# Gamma-aminobutyric acid Metabolism

# in

# *Arabidopsis thaliana*

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# Abstract

The four-carbon, non-protein amino acid γ–amino butyric acid (GABA) is found in all species, where it is involved in various signaling processes. GABA is best characterized as the main inhibitory neurotransmitter in mammalia. On the contrary, work in plants focused mainly on a metabolic role; just some recent findings indicate GABA having a possible signaling function in plants as well.

In *Arabidopsis thaliana*, mutants of both catabolic genes (*GABA transaminase (GABA-T)* and *succinic semialdehyde dehydrogenase (SSADH)*) display phenotypic deviations to wild type; the former grow vegetatively like wild type, but are less fertile due to a misguidance of pollen tubes. The latter are severely affected in growth and development, probably induced by the accumulation of GABA-shunt metabolites and/or reactive oxygen intermediates. The *ssadh* phenotype can be suppressed by interrupting the GABA-shunt upstream of the SSADH function. Based on this, two topics should be covered in this thesis. First, the substance causing the *ssadh* phenotype should be identified; hence, wild type along with GABA-shunt single and double mutants were grown on media containing GABA-shunt intermediates, and plant growth as well as metabolite content in leaf extracts was examined. Second, further genes involved in regulation or function of the GABA-shunt or in sensing of GABA-shunt related metabolites should be identified. Therefore, a population of mutagenized *ssadh* plants was screened for suppressors; among several stable, non-segregating lines, two new *gaba-t* alleles were isolated.

All GABA-shunt intermediates influence plant growth and development. First, GABA enhances plant growth by inducing expression of nitrate uptake transporters, only very high cellular concentrations have an inhibitory effect on plant growth. Second, succinic semialdehyde (SSA) considerably reduces plant growth when applied with the growth media, and higher concentrations (0.8mM and more in the media) induce dedifferentiation mainly of hypocotyl cells in a yet unknown manner. Finally, the enzymatic reduction of SSA produces γ-hydroxybutyric acid (GHB), which leads to reduced root growth in Arabidopsis without major effects on rosette size when accumulating in plant tissues.

# Zusammenfassung

Die nicht proteinogene Aminosäure γ-Aminobuttersäure (GABA) wurde in allen auf ein Vorkommen hin untersuchten Arten nachgewiesen. Im Zentralnervensystem der Säugetiere wirkt GABA als der wichtigste inhibitorische Neurotransmitter, allerdings konnte für GABA auch in Bakterien und Hefen eine Funktion als Signalsubstanz gezeigt werden. Im Gegensatz dazu beschränkte sich die Forschung an GABA in Pflanzen lange Jahre einzig auf eine Rolle im Stoffwechsel, erst einige neuere Publikationen lassen auch eine Signalfunktion in Pflanzen möglich erscheinen.

Mutanten der GABA-abbauenden Gene (*GABA-Transaminase (GABA-T)* und *Succinic Semialdehyd Dehydrogenase (SSADH)*) zeigen in *Arabidopsis thaliana* einen vom Wildtyp abweichenden Phänotyp. Pflanzen ohne GABA-T-Aktivität sind in der vegetativen Phase nicht von Wildtypen zu unterscheiden, allerdings sind die Schoten der Pflanzen kleiner und enthalten weniger Samenkörner. Dieser Phänotyp konnte durch einen erhöhten GABA-Gehalt im Fruchtknoten erklärt werden, der ein gezieltes Wachstum der Pollenschläuche zu den Samenanlagen verhindert. *ssadh*-Mutanten bilden bereits im Gewächshaus nekrotische Blätter und zeigen ein deutlich verlangsamtes Wachstum. Die Ursache dieses Phänotyps ist noch nicht vollständig geklärt, allerdings wird ein Zusammenhang mit einer hohen Konzentration von Intermediaten des GABA-Stoffwechsels und der Bildung reaktiver Sauerstoffverbindungen diskutiert. Der *ssadh*-Phänotyp kann durch Inhibierung der GABA-T unterdrückt werden, worauf zwei Fragestellungen basieren. Zum einen soll herausgefunden werden, welche Substanz für den *ssadh*-Phänotyp verantwortlich ist, weshalb Einzel- und Doppelmutanten des GABA-Stoffwechsels auf mit Intermediaten des GABA-Stoffwechsels versetzten Sterilmedien angezogen und Wachstum sowie Metabolitgehalt der Pflanzen analysiert werden. Zum anderen sollen weitere Gene identifiziert werden, die entweder indirekt als Regulatoren oder direkt am GABA-Stoffwechsel beteiligt sind bzw. an einer postulierten Signalkaskade mitwirken. Dazu wurde eine Population EMS-mutagenisierter *ssadh*-Pflanzen mit dem Ziel durchmustert, Pflanzen zu isolieren, welche den *ssadh*-Phänotyp unterdrücken. Mehrere stabile, nicht aufspaltende Linien wurden isoliert, unter denen sich auch zwei Linien befanden, welche auf Grund einer mutierten GABA-T keinen *ssadh*-Phänotyp zeigen.

Für alle im GABA-Stoffwechsel vorkommenden Intermediate konnte ein Einfluss auf Wachstum und Entwicklung von Pflanzen gezeigt werden.

Ein erhöhter GABA-Gehalt in den Zellen induziert die Expression von Genen, welche für Nitrat-importierende Proteine codieren. In Folge der erhöhten Nitrat-Aufnahme-Kapazität steht den Pflanzen mehr Stickstoff zur Verfügung, welcher ein verbessertes Wachstum ermöglicht. Dieser Effekt wird erst bei deutlich erhöhten zellulären GABA-Konzentrationen ins Gegenteil gekehrt.

Succinic Semialdehyd (SSA) verringert bereits in niedrigen Konzentrationen das Pflanzenwachstum, etwas höhere Konzentrationen (ab 0,8mM SSA im Medium) bewirken eine Dedifferenzierung von Zellen im Hypocotyl der Pflanzen, wobei der zugrunde liegende Mechanismus noch unbekannt ist.

Bei der Reduktion von SSA entsteht γ-Hydroxybuttersäure (GHB), welche ein reduziertes Wurzelwachstum ohne deutlich verringertes Sprosswachstum bewirkt.

# 1. Introduction

## 1.1. Function and metabolism of γ-amino butyric acid (GABA)

The non-protein amino acid γ-amino butyric acid (GABA) is conserved from bacteria through yeast to vertebrates. GABA was discovered nearly 60 years ago in plants [165], but research focused on vertebrates following the discovery of high GABA levels in the mammalian central nervous system and the identification of its role as the main inhibitory neurotransmitter (1.5.1). For yeast (*Saccharomyces* cerevisiae), a functional GABA-shunt is essential for oxidative stress tolerance (1.5.3), and in plant pathogenic bacteria like *Agrobacterium tumefaciens*, GABA and GABA-shunt metabolites were identified as repressors of quorum sensing signaling (1.5.2). *Escherichia coli* is dependent on the *Gad* acid resistance system, containing two Glutamate Decarboxylase genes (*GadA* and *GadB*) and the GABA exporter (*GadC*), to survive during acidic conditions [38] and expression of GABA-degrading enzymes was found to be induced under nitrogen limiting conditions [151]. In contrast, research in plants focused primarily on a role for GABA as a metabolite, mainly in the context of stress response, until some recent findings were published indicating a possible signaling function for GABA in plants as well (1.2).

In eukaryotes, the GABA-shunt is compartmentalized (Figure 1); the synthesis takes place in the cytoplasm while the degrading enzymes are localized in the mitochondrial matrix. In the cytoplasm, glutamate is irreversibly decarboxylated by the Glutamate Decarboxylase (GAD); the enzyme activity is regulated posttranslationally either by cofactor binding in mammalia or by $Ca^{2+}$/CaM-induced dimerisation.

For the catabolic steps of the pathway, GABA is imported into mitochondria, where a transamination to succinic semialdehyde occurs. The GABA-Transaminase (GABA-T) undergoes conformational changes following binding of pyridoxal phosphate near the catalytic domain, influencing enzyme activity [47-49, 51]. In mammalians, the GABA-T uses α-ketoglutarate as acceptor for the transferred amino group, whereas the plant GABA-T utilizes pyruvate. A direct interaction between GABA-T and Succinic

Semialdehyde Dehydrogenase (SSADH) was published for the mammalian proteins [82] indicating toward a substrate channeling mechanism that prevents a release of the reactive succinic semialdehyde (SSA) into the mitochondrial matrix. In the GABA-shunt, SSA is oxidized to succinate in the $NAD^+$-dependent SSADH reaction; succinate is further metabolized in the citric acid cycle. Another possible alternative for SSA degradation might be the reduction to γ-hydroxybutyric acid (GHB), a second neuroactive substance related to GABA [83].
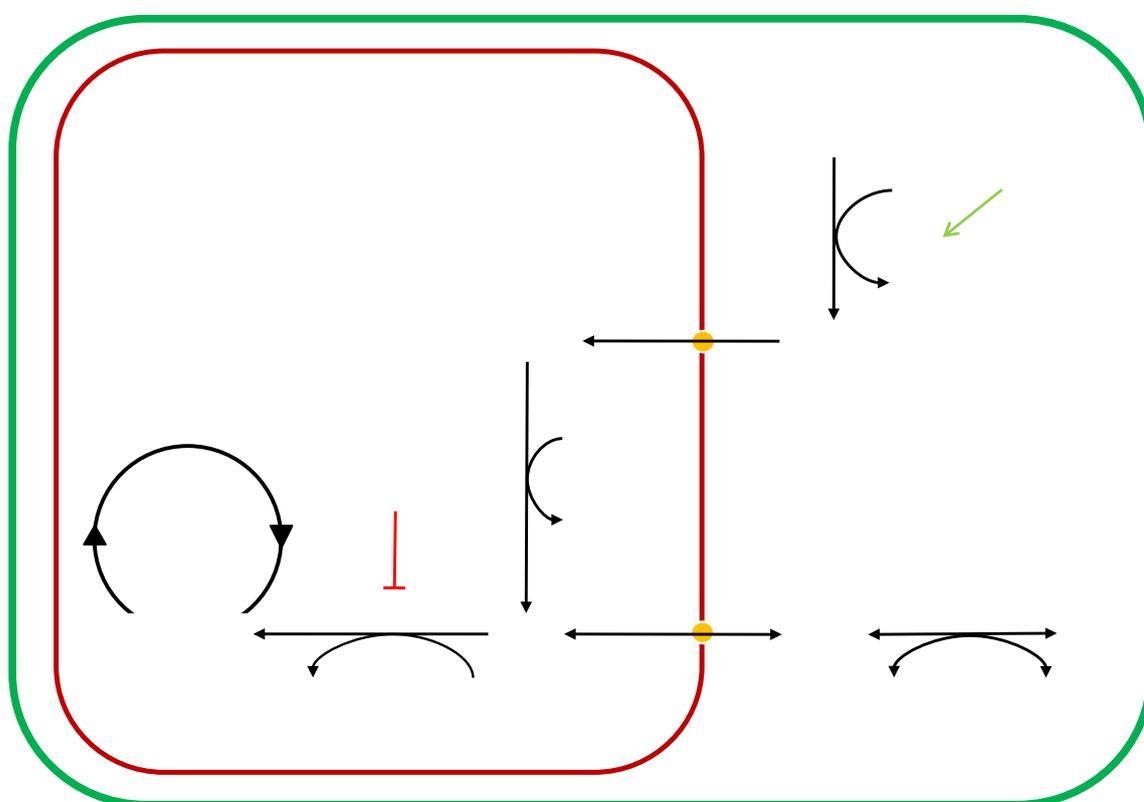


**Figure 1: Schematic representation of the GABA-shunt in plants**

Enzymes and metabolites of the GABA-shunt are shown in blue; activatory and inhibitory substances in green and red respectively.
GAD – glutamate decarboxylase; GABA – γ–amino butyric acid; GABA-T – GABA transaminase; SSA – succinic semialdehyde; SSADH – SSA dehydrogenase; GHB – γ-hydroxy butyric acid; GHBDH – GHB dehydrogenase; CaM – Calmodulin; TCA – citric acid cycle

## 1.2.  The GABA-shunt in plants

In contrast to vertebrates, the physiological role of GABA in plants remains still unclear. While a rapid GABA accumulation in plants under various stress conditions to very high concentrations is known for many years [38, 56, 62, 63, 96, 118, 151, 184], the GABA-shunt is just recently discussed to possess a signaling function in plants as well [26, 28, 130]. The observed fast increase in the cellular GABA concentration was first discussed as a regulatory mechanism for homeostasis of the cytosolic pH, since GABA synthesis is consuming protons [54]. Later, an increase of the cytosolic $Ca^{2+}$-concentration preceding the elevated GABA synthesis rate was reported, linking GABA synthesis to stress signaling [50].

Some data indicate toward a possible function of GABA in various processes like pH regulation [41], temporary nitrogen storage [97, 148, 149] or pathogen resistance. An increased expression of GABA-shunt genes and accumulation of GABA in the millimolar range was found upon plant infection with different pathogenic fungi [88, 163, 187] or in response to attack by herbivorous larvae [30, 109, 116], where GABA is discussed to interfere with signaling in the nervous system of the insects or to induce transition between larval and juvenile stages [30, 121, 154, 156].

Exogenously applied GABA affects the expression of a subset of 14-3-3 protein family members in a $Ca^{2+}$, abscisic acid (ABA) and ethylene-dependent manner [103]. A possible relation between phytohormones and GABA was assumed since exogenous GABA activates ACC synthase transcription [89, 90] as well as GABA synthesis is induced by phytohormones [68, 143]. Additionally, GABA is suggested to be involved in several plant development processes including senescence [7, 114], fertilization [130, 168, 188], and regulation of nitrate uptake by increasing expression of nitrate transporters [22].

For the functionality of the whole pathway, the existence of a GABA-uptake mechanism into the mitochondria is necessary. Therefore, the existence of a yet unknown mitochondrial GABA-Transporter must be supposed.

If GABA or GABA-shunt metabolites are acting directly as a signaling substances *in planta*, then the existence of transporters regulating import or export of the signal molecule from cells and of receptors triggering a signal is essential.

Both high and low affinity GABA transporters localized in the plasma membrane are known. Three low affinity transporters (ProT) with different expression patterns were identified in Arabidopsis [79] allowing the plants to grow on GABA as the sole nitrogen source. In studies with GABA-uptake deficient yeast, heterologous expression of these transporters was sufficient to restore yeast growth, and GABA uptake could be inhibited by proline and quaternary ammonium compounds [33]. A tomato homolog is highly expressed in pollen, where it is necessary for the uptake of compatible solutes during pollen desiccation and germination [152]. Recently, a high-affinity, plasma-membrane localized GABA transporter in Arabidopsis (AtGAT1) was isolated, displaying no sequence similarity to previously described non-plant GABA transporters. In Arabidopsis, the highest gene expression was found in flowers and under conditions inducing GABA accumulation [119].

In plants, no homologs of mammalian or bacterial GABA receptors can be identified by sequence analysis. Although GABA binding sites on the surface of living tobacco protoplasts were detected [190] and a potential modulation of ion transport in *Lemna minor* by GABA was reported [95], but no corresponding genes have been cloned thus far. Both findings indicate toward the existence of GABA-like receptor in plants. Additionally, plant glutamate receptors (GLRs) are discussed to act potentially as GABA receptors in plants [29, 45, 69, 102, 176], but experimental evidence is still lacking.

## 1.2.1. Glutamate Decarboxylase (GAD)

The first reaction of the GABA-shunt is the pyridoxal phosphate-dependent $\alpha$-decarboxylation of L-glutamate, catalyzed by the cytosolic Glutamate Decarboxylase (GAD, EC 4.1.1.15).

In plants, the *GAD* genes are differentially expressed in a developmentally controlled manner. They contain a Calmodulin-(CaM)-binding domain in the C-terminal region, allowing a fast regulation of protein activity upon increase of the cytosolic $Ca^{2+}$ concentration [15, 32, 42, 193]. *In vitro*, an enhanced GAD activity in response to the addition of $Ca^{2+}$/CaM was published for different plant species like soy bean, petunia or tobacco [8, 161, 189]. Interestingly, this stimulation of GAD activity by CaM binding could *in vitro* only be detected at neutral pH (7.0 to 7.5), whereas GAD activity is unaffected by $Ca^{2+}$/CaM at acidic pH conditions, where a sharp pH optimum at 5.8 was detected. Binding of $Ca^{2+}$/CaM or a specific monoclonal antibody induces dimerisation *in vitro*, which is essential for the activation of GADs [162, 191]. In tobacco, the overexpression of a truncated GAD without the CaM-binding domain results in plants with reduced growth due to cortex parenchyma cells fail to elongate, as well as very high GABA and low glutamate levels in plant extracts. Hence, the CaM-binding domain is discussed to possess an autoinhibitory effect on GAD activity that is relieved by addition of $Ca^{2+}$; the expression of a truncated GAD therefore leads to constitutive activity [16]. This autoinhibition was also observed in rice (*Oryza sativa*), where OsGAD1 contains the CaM binding domain in contrast to OsGAD2 [2]. The former is regulated in a $Ca^{2+}$-dependent manner, and truncation of the C-terminal extension resulted in a constitutively active enzyme [3] whereas the latter has a very low activity either in presence or absence of CaM/$Ca^{2+}$.

The regulation of GAD activity in response to cytosolic $Ca^{2+}$-levels allow plant cells a very fast response to various stress situations by initiating GABA synthesis, indicating that GABA or the GABA-shunt is involved in plant stress signaling.

In the model plant *Arabidopsis thaliana*, five GAD isoforms exist, among them two have been biochemically characterized, whereas the three additional genes were identified as GADs based on sequence homologies [155, 175]. *AtGAD1* (At5g17330) is expressed exclusively in roots and is essential for sustaining GABA levels in roots whereas the second highly expressed isoform, *AtGAD2* (At1g65960), is expressed in all tissues [27, 175]. Analysis of *AtGAD3* and *AtGAD4* (At2g02000 and At2g02010) reveals a very high sequence identity, most probably due to a recent gene duplication. In northern blots or microarray experiments transcripts are detected in generative organs, even if both genes are indistinguishable due to cross hybridizations. For the last isoform, *AtGAD5* (At3g17760), a weak expression is detected in isolated pollen (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

## 1.2.2.  GABA-Transaminase (GABA-T)

The second step of the GABA-shunt is the pyridoxal phosphate-dependent transfer of the amino group from GABA to pyruvate, yielding SSA and alanine, catalyzed by the GABA-Transaminase (GABA-T, EC 2.6.1.19). Following cell fractionation of soybean tissue, the GABA-T activity could be assigned to the mitochondrial fraction [32]. Evidence for a published α-ketoglutarate dependent GABA-T activity [177, 178] exists only from biochemical data, but thus far no corresponding gene has been cloned.

In *Arabidopsis thaliana*, one *GABA-T* isoform exists (At3g22200), and T-DNA insertion plants are indistinguishable from wild types during vegetative growth. Subsequent transition to the generative phase, *gaba-t* mutant plants reveal a reduced fertility compared to wild type, developing smaller siliques. The high number of unfertilized ovules is due to the accumulation of high GABA concentrations in the transmitting tissue of the ovary [130, 188]. In ovules of Arabidopsis wild type plants a GABA gradient is formed, reaching from the stigma to the micropyle, guiding the pollen tube along this gradient to fertilize the egg, which then might stop the production of attractants, e.g. GABA [85, 157].

In response to herbivore attack, plants produce a wide range of volatile organic compounds, among them C6-volatiles (six-carbon alcohols, aldehydes, and esters). When exposed to these compounds, even undamaged plants induce defense-regulated pathways. To elucidate the underlying mechanisms, Arabidopsis mutants with altered responses to the C6-volatile E-2-hexenal were isolated, among them a *gaba-t* mutant, termed *her1* [120]. Here, GABA seems to play a key role in E-2-hexenal detoxification; however, the underlying mechanisms are not yet fully understood.

### 1.2.3.  Succinic Semialdehyde Dehydrogenase (SSADH)

The final step of the GABA-shunt is the $NAD^+$-dependent oxidation of SSA to succinate in the mitochondrial matrix [32]. The reaction is catalyzed by succinic semialdehyde dehydrogenase (SSADH, EC 1.2.1.16), an enzyme active as homotetramer that is inhibited *in vitro* by high $NAD^+$- and adenine nucleotide concentrations [34].

In *Arabidopsis thaliana*, the *SSADH* is a single copy gene (At1g79440), *ssadh* mutant plants display a severe phenotype; they are retarded in growth and less fertile than wild type (Figure 3). The occurrence of necrotic lesions already in the greenhouse, but especially when grown under high light conditions, is discussed in relation to the disruption of the whole GABA-shunt and/or the accumulation of reactive oxygen species [26, 66].

In contrast to the assumption of an intact GABA-shunt being essential for normal plant growth, which was discussed after the isolation of *ssadh* mutants, vegetative growth of *ssadh* mutant plants is improved by inhibiting GABA-T activity. Either, *gaba-t/ssadh* double mutant plants are generated [107] or the structural GABA analog Vigabatrin (γ-vinyl-GABA) is used [66] Vigabatrin can not be metabolized by GABA-T, but interacts with the enzyme and thus irreversibly blocks GABA-T function [174]; in mammalia, it is used as therapeutic in some cases of epileptic disorders.

## 1.2.4.  GHB-Dehydrogenase (GHBDH)

Alternative to the oxidation of SSA by the mitochondrial SSADH, SSA can be reduced to γ-hydroxybutyric acid (GHB) via the GHB-dehydrogenase (GHBDH), also named as succinic semialdehyde reductase (SSR). The GHBDH is localized in the cytosol of animal cells, possibly in plants as well. In mammalians, GHB is known to be a neuroactive substance [160], whereas its role in plants is still unknown.

A gene encoding a *GHBDH* (At3g25530) in Arabidopsis was identified by the complementation of an *ssadh*-deficient yeast strain with an Arabidopsis cDNA-library [31]. GHBDH activity is discussed to be regulated by redox balance or the cellular SSA concentrations, probably complementary with SSADH activity. Sequence analysis revealed a homologous gene in Arabidopsis (At1g17650); both genes are upregulated under stress conditions like drought, salinity or oxygen deficiency [4, 5]. *In silico* analysis of the second identified GHBDH predicts the existence of a transit peptide, but the localization of the protein remains ambiguous. *In vitro* activity assays with purified recombinant enzymes revealed a high affinity for glyoxylate and, to a lesser extent, for SSA [86, 87, 158]. Therefore, both enzymes are discussed to be involved in aldehyde detoxification and in regulation of cellular redox homeostasis.

## 1.3.  Auxin related genes in Arabidopsis thaliana

The phytohormone auxin is critical for plant growth and regulates several developmental processes. Many bioassays are described to test substances for their auxin function: curvature of decapitated pea shoots, elongation of wheat coleoptiles or inhibition of root elongation and induction of lateral root emergence in Arabidopsis [182, 194]. Corresponding to these assays, mutants overproducing auxins tend to have shorter roots and abundant lateral roots. At neutral pH, all known substances with auxin activity contain a strong, negative charge at the carboxyl group and a weaker, positive charge in the aromatic ring in a distance of 0.5nm, which is a key feature of their function [171].

In plants, auxins can be synthesized in several tryptophan-dependent and -independent pathways; the tryptophan-dependent pathways are generally named according to major intermediates. Since the number of genes involved in synthesis, transport, degradation or perception of auxins is very high [139, 186], here, only those genes will be covered, where loss-of-function mutants or GUS-expressing lines are used in this work.

The nitrilase proteins belong to a family with four members in Arabidopsis. While NIT1, NIT2, and NIT3 catalyze the hydrolysis of indol-3-acetonitril (IAN) to indol-3-acetic acid (IAA) and are supposed to contribute to the maintenance of IAA homeostasis [127, 132, 138, 181, 183]. In Arabidopsis, NIT1 is the highest expressed isoform that aggregates rapidly in response to wounding as early marker of cell death  [13, 55]. *nit1* mutants are insensitive to IAN and lack obvious low-auxin phenotypes, indicating that the NIT1 function in auxin synthesis is redundant [127]. NIT2 usually has a basal expression, but induction by pathogens, leaf senescence, and exogenous IAN was reported. This induction correlates with the decreased IAN and increased IAA levels observed under these conditions [13, 80, 141]. Regarding the NIT3 gene, induced expression under sulfur starvation was published [101]. In contrast to the other family members, NIT4 is involved in cyanide detoxification, but not in auxin biosynthesis [137].

To remove the free active auxin, IAA can be conjugated to amino acids or glucose [36, 153, 164]; thus, the overexpression of the microbial IAA-conjugating enzyme IAA-L in Arabidopsis reduces the content of free IAA [192].

Auxin is mainly produced in shoot apical regions, its transport is highly regulated and necessary for various processes involving the establishment of the embryonic axis, phyllotaxis, development of vasculature and lateral root formation [11, 57, 71-73]. Both, auxin import and export from the cells are regulated processes, the former controlled by AUX1 (Auxinand the LAX proteins, the latter by PIN and MDR (multidrug-resistance like) proteins, members of the PGP (P-glycoprotein) family can be involved in either uptake or export of auxin [12, 93, 94, 100]. Proteins of the AUX/LAX family are localized asymmetrically in the plasma membrane, facilitating polar auxin transport and root gravitropism, and are involved in lateral root emergence [19, 60, 111, 169]. The auxin transporters PGP and MDR directly bind the auxin transport inhibitor N-1-naphthylphthalmic Acid (NPA) [122, 126, 173]. The polar localization of PINs is highly dynamic and responds to signal from the serin-threonin kinase PINOID [18]. PIN-mediated auxin transport can be inhibited by 2,3,5-triiodobenzoic acid (TIBA), that prevents the rapid cycling of the PIN proteins between the plasma membrane and a yet unidentified endosomal compartment by vesicle trafficking [61, 76, 91, 135].

Expression of auxin-regulated genes occurs within minutes of auxin application without a requirement for protein synthesis [1], since the promoter regions of these genes contain auxin responsive elements (AuxRE). These elements are recognized by activating transcription factors, the auxin responsive factors (ARFs) forming heterodimers with Aux/IAA proteins, which act as repressors, and prevent expression of auxin-related genes under low auxin conditions [81, 106]. Free IAA binds to a receptor, the F-box protein TIR1, an E3-ubiquitin ligase that is a member of the SCF complex. In response to auxin binding, TIR1 mediates the ubiquitination of Aux/IAA proteins, targeting them to proteosomal degradation, thus allowing the ARF proteins to initiate transcription of auxin-related genes [10, 59, 92, 133, 140].

## 1.4. Callus formation in Arabidopsis thaliana

To ensure coordinated tissue growth during plant development, events like cell division, growth, and differentiation need to be tightly controlled. Therefore, only meristems and surrounding tissues possess mitotic activity. A loss of control results in ectopic cell division and eventually in the development of tumors, i.e. unorganized growing tissue.

Dedifferentiation is in many cases due to an imbalance of the phytohormones auxin and cytokinin, this imbalance often results in tumors growing *in vitro* on media without added hormones as callus. But callus formation can not only be induced by an altered hormone content caused by an increased synthesis or a reduced degradation or inactivation. Alternatively, mutations might render the plants more sensitive toward hormones or influence signaling pathways downstream of hormone perception, which then changes gene expression or controls cell division.

The analysis of Arabidopsis cell lines obtained by X-ray mutagenesis revealed three different kinds of cell lines: the first formed shoot-like structures (shooty callus), the second formed roots (rooty callus), and the third grew without organ formation (callus). The first cell lines had low auxin and high cytokinin contents, in contrast to the second lines with high auxin and low cytokinin levels [37, 70]. Growth and differentiation of callus can be influenced by the auxin to cytokinin ratio; a low ratio leads to formation of shoots, whereas a high ratio induces root development [159, 171]. This effect is widely used in plant molecular genetics to regenerate whole plants from explants *in vitro*, e.g. to generate transgenic plant lines.

## 1.5.  GABA acts as signal molecule in various organisms

### 1.5.1.  GABA signaling in the central nervous system of vertebrates

GABA is commonly known as the main inhibitory neurotransmitter in the central nervous system of vertebrates. Excitation of GABAergic neurons results in the release of GABA from vesicles of the presynaptic neuron via $Ca^{2+}$-dependent exocytosis. By GABA binding to different GABA receptors on the membrane of the postsynaptic cell, a response is triggered; either opening of ion channels or induction of a signaling cascade. To terminate the signal, GABA is imported into the neurons or in neighboring glia cells for degradation.

GABA-mediated signaling is found to be involved in the regulation of many developmental steps from cell proliferation to synaptogenesis and circuit formation in the immature brain. Compared to adult neurons, immature cells have relatively high internal $Cl^-$ concentrations, opening of GABA-gated $Cl^-$-channels therefore results in membrane depolarization. In invertebrates, a growth-promoting effect through GABA-induced membrane depolarization was found. Taken this into account, already in ancient organisms, a GABA signaling pathway might have existed, where GABA was involved in trophic responses [129].

