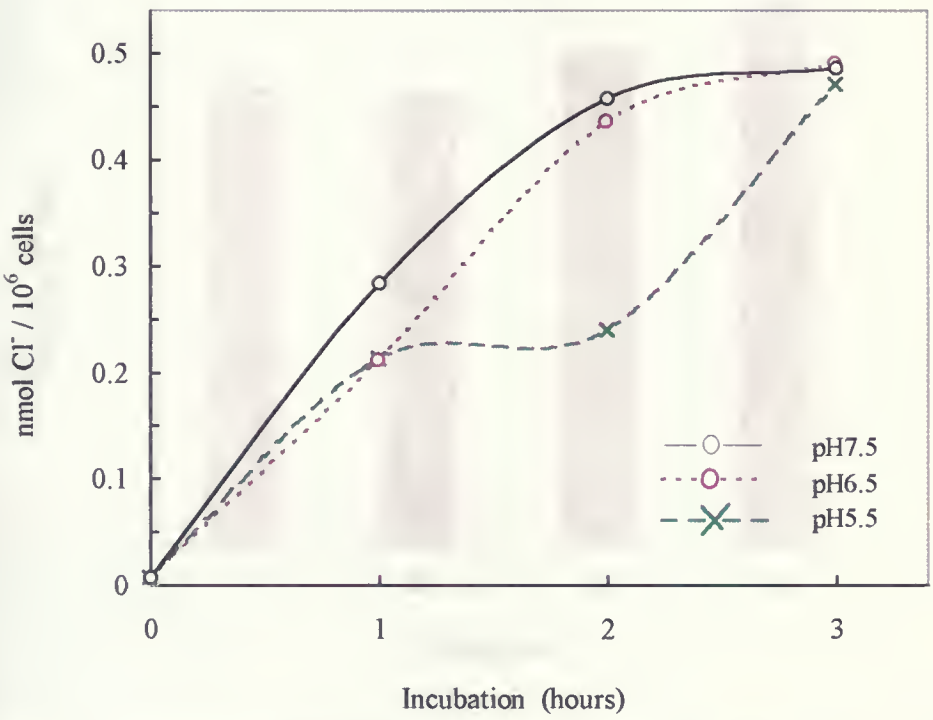
The effect of GABA on $^{36}Cl^-$ influx The aim of this investigation was to see whether or not GABA regulates chloride channels in plant cells as in animal nerve cells. Different concentrations of GABA were tested. $^{36}Cl^-$ influx was enhanced to 118.2% and 107.4% by 10.0 mM and 1.0 mM GABA, respectively. No effect of 0.1 mM GABA was found (Fig. 29). According to an F-test and the posteriori comparisons of means with Duncan's method, $^{36}Cl^-$ influx in 10.0 mM GABA treatment significantly exceeded the control level. The difference was statistically significant at the 5% level. But the influx in 1.0 mM GABA treatment did not differ significantly from the control group (Table 3-A and 3-B). In addition, in order to eliminate the possibility of attributing the observed difference of $^{36}Cl^-$ influx to the errors in dispensing $^{36}Cl^-$ solution, the total CPM of $^{36}Cl^-$ in each incubation medium was measured. The overall standard error of radioactivity among 24 incubation media was 2.08%, which means that the error in dispensing $^{36}Cl^-$ solution was not responsible for the significant difference in $^{36}Cl^-$ influx between control and the treatments. Since 10.0 mM GABA was required for any statistically significant effect, GABA might slightly and/or indirectly regulate chloride channels in plant cells.

Specificity of GABA effect on $^{36}Cl^-$ influx To determine if GABA enhanced $^{36}Cl^-$ influx is specific, amino acids (glutamate, aspartic, lysine and alanine), a GABA analog (α-isoaminobutyric acid

or AIBA), an agonist (Baclofen) and an antagonist (Saclofen) of $GABA_B$ receptors were tested. GABA, glutamate and alanine also significantly enhanced $^{36}Cl^-$ influx over control levels. Baclofen also slightly enhanced $^{36}Cl^-$ influx. On the other hand, AIBA, aspartic, lysine and Saclofen reduced $^{36}Cl^-$ influx (Fig. 30). The data show that GABA was not a specific agent for stimulating chloride influx in plant cells. Interestingly, saclofen significantly blocked $^{36}Cl^-$ influx (Fig. 30), the reason for which is unclear.

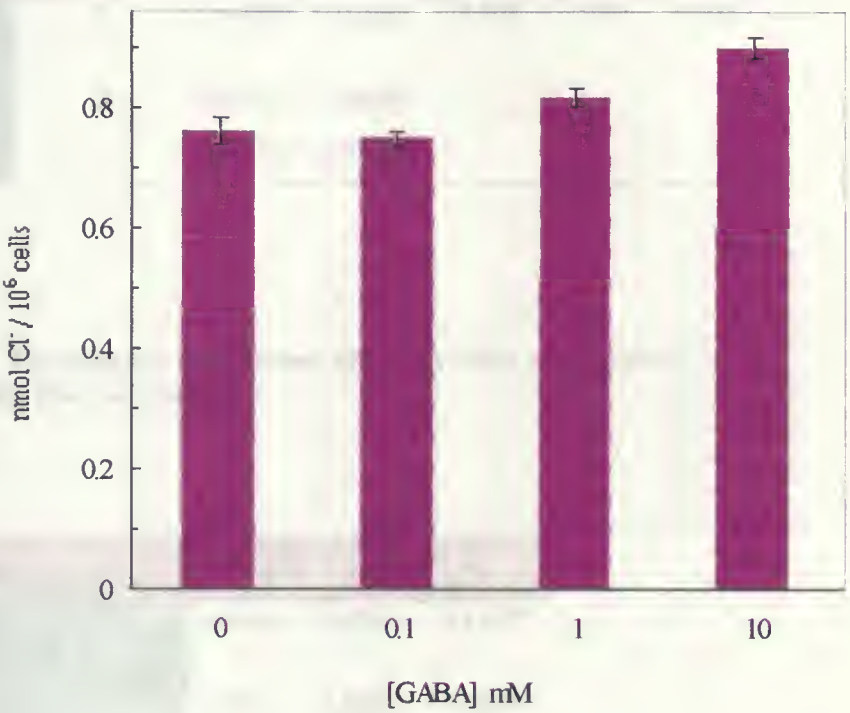
Blocking of GABA-enhanced $^{36}Cl^-$ influx by NPPB To determine whether or not GABA stimulated chloride influx through anion channels, NPPB, a blocker of anion channels in plants and animals (Cho and Spalding, 1996; Keeling *et al.*, 1991) was applied. With increases in NPPB concentration, chloride influx was reduced (Fig. 31). Meanwhile, the rates of cell damage with NPPB were examined, which showed that cell damage did not significantly increase over 6 hours if NPPB concentrations were below 250 μM (Fig. 32). For further experiments, 100 μM NPPB was used which significantly inhibited $^{36}Cl^-$ influx ($p \leq 0.05$). When 10.0 mM GABA and 100 μM NPPB were added simultaneously to the incubation medium, $^{36}Cl^-$ influx level was similar to that with NPPB alone, while $^{36}Cl^-$ influx was enhanced by the treatment with 10.0 mM GABA alone (Fig. 33). These results suggest that GABA may be involved in opening anion channels in plant cells, but its influence with the current experimental protocol is not convincing.