In vertebrates, two GAD isoforms are known, termed GAD65 and GAD67 that are differentially expressed among brain regions. In contrast to plants, short-term regulation of GAD activity in the mammalian brain is controlled by binding of the cofactor pyridoxal phosphate [25, 113], not by $Ca^{2+}$-induced dimerisation as in plants. GAD65 is localized more toward the nerve terminals, and is found in the brain in high amounts in the inactive form, suggesting it to be involved in response to short-term changes in demand for GABA. In contrast, GAD67 is found throughout the cell and its transcription is repressed by GABA, maintaining cellular GABA homeostasis [67, 144]

GABA degradation is catalyzed by the single copy genes *GABA-T* [128, 131, 136] and *SSADH* [150]. Malfunctions in GABA catabolism lead to severe mental disorders, caused by inborn gene defects. GABA-T deficiency is a rare disease, resulting in symptoms like psychomotor retardation, lethargy or electroencephalographic abnormalities [117]. Regarding human patients, a nonfunctional SSADH is more common than a mutated GABA-T. SSADH deficiency in human patients results in a disorder termed γ-hydroxybutyric aciduria, which is associated with high extracellular GABA and GHB concentrations. Again, the symptoms are behavioral disturbances and psychosis [64, 77, 134].

The short-chain fatty acid GHB occurs naturally in the mammalian brain and is formed from SSA via the succinic semialdehyde reductase (SSR or GHBDH). Additionally, this enzyme is involved in detoxification of xenobiotic carbonyl compounds [125, 150, 179]. *In vitro*, a conversion of GHB via SSA to GABA was detected by the use of ^{3}H-labeled GHB as substrate, in contrast to the *in vivo* situation, where the substrate concentrations might be too low to allow this reaction. The distribution of GHB synthesizing enzymes in the cells and high-affinity GHB binding sites in the brain suggest GHB to act as neurotransmitter or modulator of neural activity [84, 110]. The binding sites were identified as GHB receptors located in the presynaptic cells that act via initiation of G-protein coupled signal cascades [160].

GABA uptake in presynaptic neurons is catalyzed by a well-characterized GABA-Transporter, GAT1. To regulate GABAergic neurotransmission, GAT1 distribution, activity and turnover rate must be tightly controlled. Several classes of neurons are shown to express different levels of GAT1, and GFP-labeled protein is visible in different intracellular densities in the synapses. The cytosolic N-terminal domain is reported to interact with syntaxin 1A, a SNARE protein involved in neurotransmitter release and modulation of $Ca^{2+}$-and $Cl^{-}$-channels. Additionally, GAT1 activity is modulated by signaling pathways and protein-protein interactions; tyrosine-phosphorylation directs the protein to the plasma membrane, while unphosphorylated GAT1 remains cytosolic [44, 58, 104, 115].

In vertebrates, three classes of GABA-receptors with different characteristics are known, termed $GABA_A$-, $GABA_B$- and $GABA_C$- receptors [24].

The ionotropic $GABA_A$-receptors are heteropentameric $Cl^-$-channels with two GABA binding sites found in all regions of the central nervous system. The subunits are derived from several related genes or gene families, providing a very high number of potential subunit combinations; however, certain subunit combinations seem to be preferred. Each subunit comprises four transmembrane domains with an intracellular loop between TM3 and TM4, containing a consensus site for phosphorylation by protein kinases. Several chemicals acting antagonistically or modulatory on receptor transport activity are known and are used as therapeutics.

The metabotropic $GABA_B$-receptors comprise seven transmembrane helices, existing in two isoforms (R1a and R1b). Recently, evidence was found, that functional native receptors are heterodimers composed of one of the R1 isoforms and a newly identified R2 subunit. Binding of GABA results in a conformational change of the receptor, coupling activity to $Ca^{2+}$-and $K^+$-channels via heterotrimeric G-proteins and second messenger systems, initiating a signal cascade in the target cell [75].

Although being ionotropic, $GABA_C$-receptors differ from $GABA_A$-receptors in several aspects. The general membrane topology is comparable to $GABA_A$-receptors; they are homo- or heteropentameric $Cl^-$-channels, but are insensitive to $GABA_A$-receptor antagonists and modulators. $GABA_C$-receptors are mainly expressed in the vertebrate retina. Their slower activation and inactivation curves probably reflect the presence of five ligand-binding sites.

## 1.5.2. GABA metabolism in *Saccharomyces cerevisiae*

Yeast is often used as a model organism to study the function of mammalian enzymes. The role of GABA in intercellular signaling is intensely studied, whereas only little is known about its intracellular functions. In *Saccharomyces cerevisiae*, several genes are involved in GABA metabolism. GABA transport is mediated via the UGA4 gene, encoding a GABA and δ-aminolevulinic permease [20, 21], which is transcriptionally regulated in response to the available amount and quality of carbon and nitrogen sources [6, 108, 172].

In a search for yeast strains altered in GABA metabolism, an *ssadh*-deficient strain was isolated, that has a reduced growth rate compared to wild type strains and accumulates SSA. Biochemical characterization of the identified enzyme reveals a very high affinity for SSA. As conclusion, a toxicity of SSA seems to be most likely for the observed phenotypes [142].

Overexpression of GAD1 increases the resistance against different oxidants as long as the GABA catabolism remains intact, whereas a loss of either UGA1 (GABA-T) or UGA5 (SSADH) reduces oxidative stress tolerance. Additionally, the expression of UGA5 is induced by exposure to $H_2O_2$, implying a function of the GABA metabolism in buffering redox changes in yeast [53]. Oxidant resistance seems to be proportional to the gene dosage of GAD1, probably due to either the synthesis of GABA, which is involved in cellular stress response, or the production of the antioxidant NADPH during GABA degradation.

### 1.5.3.   Repression of quorum sensing in *Agrobacterium tumefaciens*

Quorum sensing (QS) is a mechanism of bacterial cell-cell communication during times of high cell density, mediated through small, diffusible signal molecules. If the population grows, then more signal molecules are produced than lost by diffusion or inactivation, this leads to an accumulation of the signal to a threshold level. This results in the activation of receptor proteins and the initialization of complex regulatory cascades inducing coordinated changes in gene expression in individual members of the whole population. In plant pathogenic bacteria, QS mechanisms are often involved in the regulation of colonization and infection of host plants, e.g. by producing cell wall degrading enzymes, altering Ti plasmid transfer, and motility of the cells. This requirement of QS regulatory systems for pathogenicity may offer an option to control bacterial diseases of plants by interfering with the QS system [14, 180].

The activity of the QS system is controlled by two systems, *tra* and *att*. TraR is an N-acyl-homoserine lactone (AHL) receptor that activates transcription of two linked *tra* operons, a *trb* and the *rep* operon [105]. The *rep* operon is necessary for replication of the Ti plasmid in response to AHL binding, and the  *tra* and *trb* operons encode structural components for conjugal transfer of the Ti plasmid [74]. AHL-bound TraR enables expression of TraI, which synthesizes AHLs, and of the TraR-repressor TraM, which regulates the activity of TraR at high AHL concentrations [170].

The *att* system is involved in QS signal decay. During the Agrobacterium growth phase, the *ALDH* gene – a succinic semialdehyde dehydrogenase – is transcribed, and the transcription of the *attKLM* operon is repressed by attJ. When the stationary phase is reached, a stress signal – ppGpp – interferes with the function of Agrobacterium RNA polymerase and *ALDH* is no longer expressed. Thus, SSA can accumulate in the cytosol, bind to attJ, and thereby release the repression of the *attKLM* operon. This operon contains three genes, a lactonase (attM), a homolog to NAD$^+$-dependent alcohol dehydrogenases (attL) and a succinic semialdehyde dehydrogenase (attK). The first protein cleaves the γ-butyrolactone ring of AHL and, together with attL, inactivates the

QS signal; whereas the third enzyme removes excessive SSA, gradually disabling the QS signal decay [39, 40, 185].

Exogenous GABA or GABA degradation products from Agrobacterium host plants, like SSA, γ-hydroxybutyric acid (GHB) and γ-butyrolactone (GBL), have been reported to bind to attJ and to interfere with bacterial QS. GABA, accumulating in wounded plant tissues and acting as signal between eukaryotes and bacteria, might offer a possibility for the development of defense strategies against bacterial pathogens [43].

## 1.6. Aims of this thesis

During this work, three main topics should be covered; first, the analysis of *Arabidopsis thaliana* GABA-shunt mutants and the effect of GABA-shunt metabolites on plant growth; second, how is succinic semialdehyde (SSA) linked to callus formation and does a connection between the GABA-shunt and phytohormones exist; and third, the identification of yet unknown genes involved in regulation or function of the GABA-shunt or in GABA sensing.

To assay the effect of GABA-shunt metabolites on plant growth and development, Arabidopsis wild type and several GABA-shunt mutants are grown on ½ MS media supplemented with SSA, GHB, GABA, or glutamate. Growth is monitored, and the metabolite contents – with special attention on substances related to GABA – are determined by GC-MS analytics.

To characterize isolated T-DNA insertion lines, their growth under various conditions is observed, and the intracellular localization is analyzed by the expression of fusion proteins tagged with fluorescent markers.

To identify further genes related to GABA-metabolism, a population of EMS-mutagenized *ssadh* plants is screened for suppressor lines, and the corresponding mutations will be identified by map-based cloning.

To test whether a linkage between SSA and phytohormones exists, the callus formation rate in response to combinations of SSA and phytohormones or phytohormone antagonists is determined. Additionally, a possible effect of SSA on the expression of phytohormone related genes is assayed using promoter::GUS lines.

# 2. Results

## 2.1. Influence of the GABA-shunt on Arabidopsis plant growth

GABA and GABA-shunt metabolites are known neurotransmitters in mammalians, and deficiencies in GABA-degrading enzymes lead to neurodegenerative disorders in patients. To test whether GABA has effects on plant growth and development, knock-out plants were isolated and in comparison to wild types analyzed for their response to exogenously applied GABA-shunt metabolites.

### 2.1.1. Examination of GABA-shunt mutants

The smaller siliques along with the reduced fertility observed in *gaba-t* mutant plants (Figure 2) was explained by an accumulation of GABA in the transmitting tissue of the ovary, leading to a misguidance of pollen tubes [130]. An increased GABA accumulation was found not only in the flowers of *gaba-t* mutant plants but also in vegetative tissues.
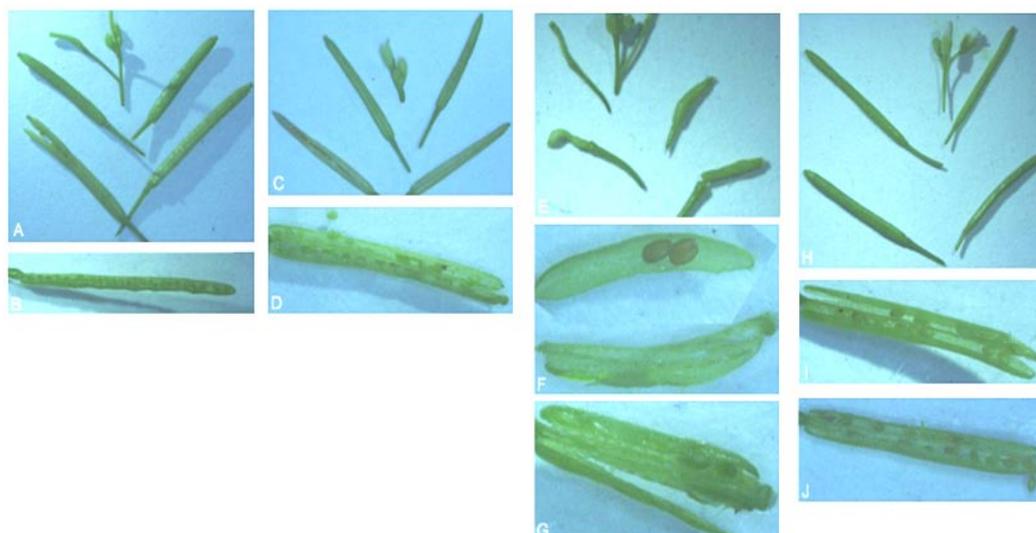


**Figure 2: Comparison of Arabidopsis wild type and *gaba-t* mutant siliques**

A to D: wild type siliques from main (A, B), and side (C, D) inflorescences
E to J: *gaba-t* mutant siliques from main (E, G), and side (H, J) inflorescences

The developmental retardation observed in *ssadh* mutant plants (Figure 3) was first discussed as a result of the disrupted GABA-shunt. Later, an interruption of the pathway at an earlier reaction was found to be sufficient to suppress the *ssadh* phenotype [66, 107]. This interruption prevents the accumulation of a toxic substance in *ssadh* mutant plants.



**Figure 3: Comparison of Arabidopsis wild type and *ssadh* mutant plants**

All plants were grown under greenhouse conditions; the pictures have the same magnification
A: 3-week-old wild type plants
B to H: 21-week-old *ssadh* mutant plants; mutants are dwarfish plants with a retarded development, they have a reduced fertility and necrotic lesions

To further analyze the mutants, the metabolite content in leaf extracts was measured, revealing about 6-fold higher GABA levels in *gaba-t* and *gaba-t/ssadh* mutant plants compared to wild type plants of the same age (Figure 4A). In *ssadh* mutants, GABA levels even accumulated to approximately 16 fold of the wild type levels.

In extracts of wild type and *gaba-t* mutant plants, the GHB levels were at or below the detection limits of the GC-MS system used; these plants did not accumulate large amounts of GHB; in extracts of *gaba-t/ssadh* mutants no major increases in GHB content were measured, levels reached about 8 fold of wild type levels. Compared to the other lines, leaf extracts of *ssadh* mutants, contained vast amounts of GHB; levels were increased 160 fold compared to wild type (Figure 4B). However, the GHB levels do not necessarily reflect the SSA levels in plants, since the GHBDH reaction might be a limiting step *in vivo*. Furthermore, these values should be handled with care, since the *ssadh* plants were about three times older than the other plants when harvested for extraction.
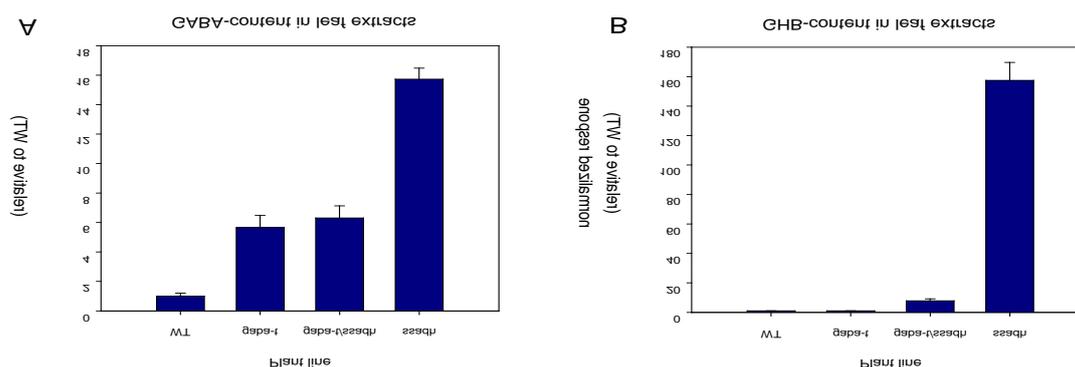


**Figure 4: Metabolite measurement of GABA and GHB in GABA-shunt mutants**

(A) GABA contents in rosette leaves of wild type, *gaba-t*, *gaba-t/ssadh,* and *ssadh* plants.
(B) GHB contents in rosette leaves of wild type, *gaba-t*, *gaba-t/ssadh,* and *ssadh* plants. The *ssadh* mutants accumulated very high amounts of GHB, whereas for the other lines GHB levels were near the detection limits.
Wild type, *gaba-t,* and *gaba-t/ssadh* plants were grown for 22 days and *ssadh* plants for 75 days on soil under greenhouse conditions. The experiment was repeated twice with six biological replicates analyzed, the bars represent the standard deviation
.

## 2.1.2. Effect of GABA-shunt metabolites on plant growth

To assay whether GABA-shunt metabolites have an effect on plant growth, wild type plants of different ecotypes (Columbia-0 – Col-0; Landsberg *erecta* – L*er*; and Wassilewskija – Ws-2) were grown on ½ MS supplemented with various amounts of GABA, GHB, and SSA respectively, and analyzed for root and shoot development (Figure 5).

GABA was found to induce only minor effects on Arabidopsis wild type plants, whereas SSA and GHB both reduced the growth rate in a concentration dependent manner. The shorter roots and yellowish leaves observed on media containing higher GABA concentrations might be a side effect of nitrogen availability. SSA induced an increased formation of lateral roots, and reduced the length of the main root; along with the shorter root, the rosette size decreased. Further increase of SSA concentrations in the media induced lesions in the leaves. Wild type plants grown on GHB displayed only minor effects on shoot growth, but the roots were notably shorter with a slightly increased number of lateral roots.

Arabidopsis wild type plants were able to grow on GABA as the sole nitrogen source, in contrast to *gaba-t* mutants that grew worse on GABA-containing media than on media without any available nitrogen (Figure 6). Compared to wild type plants, *gaba-t* mutant plants growing on ½ MS displayed a tendency toward an enhanced biomass production. The same effect could be achieved in wild type plants by replacing some inorganic nitrogen ($KNO_3$ / $NH_4NO_3$) with GABA. On nitrogen-free media, growth of both wild type and *gaba-t* mutants was reduced and the leaves were yellowish, probably due to nitrogen limitations. On media containing 20mM GABA as the sole nitrogen source, wild type plants still displayed indications of nitrogen limitation and were smaller than on control ½ MS media, but *gaba-t* mutant plants grew even worse on media containing GABA as the sole nitrogen source compared to plants grown on nitrogen-free media.
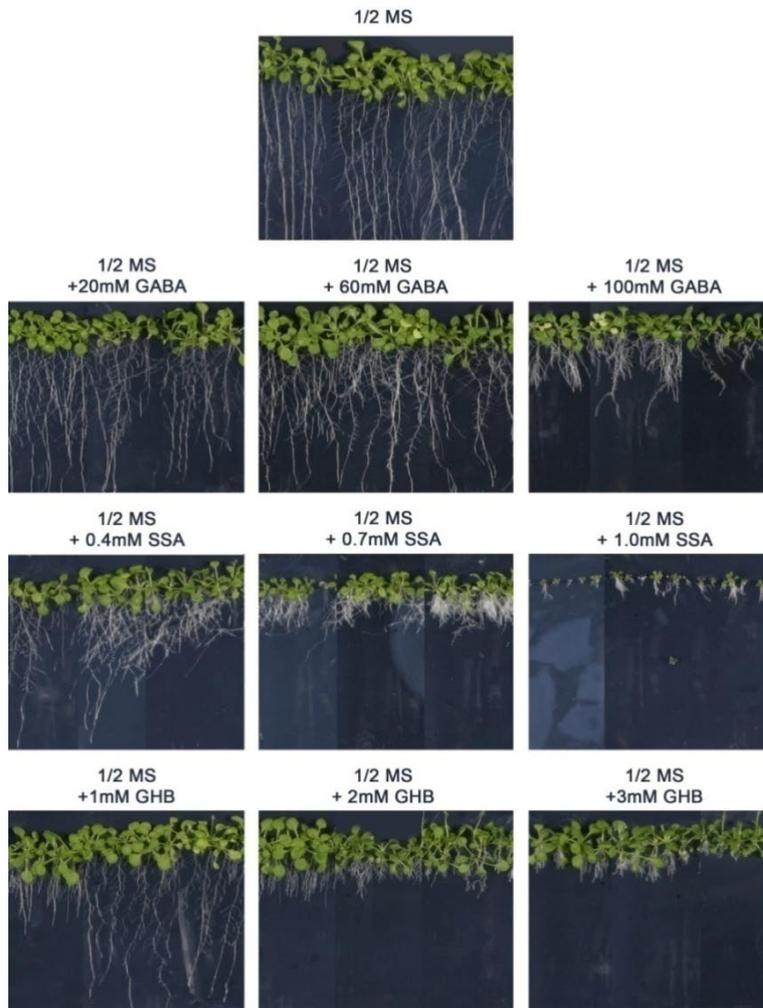
**Figure 5: Growth of various Arabidopsis ecotypes on ½ MS supplemented with different concentrations of GABA, SSA, or GHB**

Three Arabidopsis ecotypes (left: Col-0, middle: L*er,* right: Ws-2) were grown for 16 days on ½ MS supplemented with increasing concentrations of GABA, SSA or GHB and analyzed for effects on plant development.
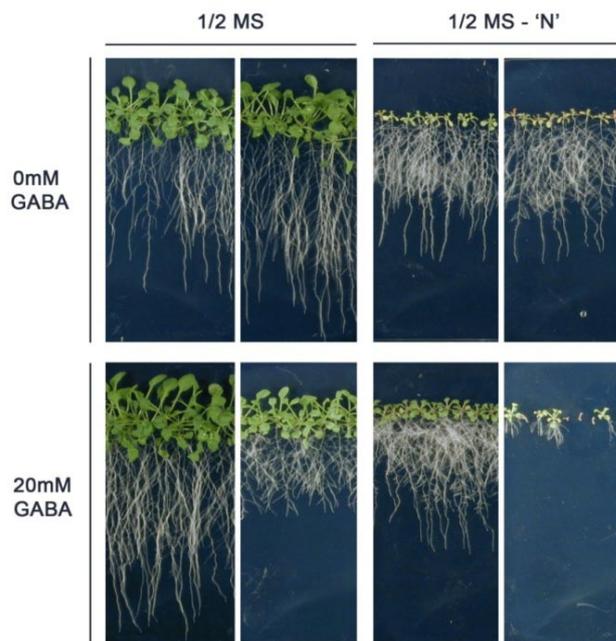


**Figure 6: Growth of wild type and *gaba-t* mutant plants on different media**

Wild type (left) and *gaba-t* (right) seedlings were grown for 14 days on ½ MS or ½ MS without inorganic nitrogen and either without GABA or supplemented with 20mM GABA. Presence of GABA in the media considerably reduced growth of *gaba-t* plants, whereas growth of wild type plants was increased by GABA. GABA as sole nitrogen source almost inhibited growth of *gaba-t* mutant plants completely.

To examine whether the inhibitory effect of GABA on *gaba-t* mutants is induced by high GABA concentration during germination, *gaba-t* and *gaba-t/ssadh* seedlings were germinated on ½ MS and transferred onto various media five days later. Neither were differences visible compared to seedlings remaining on the plates nor between wild type and *gaba-t* or *gaba-t/ssadh* mutant plants when shifted to ½ MS. Transfer of seedlings to media containing 10mM GABA improved growth of wild type plants whereas in *gaba-t* and *gaba-t/ssadh* mutants, the main root was shorter with a higher number of lateral roots, and the rosette size was slightly reduced compared to control plants.

On GABA as sole nitrogen source, *gaba-t* and *gaba-t/ssadh* plants did not develop much further; some lateral roots were initiated, but did not grow substantially. Furthermore, the leaves turned yellowish and newly formed leaves remained small. In contrast, wild type plants formed a high number of lateral roots, and both root length and rosette size were not much reduced compared to control plants (Figure 7). Germinating seeds on ½ MS supplemented with 10mM GABA for 7 days resulted in the known reduction of *gaba-t* or *gaba-t/ssadh* growth; the plants were then transferred to ½ MS and 14 days later no differences between wild type and *gaba-t* or *gaba-t/ssadh* mutants were visible any more (not shown).



**Figure 7: Growth of wild type, *gaba-t,* and *gaba-t/ssadh* mutant plants**

Wild type (left), *gaba-t* (middle) and *gaba-t/ssadh* (right) seedlings were grown on ½ MS for 5 days, followed by transfer to the indicated media. Pictures were taken 12 days after transfer.

Both, SSA and GHB affect growth of Arabidopsis wild type plants. To identify the substance responsible for the *ssadh* phenotype, the effect of the metabolites on wild type, *gaba-t,* and *gaba-t/ssadh* plants was assayed. Wild type and *gaba-t* plants were equally affected; no major differences regarding growth or metabolite content were detected. In contrast, *gaba-t/ssadh* mutant plants were highly sensitive toward SSA and GHB, small amounts of either of the substances substantially inhibited growth rates (Figure 8).

On ½ MS, no obvious growth differences were visible among the three lines. In leaf extracts of wild types and *gaba-t* mutants, GHB levels were at the detection limit, whereas levels increased up to 30-fold in *gaba-t/ssadh* mutants compared to wild types. The higher GHB levels in *gaba-t/ssadh* mutant plants in this experiment compared to the data in Figure 4 might be due to different growth conditions (½ MS vs. soil) and plant age.

Low SSA-concentrations in the media induced the formation of lateral roots in wild type and *gaba-t* mutants, but overall growth was not reduced. GC-MS measurements revealed only a minor increase in leaf GHB contents. In contrast, *gaba-t/ssadh* mutants grown under these conditions displayed a considerably reduced growth as well as increased leaf GHB contents compared to wild type. A further increase of the SSA concentrations impaired wild type and *gaba-t* mutants; the plants were smaller and accumulated higher amounts of GHB in the leaves. Growth of *gaba-t/ssadh* mutants was strongly inhibited, and an increase of GHB levels in leaf extracts up to 5000 fold of wild type levels under standard conditions was detected.

The addition of GHB to the media did not influence shoot growth of wild type and *gaba-t* mutants, although increasing the GHB concentration in the media reduced the main root length and induced lateral root formation. Growth of *gaba-t/ssadh* mutants on GHB-containing media was already strongly inhibited by low GHB concentrations. In leaf extracts of plants grown on GHB-containing media, GHB accumulation could be determined; extracts of wild type and *gaba-t* mutants contained roughly equal amounts of GHB, whereas *gaba-t/ssadh* mutants once more accumulated vast amounts of GHB.

Taken together, plant growth and GHB content in plant extracts are not necessarily linked to each other. GHB levels in plants extracts still allowing wild type and *gaba-t* mutant plants to grow fairly unaffected were sufficient to highly impair growth of *gaba-t/ssadh* mutants plants (Figure 8).
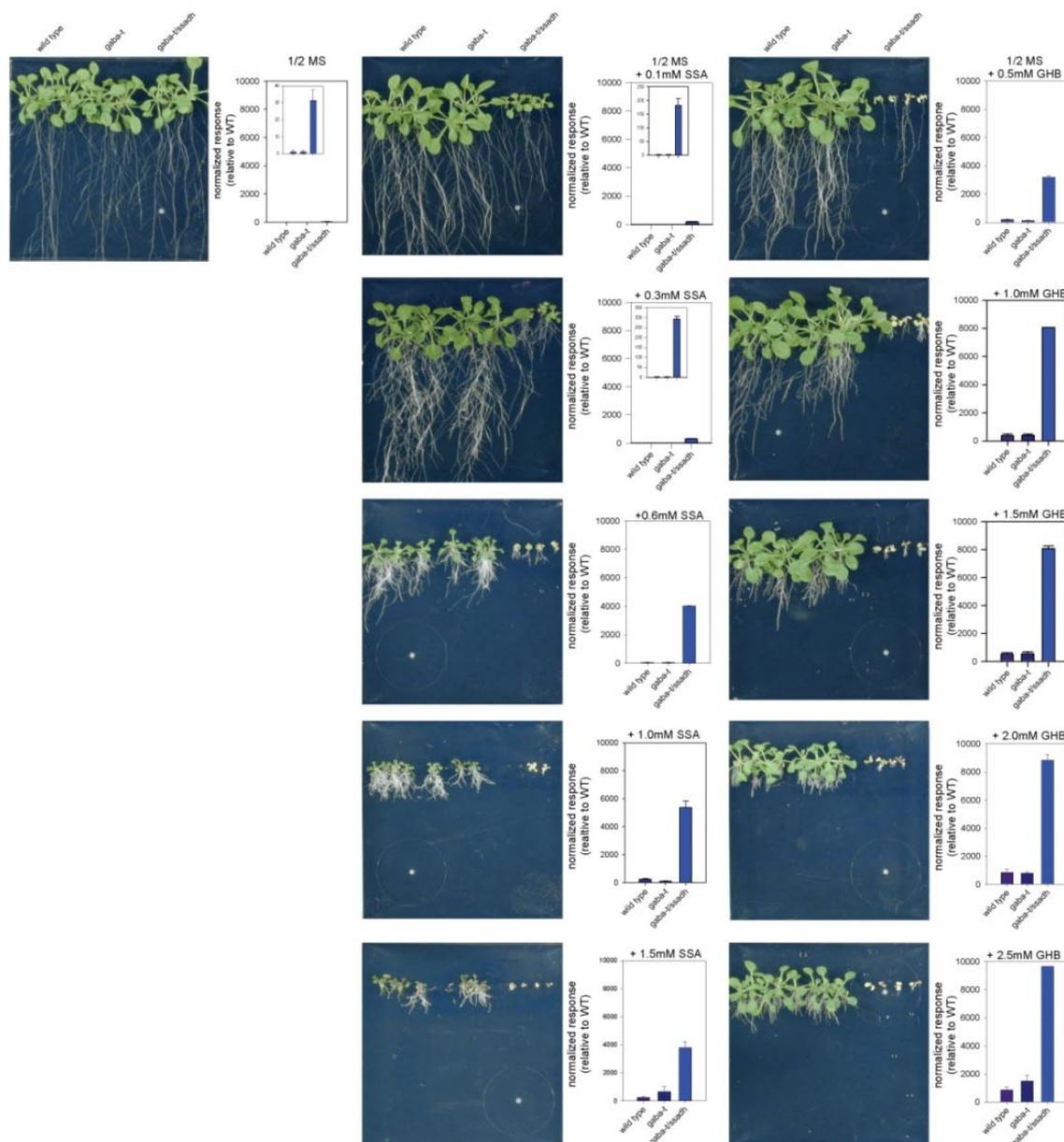


**Figure 8: Growth of wild type, *gaba-t,* and *gaba-t/ssadh* plants on ½ MS supplemented with either SSA or GHB in different concentrations**

Wild type (left), *gaba-t* (middle) and *gaba-t/ssadh* (right) plants were grown on the indicated media for 15 days until sampling for GC-MS analyses. Pictures of the plates and the determined GHB-contents relative to wild type are presented next to each other. The experiment was repeated three times and 5 biological replicates were analyzed each, with the exception of *gaba-t/ssadh* mutant plants, where the rosette weight of plants grown on higher SSA or GHB concentrations were sufficient only for three samples, the bars represent the standard deviation .

## 2.1.3. Analysis of the Arabidopsis GHBDH-genes

To address the question whether SSA or GHB causes the severe growth phenotype of *ssadh* mutant plants, *ghbdh* mutant plants were isolated and tested for their sensitivity against SSA or GHB.

An Arabidopsis cDNA encoding a GHB-Dehydrogenase was cloned a few years ago [31], and the corresponding gene (At3g25530) was identified by sequence comparison. The genomic sequence did not contain any target information; so the protein was predicted to be cytosolic. Co-localizations of AtGHBDH-1-GFP and a plasma membrane RFP marker [123] displayed a partially overlapping fluorescence (Figure 9), proving the enzyme to be cytosolic as predicted *in silico*.
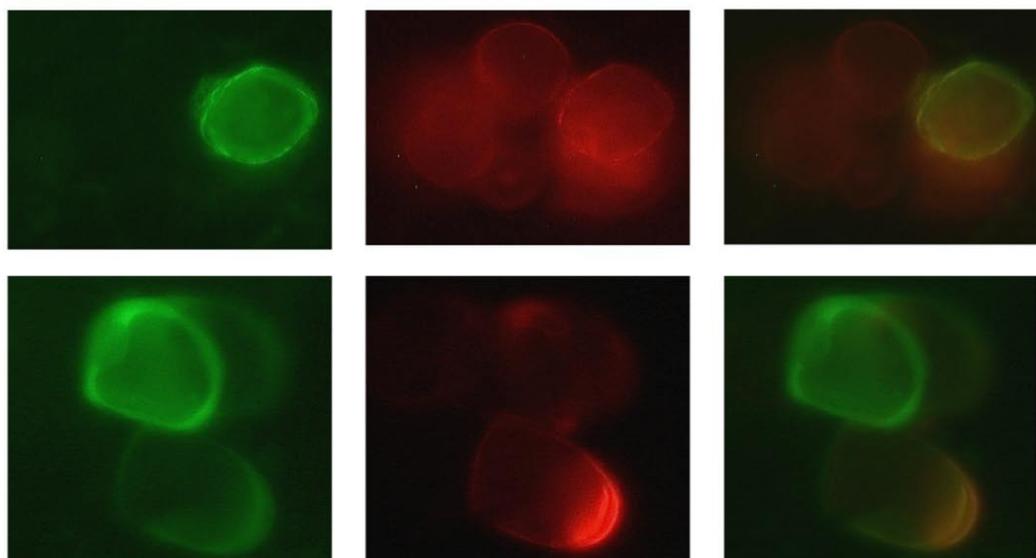


**Figure 9: Co-localization of AtGHBDH-1-GFP with a plasma membrane marker**

*Arabidopsis thaliana* cell cultures were transformed with both AtGHBDH-1-GFP and a plasma membrane RFP marker and analyzed three days later by fluorescence microscopy (4.2.1.8). A partially overlapping fluorescence was observed that corresponds to the predicted cytosolic localization of AtGHBDH-1.
Left: GFP-fluorescence, middle: RFP-fluorescence, right: merged

For *GHBDH-1*, two independent T-DNA insertion lines (GabiKat_316D05 and SALK_057410) were isolated, identified as null-mutants by RT-PCR, and no phenotypic differences compared to wild type plants were visible under greenhouse conditions.
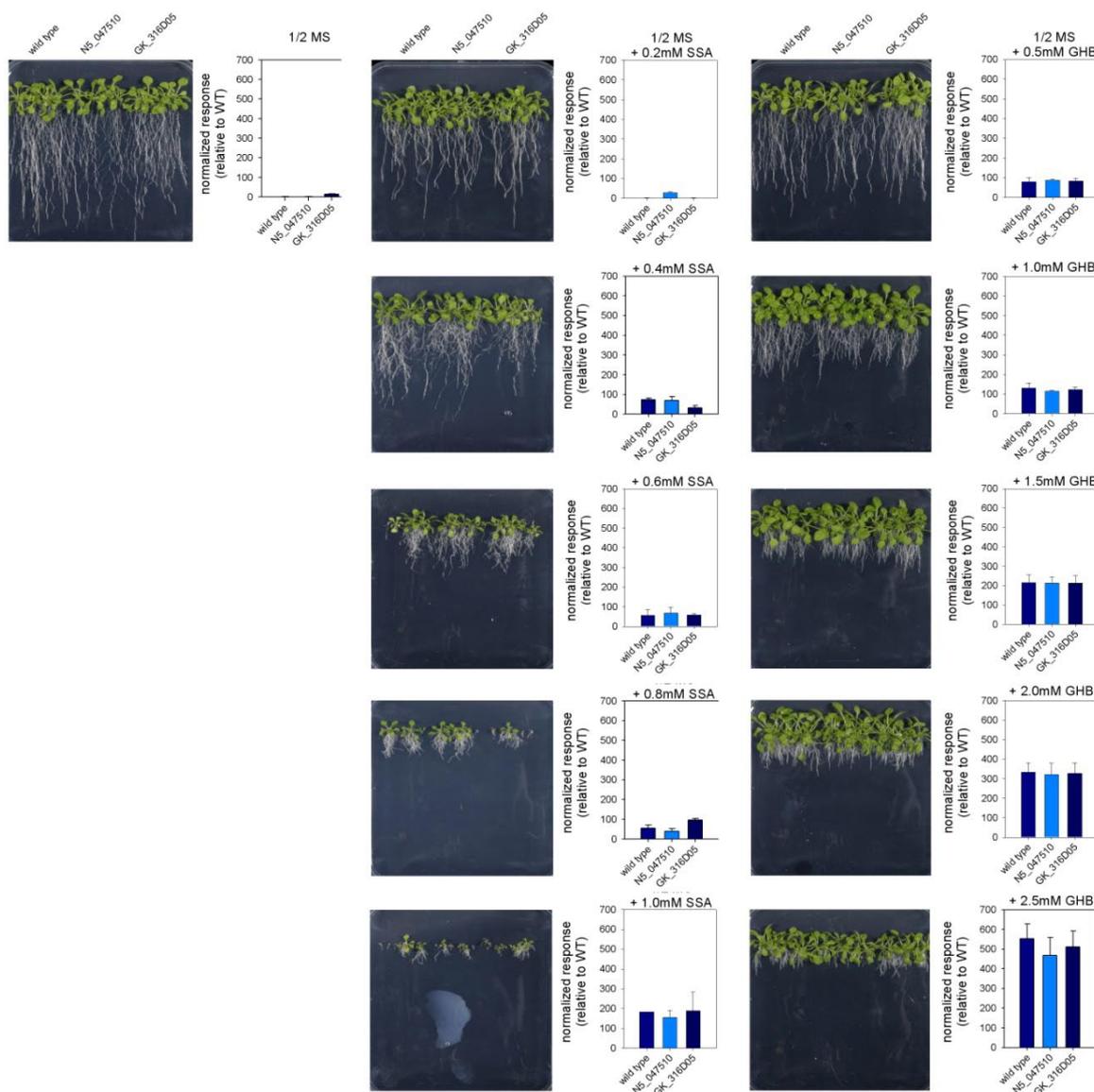
**Figure 10: Growth of wild type and two independent *ghbdh-1* lines on ½ MS supplemented with SSA or GHB**

Plants were grown on the indicated media for 15 days, and then samples for GC-MS analyses were taken. Pictures of the plates and the determined GHB-contents relative to wild type are presented next to each other. The experiment was repeated twice and 6 biological replicates were analyzed each, the bars represent the standard deviation.

Since SSA and GHB can be converted into each other by a dehydrogenase, a block of this reaction was supposed to trigger a difference in response to the substances. Under control conditions (½ MS), no differences occurred between wild type and *ghbdh-1* mutant plants. GHB levels were near detection limits of the GC-MS system used. When grown on ½ MS + SSA, both wild type and the different *ghbdh-1* mutant lines displayed a reduced root length with an increasing number of lateral roots forming and a smaller

shoot size, with slightly paler leaves compared to control plants. GC-MS analysis revealed the presence of equal amount of GHB in wild type and *ghbdh-1* mutants.

Both wild type and *ghbdh-1* mutant plants grown on ½ MS containing GHB displayed a shortened root length and an almost unchanged shoot growth. Increasing GHB-concentrations further decreased root length. No difference in GHB-content was measured in wild type and *ghbdh-1* mutant plants (Figure 10).

In contrast to the expectation, no differences between wild type and *ghbdh-1* mutant plants occurred in growth experiments on ½ MS supplemented with either SSA or GHB. Additionally, GHB was detectable in plant extracts upon SSA feeding, indicating a second enzyme capable of catalyzing the SSA-GHB conversion exists.

To identify this predicted homologous enzyme in Arabidopsis, a BLAST search with the GHBDH-1 protein sequence was performed, the similar sequences were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and a phylogenetic tree was generated (Figure 11).
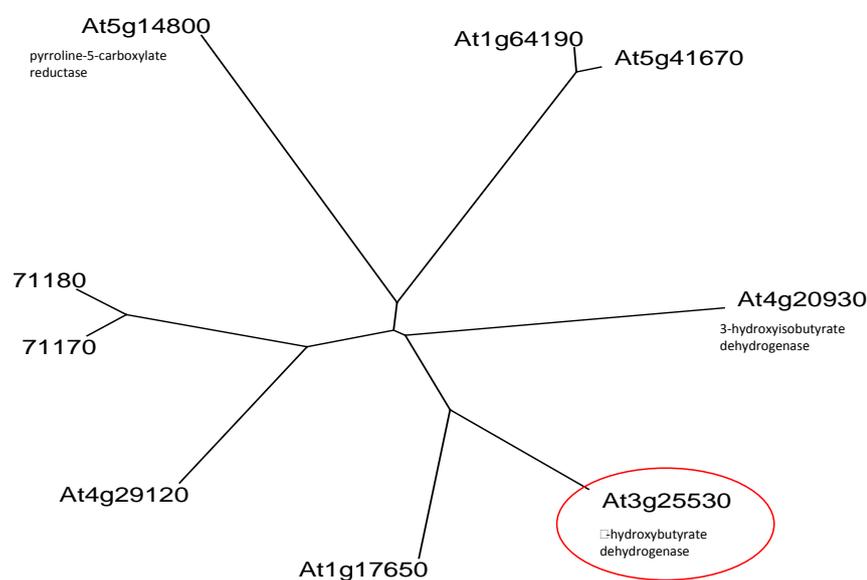


**Figure 11: Phylogenetic tree of GHBDH-homologous sequences**

Results of a BLAST search with the Arabidopsis GHBDH protein. One of the identified proteins (At1g17650) clusters together with the Arabidopsis ghbdh (At3g25530).
Protein sequences were annotated as "phosphogluconate dehydrogenase" when not labeled otherwise.

One of the proteins clustered together with GHBDH-1, and a detailed sequence analysis revealed a high identity on protein level. The new identified GHBDH-2 contained an N-terminal presequence, probably being an intracellular targeting sequence (Figure 12).

```
At3g25530    --------------------------------------------------MEVGFLGLG  9
At1g17650    MALCSICPRIPLRFRPKPISPFLSKPQICLAYRVYSSLQSTTPSTRDELGTVSIGFLGMG  60
                                                               :.:****:*

At3g25530    IMGKAMSMNLLKNGFKVTVWNRTLSKCDELVEHGASVCESPAEVIKKCKYTIAMLSDPCA  69
At1g17650    IMGSPMAQNLIKAGCDVTVWNRTKSKCDPLVGLGAKYKSSPEEVTATCDLTFAMLADPES  120
             ***..*: **:* * .******* **** **  **.  .** **  .*. *:***:** :

At3g25530    ALSVVFDKGGVLEQICEGKGYIDMSTVDAETSLKINEAITGKGGRFVEGPVSGSKKPAED  129
At1g17650    AIDVACGKNGAIFGISSGKGYVDVSTVDVASSILISKQIKDTGALFLEAPVSGSKKPAED  180
             *:.*. .*.*.:  *..****:*:****. :*: *.: *...*. *:*.**********

At3g25530    GQLIILAAGDKALFEESIPAFDVLGKRSFYLGQVGNGAKMKLIVNMIMGSMMNAFSEGLV  189
At1g17650    GQLIFLTAGDKPLYEKAAPFLDIMGKSKFYLGEVGNGAAMKLVVNMIMGSMMASFAEGIL  240
             ****:*:****.*:*::: * :*::** .****:**** ***:********* :*:**::

At3g25530    LADKSGLSSDTLLDILDLGAMTNPMFKGKGPSMNKSSYPPAFPLKHQQKDMRLALALGDE  249
At1g17650    LSQKVGLDPNVLVEVVSQGAINAPMYSLKGPSMIKSVYPTAFPLKHQQKDMRLALGLAES  300
             *::* **..:.*::::. **:. **:. ***** ** **.**************.*.:.

At3g25530    NAVSMPVAAAANEAFKKARSLGLGDLDFSAVIEAVKFSRE---  289
At1g17650    VSQSTPIAAAANELYKVAKSYGLSDEDFSAVIEALKAAKSREA  343
              : * *:****** :* *:* **.* ********:* ::.
```

**Figure 12: Alignment of the two GHBDH protein sequences**

An alignment of protein sequences revealed a high similarity on amino acid level (identical residues in red) between AtGHBDH-1 and the new classified AtGHBDH-2. A remarkable difference between both proteins is the N-terminal presequence of At1g17650, probably directing the protein into an organelle.

The N-terminal sequence of the second gene was identified as a targeting signal but predictions regarding the intracellular localization remained ambiguous. In contrast to the published plastidial localization in tobacco BY2-cells [158], transient transformation of leek by particle bombardment or transformation of Arabidopsis cells with AtGHBDH-2-GFP and a RFP-fused plastid marker [123] showed no clear plastidic localization of the GFP-fusion protein (Figure 13 and Figure 14). In contrast, co-transformation of Arabidopsis cells with AtGHBDH-2-GFP and a RFP-fused mitochondrial marker [123] or staining of AtGHBDH-2-GFP transformed cells with Mitotracker Red resulted in overlapping fluorescence, suggesting a mitochondrial localization (Figure 15).
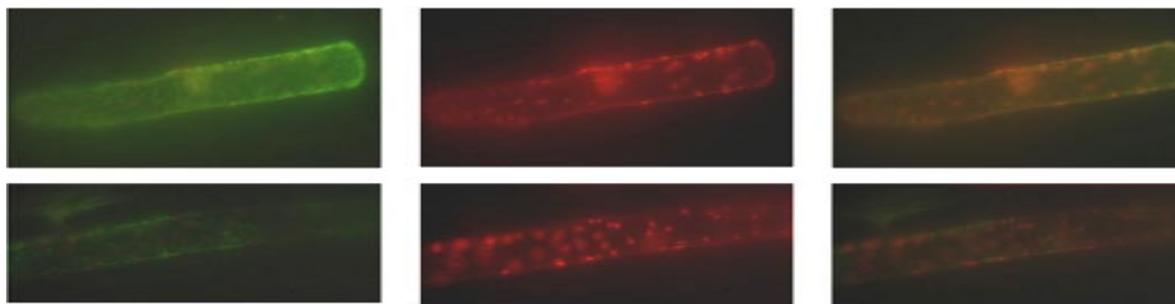
**Figure 13: AtGHBDH-2-GFP co-transformation with plastid-localized RFP**

Leek epidermal peels were transiently transformed with AtGHBDH-2-GFP and a plastid-localized RFP fusion [123] by particle bombardment (4.2.1.9) and were analyzed by fluorescence microscopy 24 hours later (4.2.1.7). GFP and RFP signals in co-transformed leek cells did not yield overlapping fluorescence patterns.
Left: GFP, middle: RFP, right: merged
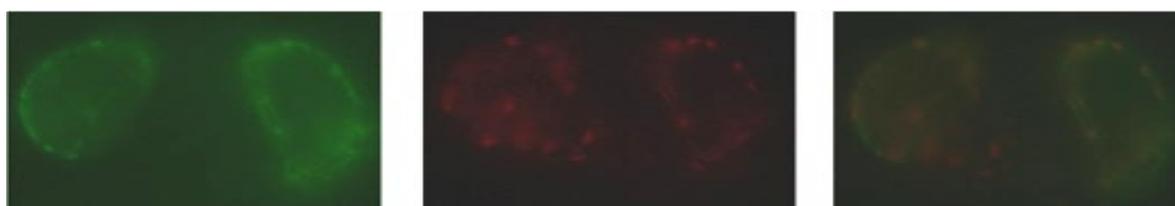


**Figure 14: AtGHBDH-2-GFP co-transformation with plastid-localized RFP**

*Arabidopsis thaliana* cell cultures were co-transformed with AtGHBDH-2-GFP and a plastid-localized RFP marker [123] and analyzed three days later by fluorescence microscopy. GFP and RFP signals in co-transformed Arabidopsis cells did not yield overlapping fluorescence patterns.
Left: GFP, middle: RFP, right: merged



**Figure 15: AtGHBDH-2-GFP co-transformation with mitochondrial-localized RFP**

*Arabidopsis thaliana* cell cultures were co-transformed with AtGHBDH-2-GFP and a mitochondria-localized RFP [123] marker and analyzed three days later by fluorescence microscopy. The GFP and RFP signals in co-transformed *Arabidopsis thaliana* cells had overlapping fluorescence patterns suggesting a mitochondrial localization.
Left: GFP, middle: RFP, right: merged
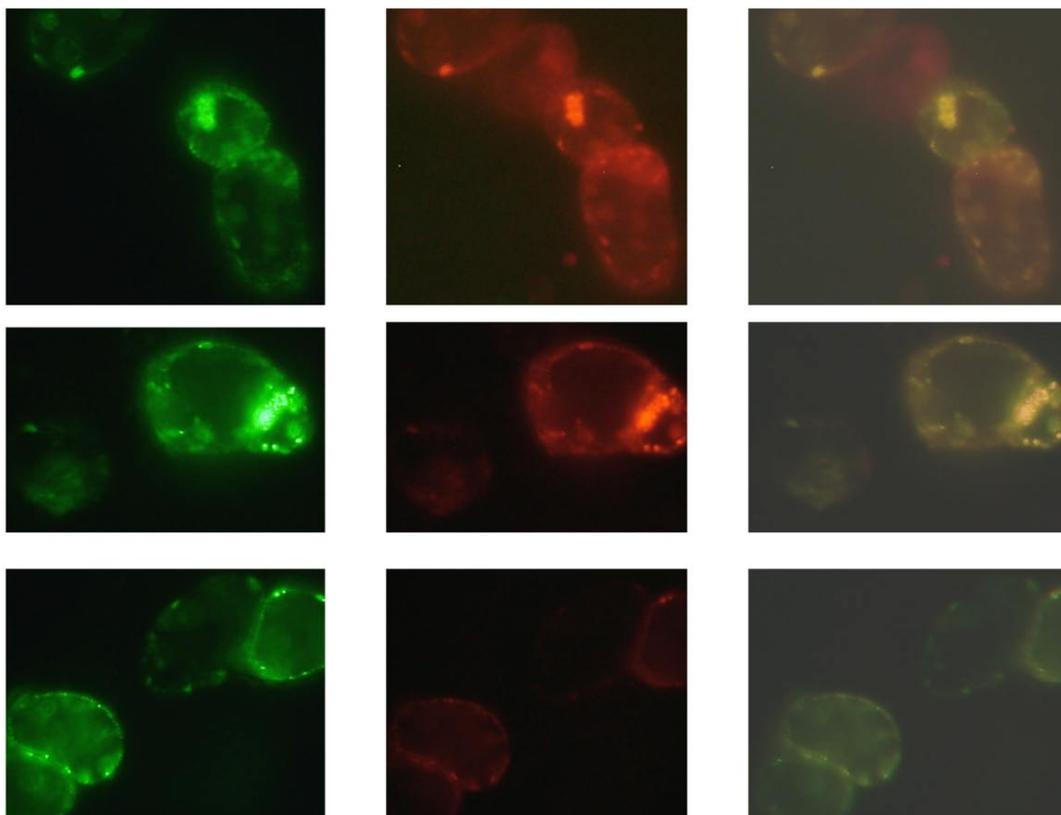
**Figure 16: AtGHBDH-2-GFP co-localization with Mitotracker Red**

Arabidopsis cells were transformed with AtGHBDH-2-GFP, incubated in the dark for 4 days, then stained with Mitotracker Red for 10min, and analyzed by fluorescence microscopy. The GFP and Mitotracker signals in transformed and stained Arabidopsis cells had overlapping fluorescence patterns, implying a mitochondrial localization of AtGHBDH-2.
Left: GFP, middle: Mitotracker Red, right: merged

An isolated T-DNA insertion line (GabiKat_933D03) for AtGHBDH-2 (At1g17650), displayed no phenotypic differences to wild type plants under greenhouse conditions. To obtain plants without GHBDH activity, *ghbdh-2* mutants were crossed to both *ghbdh-1* alleles. The resulting double homozygous lines displayed no phenotypic discrepancies to wild type plants under greenhouse conditions.

To further address the question whether SSA or GHB triggers the *ssadh* phenotype, wild type along with both *ghbdh-1/ghbdh-2* lines were grown on ½ MS supplemented with either SSA or GHB (Figure 17). Plant sizes as well as GHB levels in leaf extracts were comparable for *ghbdh-1/ghbdh-2* and wild type plants grown on ½ MS.

Addition of SSA to the media resulted in reduced root length and rosette size for wild type and mutant plants, but no growth differences between wild type and *ghbdh-1/ghbdh-2* plants were visible. In contrast to equal growth, metabolite analysis by GC-MS revealed a tendency for *ghbdh-1/ghbdh-2* mutant plants to accumulate less GHB than wild types.

No phenotypic differences were visible between wild type and *ghbdh-1/ghbdh-2* mutant plants grown on ½ MS containing GHB, all plants had a reduced root length without a substantial decrease in rosette size. GHB levels in leaf extracts from plants grown on lower GHB-concentrations were not varying among wild type and *ghbdh-1/ghbdh-2* mutant plants. Interestingly, increasing the GHB amount in the media to 5mM resulted in a minor increase of GHB levels in mutant plant extracts.
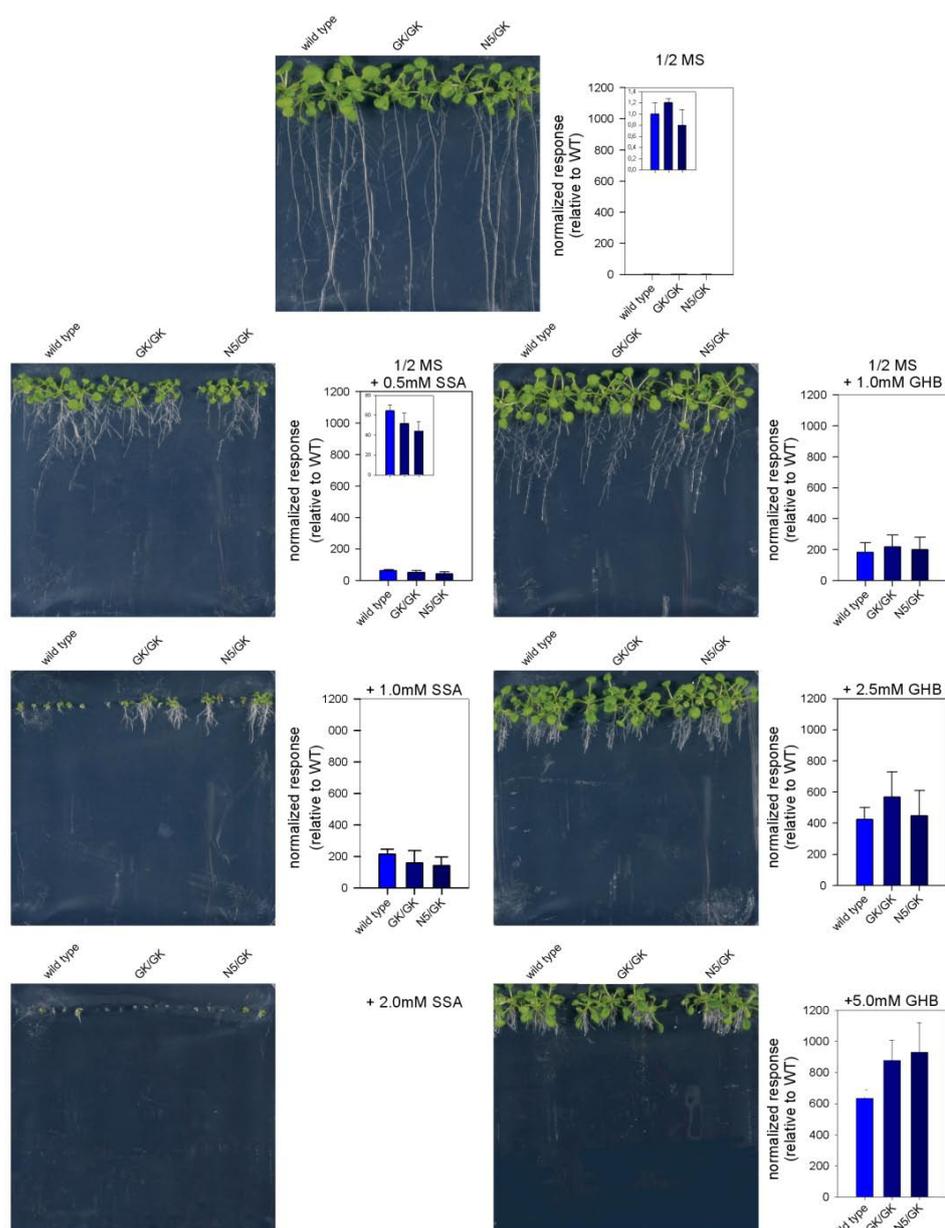


**Figure 17: Growth of wild type and two *ghbdh-1/ghbdh-2* lines on ½ MS supplemented with SSA or GHB**

Plants were grown on the indicated media for 13 days, and then samples for GC-MS analysis were taken. Pictures of the plates and the determined GHB-contents relative to wild type are given next to each other. The experiment was repeated three times and with the exception of plants grown on ½ MS + 2mM SSA 6 biological replicates were analyzed, the bars represent the standard deviation.
GK/GK: GabiKat_316D05/ GabiKat_933D03; N5/GK:  SALK_057410/ GabiKat_933D03

## 2.2. Identification of other genes involved in GABA-metabolism

The severe growth phenotype of *ssadh* mutant plants could be repressed by simultaneously inhibiting GABA-T activity, either genetically by generating *gaba-t/ssadh* double mutant plants or biochemically by applying the GABA structural analog Vigabatrin [66] which can not be metabolized and irreversibly blocks GABA-T function. These findings allow the identification of yet unknown genes involved in GABA-metabolism by searching for additional *ssadh* suppressor lines.

### 2.2.1. Screening of ssadh suppressor lines

To identify further genes involved in GABA metabolism or in a putative GABA signaling pathway in plants, additional *ssadh* suppressor mutants should be identified. Therefore, homozygous *ssadh* seeds were mutagenized using EMS (performed by Lehle Seeds; http://www.arabidopsis.com) and screened for plants with improved growth compared to the parental line. In the $M_1$ generation, out of approximately 30 000 plants, none had a considerably enhanced growth, i.e. no dominant suppressor mutation of the *ssadh* phenotype was detected. Out of approximately 60 000 $M_2$ plants, 282 *ssadh* suppressor lines displaying different phenotypes (examples in Figure 18) were initially isolated and identified as homozygous *ssadh* knock-out lines by PCR. 198 suppressor lines were rejected following maturation since the lines were not fertile or, even though they grew better their phenotypes still resembled too much the *ssadh* phenotype.

**Figure 18: Phenotypes of several independent *ssadh* suppressor mutants**

The remaining 84 lines were grown as $M_3$ generation; lines with segregating multiple EMS-induced mutations were excluded from further analysis. To identify probably allelic lines among the remaining 53 lines, plants were grown on soil under long day and short day conditions, the values recorded included rosette size, flowering time and GHB and GABA levels. Additionally, growth on ½ MS, either unchanged or supplemented with GABA or glutamate, was tested (for detailed values and pictures of plants under various growth conditions, see 6.1). Lines displaying similar developmental characteristics and metabolite contents (6.1.2) were assayed for allelic mutations; thus, plants were crossed, and growth of the offspring was examined. If the EMS induced mutations are located in the same gene, i.e. are allelic, then the $F_1$ generation should not display the *ssadh* phenotype. For two different lines, 18.B and 19.B, mutations in the same gene could be identified. The three lines chosen for mapping were tested and found not to be allelic to each other

.

## 2.2.2. Identification of new *gaba-t* alleles

As a proof-of-concept for the effectiveness of the screening, the identification of new *gaba-t* alleles was essential. For that reason, the *GABA-T* gene including 1kb upstream and 0.5 kb downstream genomic regions was sequenced in eight suppressor lines phenotypically resembling *gaba-t/ssadh* double mutants. Two of the lines contained mutations in the coding sequence (Figure 19).