Fig. 28 The time courses of chloride influx in Asparagus cells



The 1.5 ml loading medium consisted of 2 mCi $Na^{36}Cl$/ml (0.5mCi/mmol Cl^-), 5mM K_2SO_4, 5%DMSO. 5mM HEPES (pH7.5) or MES (pH6.5 or 5.5), and 4×10^7 cells/ml. 100µl cell suspension was collected at the times indicated and radioactivity was measured (n=3) (Methods).

Fig. 29 The effect of GABA concentration on chloride influx in *Asparagus* cells.



The 100µl incubation medium consisted of 2 µCi $Na^{36}Cl$/ml (0.5mCi/mmol Cl^-), 5mM K_2SO_4, 5%DMSO. 5mM Hepes (pH7.5), 6.3x 10^7 cells/ml, and the indicated concentration of GABA. After incubation for 3 hours, all cells were collected and radioactivity measured (Methods). The mean value of Cl^- influx and the standard error (SE) were obtained from3 experiments.

Table 3-A. F-test of GABA effect on Cl^- influx in Asparagus cells. †

Error sources	DF	SS	MS	F3,8	F(5%)	SE
Between	3	0.04396	0.01465	7.70*	4.07	0.0252
Within	8	0.01522	0.00190			
Total	11	0.05918	0.00538			

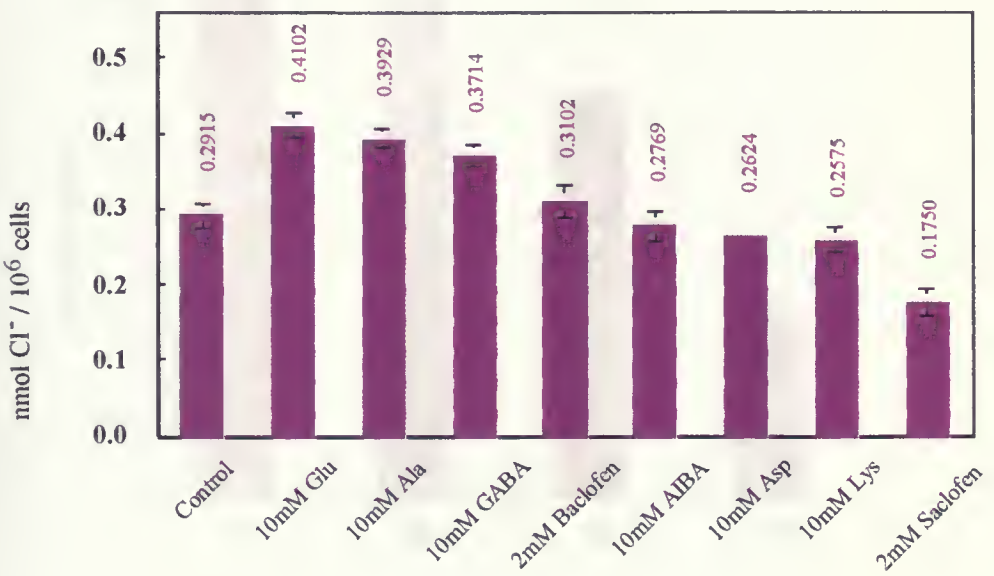
Table 3-B. The posteriori comparisons of GABA effect on Cl^- influx using Duncan's method. †

GABA (mM)		0.1	1.0	10
Differences	Observed	-0.0164	0.0561	0.1385*
from control	Critical (5%)	0.0822	0.0822	0.0857

† The analyses refer to the data in Fig. 29 using 0, 0.1, 1.0 and 10.0 mM GABA .

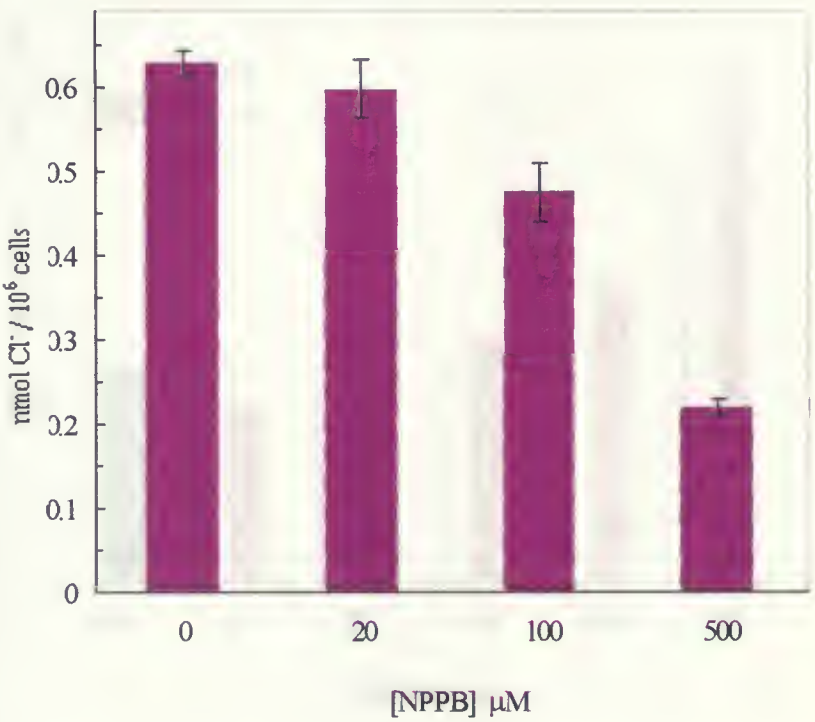
* Indicates a significant difference at $p < 0.05$.

Fig. 30 Specificity of GABA on Cl^- influx in *Asparagus* cells



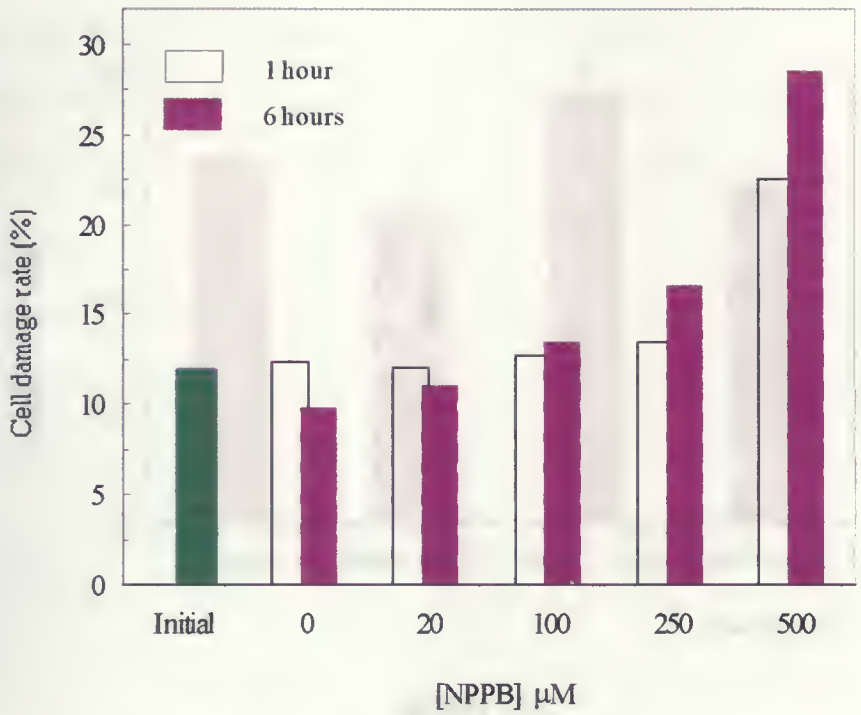
The 150 µl incubation medium contained 2 µCi $Na^{36}Cl$/ml (0.5mCi/ mmol Cl^-), 5mM K_2SO_4, 5%DMSO, 5mM Hepes (pH7.5), 3.8x 10^7 cells/ml, and an indicated concentration of the reagent added. After incubation for 3 hours, all cells were collected and radioactivity measured (Methods). The mean value with a standard error (SE) of Cl^- influx was obtained from 3 repeats.

Fig. 31 The effect of NPPB concentration on chloride influx in *Asparagus* cells.



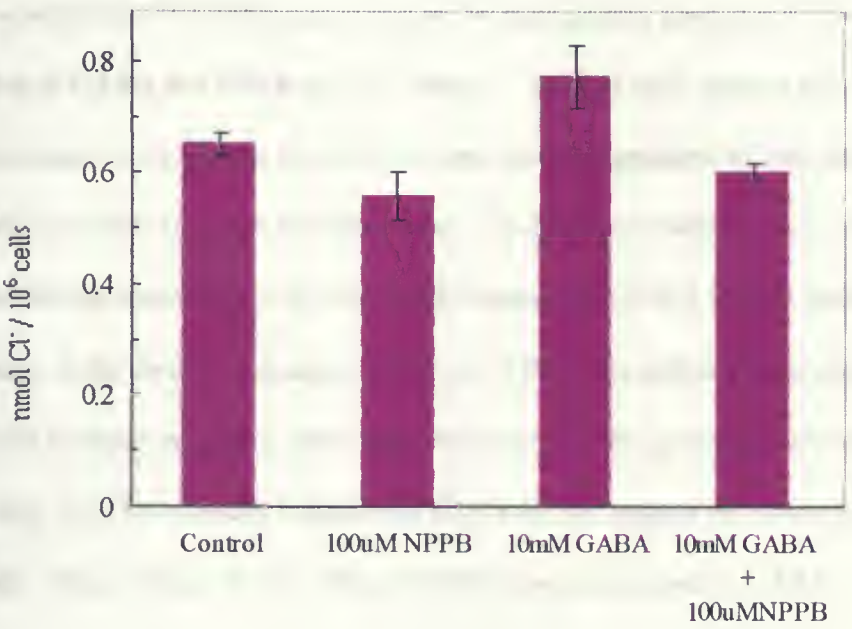
The 100µl incubation medium consisted of 2 µCi $Na^{36}Cl$/ml (0.5mCi/mmol Cl^-), 5mM K_2SO_4, 5mM Hepes (pH7.5), 4.15×10^7 cells/ml, and an indicated concentration of NPPB disolved in absolute DMSO. The final concentration of DMSO was adjusted to 5%. After incubation for 3 hours, all cells were collected and radioactivity measured (Methods). The mean Cl^- influx and the standard error (SE) were obtained from 3 repeats.

Fig. 32 The effect of NPPB on cell damage in *Asparagus* cells.



Cells were mechanically isolated with 1 mM Mes (pH5.5) containing 1 mM $CaSO_4$ (Methods). A volume of 340µl of isolated cells ($4x10^7$/ml) was incubated with 105µl H_2O, 25µl of 100mM Hepes (pH7.5), 25µl of 100mM K_2SO_4 and 5µl of a specified NPPB stock solution in DMSO. At the begining (Initial) and 1 or 6 hours after incubation at 25°C, the damage rate of cells was examined using Evan's Blue (Methods).

Fig. 33 The interaction between GABA and NPPB on chloride influx in *Asparagus* cells.