In line 37.B, a single nucleotide exchange from G to A was identified in exon 11, on amino acid level resulting in a replacement of glycine with glutamate. In line 31.A, another G-A transition was found, this time leading to the exchange of glycine by aspartate in exon 13.

A

ATG                                                          TGA

|— 1 kb —|
                                                11   13

B

Exon 11

                                                    a
                                                    ↑
attggtgcttttatagctgaaccagtcatgggtgctggggggtgtgatacctccacctgctacctactttgaaaag
 I  G  A  F  I  A  E  P  V  M  G  A  G  G  V  I  P  P  P  A  T  Y  F  E  K
                                                    ↓
                                                    E

C

Exon 13

                                                  a
                                                  ↑
tgatatgtgcatttggaaggctcgggacaatgtttggctgtgacaaatacaacattaagccagatcttgtgaccttagctaagg
   I  C  A  F  G  R  L  G  T  M  F  G  C  D  K  Y  N  I  K  P  D  L  V  T  L  A  K
                                                  ↓
                                                  D
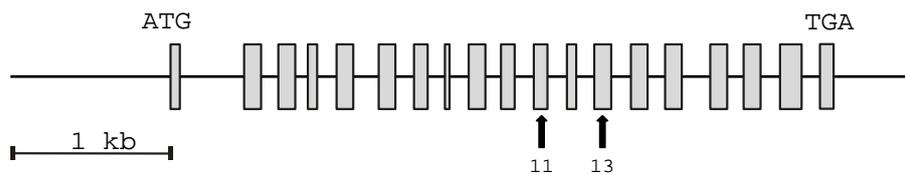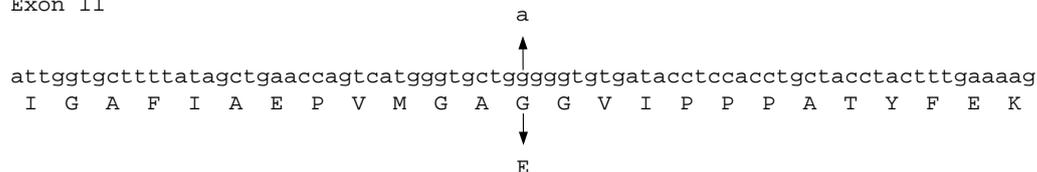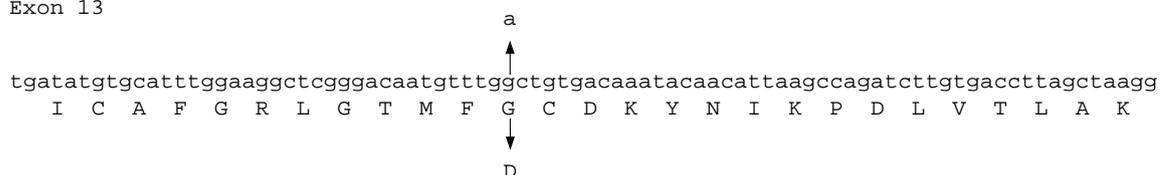
**Figure 19: Location of the EMS induced mutations in the *gaba-t* genomic sequence**

(A) Gene structure of the sequenced genomic region of At3g22200 including the regions 1kb upstream of the start and 0.5kb downstream of the stop codon. The arrows indicate the position of the identified mutations.
(B) The mutation in line 37.B was mapped to exon 11, leading to an amino acid exchange at position 271 from Gly to Glu.
(C) The mutation in line 32.A is found in exon 13; the Gly at position 312 is exchanged by Asp.

In both cases, glycine is replaced by a larger, charged amino acid, possibly leading to sterical hindrances. By comparison with crystal structures available in public databases, the mutated amino acids were located near the active site in 3D models. Additionally, the mutation in line 37.B is found in a highly conserved region. Alignments of protein sequences from species of all kingdoms revealed at this position in 97% glycine and 3% alanine, the next amino acid (glycine) was completely conserved in all species. Altogether, the mutations will most probably interfere with proper protein folding, cofactor binding, and thus protein activity (Figure 20).

```
A.t. 31.A    MVVINSLRRLARTTQVHLHSKYATCMSGNSTSRRIFTTEAAPEKKNTVGSKGHDMLAPFT  60
A.t. 37.B    MVVINSLRRLARTTQVHLHSKYATCMSGNSTSRRIFTTEAAPEKKNTVGSKGHDMLAPFT  60
GABA-T pig   -----------------------SQAAAKVDVEFDYDGPLMKTEVPGPRSRELMKQLN    35


A.t. 31.A    AGWQSADLDPLVIAK-SEGSYVYDDTGKKYLDSLAGLWCTALGGNEPRLVSAAVEQLNTL  119
A.t. 37.B    AGWQSADLDPLVIAK-SEGSYVYDDTGKKYLDSLAGLWCTALGGNEPRLVSAAVEQLNTL  119
GABA-T pig   IIQNAEAVHFFCNYEESRGNYLVDVDGNRMLDLYSQISSIPIGYSHPALVKLVQQPQNVS   95
                                                  ▲

A.t. 31.A    PFYHSFWNRTTKPSLDLAKVLLEMFTAN--KMAKAFFTSGGSDANDTQVK--LVWYYNNA  175
A.t. 37.B    PFYHSFWNRTTKPSLDLAKVLLEMFTAN--KMAKAFFTSGGSDANDTQVK--LVWYYNNA  175
GABA-T pig   TFINRPALGILPPENFVEKLRESLLSVAPKGMSQLITMACGSCSNENAFKTIFMWYRSKE  155


A.t. 31.A    LGRPEKKK-----------------FIARKKSYHGSTLISASLSGLPPLHQNFDLPAPF  217
A.t. 37.B    LGRPEKKK-----------------FIARKKSYHGSTLISASLSGLPPLHQNFDLPAPF  217
GABA-T pig   RGQSAFSKEELETCMINQAPGCPDYSILSFMGAFHGRTMGCLATTHSKAIHKIDIPSFDW  215
                                              ▲▲ ▲

A.t. 31.A    VLHTDCPHYWRFHLPGETEEEFSTRLAKNLEDLIIK--EGPETIGAFIAEPVMGAGGVIP  275
A.t. 37.B    VLHTDCPHYWRFHLPGETEEEFSTRLAKNLEDLIIK--EGPETIGAFIAEPVMGAEGVIP  275
GABA-T pig   PIAPFPRLKYPLEEFVKENQQEEARCLEEVEDLIVKYRKKKKTVAGIIVEPIQSEGGDNH  275
                                                             ▲

A.t. 31.A    PPATYFEKVQAVVKKYDILFIADEVICAFGRLGTMFDCDKYNIKP--DLVTLAKALSSAY  333
A.t. 37.B    PPATYFEKVQAVVKKYDILFIADEVICAFGRLGTMFGCDKYNIKP--DLVTLAKALSSAY  333
GABA-T pig   ASDDFFRKLRDISRKHGCAFLVDEVQTGGGSTGKFWAHEHWGLDDPADVMTFSKKMMTGG  335
                                              ▲ ▲▲                         △

A.t. 31.A    MPIGAILMSQEVADVINSHSSKLGVFSHGFTYSGHPVSCAVAIEALKIYKERNIPEYVAK  393
A.t. 37.B    MPIGAILMSQEVADVINSHSSKLGVFSHGFTYSGHPVSCAVAIEALKIYKERNIPEYVAK  393
GABA-T pig   FFHKEEFRPNAPYRIFN------------TWLGDPSKNLLLAEVINIIKREDLLSNAAH  382
                              ▲

A.t. 31.A    VAPRFQDGVKAFASGSP-IIGETRGTGLILGTEFVDNKSPNEPFPPEWGVGAFFGAECQI  452
A.t. 37.B    VAPRFQDGVKAFASGSP-IIGETRGTGLILGTEFVDNKSPNEPFPPEWGVGAFFGAECQI  452
GABA-T pig   AGKVLLTGLLDLQARYPQFISRVRGRGTFCSFDTPDESIRNKLISIARNKGVMLGGCGDK  442


A.t. 31.A    SPEEIDESIYG-KALKATEEKVKELKAQHKK  482
A.t. 37.B    SPEEIDESIYG-KALKATEEKVKELKAQHKK  482
GABA-T pig   SIRFRPTLVFRDHHAHLFLNIFSDILADFK-  472
                  ▲ ▲
```

**Figure 20: Alignment of protein sequences from Arabidopsis and pig**

Alignment of the protein sequences from the two new *gaba-t* alleles in Arabidopsis and the GABA-T sequence from pig, used for crystal structure analysis [166]. In both *gaba-t* alleles, glycine residues near the active site are replaced with a larger, charged amino acid that is most probably interfering with proper protein folding and enzyme activity.
Black triangles indicate active site residues, the white triangle the pyridoxal phosphate-binding site, the mutated residues in the Arabidopsis sequences are underlined.

## 2.2.3. **Map-based cloning of three *ssadh* suppressor lines**

To rule out the possibility of an EMS-induced mutation in known GABA-shunt related genes in the three lines analyzed, the genes encoding *GABA-T*, *GHBDH-1*, *GHBDH-2*, and the putative GABA-Transporters, including 1kb upstream of the start codon, the coding region with exon and introns, and 0.5kb downstream of the stop codon, were sequenced, but no mutations were identified.

Three non-allelic lines were chosen for mapping. Using the following SSLP markers (Figure 21), the EMS mutations could be mapped down to the chromosome arm.
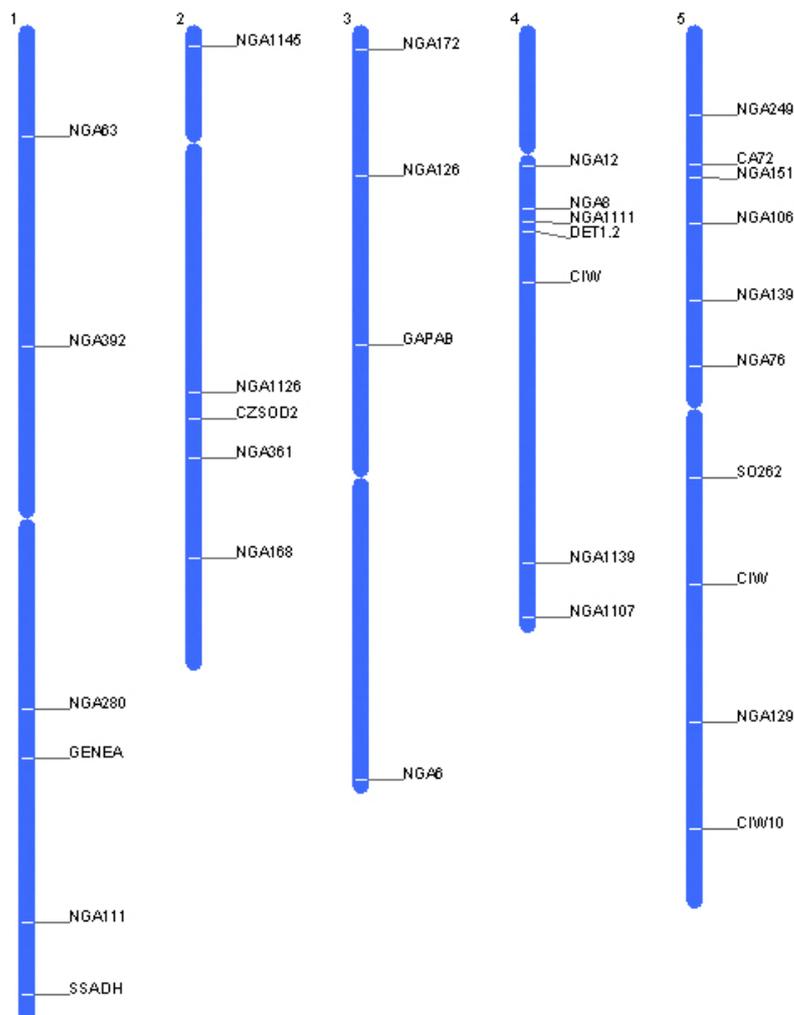


**Figure 21: Distribution of the used SSLP markers in the Arabidopsis genome**

For all markers, primer sequences and fragment sizes are given in 6.4.5.1.The marker NGA111 exhibits linkage with the *SSADH* gene.

### 2.2.3.1. Line 4.D

This plant was isolated in the $M_2$ generation in pool number 4, transferred to single pots, and grown under greenhouse conditions until maturation (Figure 22). The plant had a rather small rosette and flower buds appeared after 36 days.

In the $M_3$ generation, growth of plants under long and short day conditions and on ½ MS supplemented with GABA or Glutamate was monitored. Plants grown under long day conditions resembled the phenotype of the parental plants, whereas all leaves developing under short day conditions were very small, wrinkled and displayed a pale green color (Figure 23). Determination of the GHB and GABA content in leaf extracts of plants grown under greenhouse conditions by GC-MS revealed 324-fold increased GHB-, and 19-fold increased GABA-levels compared to wild types. This line contained the highest GHB levels in leaf extracts among all *ssadh* suppressor lines.

To further obtain information about plant characteristics, $M_3$-seeds were germinated on ½ MS supplemented with either GABA or glutamate (Figure 31). Plants grown on ½ MS had very short roots and a reduced rosette size, germination on GABA or glutamate-containing media further reduced the rosette size.

Figure 22: Phenotype of 4.D M$_2$ plant grown under greenhouse conditions for 44 days.



Figure 23: Plants of the M$_3$-generation were grown under long day (A) and short day (B) conditions for 39 days



Figure 24: Analysis of the M$_3$-generation

Plants were grown on (A) ½ MS, (B) ½ MS + 5mM GABA, or (C) ½ MS + 5mM glutamate for 16 days under long day conditions.

To identify the chromosome carrying the mutation, the F$_2$-population resulting from a cross to L*er* was screened for double homozygous plants. These plants had yellowish, slightly rounder rosette leaves compared to wild type, allowing for a phenotype-based selection. The SSLP-markers (Figure 21) were used, and the resulting recombination rates were obtained (Table 1). The mutation could be mapped to the upper half of chromosome 4 and by using additional CAPS-markers (6.4.5.2.1); the following recombination rates (Figure 25) were obtained.

**Table 1: Recombination frequencies in F$_2$ plants obtained by a cross of *ssadh* suppressor line 4.D to L*er* wild types**

| Chromosome | Marker | Number of plants | % Col-0 | % Ler |
|---|---|---|---|---|
| 1 | NGA 392 | 67 | 55,2 | 44,8 |
| | NGA 280 | 71 | 86,6 | 13,4 |
| | NGA 111 | 74 | 99,3 | 0,7 |
| 2 | NGA 168 | 56 | 51,8 | 48,2 |
| 3 | NGA 172 | 75 | 52,7 | 47,3 |
| 4 | NGA 12 | 20 | 72,1 | 27,9 |
| | NGA 8 | 67 | 85,1 | 14,9 |
| | NGA 1111 | 77 | 53,2 | 46,8 |
| | DET1.2 | 56 | 67,9 | 32,1 |
| | NGA 1139 | 77 | 48,7 | 51,3 |
| | NGA 1107 | 77 | 48,1 | 51,9 |
| 5 | NGA106 | 74 | 60,1 | 39,9 |
| | NGA139 | 77 | 66,9 | 33,1 |
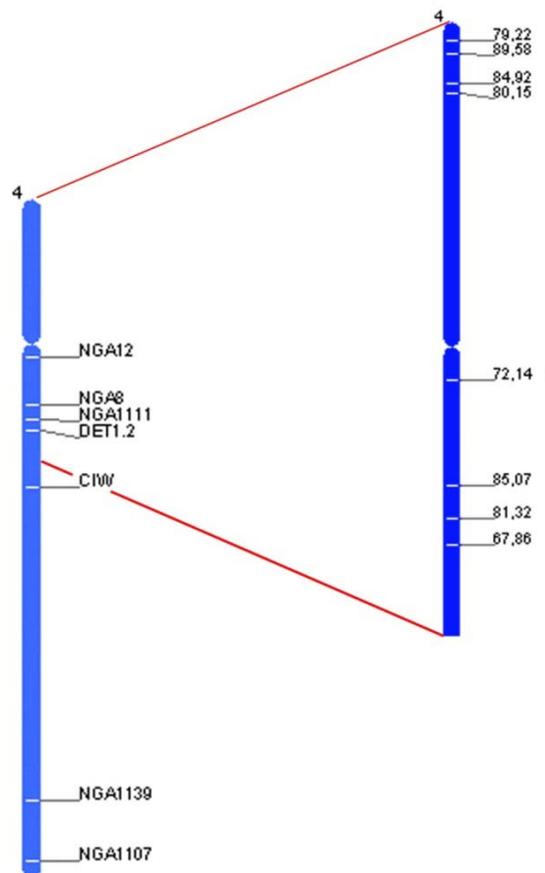


**Figure 25: Marker distribution with a high frequency of Col-0 fragments in line 4.D**

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## 2.2.3.2.    Line 17.J

This plant was isolated from the $M_2$ generation in pool number 17, transferred to single pots and grown under greenhouse conditions until maturation (Figure 26). Compared to wild type plants, no major differences were visible.

In the $M_3$ generation, growth of plants under long and short day conditions and on ½ MS supplemented with GABA or Glutamate was monitored. Plants grown under long day conditions resembled the phenotype of the parental plants, whereas all leaves developing under short day conditions were reduced in size and displayed a pale green color (Figure 27). Analyzing the GHB and GABA content in leaf extracts of plants grown under greenhouse conditions using GC-MS revealed 80-fold increased GHB-, and 5-fold increased GABA-levels compared to wild type.

To gain additional knowledge about plant characteristics, seeds were germinated on ½ MS supplemented with either GABA or glutamate (Figure 28). Plants grown on ½ MS displayed no altered root architecture, and some rosette leaves were pale green. Germination on GABA or glutamate-containing media clearly reduced rosette size and root length, plants grown on GABA-containing media were slightly smaller compared to those grown on glutamate.

**Figure 26: Phenotype of 17.J M₂ plant grown under greenhouse conditions for 44 days.**
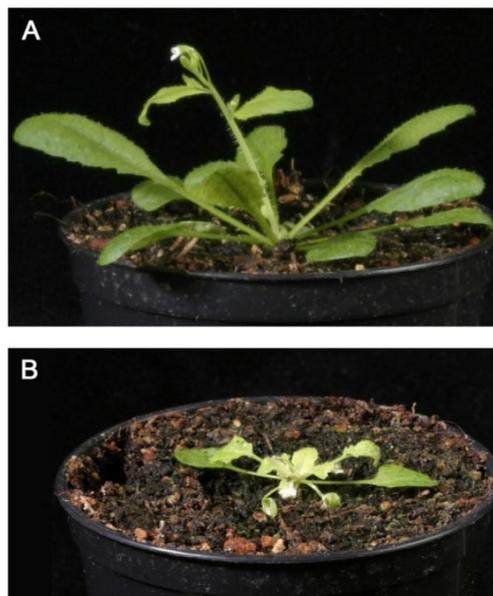


**Figure 27: Plants of the M₃-generation were grown under long day (A) and short day (B) conditions for 39 days**
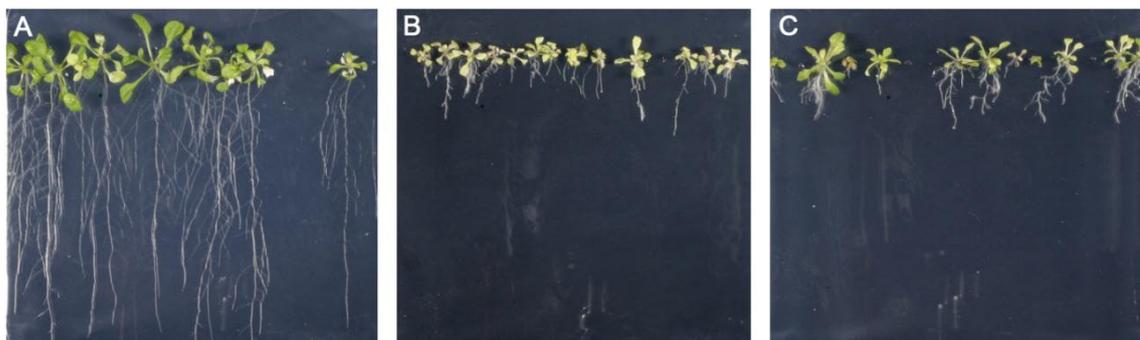


**Figure 28: Analysis of the M₃-generation**

Plants were grown on ½ MS (A), ½ MS + 5mM GABA (B), or ½ MS + 5mM glutamate (C) for 16 days under long day conditions.

To identify the chromosome carrying the mutation, the $F_2$-population resulting from a cross to L*er* was screened for double homozygous plants. These plants were phenotypically indistinguishable from wild types, with the exception of a paler petiole in young, developing leaves. Therefore, all $F_2$ plants had to be screened by PCR. The SSLP-markers (Figure 21) were used, and the resulting recombination rates were obtained (Table 2). The mutation could be mapped to chromosome 5. Additional CAPS-markers (6.4.5.2.2) were selected to further narrow down the region containing the EMS mutation, and the following recombination rates (Figure 29) were identified.

**Table 2: Recombination frequencies in F$_2$ plants L*er/17.J***

| Chromosome | Marker | Number of plants | % Col-0 | % Ler |
|---|---|---|---|---|
| 1 | NGA 392 | 35 | 44,3 | 55,7 |
| 2 | NGA 1145 | 38 | 59,2 | 40,8 |
|   | NGA361 | 49 | 57,1 | 42,9 |
| 3 | GAPAB | 39 | 56,4 | 43,6 |
| 4 | NGA 8 | 40 | 52,5 | 47,5 |
| 5 | NGA 151 | 38 | 60,5 | 39,5 |
|   | NGA106 | 40 | 62,5 | 37,5 |
|   | CDPK9 | 39 | 65,4 | 34,6 |
|   | NGA76 | 37 | 80,0 | 20,0 |
|   | SO262 | 38 | 72,4 | 27,6 |
|   | NGA129 | 35 | 80,0 | 20,0 |
|   | CIW10 | 42 | 82,1 | 17,9 |
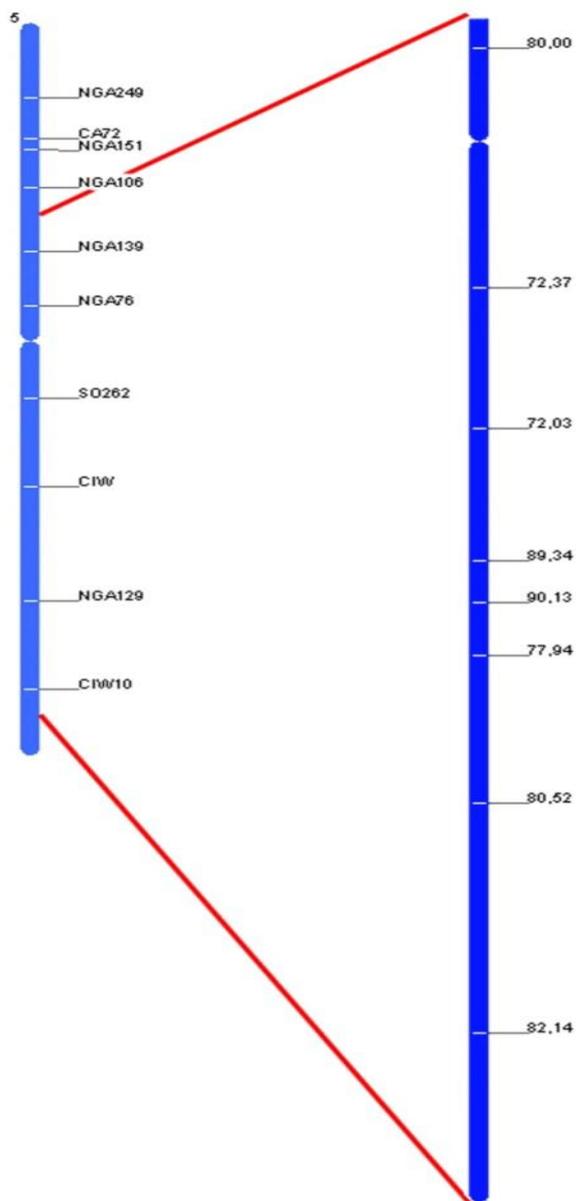


**Figure 29: Marker distribution with a high frequency of Col-0 fragments in line 17.J**

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### 2.2.3.3.    Line 21.H

This plant was isolated in the $M_2$-generation in pool number 4, transferred to single pots, and grown under greenhouse conditions until maturation (Figure 30). The plant had slightly smaller, pale green rosette leaves, and first flower buds appeared after 41 days.

In the $M_3$-generation, growth of plants under long and short day conditions and on ½ MS supplemented with GABA or Glutamate was monitored. Plants grown under long day conditions resembled the phenotype of the parental plants whereas plants grown under short day conditions were highly affected, new developing leaves remained very small (Figure 31). Determining the GHB and GABA content in leaf extracts of plants grown under greenhouse conditions by GC-MS revealed 230-fold increased GHB-, and 8-fold increased GABA-levels compared to wild type.

To obtain additional information about plant features, seeds were germinated on ½ MS supplemented with either GABA or glutamate (Figure 32). Plants grown on ½ MS had no altered root morphology, but pale green rosette leaves, germination on GABA or glutamate-containing media, the plant size was plainly reduced.



**Figure 30: Phenotype of 21.H $M_2$ plant grown under greenhouse conditions for 44 days.**



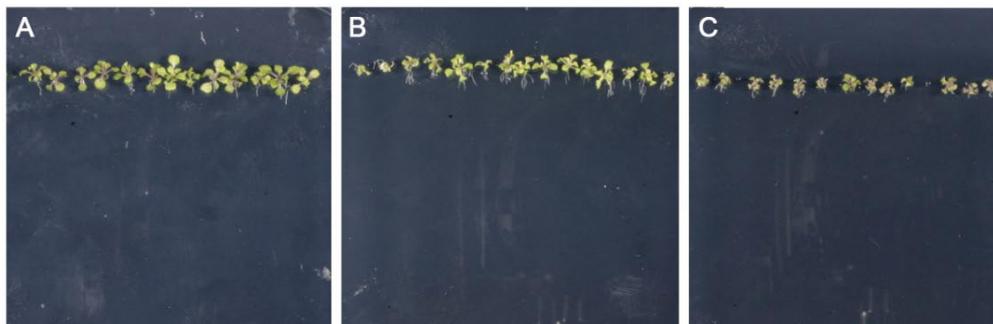**Figure 31: Plants of the $M_3$-generation were grown under long day (A) and short day (B) conditions for 39 days**
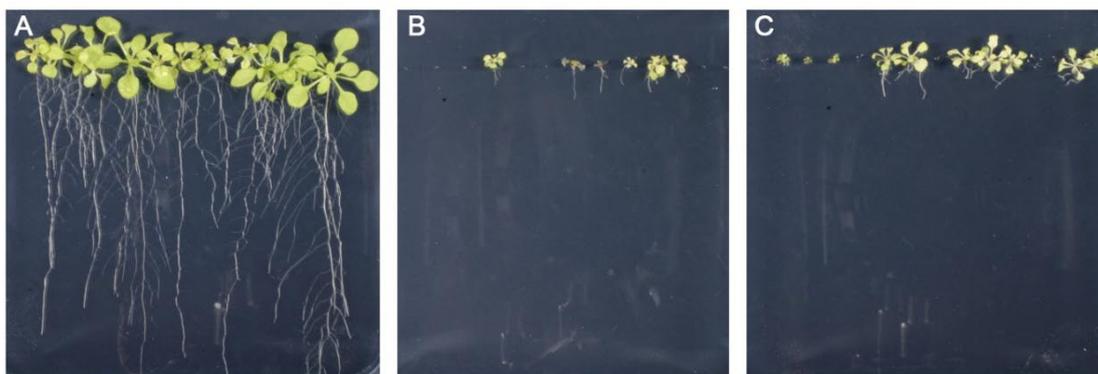
**Figure 32 Analysis of the M$_3$-generation**

Plants were grown on ½ MS (A), ½ MS + 5mM GABA (B), or ½ MS + 5mM glutamate (C) for 16 days under long day conditions.

To identify the chromosome carrying the mutation, the F$_2$-population resulting from a cross to L*er* was screened for double homozygous plants. These plants had yellowish, slightly smaller rosette leaves compared to wild type, allowing for a phenotype-based selection. Using the SSLP-markers (Figure 21), the mutation could be mapped to the lower arm of chromosome 2 (Table 3). By using additional CAPS-markers (6.4.5.2.3), the region carrying the mutation could be further narrowed (Figure 33).