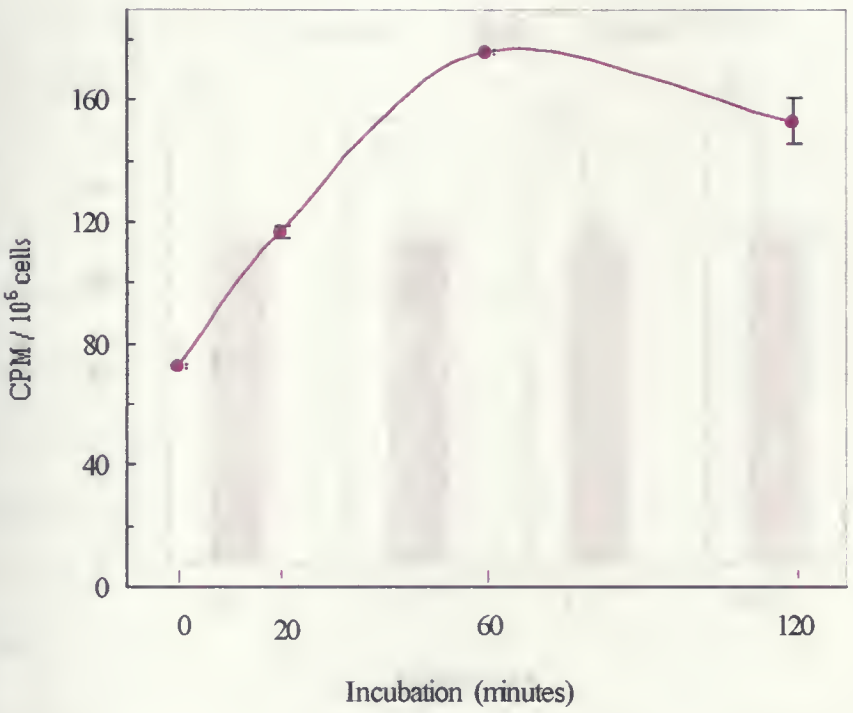
The 100µl incubation medium consisted of 2 µCi $Na^{36}Cl$/ml (0.5mCi/mmol Cl^-), 5mM K_2SO_4, 5mM Hepes (pH7.5), 3 x 10^7 cells/ml, and the indicated concentration of GABA or /and NPPB which was dissolved in absolute DMSO. The final concentration of DMSO was adjusted to 5% in all treatments. After incubation for 3 hours, all cells were collected and radioactivity measured (Methods). The mean values of Cl^- influx and standard errors (SE) were obtained from 3 experiments.

2. Efflux of chloride

Time course of chloride efflux To obtain a suitable duration of chloride efflux from the $^{36}Cl^-$-loaded cells, the time course of $^{36}Cl^-$ efflux was investigated. Efflux was initiated by placing $^{36}Cl^-$-loaded cells in Cl^--free buffer at pH7.5. $^{36}Cl^-$ was released from the cells during the first 60 minutes(Fig. 34). In further experiments, $^{36}Cl^-$ efflux was monitored 60 minutes after placing in Cl^--free medium.

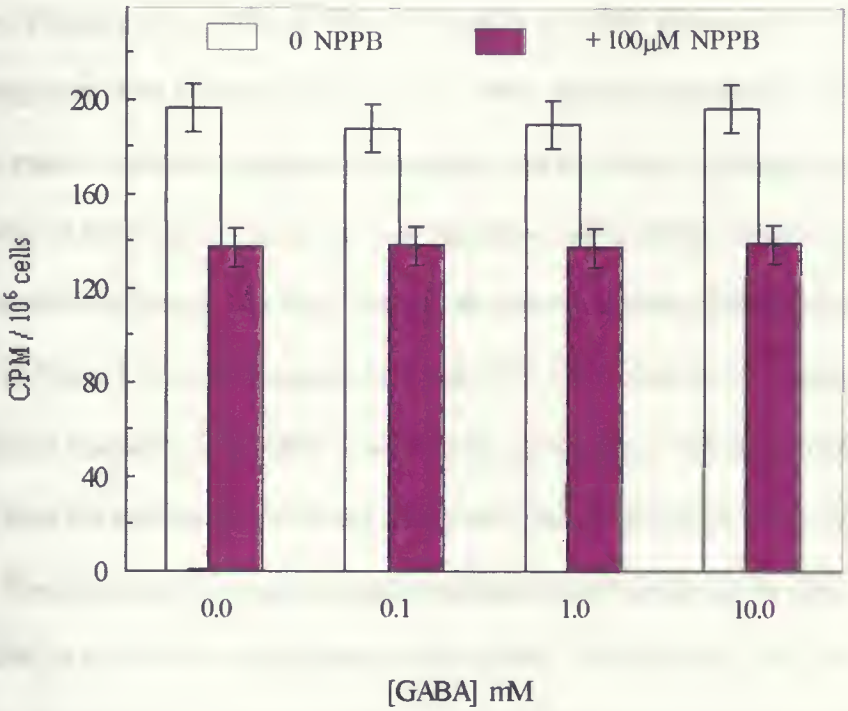
The effect of GABA and NPPB on $^{36}Cl^-$ efflux In plant cells, chloride ions are usually released from the cytosol to the outside, chloride ions move down the gradients of both chloride concentrations and electric potential across plasma membranes. To determine whether or not GABA regulates outward-chloride channels in plant cells, GABA concentrations of 0.1mM, 1.0mM and 10.0mM were tested either in the absence or presence of 100 μM NPPB. No difference was observed between control and a GABA-treated samples in either case. However, NPPB significantly inhibited $^{36}Cl^-$ efflux by 28.2% (Fig. 35). These results indicate that GABA did not regulate the outward chloride channels in plant cells. The inhibition of $^{36}Cl^-$ efflux by NPPB was not reversed by GABA.

Fig. 34 The time courses of chloride efflux from *Asparagus* cells



$^{36}Cl^-$ -loaded cells (9.4 x 10^7) were washed with 2mM Hepes buffer (pH7.5) containing 100mM NaCl as described (Methods). The cells were then resuspended in 2.5 ml of 5 mM Hepes (pH7.5) containing 5 mM K_2SO_4. A 300µl volume of the cells was transferred to an Eppendorf tube at the times indicated and spun for 5 seconds to pellet the cells. Subsequently, 220µl of the resulting clear fluid was immediately pipetted into a scintillation vial for radioactivity measurement (Methods). The value indicated represents the mean value from2 experiments. Error bars represent the standard error.

Fig. 35 The effect of GABA and NPPB on chloride efflux in *Asparagus* cells.



$^{36}Cl^-$ -loaded cells (8.7 x 10^7) were washed with 2mM Hepes buffer (pH7.5) containing 100mM NaCl as described (Methods). The cells were then resuspended in 2.5 ml of 5 mM Hepes (pH7.5) containing 5 mM K_2SO_4 and 0 or 100μM NPPB. A 300μl volume of the cells was transferred to an Eppendorf tube after incubation for 60 minutes and spun for 5 seconds to pellet the cells. Subsequently, 250μl of the resulting clear fluid was immediately pipetted into a scintillation vial for radioactivity measurement (Methods). The value indicated represents the mean value from 2 experiments. Error bars represent the standard error.