**Table 3: Recombination frequencies in F2 plants obtained by a cross of *ssadh* suppressor line 4.D to Ler wild types**

| Chromosome | Marker | Number of plants | % Col-0 | % Ler |
|:---:|:---:|:---:|:---:|:---:|
| 1 | NGA 280 | 26 | 63,5 | 36,5 |
| 2 | CZSOD 2 | 19 | 63,2 | 36,8 |
|   | NGA361 | 31 | 59,7 | 40,3 |
|   | NGA 168 | 20 | 90 | 10 |
| 3 | NGA 172 | 22 | 59,1 | 40,9 |
|   | NGA126 | 21 | 61,9 | 38,1 |
| 4 | NGA 8 | 27 | 59,3 | 40,7 |
| 5 | NGA139 | 28 | 58,9 | 41,1 |
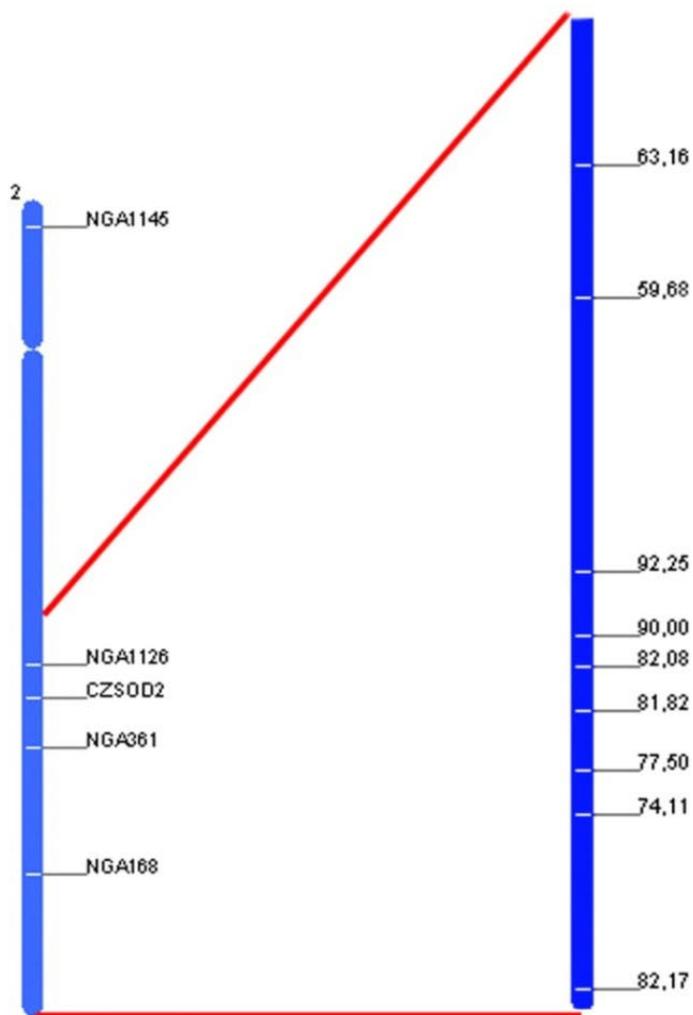
**Figure 33: Marker distribution with a high frequency of Col-0 fragments in line 21.H**

To further map down the mutations to a smaller chromosomal region, a larger mapping population has to be isolated and more markers have to be available.

## 2.3. SSA induces callus formation in Arabidopsis

To enhance the survival rates of *ssadh* mutant plants, seeds were germinated on ½ MS and transferred to soil into the greenhouse about eight weeks later. At this time point, the occurrence of dedifferentiated tissue at the hypocotyls of some *ssadh* seedlings was visible (Figure 34). Whether the hypocotyl dedifferentiated or not did not influence overall plant growth. In contrast to Col-0 wild types that formed no dedifferentiated tissue when germinated on ½ MS, 128 out of 1000 *ssadh* mutant plants developed callus under the same conditions.



**Figure 34: *ssadh* mutant plants develop callus on ½ MS**

(A) Col-0 wild type plants 10 days after germination, none with callus
(B) *ssadh* mutant plants 9 weeks after germination, several plants developed dedifferentiated tissues
(C-E) close-up view on dedifferentiated cells

### 2.3.1. Callus formation is dependent on the SSA concentration

To test whether dedifferentiation is dependent on the cellular SSA concentration, wild type seeds of three different Arabidopsis ecotypes (Col-0, L*er,* and Ws-2) were germinated on ½ MS supplemented with SSA. In all ecotypes analyzed, callus formed at the hypocotyl in a linear response to the SSA concentration in the media; only minor variations appeared among the ecotypes (Figure **35**). Notably, the callus formation rate of *ssadh* mutant plants on ½ MS and of wild types on ½ MS + 0.8mM or 1.0mM SSA are comparable.
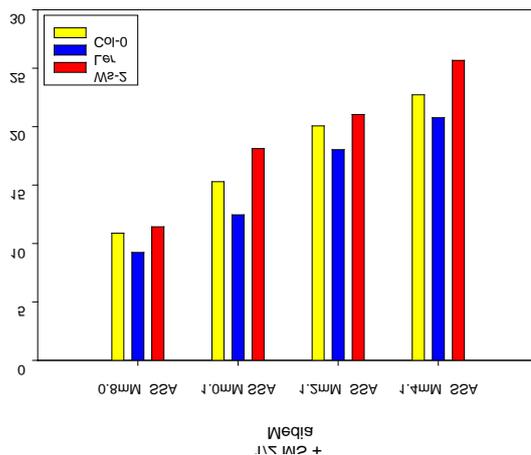
**Figure 35: Rate of callus formation in Arabidopsis wild type plants is dependent on the SSA concentration in the media.**

Three Arabidopsis ecotypes (Col-0, L*er,* and Ws-2) were grown on ½ MS containing different amounts of SSA for 36 days, and among the germinated seeds the number of plants developing dedifferentiated tissue was determined. The experiment was repeated once, each with approximately 300 plants per ecotype analyzed. The given values are the average of both experiments.

Comparing the rate of callus formation among seeds germinating on SSA-containing media with or without added sucrose, no big differences arose. Interestingly, when growing wild type plants on ½ MS + SSA in darkness, no dedifferentiation occurred, probably being an indication toward an underlying light-dependent process.

When *ssadh* mutant seeds were germinated on ½ MS + SSA, all plants developed dedifferentiated tissue, but without restriction to the hypocotyl (Figure 36). Again, growth in darkness prevented callus formation.

To test whether the dedifferentiated cells can be used for regeneration of whole plants, the dedifferentiated cells were isolated and transferred first on shoot-, then on root-induction media; the former containing a lower, the latter a higher auxin-to-cytokinin ratio (Figure 37). The resulting plants were paler than normal wild type plants, but produced seeds in the greenhouse; and their offspring presented no differences to other wild type plants.
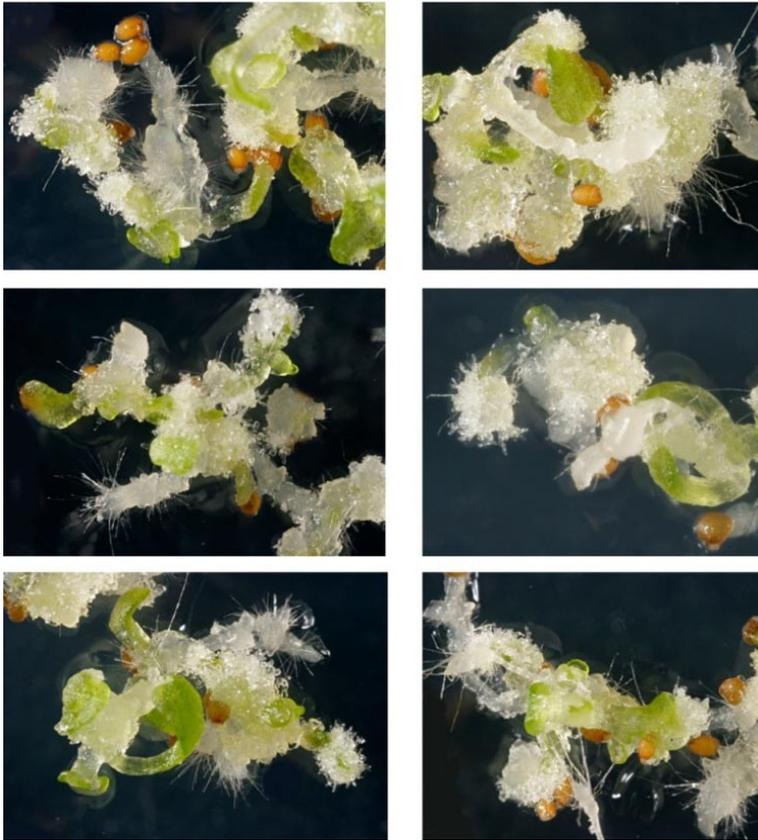
**Figure 36: Phenotype of *ssadh* seeds germinated on ½ MS containing SSA**

*ssadh* mutant seeds were germinated on ½ MS+1.4mM SSA and grown for 28 days. The seedlings developed dedifferentiated tissue not only localized to the hypocotyl. The nature of the root hair like structures emerging from the calli remains unclear.
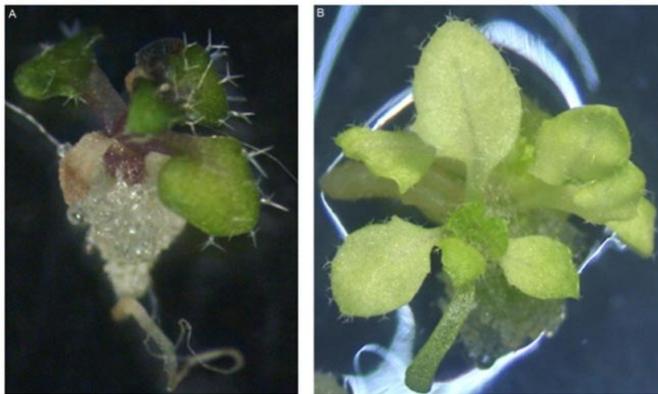


**Figure 37: Whole plants can be regenerated from SSA-induced callus**

(A) Col-0 wild type developed callus from the hypocotyl after 4 weeks on ½ MS + 1.4mM SSA.

(B) Shoots were regenerated from isolated callous cells on media containing a low auxin to cytokinin ratio. The picture was taken 22 days after transfer to shoot-regeneration media.

## 2.3.2. Does a linkage between SSA-induced callus formation and phytohormones exist?

It has been reported that callus formation occurs dependent on the auxin to cytokinin ratio in tissue and further differentiation can be induced by altering the ratio.

To test whether SSA does influence phytohormone sensitivity in Arabidopsis, the effect of a combination of SSA and auxin or cytokinin on the callus formation rate was assayed. Neither were phenotypic discrepancies visible nor was the callus formation rate altered dramatically between plants grown on the different media (Figure 38).
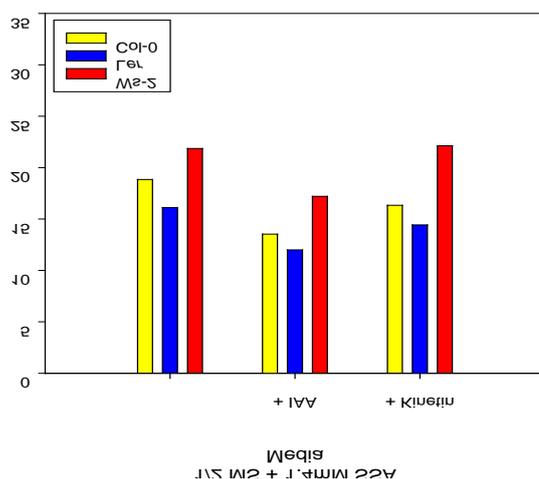


**Figure 38: Callus formation is not dependent on the auxin to cytokinin ratio**

Three Arabidopsis ecotypes (Col-0, L*er,* and Ws-2) were grown on ½ MS containing 1.4mM SSA, or on ½ MS + 1.4mM SSA supplemented with either 10µM IAA or 10µM kinetin for 36 days. Among the germinated seeds, the number of plants developing dedifferentiated tissue was determined. Addition of IAA and kinetin respectively, did not alter the rate of callus formation much. The experiment was performed two times, each with approximately 300 plants per ecotype analyzed. The given values are the average of both experiments.

Although the addition of IAA or kinetin to ½ MS containing SSA did not notably affect the callus formation rate, the appearance of the calli - especially the phenotype of the *ssadh* calli with root hair like structures emerging from the dedifferentiated cells (Figure 36) - point toward an auxin related mechanism. Therefore, the auxin transport inhibitors NPA and TIBA as well as the antiauxin PCIB [23] were tested regarding their capacity to affect the rate of SSA-induced callus formation. Neither addition of PCIB nor of NPA showed a drastic reduction in callus formation, but the addition of TIBA completely inhibited SSA-induced callus formation (Figure 39).
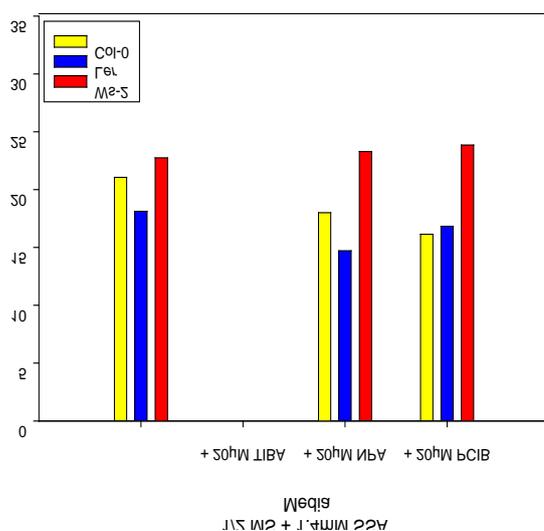
**Figure 39: Influence of inhibitors of auxin transport or activity on SSA-induced callus formation**

Three Arabidopsis ecotypes (Col-0, L*er* and Ws-2) were grown on ½ MS containing 1.4mM SSA and either 20µM TIBA, 20µM NPA or 20µM PCIB for 36 days. Among the germinated seeds, the number of plants developing dedifferentiated tissue was determined. Addition of PCIB or NPA – NPA even in 5-fold higher concentrations - did not alter the rate of callus formation much. In contrast, the auxin-transport inhibitor TIBA completely prevented SSA-induced callus formation. The experiment was repeated once, each with approximately 400 plants per ecotype analyzed, the given values are the average of both experiments.

The TIBA-mediated inhibition of SSA-induced callus formation in Arabidopsis wild type plants refers to auxin-related processes. To assay the expression of various genes related to auxin synthesis, transport, and perception, transgenic Arabidopsis lines expressing GUS under control of auxin-associated promoters were germinated on ½ MS containing SSA, and the calli were stained for GUS activity (Figure 40). A strong signal was detected in plants expressing GUS under control of the NIT1, NIT2, LAX1, and LAX2 promoters. Compared to these lines, plants carrying NIT3::GUS or LAX3::GUS constructs had a weaker expression level. SSA induced promoter activity in plants, visualized by GUS staining for genes involved in import and synthesis of indol-3-acetic acid (IAA). No GUS activity was visible in calli of the other lines tested, which expressed GUS under control of native promoters or the synthetic auxin responsive element DR5, where GUS expression was only detected in root tips of plants grown on media with or without SSA.

In parallel to the promoter-activity assays, the effect of SSA on callus-induction in several auxin-related mutants was assayed (Figure 41). A reduction of callus formation was observed in plant lines deficient in auxin carriers (*aux1*, *lax1*, *lax3*, and *pin3*) or the *arf7/arf19* double mutants and in plants overexpressing an IAA ligase that is involved in removal of active auxin. As control for other phytohormone signaling pathways, plants overexpressing nahG, a bacterial salicylate hydrolase, were assayed, but no difference to the corresponding wild type was detectable.
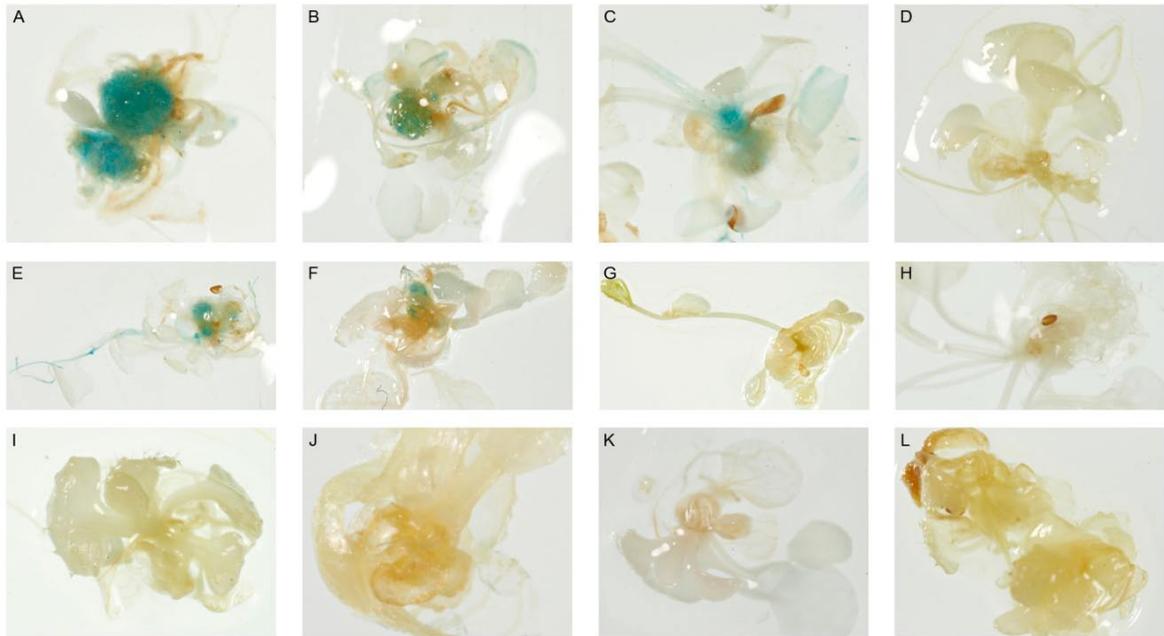
**Figure 40: GUS-staining of SSA-induced calli**

Plants expressing different Promoter::GUS fusion constructs were germinated on ½ MS + SSA and stained for GUS activity.

Expression was found for NIT1 (A), NIT 2 (B), NIT3 (C), LAX1 (E), LAX2 (F) and very weak for LAX3(G), whereas no staining was visible for NIT4 (D), AXR1 (H), IAA2 (I), PIN1 (J), PIN6 (K) and AUX1 (L)
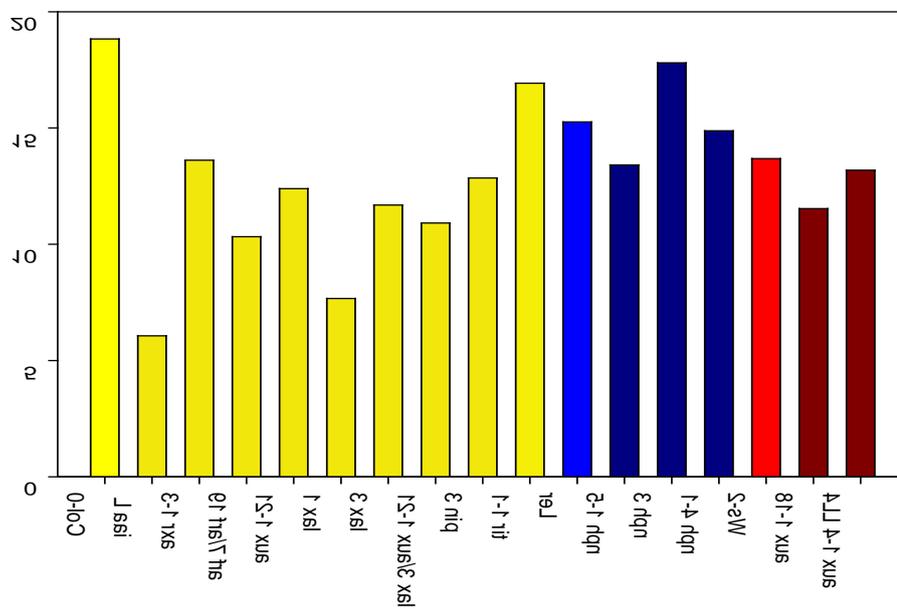


**Figure 41: Callus formation rate in auxin-related mutants**

Plants mutated in genes involved in different phytohormone pathways were analyzed for their response to SSA. Therefore, the mutants and the corresponding wild type lines were grown for 28 or 31 days on ½ MS + 1.4mM SSA, and the number of plants developing callus was determined. The experiment was repeated twice with 100 to 400 plants analyzed, the given values are the average from all experiments.

## 2.3.3. Does SSA itself act as auxin?

Several assays are published to test chemicals for their auxin-activity, one among them is the determination of root length in Arabidopsis plants [186]. Therefore, Arabidopsis wild type seeds from three ecotypes were germinated on media containing either different concentrations of SSA or 2,4-D as positive control for a substance with known auxin activity (Figure 42), and root length was determined 8 days after germination. A reduced root growth was measured for seedlings grown on both SSA and 2,4-D, however the SSA concentrations efficiently inhibiting root growth were about 10000 fold higher than the 2,4-D concentrations.
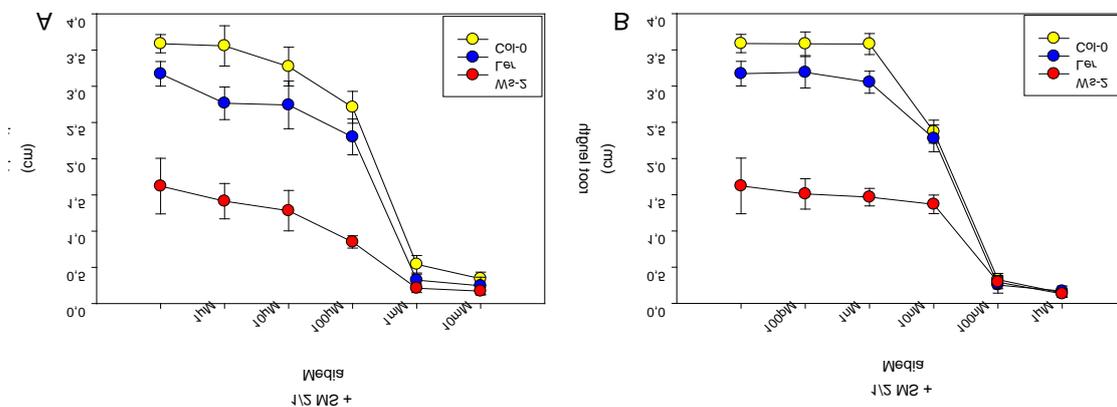


**Figure 42: Assay for auxin activity in Arabidopsis**

Three Arabidopsis ecotypes (Col-0, L*er* and Ws-2) were grown on ½ MS supplemented with different concentration SSA (A) or the auxin 2,4-D (B). Root growth of 40 plants each was measured 8 days after germination; the given values are the average and standard deviation.

# 3. Discussion

## 3.1. Influence of GABA-shunt metabolites on plant growth

To assay the effect of the GABA-shunt or GABA-related metabolites, growth and development of several GABA-shunt mutants was analyzed in comparison to wild type. Additionally, the effect of exogenously supplied GABA-shunt metabolites on plant growth *in vitro* was examined.

On standard ½ MS media, *gaba-t* mutants display a trend toward an increased shoot growth without an increase in root length. The same effect can be induced in wild type plants by the addition of GABA to the media (Figure 6 and Figure 7). The availability of inorganic nitrogen in the media is essential for plant growth, and nitrate uptake in the roots is a limiting step of nitrate assimilation. In *Brassica napus,* GABA enhances nitrate transporter gene expression, resulting in increased root nitrate uptake capacities [22]. Since *Brassica napus* and *Arabidopsis thaliana* are closely related species, a GABA-induced upregulation of nitrate uptake transporters seems conceivable in Arabidopsis. This regulation would lead to an increased nitrate-uptake capacity and as a result to enhanced growth. Taken together, the endogenous GABA concentrations in *gaba-t* mutant plants might be sufficient to induce expression of nitrate uptake transporters that increases plant growth under standard conditions. In contrast, the cellular GABA concentrations required for the enhanced expression of nitrate uptake transporters might be reached in wild type plants only by the exogenous supply of GABA.

The increasing number of lateral roots forming in wild type plants growing on GABA as sole nitrogen source might be explained in two different ways. GABA – even when available in equal concentrations to inorganic nitrogen in control plates – might be an inappropriate nitrogen source compared to inorganic nitrogen, with limitations regarding GABA-uptake capacities and metabolism. The high affinity GABA transporter GAT1 is very low expressed in the whole plant, with hardly any expression detected in roots. The ProT proteins are low affinity GABA transporters that are not expressed in roots. As conclusion from the expression data (available at https://www.genevestigator.ethz.ch and http://www.bar.utoronto.ca/efp), only minor GABA uptake rates are found in

Arabidopsis roots under physiological conditions. Ammonia can directly be incorporated into amino acids, whereas nitrate has to be reduced in the plastids before being incorporated in α-ketoglutarate, producing glutamate. In contrast, GABA-degradation is localized in the mitochondria and the amino group is transferred to pyruvate, yielding alanine. To obtain all other amino acids starting from GABA, will necessarily include several accessory reactions. Taken together, GABA as sole nitrogen source might induce reduced growth and indications of nitrogen deprivation in plants[112]. The increased number of lateral roots might be required to allow for sufficient GABA uptake capacities. Furthermore, GABA is known to induce ethylene production, a main regulator of root hair and lateral root formation also in Arabidopsis [35, 52, 90].

The impaired growth of *gaba-t* mutant plants on media supplied with GABA could be due to the accumulation of GABA in the plants, initiating a yet unidentified mechanism provoking a developmental inhibition. This effect might argue for GABA acting as signaling molecule in Arabidopsis, where a function in pollen tube guidance and fertilization is known (Figure 2 and [29, 130]); additionally, GABA is long known as signal molecule in several other species (1.5).

The suppression of the *ssadh* phenotype is possible using either a biochemical or a genetic approach to prevent GABA-T function. First, the application of Vigabatrin to *ssadh* mutant plants improves plant growth [66], since Vigabatrin interacts with the GABA-T and irreversibly blocks the protein function. Second, *gaba-t/ssadh* homozygous plants grow like *gaba-t* single mutants under greenhouse conditions, proving *GABA-T* to be epistatic to *SSADH* (2.1.1).

GABA-shunt metabolites can be measured in plant extracts using GC-MS, with the exception of SSA, where - in contrast to human medicine [167] - currently no method is available. To assay whether GABA-shunt metabolites influence plant development, GABA and GHB levels in wild type and several mutant plant lines are determined (2.1.1).

The accumulation of GABA (Figure 4a) in *gaba-t* and *gaba-t/ssadh* mutants is due to GAD activity, while GABA degradation is blocked. In contrast, *ssadh* mutants

accumulate twice as much GABA as the aforementioned lines. In *ssadh* mutants SSA-synthesis from GABA still can occur, resulting in SSA accumulation. In order to remove excess SSA, when a threshold level is reached, an amino group might be transferred from alanine to SSA, thereby producing GABA and further increasing the GABA concentrations; in mammalia, the GABA-T is capable of acting in the reverse direction [179].

GHB levels (Figure 4b) in wild type and *gaba-t* mutants are near the detection limits of the GC-MS system used. Most probably, if GHB is synthesized in the aforementioned plant lines, then it is degraded via SSA to succinate under normal growth conditions, comparable to data published for rat [179]. If GHB-synthesis occurring independently from the GABA-shunt in Arabidopsis is assumed, then the detection of GHB in *gaba-t/ssadh* mutant plants could be explained. The vast amounts of GHB determined in leaf extracts of *ssadh* mutants might be a mechanism comparable to one appearing in human patients with SSA-deficiency, resulting in an accumulation of GHB [77]. Suspecting SSA to be toxic for cells, then GHB synthesis might be an attempt of plants to remove SSA in situations with no or too little functional SSADH protein in the cells. However, the GHBDH would – at least under these conditions - prefer the reduction of SSA to GHB and not the oxidation of GHB to SSA, as known from mammalia.

Given that *ssadh* mutant plants accumulate both SSA and GHB which, either alone or in combination, reduce plant growth and induce the accumulation of reactive oxygen intermediates (ROIs), then the substance causing the *ssadh* phenotype might be identified by the analysis of *gaba-t/ssadh* plants [66, 107]. To address the question whether SSA or GHB has a larger impact on plant growth, *gaba-t*, *gaba-t/ssadh* and wild type plants grown on media containing SSA or GHB were analyzed regarding growth and metabolite content. In these experiments, no differences occurred between wild type and *gaba-t* mutants since both are able to degrade SSA and GHB. In contrast, *gaba-t/ssadh* mutants were highly affected by either SSA or GHB (Figure 8). Interestingly, no correlation was found between GHB content measured in leaf extracts and plant growth observed on the different media. Wild type and *gaba-t* mutant plants are less impaired in growth than *gaba-t/ssadh* plants that contain comparable amounts of GHB in leaf extracts. Additionally, *gaba-t/ssadh* plants with similar GHB amounts in leaf extracts are smaller

when grown on media containing SSA. Several additional findings favoring SSA to be toxic can be stated. First, compared to GHB, lower concentrations of SSA induce severe growth reduction in wild type plants. Second, GHB in the media mainly decreases root length, whereas the rosette size is not altered; supplementation with SSA leads to growth of very small rosettes and a shorter main root with an increased number of lateral roots. Third, in the screening for *ssadh* suppressor lines, several plants lines with improved growth were isolated containing higher GHB amounts in leaf extracts than the *ssadh* mutants (6.1.1). Forth, in *Agrobacterium tumefaciens*, two differentially expressed *SSADH* genes were identified that are probably necessary for SSA detoxification [185], and finally, SSADH deficiency in humans lead to neurological disorders, but no reports of GHBDH deficiencies are published.