Discussion

A. Development of a method for the rapid determination of GABA in plants

Determination of GABA levels in plant tissues is performed instrumentally using amino acid analyzers (Wallace *et al.*, 1984) or HPLC (Carroll *et al.*, 1994; Baum *et al.*, 1996), or enzymologically using Gabase (Crawford *et al.*, 1994; Ramputh and Bown, 1996). The instrumental methods require expensive apparatus and reagents, and are time consuming as well. The enzymological method does not rely on such apparatus, which makes it more convenient and less expensive than the former. The latter employs the enzyme complex Gabase which is a commercial product of Sigma Chemical Company, St. Louis, MO, USA. Gabase is a mixture of GABA : α-ketoglutarate transaminase [GABG-T] and succinic semialdehyde dehydrogenase [SSDH], both isolated from the bacteria *Pseudomonas fluorescens* (Jakoby and Scott, 1959; Scott and Jakoby, 1959). These two enzymes catalyze similar reactions to those catalyzed by plant-derived GABA-T and SSDH, as described in the literature review section. The exception is the use of $NADP^+$ as the coenzyme for SSDH in bacteria. The principle of this method is that GABA consumption is coupled to produce NADPH with a stoichiometric ratio of 1:1. The resulting NADPH can be detected at 340 nm spectrometerically. The amount of GABA in a sample is deduced from the increase in absorbance at 340 nm (ΔA340) due to NADPH production using a calibration curve (Fig. 4). The extinction coefficient of NADPH ($E^{mM}_{340nm} = 6.22$) can also be used, but this assumes that the reactions go to completion. The former was used in this study.

The enzymological method for GABA determination was first developed in 1959 (Jakoby and Scott, 1959) and performs well with animal tissues. Plant materials, particularly old plant tissues, however, contain a variety of water-soluble pigments (*e.g.* phytophenolics) which have a substantial

absorbance around 340 nm (Fig. 9). This high background absorbance interferes severely with the performance of this method. Removal of these interfering pigments either by extraction with organic solvents or by chromatography with ion-exchange columns is time-consuming and not complete as well (data not shown).

Removal of water-soluble pigments from tissue extract by $LaCl_3$ During the classic period of phenolic studies, Pb^{2+} (lead acetate) was commonly used to bind and separate the phenolic fraction of a plant extract (Harborne, 1989). However, Pb^{2+} , a heavy metal ion, is highly toxic to enzymes. In trials with lead acetate, Pb^{2+} inactivated Gabase with an IC_{50} of 0.5 mM. Acetate also inhibited Gabase activity with an IC_{50} of 30 mM. La^{3+} is also a heavy metal ion and carries one more positive charge and has a bigger ionic radius in the hydrated form than Pb^{2+} does. Thus, La^{3+} is theoretically capable of binding pigments. It was observed experimentally that yellow pigments were precipitated by La^{3+} in a $LaCl_3$ -treated plant extract; after centrifugation, the resulting supernatant fluid was clear. The absorbances of the plant extracts over the wavelength range from 260 to 500 nm were greatly reduced by $LaCl_3$ treatment (Fig. 9). These data support the idea that water-soluble pigments can be removed by La^{3+} (Fig. 9). To determine the effective concentration of $LaCl_3$, absorbances of the resulting extracts were plotted against $LaCl_3$ concentrations employed during extraction (Fig. 10). To ensure pigment removal, 1.0 ml of 70 mM $LaCl_3$ was chosen. This was effective with samples of up to 0.2 g fresh weight.

Excessive La^{3+} was eliminated from the tissue extracts treated with 1.0 ml of 70 mM $LaCl_3$ to prevent a lanthanum pyrophosphate precipitate during the GABA assay and the possible inhibition of Gabase activity by La^{3+} . One hundred and sixty µl of 1.0 M KOH was added to the $LaCl_3$-treated extracts to form a pellet of $La(OH)_3$. This was followed by centrifugation to remove $La(OH)_3$. The

resulting clear fluid was added to the GABA assay medium. It was experimentally estimated that the residual concentration of La^{3+} in the assay medium was less than 1mM (see Results). Theoretically, the dissociation constant K_d of $La(OH)_3$ equals 18.9. Thus, the calculated concentration of La^{3+} remaining in the fluids after centrifugation is 1.88×10^{-2} mM. Moreover, the inhibition of GAD activity by La^{3+} indicates that at least 90% of Gabase activity remains if La^{3+} concentrations are below 20 mM (Table 1). Therefore, the residual La^{3+} would only slightly inhibit Gabase activity if at all. In addition, Cl^-, in the form of KCl, did not inhibit Gabase activity up to 250 mM (data not shown). This indicates that Cl^- which was introduced into the assay medium by 70 mM $LaCl_3$ during extraction would not inhibit Gabase activity. These data, together with the similarity of the time courses of the Gabase reactions between control and $LaCl_3$-treated standard GABA samples (Fig. 11), demonstrate that the enzyme for GABA determination will not be inhibited significantly by $LaCl_3$ treatment for pigment removal from tissue extracts.

The necessity for methanol treatment of the samples prior to addition of $LaCl_3$ Plant GAD can be activated dramatically by H^+ and Ca^{2+}/calmodulin (Crawford *et al.*, 1994; Ling *et al.*, 1994; Snedden *et al.*, 1995). Homogenization of plant tissues causes release of H^+ and Ca^{2+} from vacuoles and activates GAD and GABA synthesis. This is indicated by reports that mechanical stimulation (Wallace *et al.*, 1984) or mechanical damage (Ramputh *et al.*, 1996) results in a many-fold increase of GABA levels in soybean leaves within 4 minutes. The present data strongly support this hypothesis. Five minutes after homogenization of tissues, GABA levels rose from 7 to 34 or 2122 nmol/g fresh weight in the tissue homogenates at 0°C or 25°C, respectively (Fig. 13). Thus, it is necessary to avoid the production of GABA in tissue homogenates. To do so, tissue homogenates were kept frozen before inactivating GAD, and GAD was inactivated completely prior to addition of $LaCl_3$ solution. To inactivate GAD, in animal

tissues hot (60 °C) 10 mM HCl has been used (Okada *et al.*, 1976). In the present study, three treatments of tissue homogenates were employed, immediate addition of methanol, hot 30 mM HCl (60 °C) or hot 70 mM $LaCl_3$ (60 °C), to the frozen tissue homogenates after grinding in liquid nitrogen were compared. The data indicate that methanol most efficiently inactivated plant GAD (Fig. 12).

In summary, $LaCl_3$ could remove most of the pigments from plant tissue extracts, which interfere severely with spectrometric measurements of NADPH at 340 nm. Unlike Pb^{2+}, La^{3+} slightly inhibits Gabase activity. In addition, the low residual La^{3+} concentration in the GABA assay medium does not interfere with GABA determination. Compared to other protocols for GABA determination in plants (Wallace *et al.*, 1984; Crawford *et al.*, 1994; Ramputh and Bown, 1996), the extraction protocol of GABA from plant tissues developed in the present study could be performed efficiently, quickly and less expensively without loss of GABA (Table 2). Due to the substantial accumulation of GABA induced by tissue damage or crushing, the following two points should be kept in mind so that GABA accumulation after sampling is prevented. (a) A harvested tissue sample should be frozen in liquid nitrogen immediately (within seconds); (b) Methanol should be added to the frozen tissue powder without allowing it to thaw.

B. The relationship between GABA levels and hypocotyl elongation in soybean seedlings

During the last few years, several findings (see Literature review) suggest that GABA may be involved in regulating plant development. In order to answer this question, the present study employed different approaches to modulate hypocotyl elongation and *in vivo* GABA levels in soybean seedlings in an attempt to assess whether or not GABA plays a role in hypocotyl elongation of soybean seedlings.

Mechanical stimulation inhibited hypocotyl elongation with increases in vivo *GABA levels*

The fact that mechanical stimulation induces growth inhibition has been well documented in plant species, such as *Arabidopsis thaliana* (Braam and Davis, 1990), pea (Goeschl *et al.*, 1966), *Liquidambar styraciflua* trees (Neel and Harris, 1971), *Phaseolus vulgaris* L. (Jaffe, 1976), *Cucurbita melopepo* (Turgeon and Webb, 1971), *Bryonia dioica* (Thonat *et al.*, 1997), tomato plants (Björlman and Garner, 1997), and soybean plants (Jones and Mitchell, 1989). In this study, a significant inhibition of hypocotyl elongation over 24 hours in either dark-grown or light-grown soybean seedlings subjected to the mechanical stimulation of 20 strokes was observed (Fig. 15). In experiments designed to determine the site of elongation inhibition, the most inhibition was seen within the top 17mm in dark-grown hypocotyls (Fig. 14). These data indicate that this segment is most responsive to mechanical stimulation. It was therefore selected for all treatments and sample analyses throughout this study. The inhibition is due most likely to the reduction in cell elongation rather than cell mitosis. This is supported by observations in *Arabidopsis thaliana* seedlings which demonstrated that the growth of hypocotyls mainly involves cell elongation, and that cell division is not significantly involved in this process (Gendreau *et al.*, 1997). To determine how soon the elongation inhibition occurs after mechanical stimulation, the kinetics of hypocotyl elongation were investigated using a mechanical transducer (Fig. 5). In either dark-grown or light-grown hypocotyls, elongation inhibition occurred within 40 seconds of initiation of stroking, which was performed over a period of about 20 seconds (Fig. 16). These data differ from those reported in light-grown common bean seedlings (*Phaseolus vulgaris* L) which show a transient acceleration in growth within the first 3 minutes followed by a complete cessation for at least 18 minutes after 10 strokes (Jaffe, 1976). This difference may reflect the sensitivity of different plants to mechanical stimulation. The mechanism of this rapid inhibition is not clear. There are probably electrochemical events taking place as indicated by the rapid changes in the electrical resistance of the stimulated tissues (Jaffe, 1976). These may result in

changes in cell turgor pressure which in turn cause cell shrinking, as indicated by the blue light-induced shrinking of maize coleoptile protoplasts and the induced inhibition of coleoptile elongation (Wang and Iion, 1997). Alternatively, ion transport processes may result in cell wall alkalization and growth inhibition (Cho and Spalding,1996). The elongation inhibition could last 24 hours after 20 strokes (Fig. 17). This may be controlled by certain genes. It has been found that mechanical stimulation initiates the expression of the 1-aminocyclo-propane-1-carboxylic acid (ACC) synthase gene (Botella *et al.*, 1995; Arteca and Arteca, 1997) and 5 touch-induced (TCH) genes (Braam and Davis, 1990). ACC synthase controls the production of ethylene, whose production was also seen in response to mechanical stimulation (Goeschl *et al.*, 1966) and which generally causes growth inhibition. Among 5 TCH genes, interestingly, TCH4 encodes a cell-wall-modifying enzyme xyloglucan endotransglycosylase (XET); TCH1, 2 and 3 encode calmodulin and Ca^{2+} binding proteins which may also take part in growth regulation (Braam *et al.*, 1996).

Accumulation of GABA was found in soybean leaves (Wallace *et al.*, 1984) and etiolated hypocotyls (Grognet, 1996) in response to mechanical stimulation. These phenomena imply that GABA may be involved in the elongation inhibition of hypocotyls stressed by mechanical stimulation. Therefore, the kinetic accumulation of *in vivo* GABA was investigated in the mechanically-stimulated soybean hypocotyls. Rapid and large accumulations of GABA were observed after stroking dark- and light-grown hypocotyls (Fig. 18). Although GABA levels declined from maxima at 8 minutes, the overall levels of GABA in stroked hypocotyls remained approximately double those observed prior to stroking. This increase could last for at least 16 hours (data not shown). Therefore, there seems to be a correlation between increased GABA levels and the inhibition of hypocotyl elongation. We hypothesized that there might be a causal relationship between them.

To understand whether or not a reduction in glutamate levels is responsible for inhibition of hypocotyl elongation in the stroked seedlings, glutamate levels in stroked hypocotyls were measured.