Since SSA and GHB can rapidly be converted into each other, a central point for the identification of the substance causing the *ssadh* phenotype is to inhibit this conversion. In Arabidopsis, one gene encoding a *GHBDH* has been published [31] and homozygous T-DNA lines are available. Although these plants were supposed to be unable to catalyze the SSA-GHB conversion and hence growth should vary compared to wild type grown on SSA- or GHB-containing media, surprisingly no differences were observed (Figure 10). The identification of a sequence homologous to the GHBDH protein explained this finding, and the analysis of double homozygous plants revealed minor variations in GHB content (Figure 17). Compared to wild type, no growth differences occurred on the different media, but mutant plants accumulate less GHB upon growth on SSA containing media, and more GHB when grown on media supplemented with GHB. These findings indicate a possible SSA or GHB threshold level in plants that must be reached to induce activity of the GHBDH proteins *in vivo*. To test this assumption, GHBDH activity levels of recombinant and native protein should be assayed. As an alternative hypothesis, the existence of a third protein catalyzing the SSA-GHB conversion can be assumed, probably the more distantly related gene At4g20930, annotated as 3-hydroxyisobutyrate dehydrogenase. So, the isolation of knockout lines for this gene might be helpful in this context.

Taken together, the analysis of a yet not existing *ghbdh* triple mutant or a *gaba-t/ssadh/ghbdh-1/ghbdh-2* quadruple mutant and the development of a method to measure SSA in plant extracts is essential to answer the question which substance is causing the *ssadh* phenotype.

For both GHBDH proteins, the intracellular localization was analyzed using GFP fusion proteins in co-localization studies with RFP-tagged organelle markers [123]. Corresponding to prediction programs, GHBDH-1 localizes to the cytosol (Figure 9). Biochemical analyses of recombinant protein in a published report suggest a preference for SSA as well as for glyoxylate reduction over the corresponding oxidation reactions [86, 87]. If the GHBDH-1 reduces SSA, then the substrate for a cytosolic enzyme can only be present, when SSA export from the mitochondria occurs.

The second protein reveals indifferent localizations when comparing the output of several prediction programs. In contrast to published data, where a plastidial localization is reported [158], neither in Arabidopsis cell culture co-transformed with GHBDH-2-GFP along with a plastidial RFP marker (Figure 14) nor following particle bombardment of leek epidermal peels (Figure 13) co-localization of GHBDH-2-GFP with a plastidial marker is observed. However, since glyoxylate is found predominantly in plastids and peroxisomes, a plastidial localization might be necessary for the enzyme to act *in vivo* as glyoxylate reductase. If SSA reduction is the preferred reaction, then a plastidial localization of the enzyme would not be essential.

In transient transformation of Arabidopsis suspension cells with GHBDH-2-GFP, co-localization is perceived either with a co-transformed mitochondrial RFP marker (Figure 15) or in cells stained with Mitotracker Red (Figure 16). In the mitochondrial matrix, the general reduction potential favors oxidation of substrates, whereas in plastids, substrates are predominantly reduced. This supports the hypothesis that GHB might additionally be produced in a presumed GABA-independent pathway, and is oxidized in the mitochondria via SSA to succinate, comparable to the degradation of the neurotransmitter GHB in the mammalian brain [77, 179].

## 3.2. Identification of ssadh suppressor lines

Since the suppression of the *ssadh* phenotype is possible as presented in 2.1.1 and [66, 107], a population of EMS-mutagenized *ssadh* plants was screened for plants with improved growth, to identify further genes involved in regulation or function of the GABA-shunt. Growth characteristics and metabolite contents of several lines resembled *gaba-t/ssadh* mutants, and among these, two new *gaba-t* alleles were identified by sequencing (2.2.2).

By comparing the Arabidopsis protein sequence to sequences of crystal structures available in public databases, the mutation in line 37.B is identified next to an active site residue, whereas in line 31.A a single residue located both in the recognition site for cofactor binding and near an active site residue is exchanged [166, 174]. These substitutions by larger, negatively charges amino acids will most probably result in an altered protein structure. Minor modifications of the protein structure might result in changed catalytic properties or substrate specificities. In contrast, serious structural modifications will lead to misfolded proteins that are recognized and degraded in ubiquitin-dependent proteolysis [124]. Here, both mutations are supposed to interfere with both the formation of the active site and proper folding of the whole enzyme, thus resulting in protein inactivation and probably even degradation. Therefore, the two *ssadh* suppressor lines most likely contain no functional GABA-T enzyme.

Currently, three other *ssadh* suppressor lines are under investigation to identify the location of the EMS mutation (2.2.3). All available SSLP-markers are used to identify a larger region on the chromosome, where the EMS-mutation might reside. The CAPS-markers proposed by the Marker Tracker (http://www.bar.utoronto.ca/markertracker/) are based on the published Col-0 and L*er* genomic sequences, and are derived from *in silico* analyses, but the sequences were found to produce not necessarily polymorphic fragments following PCR and restriction. To identify a smaller chromosomal region allowing to select a number of possible candidate genes, two points have to be covered: first, more double homozygous $F_2$ plants need to be isolated and second, more markers – probably also without restriction to PCR-based markers – have to be defined and assayed for polymorphisms.

Plants of line 4.D (2.2.3.1) are those accumulating the highest amounts of GHB in leaf extracts among all *ssadh* suppressor lines analyzed. Corresponding to the metabolite data, growth of the plants on ½ MS plates resemble the phenotype of wild type plants grown on ½ MS supplemented with GHB: the rosette size is not much affected, while the roots are considerably shorter. In comparison to wild types, the reduced rosette size might be due to the *ssadh* mutant background of the plants. A mutation in one of the known *GHBDH* genes could be excluded since the mutation maps to chromosome 4 and the *GHBDH* genes are localized on the chromosomes 2 and 1 respectively. However, the putative third possible GHBDH in Arabidopsis (At4g20930) is located on chromosome 4 within the region possibly containing the EMS-mutation and might induce suppression of the *ssadh* phenotype.

## 3.3. SSA induces callus formation in Arabidopsis thaliana

Arabidopsis *ssadh* mutant plants growing on ½ MS or wild type plants growing on ½ MS supplemented with SSA develop dedifferentiated tissue in the hypocotyl. The regeneration of whole plants from the isolated dedifferentiated cells is considered as a proof for these cells being callus.

Analyzing the rate of callus formation in wild type plants of three different Arabidopsis ecotypes (Figure 35) reveals a positive correlation of callus formation on the SSA concentration in the media. Furthermore, the rate of callus formation was similar in wild type growing on ½ MS supplemented with 0.8mM or 1.0mM SSA and in *ssadh* mutants growing on ½ MS. Probably, a certain cellular SSA concentration is necessary to induce dedifferentiation, which is reached in *ssadh* mutants naturally, whereas in wild type plants this concentrations is only accessed upon SSA-feeding. Further arguments supporting callus formation to be a concentration- and light-dependent mechanism are three observations; first, dark grown plants never developed callus; second, increasing the SSA-concentration in the media enhances callus formation in the hypocotyl of wild types; and third, germinating *ssadh* mutants on media containing SSA results in dedifferentiation occurring in several tissues without a restriction to the hypocotyl (Figure 36).

Callus formation in plants is long known to occur in response to the ratio of the phytohormones auxin and cytokinin [37, 159]. However, growing plants on media containing SSA and IAA or SSA and kinetin has no large effect on the rate of callus formation in wild type (Figure 38), indicating that SSA might induce callus formation independent of the auxin or cytokinin supplied in the media. Nevertheless, the phenotype – especially of the *ssadh* calli with emerging root hair like structures (Figure 36) – indicates an auxin-related process.

In contrast to NPA and PCIB – an auxin transport inhibitor and an antiauxin - only the second tested auxin-transport inhibitor TIBA has the ability to prevent SSA-induced callus formation when supplied to the media (Figure 39). TIBA inhibits vesicle

trafficking and thus the recycling of auxin transporters belonging to the AUX/LAX and PIN protein families [61, 98].

In addition to the inhibition of callus formation by preventing cycling of auxin transporters, a considerably reduced rate of callus development was observed in plants with mutations in different auxin transport genes (*aux1, lax1, lax3,* and *pin3*) as well as in plants overexpressing an IAA ligase (IAA-L plants) that contain lesser amounts of free active auxin (Figure 41). Furthermore, assaying the GUS expression driven by promoters of auxin-related genes reveals SSA-induced promoter activity for the *LAX* genes and for the *NIT1* to *NIT3* genes. The SSA-induced expression of *LAX* genes corresponds to the reduction of callus formation in *lax* mutants, might present evidence for a mechanism related to auxin-transport. The nitrilase proteins 1 to 3 catalyze the hydrolysis of IAN to IAA, increasing the concentration of free, active auxin in the cells. Activation of the *NIT* genes in response to SSA will result in an enhanced IAA production in the dedifferentiated tissue. If SSA-induced callus induction involves an IAA-related mechanism, then expression of the synthetic auxin-responsive promoter DR5 should be detectable in the tissue. Recently published data revealed that DR5 expression is highest in the early stages of callus development and depletes over time. About one week after the transfer to callus induction medium, no DR5 expression was detectable in the calli any more [9, 78]. When assigning this discovery to the SSA-induced callus formation, then DR5 expression should be detectable early during callus development, but this remains to be tested.

In a root elongation assay, a test for auxin activity in Arabidopsis, SSA inhibits root growth, but the required concentrations are much higher than those published for auxins (2.3.3). This supports the hypothesis, that SSA induces the synthesis of auxins in a yet unknown way.

Taken together, the combination of increased IAA synthesis and enhanced transport – especially uptake – might result in the accumulation of free auxin in the cells of the hypocotyl, and as a consequence induce callus.

# 4. Materials and Methods

## 4.1. Materials

### 4.1.1. Chemicals

Chemicals were obtained from the companies:
Biomol (Hamburg, D), Difco (Hamburg, D), Duchefa (Haarlem, NL), Eurobio (Les Ulis Cedex, F), Fluka AG (Buchs, CH), Merck (Darmstadt, D), Roche (Mannheim, D), Roth (Karlsruhe, D), Fisher Scientific GmbH (Schwerte, D), Chromatographie Service GmbH (Langerwehe, D) and Sigma-Aldrich (Deisenhofen, D).
Purity was "research grade" or the highest available.

### 4.1.2. Enzymes

Enzymes for molecular biology were obtained from the companies:
Invitrogen (Karlsruhe, D), MBI Fermentas (St. Leon-Rot, D), Promega (Mannheim, D), Qiagen (Hilden, D), Roche (Mannheim, D) and Sigma (Deisenhofen, D).

### 4.1.3. Kits

The following Kits were used following the manufactures protocols:
TRIsure (Bioline)
Big Dye ® Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, USA)
CompactPrep Plasmid Midi (Qiagen GmbH, Hilden, D)
QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, D)
Quantum Prep ® Plasmid Miniprep Kit (BioRad, München, D)
Super ScriptTM II - Reverse Transcriptase (Bioline)

### 4.1.4. Antibiotics

The following antibiotics were used:

| Antibiotic | stock solution | | working concentration |
|---|---|---|---|
| Ampicillin | 100 mg/mol | in water | 100 µg/mol |
| Carbenicillin | 50 mg/mL | in water | 50 µg/mL |
| Kanamycin | 50 mg/mL | in water | 50 µg/mL |
| Gentamycin | 25 mg/mL | in water | 25 µg/mL |
| Hygromycin | 50 mg/mL | in water | 50 µg/mL |
| Chloramphenicol | 75 mg/mL | in ethanol | 75µg/mL |
| Rifampicin | 20 mg/mL | in DMSO | 100 µg/mL |
| Rifampicin | 30 mg/mL | in DMSO | 150 µg/mL |

## 4.1.5. Bacteria

The following bacteria strains were used:

Escherichia coli:

DH5α

*F- supE44 ΔlacU169 (Φ80, lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*
for plasmid amplification

XL10-Blue

*F- supE44, hsdR17, recA1, endA1, gyrA46, thi, relA1, lac, F´[proAB+, lacIq, lacZΔM15, Tn10(tetr)]*
for plasmid amplification

Top10

*F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*
for plasmid amplification

DB3.1

*F- gyrA462 endA1 (sr1-recA) mcrB mrr hsdS20(rB-, mB-) supE44 ara-14 galK2 lacY1 proA2 rpsL20(SmR) xyl-5 - leu mtl1*
for amplification of plasmids containing the ccdB gene (GATEWAY-cloning)

Agrobacterium tumefaciens:

GV3101 (pMP90)
GV3101 containing the helper plasmid pSoup
SV 0 (super virulent strain for cell culture transformation*) LBA4404.pBBR1MCS virGN54D*

## 4.1.6. Plant lines

Wild type *Arabidopsis thaliana* plants were of the ecotypes Columbia-0 (Col-0) and Landsberg erecta (Ler). The following T-DNA insertion lines were all in Col-0 background and were obtained from the following sources:
SIGnAL (SALK Institute Genomic Analysis Laboratory, http://signal.salk.edu)
MPIZ (http://www.mpiz-koeln.mpg.de/GABI-Kat/GABI-Kat_homepage.html)
FLAG-collection from INRA (http://www-ijpb.versailles.inra.fr)
Syngenta (http://www.tmri.org/en/partnership/sail_collection.aspx)

| Gene | At number | Line |
|------|-----------|------|
| SSADH | At1g79440 | SALK_003223 |
| | | Gabi-Kat 737E05 |
| | | SAIL_1278 B12 |
| GABA-T | At3g22200 | Gabi-Kat 157D10 |
| | | SALK_007661 |
| | | SAIL_1230C03 |
| GAD 1 | At5g17330 | SALK_017810 |
| | | SALK_017931 |
| GAD 2 | At1g65960 | SALK_028819 |
| | | SALK_033689 |
| GAD 3 | At2g02000 | SALK_033307 |
| GAD 4 | At2g02010 | SALK_146398 |
| GAD 5 | At3g17760 | FLAG_460F07 |
| | | GABI_326C03 |
| GHBDH-1 | At3g25530 | Gabi-Kat 316D05 |
| | | SALK_057410 |
| GHBDH-2 | At1g17650 | Gabi-Kat 933D03 |
| | | SALK_047412 |

## 4.1.7. Software

| | |
|---|---|
| Acrobat Reader Version 7.0 | |
| Photoshop Version CS3 | Adobe |
| | |
| EndNote Version 9.0 | ISI ResearchSoft (USA) |
| | |
| Enhanced Chem Station G1701 CA Dec. 1999 | Agilent (USA) |
| | |
| Microsoft Office 2000/2007 | |
| Windows XP | Microsoft |
| WinNT Version 4.0 | |
| | |
| Multi Experiment Viewer 4.2 | TIGR |
| | |
| Mozilla Firefox 3.0 | Mozilla Foundation |
| | |
| SeqMan II | DNA-Star Inc |
| Edit Seq | |
| | |
| Sigma Plot Version 10.0 | SPSS |
| | |
| Vector NTI Advanced 10.0 | Invitrogen |

## 4.1.8. Used Equipment

| | | |
|---|---|---|
| Autoclave | Sanoklav L 11201 | Wolf, Geislingen (D) |
| Balances | Sartorius FX-40 | Sartorius, Leichlingen |
| | Sartorius 1264 MP | |
| | Ohaus GT 4800 | Ohaus, Florham Park (USA) |
| Centrifuges | Eppendorf 5417, 5417 R, 5417 C and 5402 | Eppendorf, Hamburg |
| Clean Bench | Heraeus LaminAir HB 2448 | Heraeus, Düsseldorf (D) |
| Climate Chambers | Percival Scientific CU-36L5 | CLF, Emersacher |
| | Percival Scientific AR-66L | |
| Crimper/Decapper | 5181-1213 (open) | Agilent (USA) |
| | 710-0979 (close) | |
| Dewar | | KGW Isotherm |
| Electroporator | Genepulser II | BioRad, München, D |
| Freezer | +4 °C, -20 °C | Liebherr |
| | -80 °C Hera freeze | Heraeus |
| | -80 °C Ultima II | Revco |
| GC/MS | Auto Sampler # 7683 | Agilent (USA) |
| | Injector # 7683 | |
| | GC # 6890N | |
| | MS # 5973N | |
| | Ionization Gauge Controller # 59864B | |
| | Forline Pump | Edwards (Agilent) |
| Heater | | Eppendorf, Hamburg (D) |
| | Thermo mixer 5436 | |
| | Thermostat 5320 | HLC Bovenden (NL) |
| | HTM-130L | |
| Magnetic Stirrer | Ikamag REO or. RET | IKA-Werke, Staufen (D) |
| NanoDrop | ND 1000 | NanoDrop Technologies, Wilmington (USA) |
| Particle Gun | PDS-1000/He System | BioRad, München, D |
| | Macrocarriers | |
| | 900psi rupture disks | |
| | Stopping screens | |
| | 1µm gold microcarriers | |
| PCR Cycler | PTC 100 and PTC 200 | MJ Research, distributed by Biozym, (Hess. Oldendorf, D Weilheim |
| pH-Meter | pH 521 WTW | |
| Photometer | Pharmacia LKB Novaspec II | Pharmacia, Freiburg |
| | Pharmacia Ultraspec III | |
| Pipettes | pipetman | Gilson, Lewis Center (USA) |
| | Research | Eppendorf, Hamburg (D) |
| Printer | HP Color Laserjet 3600 | |
| | HP Laserjet 2300 | |
| | HP Laserjet 4100 | |
| Shaker | G25 | Fa. New Brunswick Scientific GmbH, Nürtingen (D) |

| Spectrophotometer | SpectraFluor plus SIL | Tecan, Crailsheim |
| Speedvac | Vacuum Concentrator | Bachofer, Reutlingen (D) |
| Syringes | Microliter # 705 or. #710 | Hamilton, Bonaduz (CH) |
| | 5 or 10 µL FN 23/42/HP | Agilent (USA) |
| TissueLyser | | Qiagen, Hilden (D) |
| Ultrasonic bath | Sonorex-RK255H | Bandelin |
| Vortexer | Heidolph Reax 2000 | Heidolph, Hartenstein |
| | Vortex Genie 1 or. 2 | Bender & Hobein, Zürich (CH) |
| Water bath: | Type 1003 | Firma GFL, Burgwedel (D) |

## 4.1.9.  Consumables

| Autoclave tape | | 3M, Neuss (D) |
| Adhesive tape for Petri dishes | | Servopor Eurofarm (I) |
| Falcon Tubes | 15 mL & 50 mL | Corning Inc. (USA) |
| fluorescent lamps | Greenhouse: | |
| | L58W/31-830, L58W/76 | Osram |
| | Climate Chamber: | |
| | F17T8/TL741          17W, | Philips |
| | F23T8/TL741, TL70 | |
| Cuvette | | Müller Ratiolab, Dreieich (D) |
| Microtiter Plate | | Greiner, Solingen (D) |
| Para film | | Am. National Can, Nenasha (USA) |
| Pasteur pipettes | | Brand, Wertheim (D) |
| PCR-tubes | 8$^{er}$ strips | 4titude, (UK) |
| | 96$^{er}$ plates | |
| Petri dishes | | Greiner, Solingen (D) |
| Pipette tips | 10µL, 200µL, 1mL | Sarstedt, Nümbrecht (D) |
| | 10µL, 5mL | Eppendorf, Hamburg (D) |
| Reaction tubes | 1.5mL & 2mL | Sarstedt, Nümbrecht (D) |
| Scalpels | | Braun, Melsungen (D) |

**Particle Gun**

| Macrocarriers | 165-2335 | BioRad (München) |
| 900psi rupture disks | 165-2328 | |
| Stopping screens | 165-2336 | |
| 1µm gold microcarriers | 165-2263 | |

**GC-MS**

| | | |
|---|---|---|
| Ferule | Injector: 5181-3323 0.4mm ID Vespel, Graphite<br>Interface: 5862-3508, 0.4mm; 200, 250μm | Agilent (USA) |
| Filament Assembly | 50972-60053 | Agilent (USA) |
| GC-Column | HP-50+<br>L 30m, ID 0.25mm, Film 0.25μm<br>Temp. Limits: 40°C – 280°C | J&W Scientific (USA) |
| Liner | 5183-4711 4 mm ID Split/Split less containing glass wool | Agilent (USA) |
| Oil | Diffusion Pump: Santovac 5P Ultra<br>Foreline pump: Rough pump oil | Agilent (USA)<br>Edwards (USA) |
| O-Rings | 5180-4182 | Agilent (USA) |
| Septum | 5181-3383 1mm prtl. | Agilent (USA) |
| Sample Handling (GC-MS) | Crimp cap and vials<br>Micro inlets G 30/5mm.<br>Screw cap G8<br>Vial G1 clear | CS-Chromatographie Service GmbH, Langerwehe (D) |

## 4.2. Methods

### 4.2.1. Work with *Arabidopsis thaliana*

#### 4.2.1.1. Growth conditions

##### 4.2.1.1.1. Greenhouse

Arabidopsis seeds were sown in 9cm plastic pots on a substrate mixture containing three parts soil and one part Vermiculit For vernaliztion, the pots were kept for a minimum of 48 hours at 4°C in the dark, and then the pots were transferred to the greenhouse for germination. Approximately 2 weeks after germination, the plants were pricked out.
In the greenhouse, plants were grown under long day conditions with a light/dark rhythm of 16:8 hours, a temperature of 21°/18°C and a relative humidity of approximately 40%.

##### 4.2.1.1.2. Climate chamber

*Arabidopsis thaliana* plants were grown on culture plates in a climate chamber (Percival Scientific, Model CU-36L5 or AR-66L_S) with o humidity of 50% and a temperature of 22°C during light period and 18°C during dark period respectively. For growth under long day conditions, the plants were subjected to a day/night rhythm of 16:8 hours, for short day conditions of 8:16 hours.

#### 4.2.1.2. Screening of T-DNA Insertion lines

The genotype of T-DNA insertion lines was determined using PCR-based methods. Therefore, genomic DNA of single plants was isolated and used as template for PCR with two gene specific primers or one gene specific primer and one primer complementary to the flanking sequence of the T-DNA in separate reactions (primer sequences are listed in 6.4.1). Homozygous plants were kept for further analyses.

#### 4.2.1.3. Crossing of *Arabidopsis thaliana* plants

For crossing to other lines, homozygous plants of about 6 weeks age were used with exception of *ssadh* plants, where heterozygous plants were used due to the reduced growth of homozygous plants.
All open flowers and developed siliques were removed from the plants to be pollinated and of the remaining flower buds the three largest buds were opened carefully under the binocular and all organs except the carpels were removed. Stamens of the plants donating pollen were isolated and pollen was transferred onto the carpel of the other line. The flowers used for crossing were labeled, harvested when ripened and were sown on soil. $F_1$ plants were screened for occurrence of the respective T-DNA inserts in heterozygous state, these were allowed to set seeds, and in the $F_2$ generation, the offspring was screened in PCR reactions for double homozygous plants.

#### 4.2.1.4. Map-based cloning in *Arabidopsis thaliana*

Sequencing the genome of *Arabidopsis thaliana* ecotypes revealed variations among them: single nucleotide exchanges, a different number of simple sequence repeats or insertions and deletions. For the ecotypes Columbia (Col-0) and Landsberg erecta (Ler), several types of markers have been published [17, 46, 65, 99, 145]. Markers are available via TAIR (http://www.arabidopsis.org/ > search > markers) or can be designed using Marker Tracker (http://www.bar.utoronto.ca/markertracker/). To actually map a single trait to a genomic locus, ssadh suppressor lines were crossed with Ler wild type plants. In the F2 generation, plants without ssadh phenotype were screened for homozygous occurrence of the T-DNA insertion in the ssadh gene. These plants also carry the EMS mutation in homozygous state; otherwise, the plants would display the reduced growth of ssadh plants. The identified double homozygous plants constitute the mapping population.

Since the mapping population was selected was selected for homozygousity of both mutations and both are in Col-0 background, a higher percentage of Col-0 specific fragments will be obtained near the mutations. All distant markers will be equally distributed between Col-0 and Ler specific fragment.

#### 4.2.1.5. GUS Staining

Plants to be tested for promoter activity were vacuum-infiltrated with GUS Staining solution, until plants are completely submerged in the solution and the leaves are slightly translucent. Then, the plants are incubated at 37°C until staining was visible. To enhance visibility of the GUS signal, chlorophyll was extracted by incubating the plants in 80% ethanol at 65°C.

| GUS Staining Working Solution | | |
|---|---|---|
| | 0.1M | NaPO4 pH7 |
| | 10mM | EDTA |
| | 0.5mM | K-Ferricyanid |
| | 0.5mM | K-Ferrocyanid |
| | 1mM | X-Gluc |
| | 0,1% | Triton X-100 |

#### 4.2.1.6. Growth of Arabidopsis plants on sterile media

##### 4.2.1.6.1. Surface sterilization of Arabidopsis seeds

For growth of Arabidopsis plants on sterile media, the seeds were surface sterilized before sowing on plates.

About 100 seeds per line were placed in 1.5mL reaction tubes, which were then placed open in an exsiccator containing a beaker with 100ml sodium hypochlorite. 3mL $HCl_{conc.}$ was added and the lid of the exsiccator was kept closed or approximately 4 hours. Then, the tubes were closed and transferred to the sterile hood, where they were kept open for one hour to allow remaining chlorine gas to evaporate.

### 4.2.1.6.2. Preparation of MS plates

For plant growth under controlled nutrient conditions *in vitro*, half strength MS media was used, prepared from several stock solutions.

| | | |
|---|---|---|
| MS Macro I: | 21.06mM | $NH_4NO_3$ |
| | 18.8mM | $KNO_3$ |
| | 1.5mM | $MgSO_4 * 7 H_2O$ |
| | 1.249mM | $KH_2PO_4$ |
| | | |
| MS Macro II | 2.979mM | $CaCl_2 * 2H_2O$ |
| | | |
| FeNaEDTA | 100µM | FeNaEDTA |
| | | |
| MS Micros | 100.2µM | $H_3BO_3$ |
| | 70.4µM | $MnSO_4 * H_2O$ |
| | 29.9µM | $ZnSO_4 * 7H_2O$ |
| | 1.033µM | $Na_2MoO_4 * 2H_2O$ |
| | 0.1µM | $CuSO_4 * 5H_2O$ |
| | 0.154µM | $CoCl_2$ |
| | | |
| Vitamins (modified MS): | 100mg/L | Myo-Inositol |
| | 0.5mg/L | Nicotinic acid |
| | 0.5mg/L | Pyridoxine-HCl |
| | 1.0mg/L | Thiamine-HCl |
| | 2mg/L | Glycine |
| | | |
| | 0.5g/L | MES |
| | 29.2mM | Sucrose (facultative) |

Vitamins were obtained from Duchefa, to prepare the stock solution; 1.038g was dissolved in 10mL $H_2O_{bd}$.
MS Macro I and II were prepared as 10x stock solution, FeNaEDTA as 100x stock solutions and Vitamins and MS Micros as 1000x stock solution.

For growth experiments with different nitrogen / GABA concentrations, MS Macro I was prepared without $NH_4NO_3$ and $KNO_3$. GABA and organic nitrogen ($NH_4NO_3$ and $KNO_3$ in the molar ratio of the standard medium) were added to the media from stock solutions. The pH of the solution was set to 5.7 with 1M KOH, for preparation of plates, $0.8_{(w/v)}$ Gelrite (Duchefa) was added. The media were autoclaved, and poured in square culture plates (Greiner).

### 4.2.1.6.3. Sowing of Arabidopsis seeds on sterile media

Surface sterilized (4.2.1.6.1) Arabidopsis seeds were used for growth on sterile MS media. Therefore, dry seeds were spread on autoclaved paper and transferred onto the plates using a sterile toothpick. The seeds were vernalized for 48 hours at 4°C in the dark, and then the plates were transferred in a climate chamber. All plates were sealed using gas permeable adhesive tape.

## 4.2.1.7.    Fluorescence Microscopy

Protein localizations were assayed using the fluorescent proteins GFP of the RFP-derivative  mCherry [123]. The optimal excitation and emission wavelengths were 488nm/507nm for GFP and 587nm/610nm for mCherry.

Transformed Arabidopsis cells were pelleted by short centrifugation (5min, 1000rpm) and then spread on a microscope slide, in the case of leek, epidermal peels were prepared and used for microscopy.

## 4.2.1.8.    Arabidopsis thaliana cell culture

### 4.2.1.8.1.   Maintenance of Cell Culture

*Arabidopsis thaliana* cells were grown in suspension culture in the dark at 18 to 22°C while shaking at about 1560rpm. Once a week, cells were diluted 1:4 with fresh, room temperature media

Approximately every 4 weeks, the cell culture was screened for bacterial contamination and backup plates were prepared. In case of visible bacterial contamination, the diluted cultures were treated with Ticarzidin, before using cells for transformation, they should be diluted twice. For backup plates, 50mL cell culture was centrifuged (15min, 500rpm), the supernatant discarded, the pellet resuspended in some media and spread on plates.

| | |
|---|---|
| 4.3g | Duchefa MS basal salt mixture (M 0221) |
| 4mL | B5 vitamins |
| 400mg | Myo-Inositol |
| 30g | Sucrose |
| ad 1L | $H_2O$ |

The pH was set to 5.8 with KOH; for plates, 0.8% Gelrite was added, 0.5mL 2mg/mL 2,4D was added after autoclaving.

### 4.2.1.8.2.   Cell Culture Transformation

For cell culture transformation, *Agrobacterium tumefaciens* strain SV-0 carrying the binary vector and colonies of the antisilencing strain RK19 were picked from fresh plates and were grown for 24h at 28°C in 5mL cultures in YEB containing the appropriate antibiotics (20µg/mL Rifampicin, 75µg/mol Chloramphenicol, 50µg/mL Kanamycin for selection of the binary plasmid). The cells were pelleted by centrifugation (15min, 4000rpm) and resuspended in 1mL Arabidopsis cell culture media.

For coculture, freshly diluted Arabidopsis suspension culture were mixed 50:1 with the different Agrobacterium strains, grown for 3 to 5 days in the dark at 25°C, and then screened for expression of the transgene.

### 4.2.1.9. Biolistic transformation

### 4.2.1.9.1. Preparation of Microcarriers

For the preparation of the microcarriers, 30mg of 1µm gold particles were weighed into a microcentrifuge tube. 1mL 70% EtOH was added, the suspension was vortexed vigorously and then soaked for 15min in 70% EtOH. The gold particles were pelleted by 15 sec centrifugation at 13000rpm and the supernatant was discarded. Then, 1mL $H_2O_{dest}$ was added, the suspension was vortexed and the gold particles pelleted by 10 sec centrifugation at 10000rpm. The supernatant was removed and the washing step was repeated once. Finally, the gold particles were resuspended in 1mL $H_2O_{dest}$ and sonicated for 3sec.
This suspension was divided into 50µL aliquots while vortexing and were stored at -80°C.

### 4.2.1.9.2. DNA Coating of Microcarriers

For each shot, 5µL gold suspension (4.2.1.9.1) with a concentration of 30mg/mL was used. To the gold suspension 300ng of each DNA, 10mL 2.5M CaCl2 and 4µL 0.1M spermidine (free base) were added while vortexing. The volume was set to a final volume of 25µL with water and the tubes were closed and vortexed for 5 to 10min, during this time, the gold particles were coated with DNA. Then, the gold particles were pelleted by 5sec centrifugation at 10000rpm and the supernatant was discarded. The particles were washed twice, first with 50µL 70% EtOH, then with 20µL 100% EtOH. Finally the particles were resuspended in 12µL 100% EtOH by vortexing. To deagglomerate the particles, the suspension was sonicated for 3sec and the suspension was stored on ice until use.

### 4.2.1.9.3. Bombardement

For bombardment, the macrocarriers were placed into the macrocarrier holders. 10µL of the coated microcarriers were pipetted onto a macrocarrier and spreaded equally. The macrocarriers were dried in vacuum, leaving a dry monolayer of microcarriers.
The conditions for bombardment were: 900psi rupture disk, vacuum 26mm Hg and the sample tray carrying a petri dish with leek epidermis on the third level from the bottom.
Subsequent to the bombardment, the samples were kept in a dark, humid environment at room temperature over night and were analyzed by fluorescence microscopy the following day.

## 4.2.2. Microbiology

### 4.2.2.1. Work with E. coli

#### 4.2.2.1.1. Media

| LB | 10g | Trypton |
|----|-----|---------|
| | 5g | Yeast Extract |
| | 10g | NaCl |
| | ad 1L | $H_2O_{bd}$ |

| 2YT | 16g | Trypton |
|-----|-----|---------|
| | 10g | Yeast Extract |
| | 5g | NaCl |
| | ad 1L | $H_2O_{bd}$ |

| SOC | 20g | Trypton |
|-----|-----|---------|
| | 5g | Yeast Extract |
| | 0.585g | NaCl |
| | 0.186g | KCl |
| | 0.953g | $MgCl_2$ |
| | 1.2g | $MgSO_4 * 7H_2O$ |
| | 36g | Glucose |
| | ad 1L | $H_2O_{bd}$ |

For preparation of agar plates, 1.5 %$_{(w/v)}$ bacto agar was added.
The media were autoclaved and the required antibiotics were added after cooling of the media to about 40°C.

#### 1.1.1.1.1. Growth of E. coli

Cells were grown either on agar plates in a 37°C incubator or as liquid cultures in a Shaker.
For liquid cultures, single colonies were picked from LB plates with a sterile toothpick, inoculated in 3mL media and grown over night at 37°C while shaking with 200-250rpm.
For growth of transgenic bacteria, the corresponding antibiotics were added for selection.
Bacteria could be kept for several weeks at 4°C on YEB plates or at -80°C as glycerol culture (500μL culture and 500μL 65% Glycerol, 100mM $MgSO_4$, 25mM Tris-HCl, pH 8.0).

### 4.2.2.1.2. Preparation of competent cells (E. coli)

A 5mL culture was inoculated with a single *E.coli* culture and incubated over night in a 37°C shaker. The next day, 1mL of the overnight culture was used to inoculate a 100mL culture which was grown until an $OD_{550}$ of 0.48 was reached.

The culture was transferred into precooled 50ml tubes, then incubated on ice for 15min and finally the cells were spinned down (2000 rpm at 4°C for 10min). All further steps were performed at 4°C. The supernatant was removed; the pellet was resuspended in 15mL TfB1 and incubated on ice for 2 hours. Then, the cells were centrifuged again (2000 rpm at 4°C for 10min) and the supernatant was removed. The cells were resuspended in 4mL TfB2 and transferred in 100µL aliquots into precooled 1.5mL tubes and immediately frozen in liquid nitrogen. The cells were then stored at -80°C until transformation.

To test for transformation efficiency, one aliquot of competent cells was transformed with 10pg PUC19 DNA and the number of grown colonies was determined on the next day.

| TfB1 | 100mM | $RbCl_2$ |
|---|---|---|
| | 50mM | $MnCl_{2*}4\ H_2O$ |
| | 30mM | KAc |
| | 10mM | $CaCl_2$ |
| | 15% $_{(v/v)}$ | Glycerin |

The pH was adjusted to 7.0 and the media was filter sterilized.

| TfB2 | 10mM | $RbCl_2$ |
|---|---|---|
| | 75mM | $CaCl_2$ |
| | 10mM | MOPS |
| | 15% $_{(v/v)}$ | Glycerin |

The pH was adjusted to 7.0 and the media was filter sterilized.

### 4.2.2.1.3. E. coli transformation

Competent cells were thawed on ice, mixed with plasmid DNA, and incubated on ice for 15min. The cells were then transformed by heat shock (45 to 60 seconds at 42°C). After addition of 1mL SOC, the cells were incubated at 37°C for about 1 hour, and then the cells were plated in different concentrations on selective media and kept at 37°C over night.

## 4.2.2.2.    Work with Agrobacterium tumefaciens

### 4.2.2.2.1.   Media

| YEB | 5g | Bacto Peptone |
|---|---|---|
| | 5g | Beef Extract |
| | 1g | Yeast Extract |
| | 5g | Sucrose |
| | 0.5g | $MgSO_4 * 7 H_2O$ |
| | ad 1L | $H_2O_{bd}$ |

| MGL | 5g | Bacto Tryptone |
|---|---|---|
| | 2.5g | Yeast Extract |
| | 5g | NaCl |
| | 5g | Mannitol |
| | 1.16 | Na glutamate |
| | 0.25g | $KH_2PO_4$ |
| | 0.1g | $MgSO_4 * 7 H_2O$ |
| | 1mg | Biotin |
| | ad 1L | $H_2O_{bd}$ |

The media were autoclaved and the required antibiotics were added after cooling of the media to about 40°C.

### 4.2.2.2.2.   Growth of *Agrobacterium tumefaciens*

For liquid cultures, single colonies were picked from YEB plates with an inoculation loop, inoculated in 3mL media and grown for one to two days at 28°C while shaking with 200-250rpm.
For growth of transgenic bacteria, the corresponding antibiotics were added for selection. Bacteria could be kept for several weeks at 4°C on plates or at -80°C as glycerol culture (500µL culture and 500µL 65% Glycerol, 100mM $MgSO_4$, 25mM Tris / HCl, pH 8.0).

### 4.2.2.2.3.   Preparation of competent cells (*Agrobacterium tumefaciens*)

A 5mL MGL preculture was inoculated with Agrobacterium tumefaciens and grown over night, then the culture was diluted in 100mL MGL and grown until a density of OD600 of about 0.5 was reached. The culture was centrifuged (5min at 4°C and 5000rpm) and the cells were resuspended in 40mL cold buffer (1mM HEPES pH 7.0) followed by another centrifugation step (5min at 4°C and 5000rpm). Then, the cells were washed in 2mL cold buffer (1mM HEPES pH 7.0 containing 10%$_{(V/V)}$ glycerol) and finally resuspended in 200µL 1mM HEPES pH 7.0 containing 10%$_{(V/V)}$ glycerol. 50µL aliquots were frozen directly in liquid nitrogen and stored at -80°C.

### 4.2.2.2.4. Transformation of Agrobacteria

### 4.2.2.2.4.1. Electroporation

Competent cells were thawed on ice, mixed with about 200ng plasmid DNA, and kept on ice for 2min. Then, the cells were transferred into precooled 0.2cm cuvettes. The electroporation conditions were 25µF, 400Ω, 2.5 kV pulse with a retention time of 8-9msec. Following electroporation, 1mL room temperature YEB containing the antibiotics used for selection was added and the culture was incubated at 28°C whilst shaking with 100-150rpm.

The cells were plated in different concentrations on antibiotics containing YEB plates and were grown at 28°C for two days.

### 4.2.2.2.4.2. Freeze-Thaw Method

Competent cells were thawed on ice, mixed with about 200ng plasmid DNA, and incubated on ice for 5min, followed by 5min frozen in liquid nitrogen and 5min at 37°C. Then, 1mL YEB containing antibiotics was added and the cells were incubated at 28°C for about 2 hours, then the cells were plated in different concentrations on selective media and kept at 28°C for about 2 days.

### 4.2.3.  Molecular biology techniques

#### 4.2.3.1.  Plasmid Preparation

##### 4.2.3.1.1.  Mini Preparation

Plasmid DNA from E.coli in Miniprep scale was isolated using the QantumPrep Kit (BIORAD) following the manufacturer's protocols. A part of the overnight culture could be used for storage as glycerol stock cultures at -80°C.

##### 4.2.3.1.2.  Midi Preparation

Plasmid DNA from E.coli in Midiprep scale was isolated using the CompactPrep Plasmid Midi Kit (QIAGEN) following the manufacturer's protocols. A part of the overnight culture could be used for storage as glycerol stock cultures at -80°C.

#### 4.2.3.2.  Preparation of *Arabidopsis thaliana* genomic DNA

##### 4.2.3.2.1.  Fast Prep – Method a

Plants used for identification of the mapping population were screened by PCR, using genomic DNA isolated from single plants. One leaf from each seedling was harvested into a tube from an 8-tube strip. Then, 300µL extraction buffer and a 5mm stainless steel bead were added. The strips were inserted into the adapters of the tissue lyser and shaked at 30Hz for 90sec. The solution could be stored at -20°C until further use or 1µL was used as template for a 25µL PCR reaction.

| Extraction Buffer | 50mM | Tris HCl pH 7.2 |
|---|---|---|
| | 300mM | NaCl |
| | 10% | sucrose |

The buffer was autoclaved and stored in aliquots.

##### 4.2.3.2.2.  Fast Prep – Method b

One leaf from each plant was harvested into a 2mL tube, frozen in liquid nitrogen, and stored at -80°C until preparation. The tissue was pulverized with 400µL extraction buffer. Cell debris was removed by centrifugation and the supernatant was transferred into a new 1.5mL tube. The DNA was precipitated with Isopropanol, pelleted by centrifugation and the supernatant was discarded. The resulting pellet was dried and resuspended in 50-100µL H2O$_{bd}$ and could be stored at -20°C.

| Extraction Buffer | 200mM | Tris HCl pH 7.5 |
|---|---|---|
| | 250mM | NaCl |
| | 25mM | EDTA |
| | 0.5% | SDS |

### 4.2.3.2.3.  Standard Preparation

For isolation of genomic DNA with a higher purity, tissue (usually flowers and buds) from single plants was harvested into reaction tubes, frozen in liquid nitrogen, and stored at -80°C until preparation.

Samples were pulverized and taken up in 500µL extraction buffer. Then, 400µL PCI (phenol, chloroform, and isoamyl alcohol in a ratio of 25:24.1) was added and the samples were mixed. The samples were centrifuged for 15min at 3500rpm to remove proteins and plant material from the aqueous phase, which was transferred into a new tube. Following addition of 300µL Isopropanol, the samples were incubated at room temperature for 10min, and then DNA was pelleted by centrifugation (20min, 3500rpm). The pellet was washed twice with 70% ethanol, dried, and resuspended in 50µL RNAse containing water (1:100 RNAse dilution).

| 2x Buffer | 0.6M | NaCl |
|---|---|---|
| | 100mM | Tris HCl pH 7.5 |
| | 40mM | EDTA |
| | 4% $_{(w/v)}$ | Sarkosyl |
| | 1% $_{(w/v)}$ | SDS |
| Extraction Buffer | 25mL | 2x Buffer |
| | 20mL | 12M Urea |
| | 2.5mL | Phenol |
| | 2.5mL | $H_2O$ |

### 4.2.3.3.  Expression Analysis

### 4.2.3.3.1.  RNA-Isolation

For RNA isolation, about 100mg plant tissue was harvested into 1.5mL tubes and directly frozen in liquid nitrogen. The samples were homogenized with 1mL TRIsure reagent and incubated at room temperature for 5min. Then, 200µL chloroform was added to the samples, which were kept at room temperature for 2min and then centrifuged at 4°C for 10min at 14000rpm. The supernatant was transferred into a new tube, mixed with 500µL Isopropanol, incubated at room temperature for 10min. The RNA was precipitated by centrifugation (4°C, 5min, 7500rpm), washed with 75% ethanol and air dried, then the RNA pellet was resuspended in DEPC-$H_2O$ and the concentration was determined photometrical (4.2.3.4).

### 4.2.3.3.2.  DNAse digestion

To avoid false-positive results when testing the expression level of a gene, genomic DNA was removed by DNAse digestion prior to cDNA synthesis.

The reaction mix contained 2.5µg total RNA, 4µL RT-buffer and 1µL DNAse I (10U) in a total volume of 20µL. The samples were incubated at 37°C for 20 to 30min. For DNAse inactivation, 2µL 25mM EDTA was added and the samples were heated to 65°C for 10min.

#### 4.2.3.3.3. Reverse Transcription

For cDNA synthesis, 11µL DNAse digestion and 1µL oligo-dT primer were incubated for 5min at 70°C and were then transferred directly into ice. Then, 1µL 10mM dNTP's, 4µL 5xRT-buffer, 2.75µL DEPC-$H_2O$ and 0.25µL BioScript Reverse Transcriptase were added. cDNA synthesis took place at 37°C for about 1hour, then the enzyme was deactivated by heating to 70°C for 10min.

### 4.2.3.4. Photometric determination of nucleic acid concentrations

To determine the concentration of isolated nucleic acids (4.2.3.2.3 and 4.2.3.3.1), 1µL sample was used for photometric measurement using a nanodrop machine. The concentration of the sample was calculated from the absorption at a wavelength of 260nm.

### 4.2.3.5. PCR

The PCR is a method to amplify a DNA fragment using complementary oligonucleotides (primers), and a thermo stable DNA polymerase. The annealing temperature is dependent on the primer sequence, the elongation time and temperature on the fragment length and the used polymerase.

| Step | Temperature | Time |
|------|-------------|------|
| Denaturing | 95°C | 5min |
| Denaturing | 95°C | 0:30min |
| Annealing | Primer dependent | 0:30min |
| Elongation | 72°C (68°C for *Pfx*) | 1min/kb (2min/kb for *Pfu*) |
| Elongation | 72°C (68°C for *Pfx*) | 5:00min |
| | 16°C | endless |

Commercially available polymerases were used with the buffer supplemented, for the homemade *Taq*; the following final concentrations were applied:

| | |
|------|------|
| 67mM | Tris HCl, pH 8.8 |
| 16mM | $(NH_4)_2SO_4$ |
| 0.1% | Tween |
| 1.5mM | $MgCl_2$ |
| 0.2mM | dNTP's (each nucleotide) |
| 0.2mM | Primer 1 |
| 0.2mM | Primer 2 |

### 4.2.3.6.    Gel electrophoresis

DNA fragments of different size were separated using agarose gel electrophoresis and visualized by ethidium bromide staining under UV-light (254nm wavelength). Agarose concentration was chosen dependent on the fragment size, smaller fragments were separated using high percentage gels (3-5% agarose in TBE), larger fragments in low percentage gels (0.8-2.5% agarose in TAE).
Agarose was boiled in the corresponding buffer in a microwave and ethidium bromide was added before pouring the gel.
For electrophoresis, size marker (1kb ladder, Invitrogen or 20bp ladder, MBI) and samples mixed with loading dye were pipetted into the gel slots and the gel was run at an amperage of approximately 150mA, corresponding to a voltage between 80 and 130V, dependent on size and percentage of the gel.

| 50x TAE | 2M | Tris/ Acetic Acid, pH 7.5 | |
|---|---|---|---|
| | 1M | Sodium acetate | |
| | 50mM | EDTA | |

| 10x TBE | 0.89M | Tris/NaOH, pH 8.3 | |
|---|---|---|---|
| | 0.89M | Boric Acid | |
| | 20mM | EDTA | |

| Ethidium bromide | 0.5%$_{(w/v)}$ | Ethidium bromide in methanol | |
|---|---|---|---|

| 6x Loading Dye | 10mM | Tris-HCl (pH 7.6) | |
|---|---|---|---|
| | 60mM | EDTA | |
| | 60% | Glycerol | |
| Plus one or two of | | | migrates in 1% gel at: |
| the following dyes | 0.15% | Orange G | 50-100bp |
| | 0.03% | Bromophenol blue | 200-300bp |
| | 0.03% | Cresol Red | ~3000bp |
| | 0.03% | Xylene cyanol FF | ~4000bp |

### 4.2.3.7.    Gateway-Cloning

GATEWAY®-Cloning uses the site-specific recombination activity of bacteriophage λ for all steps during cloning.
The sequence of interest is amplified with either gene specific primers containing an *att*B1 site 5' of the forward primer and an *att*B2 site 5' of the reverse primer or primers containing a 5' CACC-sequence in the forward primer and the unmodified reverse primer. The PCR fragments are then recombined using BP-Clonase into a pDONR vector or via TOPO-Cloning into pENTR/D-TOPO™. Positive clones of the *Entry Vectors* are then used for LR-recombination into the selected *Destination Vectors*.
For the TOPO-Cloning, 0.2µL Salt Solution, 0.2µL TOPO-pENTR™, 0.1µL PCR fragment and 0.7µL sterile water were mixed, incubated at room temperature for approximately 30min and then transformed into *E.coli*.

BP reactions were set up as following: 10ng-50ng PCR product, 50ng Entry Vector, 1µl 5xBP-Reaction Buffer and 1µL BP-Clonase and filled up with sterile water to 5µL. LR reactions were set up as following: 10ng-50ng Entry Clone, 50ng Destination Vector, 1µl 5x LR-Reaction Buffer and 1µL LR-Clonase and filled up with sterile water to 5µL. The reaction were incubated at room temperature over night, stopped by addition of 1µL Proteinase K, and then transformed into *E.coli*.

### 4.2.3.8.    Restriction Digestion of Nucleic Acids

Restriction endonucleases recognize stretches of 4 or more nucleotides in a palindrome sequence and introduce double strand breaks specifically at these sites.
A reaction contained DNA, one or several restriction endonucleases, the corresponding reaction buffer, and sterile water in a volume of 10µL to 50µL. The reaction temperature was dependent on the optimal reaction temperature of the enzyme; the reaction time was between 2 hours and 16 hours.

### 4.2.3.9.    Precipitation of Nucleic acids

Nucleic acid can be precipitated from an aqueous solution by addition of alcohol and salts by reducing solubility in water. Therefore, a DNA containing solution was mixed with 2.5 volume 100% ethanol and 0.1volume 3M Sodium acetate pH 5.2. DNA was pelleted by centrifugation (14 000rpm for 30min), washed with 500µL 75% ethanol and air-dried. The dry pellet was dissolved in 25µL $H_2O_{bd}$ and the concentration was determined (4.2.3.4).

### 4.2.3.10.    Sequencing

Sequence reactions were performed using the chain termination method [146, 147], using the Big Dye® Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems).
A sequencing reaction contained 100ng to 150ng plasmid DNA or 0.5ng to 25ng PCR product as template, one primer with a final concentration of 1mM and 2µL Big Dye v3.1 in a total volume of 10µL. This mix was filled up with HPLC-$H_2O$ to 20µL after the sequence reaction.
The PCR program used for sequencing consists of the following steps:

Sequence runs were performed at the Institute for Genetics of the University of Cologne and the data was available via network.

| Step | Temperature | Time |
|------|-------------|------|
| Denaturing | 96°C | 0:20min |
| Denaturing | 96°C | 0:10min |
| Annealing | 50°C | 0:05min |
| Elongation | 60°C | 4:00min |
| Elongation | 60°C | 5:00min |
|  | 16°C | endless |

## 4.2.4. Metabolite Analysis

### 4.2.4.1. GC-MS measurements

#### 4.2.4.1.1. Extraction

60 to 100mg plant tissue per sample was harvested in 2mL tubes and frozen immediately in liquid nitrogen. For metabolite extraction, the tissue was pulverized and mixed with 300µL cold methanol and 30µL Ribitol (0,3mg/mL in methanol). Samples were stored on ice during extraction followed by incubation at 70°C for 15min. 200µL chloroform was added, then mixed and incubated for 5min at 37°C. Finally 400µL HPLC water was added, mixed and centrifugation for 5min at 14.000rpm.

For each aliquot, 160µL of the upper polar phase was removed and transferred into 1.5mL glass flasks and vacuum dried in a speed vac for about 90min without heating. Samples could then be stored at -20°C until derivatisation.

#### 4.2.4.1.2. Derivatisation

Derivatisation took place in two steps: first, samples were suspended in 40µL Methoxyamine hydrochloride (20mg/mL in pyridine) and kept at 30°C for 90min, then 70µL MSTFA was added and samples were incubated at 37°C for 30min. Then, the derivatized extracts were transferred in glass flasks with micro inserts and analyzed.

#### 4.2.4.1.3. Measurement

Measurements were performed using a GS-MS system from Agilent Technologies. As carrier gas, He 5.0 was used. 2µL derivatized sample were injected split less onto the column. A run consisted of the following steps: first, the oven temperature was kept at 70°C for 5min, then the temperature was increased with a ramp of 5°C/min up to 280°C, kept there for 7min and finally, the oven was cooled to 70°C and kept there for 5min before injecting the next sample. During the whole runtime, the inlet was kept on 250°C, the mass spectrometer at 280°C.

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# 6. Appendix

## 6.1. Features of selected ssadh suppressor lines

### 6.1.1. Growth characteristics and metabolite contents

**Table 4: Analysis of M₃ plants**

Rosette diameter conditions and relative GHB- and GABA-contents in leaf extracts from M₃ plants grown under greenhouse from plants conditions

| Line | rosette diameter [cm] | GHB-content (relative to WT) | GABA-content (relative to WT) |
|---|---|---|---|
| *gaba-t* | 5.0 | 1.00 | 9.43 |
| *gaba-t/ssadh* | 4.9 | 14.20 | 10.08 |
| wild type | 5.1 | 1.00 | 1.00 |
| 1F | 2.76 | 113.20 | 4.64 |
| 3C | 4.7 | 95.02 | 4.47 |
| 3F | 2.4 | 86.88 | 6.46 |
| 3L | 4.42 | 132.12 | 4.85 |
| 4B | 2.88 | 62.64 | 2.84 |
| 4D | 2.44 | 323.71 | 19.32 |
| 5E | | 159.76 | 4.43 |
| 6B | 2.22 | 53.26 | 6.43 |
| 7D | 3.69 | 40.15 | 1.22 |
| 7F | 1.74 | 69.75 | 2.82 |
| 8G | 1.98 | 100.50 | 5.02 |
| 10F | 2.88 | 201.70 | 7.81 |
| 12B | | 60.13 | 2.10 |
| 12F | 3.36 | 125.50 | 4.99 |
| 13K | 3.09 | 107.22 | 2.65 |
| 13M | 2.19 | 170.62 | 2.75 |
| 13O | 3.3 | 150.23 | 7.08 |
| 15A | 4.41 | 82.09 | 2.94 |
| 17J | 4.32 | 82.25 | 5.07 |
| 18B | 5.43 | 1.94 | 0.82 |
| 18J | 1.77 | 44.33 | 2.56 |
| 18N | 1.59 | 61.78 | 4.10 |
| 19B | 7.24 | | 0.84 |
| 20H | 2.37 | 99.49 | 3.80 |
| 21H | 3.21 | 231.94 | 7.77 |
| 23F | 1.98 | 257.09 | 10.52 |
| 24E | 2.22 | | 0.00 |
| 24F | 3.36 | 196.44 | 11.11 |
| 24G | | 131.12 | 5.13 |
| 26F | 1.72 | 168.66 | 5.82 |
| 29B | 3.36 | 129.13 | 4.39 |
| 29C | 2.97 | 148.82 | 4.34 |

| | | | |
|---|---|---|---|
| 30L | 3.21 | 9.54 | 4.90 |
| 30M | 2.67 | 1.00 | 1.72 |
| 31A | 2.31 | 12.48 | 12.08 |
| 32F | 2.43 | | |
| 34D | | | |
| 34G | 2.1 | | |
| 35A | 2.61 | 99.06 | 2.50 |
| 35D | 5.07 | 20.11 | 8.55 |
| 35F | | | |
| 35J | 3.42 | | |
| 35K | 2.24 | 119.10 | 1.53 |
| 37H | 2.18 | 119.79 | 5.15 |
| 37J | | 136.03 | 4.88 |
| 38H | 3.1 | 213.68 | 2.73 |
| 39C | 2.31 | 70.58 | 2.90 |
| 39D | 2.49 | 139.50 | 2.40 |
| 39E | 4.35 | 160.19 | 1.18 |
| 39H | 2.1 | 129.06 | 2.18 |

**Table 5: M$_4$ plants grown under long day conditions**

Rosette diameter 36 and 47 days after germination, appearance of first flower buds and the number of leaves when flowering for M$_4$-plants grown under long day conditions.

| Line | rosette diameter [cm] 36 days | rosette diameter [cm] 47 days | flowering time [days] | number of leaves |
|---|---|---|---|---|
| 1F | 3.53 | 5.19 | 38 | 13 |
| 3C | 2.41 | 4.88 | 41 | 13 |
| 3F | 1.30 | 4.36 | 43 | 12 |
| 3L | 1.94 | 1.75 | 41 | 15 |
| 4B | | | | |
| 4D | 2.20 | 3.86 | 39 | 14 |
| 5E | 2.71 | 5.89 | 46 | 12 |
| 6B | 2.30 | 4.69 | 46 | 12 |
| 7D | 3.11 | 5.56 | 40 | 11 |
| 7F | 2.05 | 4.69 | 43 | 14 |
| 8G | 2.23 | 3.00 | 40 | 9 |
| 10F | 1.83 | 1.18 | 44 | 12 |
| 12B | | | | |
| 12F | 1.79 | 4.18 | 37 | 11 |
| 13K | | | | |
| 13M | 1.11 | 1.96 | 48 | 13 |
| 13O | 1.92 | 3.27 | 45 | 13 |
| 15A | 2.84 | 4.58 | 48 | 13 |
| 17J | 3.43 | 6.55 | 42 | 14 |
| 18B | 6.14 | 10.50 | 38 | 17 |

| | | | |
|---|---|---|---|
| 18J | 2.10 | 4.25 | 52 | 17 |
| 18N | 1.89 | 4.91 | 45 | 16 |
| 19B | 6.54 | 9.00 | 37 | 17 |
| 20H | 1.18 | 1.40 | 43 | 11 |
| 21H | 2.32 | 3.93 | 44 | 13 |
| 23F | 1.45 | 2.40 | 43 | 14 |
| 24E | 2.15 | 4.91 | 41 | 13 |
| 24F | 1.68 | 2.51 | 43 | 12 |
| 24G | 0.74 | 1.96 | | |
| 26F | 1.56 | 3.27 | 42 | 11 |
| 29B | 3.55 | 4.58 | 39 | 14 |
| 29C | 1.79 | 5.02 | 45 | 16 |
| 30L | 2.28 | 4.04 | 43 | 14 |
| 30M | 1.41 | 2.73 | 35 | 10 |
| 31A | 1.59 | 2.07 | 38 | 9 |
| 32F | 2.30 | 2.62 | 41 | 13 |
| 34D | 1.72 | 2.07 | 50 | 18 |
| 34G | 2.09 | 3.38 | 39 | 11 |
| 35A | 2.64 | 4.47 | 41 | 11 |
| 35D | 2.57 | 3.38 | 43 | 16 |
| 35F | 3.19 | 5.75 | 48 | 15 |
| 35J | 2.13 | 5.24 | 42 | 14 |
| 35K | 1.80 | 3.38 | 41 | 14 |
| 37H | 1.44 | 2.07 | | |
| 37J | 0.98 | 2.29 | | |
| 38H | 2.28 | 4.15 | 47 | 17 |
| 39C | 2.43 | 4.25 | 48 | 18 |
| 39D | 2.70 | 3.16 | 42 | 11 |
| 39E | 3.77 | 8.14 | 37 | 14 |
| 39H | 1.33 | 1.31 | 45 | 12 |

**Table 6: $M_4$ plants grown under short day conditions**

Rosette diameter 36 and 47 days after germination, appearance of first flower buds and the number of leaves when flowering for $M_4$-plants grown under short day conditions.