GABA is derived from glutamate through decarboxylation by GAD (see literature review), and elevation of GABA levels and decline of glutamate levels concur in transgenic tobacco plants expressing a truncated GAD gene (GADΔC). The GADΔC gene lacks the calmodulin binding domain. These transgenic tobacco plants over-express GAD activity and over -produce GABA (Baum *et al.*, 1996). Surprisingly in this study, glutamate levels increased during the first 8 minutes after stroking though the values decreased during the subsequent 2 hours (Fig. 27). During the first 8 minutes, the deamination of glutamate by glutamate dehydrogenase (GDH) might be inhibited by the mechanical stress, resulting in a reduction of glutamate consumption which would facilitate glutamate accumulation. The deamination of glutamate is supposed to be a normal pathway for glutamate catabolism into the Kreb's cycle. The inhibition of GDH activity caused by water stress was reported in rape seedlings (Srivastava and Singh, 1987). These results show no consistent relationship between glutamate levels and elongation inhibition. Thus, glutamate does not appear to mediate the inhibition of hypocotyl elongation induced by mechanical stimulation.

Increase in in vivo *GABA levels in response to exogenous GABA does not affect elongation of hypocotyls* Experiments were designed to investigate the rate of hypocotyl elongation in response to elevated *in vivo* GABA levels resulting from exogenous GABA application. First, it was demonstrated that externally applied radioactive GABA could penetrate into the hypocotyls. Most of the applied GABA remained within the growth sensitive region where it was applied, and a small portion was transported to the cotyledons likely through the xylem (Fig. 19). Second, *in vivo* GABA levels in dark-grown seedlings were raised by application of 10 µl of 10 mM GABA from initial levels around 48.2 nmol/gfw to 199.8 and 274.3 nmol /gfw in 2 and 4 hours, respectively (Fig. 20). These levels of GABA are comparable to those in the stroked dark-grown hypocotyls (Fig. 18). Unlike

mechanical stimulation, however, the external application of 10 µl of 10 mM GABA failed to cause a significant inhibition or stimulation in hypocotyl elongation (Fig.26). It was also proposed that GABA might function at a low concentration. The application of 10 µl of 0.1, 0.25, 0.5 mM GABA did not result in any significant effect on hypocotyl elongation either (data not shown). In contrast to this result, Reid's group at the University of Calgary observed that GABA applied to culture media at less than 1.0 mM stimulated sunflower hypocotyl elongation over a 21-day incubation (A. W. Bown's personal communication). Our data indicate that GABA alone is not sufficient to inhibit soybean hypocotyl elongation within 24 hours.

Inhibition of hypocotyl elongation by $LaCl_3$ or blue light is not associated with an increase in in vivo *GABA levels* Up to this point, it has been shown that an increase in GABA is not sufficient to inhibit hypocotyl elongation. Conversely, it is not clear whether inhibition of hypocotyl elongation can cause any accumulation of GABA. To address this question, two approaches were used. Application of $LaCl_3$ and irradiation with blue light were employed to induce inhibition of hypocotyl elongation in dark-grown soybean seedlings.

Ca^{2+} has been thought to be the second messenger in the signal transduction pathway leading from mechanical stimulation to growth inhibition. This hypothesis is supported by the following evidence. First, touch induces a rise in the cytosolic free Ca^{2+} concentration in transgenic plants which contain a Ca^{2+} reporter gene --- apoaequorin (Knight *et al.*, 1991). Second, Jones and Mitchell (1989) demonstrated Ca^{2+} involvement in the growth inhibition of mechanically stressed soybean seedlings. Third, TCH1 is a calmodulin protein and TCH2 and TCH3 are Ca^{2+} binding proteins in *Arabidopsis thaliana*. These proteins are induced by touch (Braam *et al.*, 1996). Fourth, in *Bryonia dioica* there is a rapid influx of Ca^{2+} from the plasma membrane surface after mechanical stimulation and evidence

for the involvement of several Ca^{2+} -binding proteins in the early responses to mechanical stimulation (Thonat *et al.*, 1993, 1997). Fifth, in terms of GABA production, plant GAD can be activated by Ca^{2+}/CaM (Ling *et al.*, 1994; Snedden *et al.*, 1995). Therefore, it is feasible that elevated Ca^{2+} levels inhibit growth by elevating GABA levels. La^{3+} is known as a calcium channel blocker. In other words, La^{3+} restrains the influx of Ca^{2+} from the outside of cells and inhibits increase in the cytosolic [Ca^{2+}] after certain stimuli. In the present study, La^{3+} inhibited both hypocotyl elongation and GABA production (Fig. 21). This result agrees with the report that La^{3+} inhibited growth of etiolated soybean hypocotyls (Jones and Mitchell, 1989). These data indicate that Ca^{2+} may be involved in the signal transduction pathway leading from mechanical stimulation to inhibition of hypocotyl elongation and GABA synthesis. Moreover, this experiment demonstrated that elongation inhibition occurred without an increase in GABA levels (+La/-Stroke; +La/+Stroke). Thus, the data indicate that La^{3+}-induced elongation inhibition does not result in GABA accumulation, nor does it results from GABA accumulation.

The inhibition of growth by blue light and by mechanical stimulation are old observations. The experimental investigations regarding blue light inhibition of growth can be dated back more than 60 years (Briggs, 1993). The rapid inhibition of stem elongation in dark-grown seedlings by blue light is well known (Kaufman, 1993; Cosgrove, 1981). In the present study, irradiation with blue light of 340 to 480 nm wavelengths induced a 55% or 72% reduction in the rates of hypocotyl elongation of dark-grown soybean seedlings with continuous irradiation or a 5-minute-pulse irradiation, respectively (Fig. 23 and 24). However, *in vivo* GABA levels were decreased with blue light, instead of an increase (Fig. 25). As previously described in the Literature review, GABA synthesis is stimulated by two factors: increases in cytosolic Ca^{2+} and H^+ levels. Using transgenic *Arabidopsis thaliana* cytoplasmically expressing aequorin, it was demonstrated that blue light did not trigger Ca^{2+} influx (Lewis *et al.*, 1997). In addition, in dark-grown cucumber hypocotyls, inhibition of Ca^{2+} channels by

La^{3+} or verapamil (Ca^{2+} channel blockers) had no effect on the depolarization which is triggered by blue light. Neither did the depletion of extracellular Ca^{2+} by EGTA (Spalding and Cosgrove, 1992). There is scant evidence implicating Ca^{2+} involvement in the responses to blue light (Jenkins *et al.*, 1995). Therefore GAD is not likely to be activated by blue light through Ca^{2+}. In other words, GABA synthesis should not be enhanced by irradiation with blue light. In terms of cytosolic pH, because of the optimal pH 5.8 of GAD, a reduction of cytosolic pH may also stimulate GAD activity and GABA production. In stomatal guard cells of fava beans (*Vicia faba*), blue light activated H^+-ATPase and promoted H^+ extrusion (Shimazaki *et al.*, 1986). This would raise cytosolic pH and down-regulate GABA synthesis. In dark-grown cucumber hypocotyls, on the other hand, blue light might inhibit H^+-ATPase (Spalding and Cosgrove, 1992), which may reduce cytosolic pH and up-regulate GABA synthesis. This was supported by the observation that H^+-ATPase inhibitors, KCN and vanadate, could diminish blue light-induced plasma membrane depolarization (Spalding and Cosgrove, 1992). Thus, there seems to be different mechanisms of regulating H^+-ATPase activity in stomatal cells and stem cells in response to blue light. In this investigation, inhibition of hypocotyl elongation by blue light did not result in GABA production. This result indicates that GABA synthesis does not occur in response to the elongation inhibition occurring in response to blue light.

In summary, mechanical stimulation induced inhibition in hypocotyl elongation and rapid GABA accumulation. However, GABA accumulation resulting from external application of GABA failed to inhibit hypocotyl elongation, and inhibition of hypocotyl elongation induced by $LaCl_3$ or blue light did not cause GABA accumulation. Thus, the conclusion can be drawn that there is no causal relationship between GABA accumulation and inhibition of hypocotyl elongation in soybean seedlings. Since Ca^{2+} increases occur in response to mechanical stimulation (Jones and Mitchell, 1989; Knight *et al.*, 1991), and since Ca^{2+} activates GAD (Snedden *et al.*, 1995; Baum *et al.*, 1996), it is suggested

that GABA accumulation most likely occurs in response to an increase in cytosolic free Ca^{2+} levels. However, GABA increases on their own are not sufficient to inhibit growth.

In addition, it was postulated that plants have GABA receptors which may resemble $GABA_A$ and $GABA_B$ receptors in animals. Bicuculline (a $GABA_A$ antagonist), saclofen (a $GABA_B$ antagonist) and baclofen (a $GABA_B$ agonist) had no effect on 24 hour hypocotyl elongation in light grown- or dark-grown soybean seedlings (Fig.26), however, the penetration of these agents into plant tissues was not demonstrated. This result suggests that GABA receptors in plants, if they occur, are not involved in the regulation of growth.

C. The influence of GABA on chloride flux in mesophyll cells

Newly synthesized GABA in isolated asparagus cells is released to the medium. This may imply that GABA is an intercellular messenger (Chung *et al.*, 1992). In the animal CNS, GABA functions as a major inhibitor of GABA -gated chloride channels (Literature review). This information led to an investigation of the influence of GABA on chloride influx and efflux in asparagus cells. In plant cells, intracellular concentrations of Cl^- are around 30 mM and are much higher than those outside. Chloride efflux from cells is passive down the physiological electrochemical gradient. Most of the understanding about anion channels in plants derives from studies with guard cells. Briefly, there are two types of anion channel in higher plants. One type is the voltage-gated channel activated by depolarization, the other one is Ca^{2+} -activated (Schroeder, 1992). Stretch-induced anion channels have also been found in tobacco protoplasts (Falke *et al.*, 1988). As with animal chloride channels in intestine and kidney cells (Keeling *et al.*, 1991), plant anion channels can be inhibited by NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid) (Schroeder *et al.*, 1993; Cho and Spalding, 1996). In animal cells, chloride channels play important functions in many aspects of cell physiology including

volume regulation, ion transport and stabilization of membrane potential (Valverde *et al.*, 1995). In plant guard cells, activation of anion channels causes efflux of anions (*i.e.* malate and Cl^-), decrease in turgor pressure, shrinkage of cells and finally stomatal closing (Ward *et al.*, 1995). In non-stomatal tissues, it was found that activation of anion channels by blue light could cause cell shrinkage in maize coleoptiles (Wang and Iino, 1997) as well as growth inhibition in both maize coleoptiles (Wang and Iino, 1997) and dark-grown cucumber hypocotyls (Cho and Spalding, 1996). In this investigation with isolated asparagus cells, GABA had no effect on Cl^- efflux (Fig. 35), whereas GABA could enhance Cl^- influx. Ten mM GABA stimulated Cl^- influx by an unknown mechanism (Fig. 29 and Table 3). However, this concentration of GABA is above the physiological level of it, and no specificity for the effect of GABA was observed when compared with other applied agents (glutamate, alanine, asparate, lysine, α-aminoisobutyric acid (AIBA), baclofen and saclofen) (Fig.30). NPPB inhibition of Cl^- efflux has been demonstrated in plant cells (Schroeder *et al.*, 1993; Cho and Spalding, 1996). One hundred μM concentration of NPPB could significantly inhibit both Cl^- influx and efflux (Fig. 31,33 and 35). This concentration of NPPB is higher than those used by other workers (10μM used by Schroeder *et al.*, 1993; and 20 μM used by Cho and Spalding, 1996). The difference between the effective concentrations of NPPB in the present study and those in other reports might be due to differential sensitivity of different plant species to NPPB. However, 100 μM NPPB did not cause any significant increase in cell damage over 6 hours (Fig. 32). Since NPPB could only partially inhibit Cl^- influx and efflux (Fig.33 and 35), Cl^- movement may be through other pathways besides NPPB-sensitive anion channels. When treated with both GABA and NPPB, NPPB almost completely eliminated the increases in Cl^- influx in response to GABA (Fig. 33). This indicates that GABA may only stimulate NPPB-sensitive anion channels. However, whether or not GABA-activated anion channels exist in plants requires further investigations.

Summary In this study, a method for the rapid determination of GABA was developed, in which 70 mM $LaCl_3$ is used to remove interfering pigments from plant tissue extracts, and immediate addition of methanol to frozen samples is required to inactivate GAD and prevent GABA production during extraction. Second, different approaches (mechanical stimulation, application of external GABA, application of $LaCl_3$, and blue light irradiation) to regulate elongation and *in vivo* GABA levels in the hypocotyls of soybean seedlings were employed. It was demonstrated that GABA alone is not sufficient to inhibit hypocotyl elongation. Third, working with isolated asparagus cells, GABA could inhibit Cl^- influx at 10 mM, but had no effect on Cl^- efflux. NPPB, a chloride channel inhibitor, inhibited Cl^- influx and efflux. However, GABA did not reverse NPPB-induced inhibition of Cl^- influx and efflux. This may indicate that NPPB-sensitive Cl^- channels are present in plants. However no evidence for GABA-gated Cl^- channels was obtained.

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