| Line | rosette diameter [cm] 36 days | rosette diameter [cm] 47 days | flowering time [days] | number of leaves |
|---|---|---|---|---|
| 1F | | | 46 | 14 |
| 3C | 2.70 | 1.88 | * | |
| 3F | | | * | |
| 3L | 1.41 | 3.66 | * | |
| 4B | | | | |
| 4D | | | * | |

| | | | | |
|------|------|------|-----|-----|
| 5E   | 0.55 | 1.02 | *   |     |
| 6B   |      |      | *   |     |
| 7D   | 0.42 | 0.90 | *   |     |
| 7F   | 2.74 | 3.07 | *   |     |
| 8G   |      |      | *   |     |
| 10F  | 2.08 | 0.54 | *   |     |
| 12B  |      |      | *   |     |
| 12F  |      | 3.44 | 14  | 47  |
| 13K  |      |      | *   |     |
| 13M  | 2.85 | 1.94 | *   |     |
| 13O  | 1.82 | 3.07 | *   |     |
| 15A  | 1.52 | 1.83 | *   |     |
| 17J  | 3.27 | 0.98 | *   |     |
| 18B  | 6.78 |      | *   |     |
| 18J  | 1.27 | 1.92 | *   |     |
| 18N  | 1.01 | 1.83 | *   |     |
| 19B  | 6.86 |      | 20  | 46  |
| 20H  | 3.43 | 1.71 | *   |     |
| 21H  | 1.92 | 1.41 | *   |     |
| 23F  | 2.36 | 1.64 | 8   | 33  |
| 24E  | 3.11 | 2.40 | *   |     |
| 24F  |      | 2.10 | *   |     |
| 24G  | 1.50 | 0.74 | *   |     |
| 26F  | 1.45 | 1.59 | *   |     |
| 29B  | 2.14 | 2.08 | *   |     |
| 29C  | 2.05 | 2.44 | *   |     |
| 30L  | 1.77 |      | *   |     |
| 30M  |      | 2.25 | 14  | 47  |
| 31A  | 2.04 | 1.60 | 14  | 50  |
| 32F  | 2.31 | 1.60 | *   |     |
| 34D  | 1.87 | 1.28 | *   |     |
| 34G  | 1.66 | 1.35 | *   |     |
| 35A  | 2.61 | 3.68 | *   |     |
| 35D  | 1.75 | 1.68 | *   |     |
| 35F  | 3.51 | 3.02 | *   |     |
| 35J  | 1.38 | 1.20 | *   |     |
| 35K  | 2.82 | 2.29 | 14  | 48  |
| 37H  | 0.97 | 1.05 | *   |     |
| 37J  |      |      | *   |     |
| 38H  | 1.33 |      | *   |     |
| 39C  | 2.74 | 2.29 | *   |     |
| 39D  | 1.82 | 1.55 | *   |     |
| 39E  | 4.79 |      | *   |     |
| 39H  | 2.29 | 2.95 | *   |     |

## 6.1.2. Cluster analysis

The cluster analysis was performed on growth characteristics and metabolite contents of *ssadh* suppressor lines using the MeV software, available at the TIGR homepage:
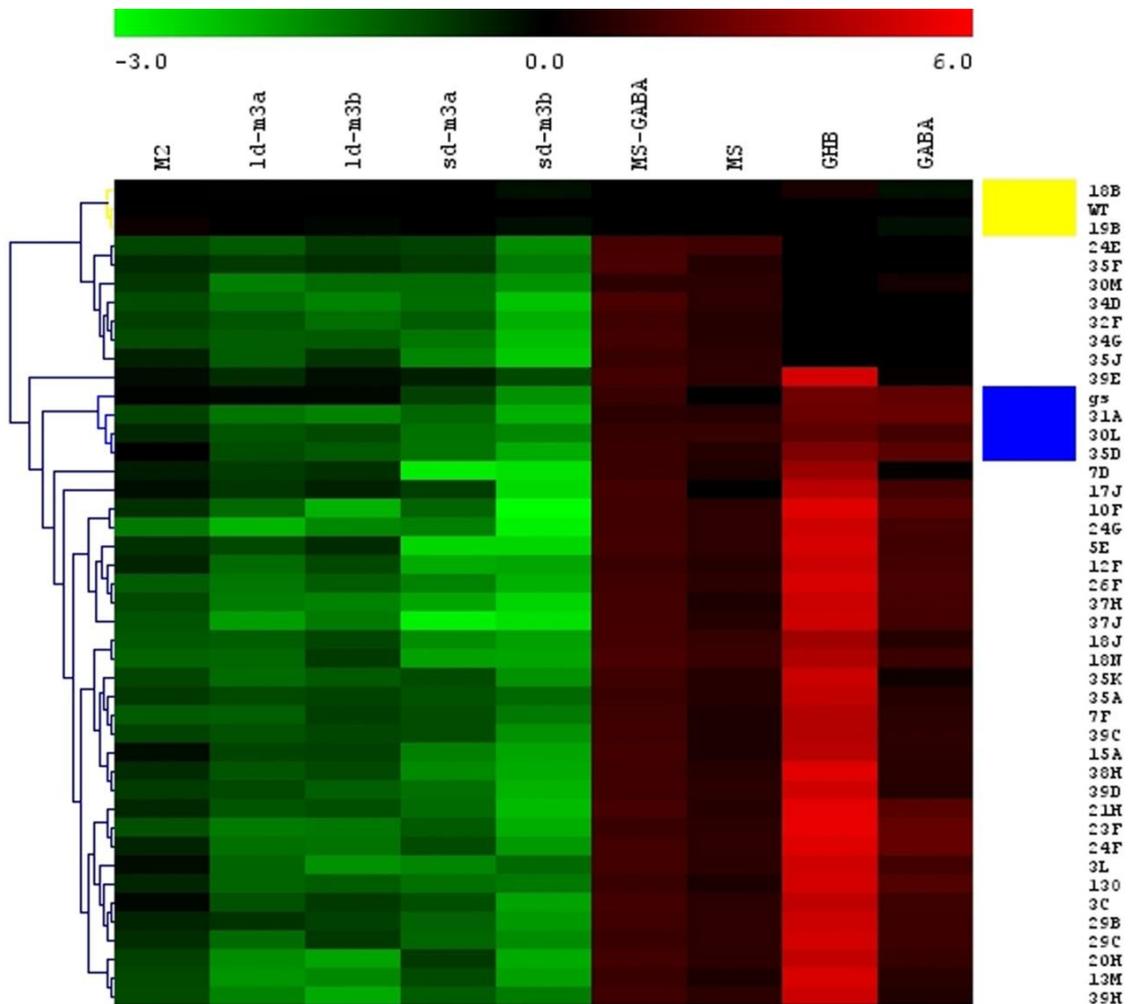


**Figure 43: Cluster Analysis of *ssadh* suppressor lines**

Plant lines were clustered based on growth characteristics and metabolite content. The values are relative to wild type and logarithmic. The yellow block indicated plants highly similar to wild types; the blue corresponds to *gaba-t/ssadh* comparable plants.

M2: rosette diameter of $M_2$-plants
ld-m3: rosette diameter of $M_3$-plants grown under long day conditions for 20 (a) or 39 (b) days
sd-m3: rosette diameter of $M_3$-plants grown under short day conditions for 20 (a) or 39 (b) days
MS-GABA: plant size of $M_3$-plants grown on ½ MS supplemented with GABA
MS: plant size of $M_3$-plants grown on ½ MS
GHB: GHB-content in leaf extracts of $M_3$-plants grown under greenhouse conditions
GABA: GABA-content in leaf extracts of $M_3$-plants grown under greenhouse conditions

## 6.1.3. Phenotypes

For all lines, the pictures provide details on plant growth in the following order:

First row: $M_2$ plant grown under greenhouse conditions for 44 days, a representative $M_3$ plant grown for 39 days under long day conditions, a characteristic $M_3$ plant grown under short day conditions for 39 days

Second row: $M_3$ plants grown for 16 days under long day conditions on the indicated media, ½ MS, ½ MS + 5mM GABA, and ½ MS + 5mM glutamate

1.A

D

E

3.C

3.L

6.B

7.D



7.F



8.G



10.F



12.F



.J
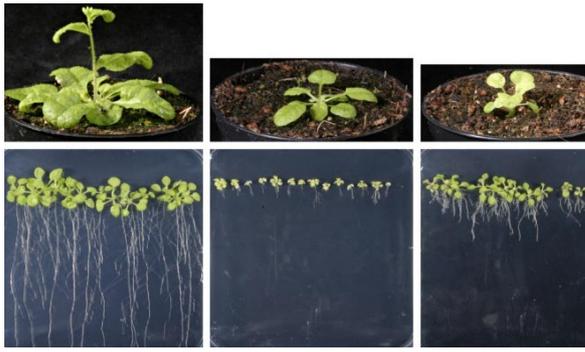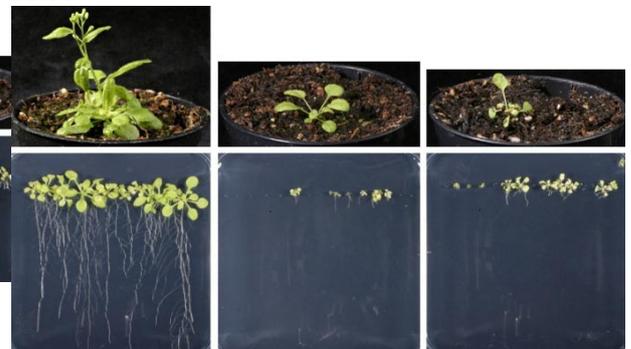


.M



J
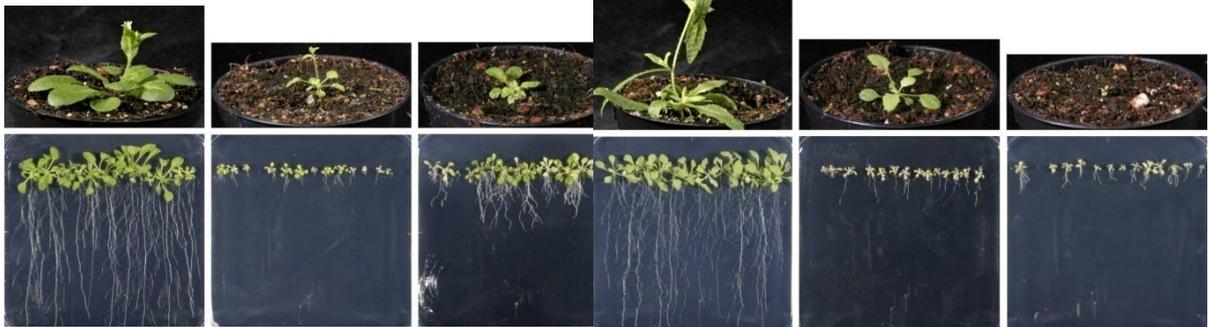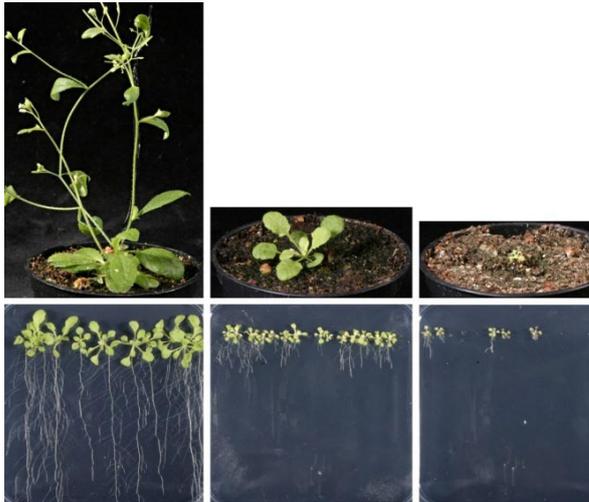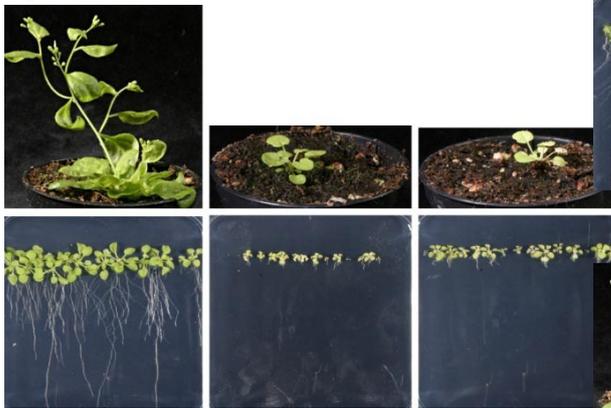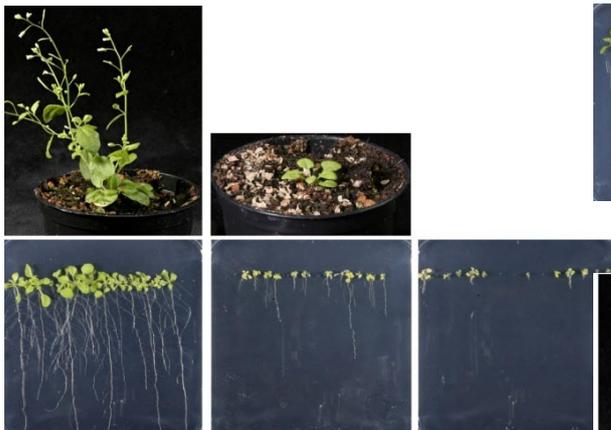
15.A



19.B



18.B



20.H



18.J



21.H



18.N



23.F

24.E



29.B



24.F



.C



24.G



.M



26.F
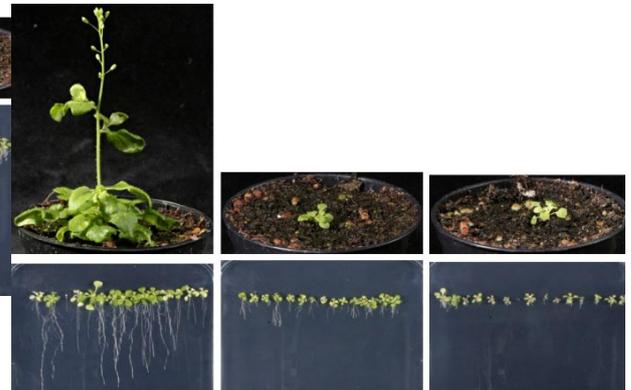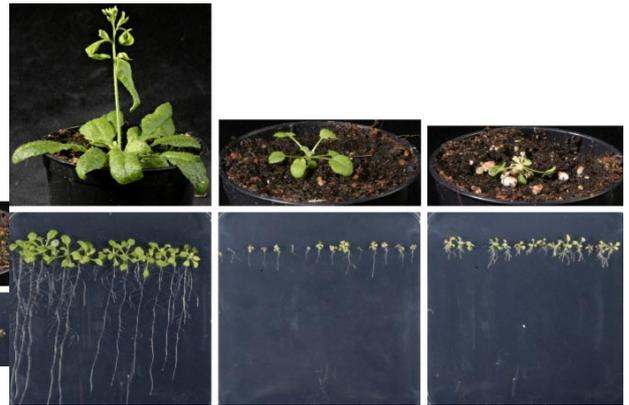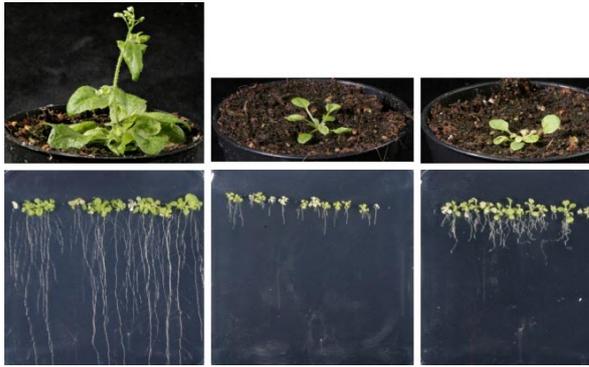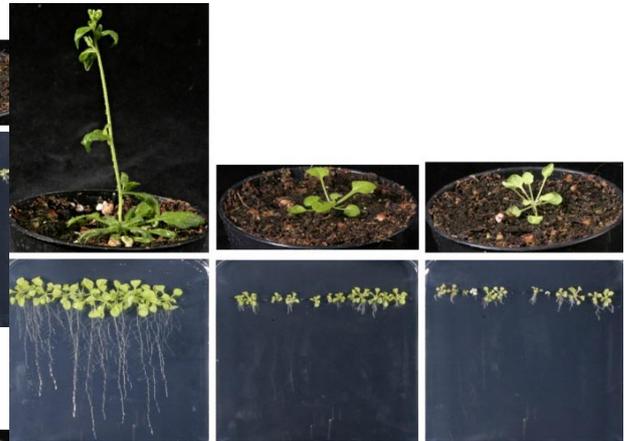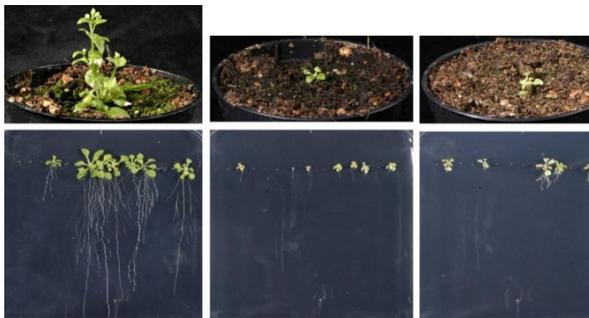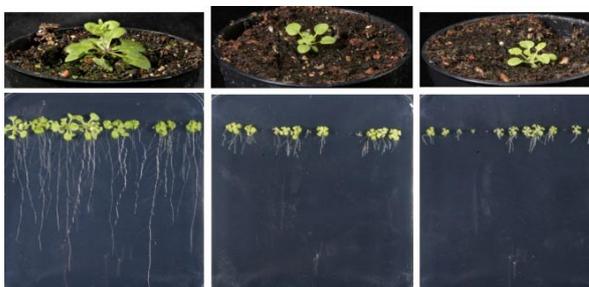


.F
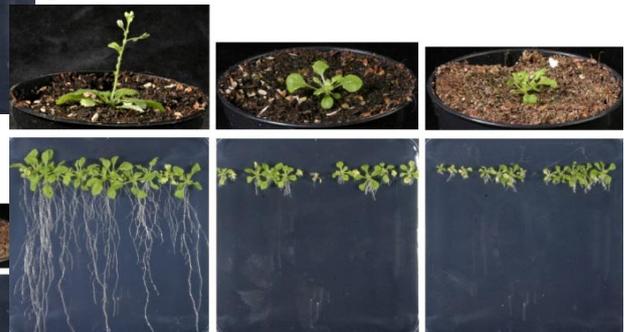


30.L

31.A
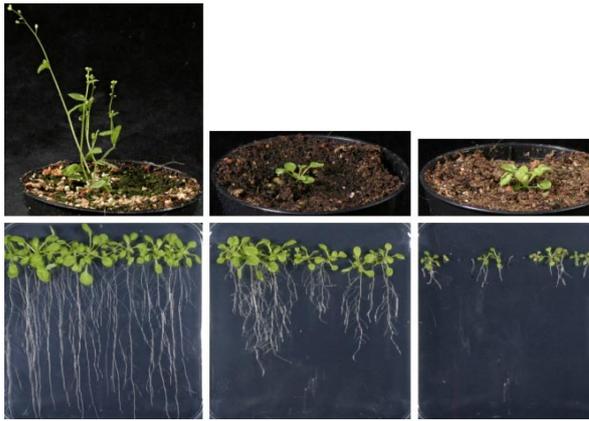


35.A
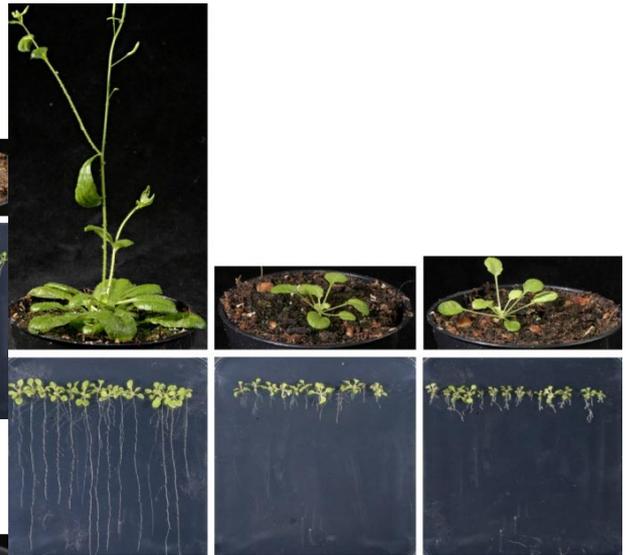


34.D
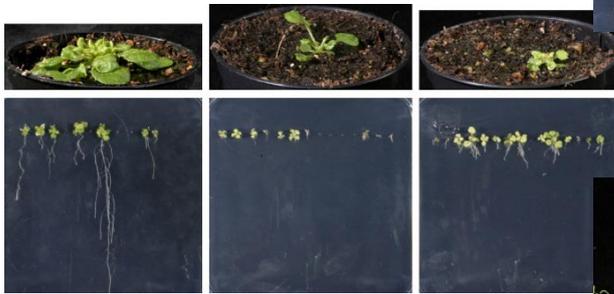


.D



34.G



.F



35.J

35.K



38.H



37.H



.D



39.E



37.J



39.C



39.H
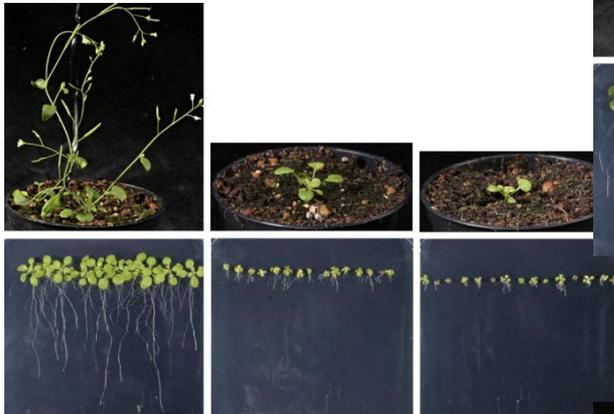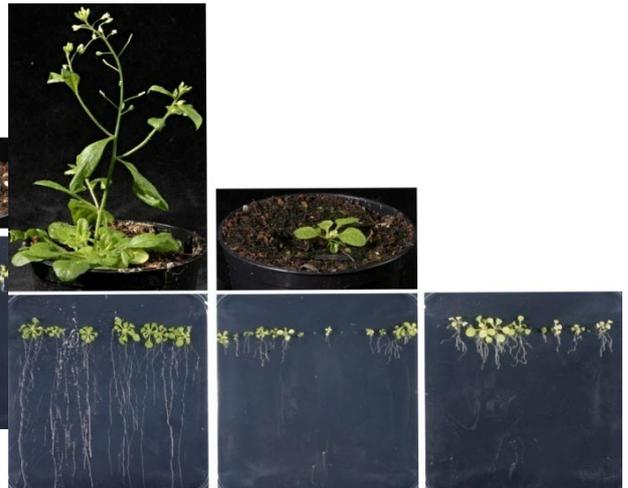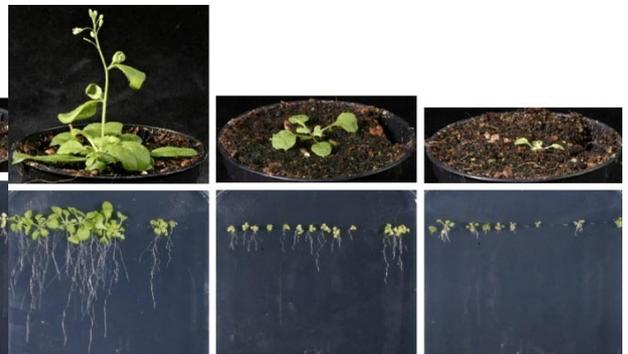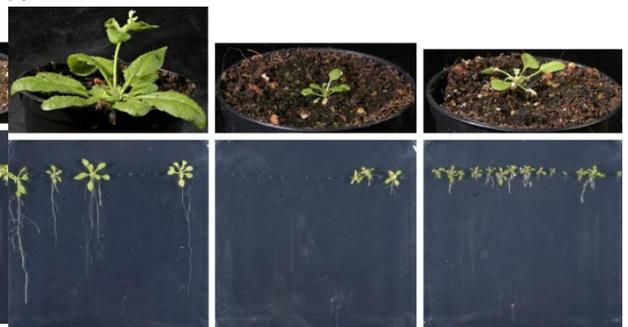
## 6.2. Reporter lines

| Clone | Abbreviation | Localization |
|-------|--------------|--------------|
| CD3-960 | ER-rb | ER-mCherry |
| CD3-968 | G-rb | Golgi-mCherry |
| CD3-976 | vac-rb | tonoplast-mCherry |
| CD3-984 | px-rb | peroxisome-mCherry |
| CD3-992 | mt-rb | mitochondria-mCherry |
| CD3-1000 | pt-rb | plastid-mCherry |
| CD3-1008 | pm-rb | plasma membrane-mCherry |

All constructs [123] are available via TAIR.

| Plant Line | | donated from |
|------------|---|--------------|
| AKT1prom-GUS | root hair specific promoter | Uta Anschütz, Würzburg |
| ARR7::GUS | Response Regulator 7 (At1g19050) | Rosalia Deeken, Würzburg |
| AtKC::GUS | root hair specific promoter | Uta Anschütz, Würzburg |
| aux1p::GUS (ll4) | Auxin Resistant 1 (At2g38120) | Rosalia Deeken, Würzburg |
| DR5::GUS | artificial auxin-responsive promoter | Klaus Palme, MPIZ |
| GSTF2::GUS | hypocotyl specific expression, ethylene response | Markus Geisler, Zürich |
| IAA2::GUS | auxin-inducible transcription factor, promoter-GUS-fusion | Klaus Palme, MPIZ |
| LAX1::GUS | Auxin Transporter-like 1 (At5g01240) | Rosalia Deeken, Würzburg |
| LAX2::GUS | Auxin Transporter-like 2 (At2g21050) | Rosalia Deeken/Würzburg |
| LAX3::GUS | Auxin Transporter-like 3 (At1g77690) | Rosalia Deeken, Würzburg |
| NIT1::GUS | Nitrilase, IAA sensitive | Klaus Palme, MPIZ |
| NIT2::GUS | Nitrilase, IAA sensitive | Klaus Palme, MPIZ |
| NIT3::GUS | Nitrilase, IAA sensitive | Klaus Palme, MPIZ |
| NIT4::GUS | Nitrilase | Klaus Palme, MPIZ |
| Pin1::GUS | auxin efflux carrier | Klaus Palme, MPIZ |
| Pin6::GUS | auxin efflux carrier | Klaus Palme, MPIZ |

## 6.3. Phytohormone mutants

| Plant Line | | donated from |
|---|---|---|
| aux1/lax3 | aux1/lax3-Doppelmutante, | Malcolm Bennett, Nottingham |
| aux1-100 | Auxin Resistant 1 (At2g38120) | Rosalia Deeken, Würzburg |
| aux1-21 | Auxin Resistant 1 (At2g38120) | Malcolm Bennett, Nottingham |
| aux1-7 (ll4) | Auxin Resistant 1 (At2g38120) | Rosalia Deeken, Würzburg |
| axr1-3 | Auxin Resistant 1 (At1g05180) | Rosalia Deeken, Würzburg |
| lax1 | Auxin Transporter-like 1 (At5g01240) | Rosalia Deeken, Würzburg |
| lax2 | Auxin Transporter-like 2 (At2g21050) | Rosalia Deeken, Würzburg |
| lax3 | Auxin Transporter-like 3 (At1g77690) | Rosalia Deeken, Würzburg |
| lax3 | Auxin Transporter-like 3 (At1g77690) | Malcolm Bennett, Nottingham |
| N24607 | At5g20730 (arf7), Auxin regulated TF | Eva Winkelbauer, Tübingen |
| N24617 | At1g19220 (arf19), Auxin response factor | Eva Winkelbauer, Tübingen |
| N24629 | arf7/arf19-double mutant | Eva Winkelbauer, Tübingen |
| nahG | Overexpression of a bacterial Salicylate Hydroxylase | Rosalia Deeken, Würzburg |
| nph1-5 | Non Phototropic Hypocotyl 1 (At3g45780) | Rosalia Deeken, Würzburg |
| nph3 | Non Phototropic Hypocotyl 3 (At5g64330) | Rosalia Deeken, Würzburg |
| nph4-1 (ARF7) | Non Phototropic Hypocotyl 3 (At5g20730) | Rosalia Deeken, Würzburg |
| pin1oid | Pin-formed 1 (At1g73590) | Rosalia Deeken, Würzburg |
| pin3 | Pin-formed 3 (At1g70940), Col-0 | Rosalia Deeken, Würzburg |
| shy2-2 | SHY2/IAA3 (At1g04240) | Rosalia Deeken, Würzburg |
| tir1-1 | Transport Inhibitor Response 1 (At3g62980) | Rosalia Deeken, Würzburg |

## 6.4. Primer Sequences

All primer sequences are given in 5'→ 3' orientation.

### 6.4.1. Screening

| Primer | Sequence |
|---|---|
| LB-F (SALK) | GTCCGCAATGTGTTATTAAGTTGTC |
| RB-F (SALK) | CAACGTTGCGGTTCTGTCAGTTCC |
| RB-RB (SALK) | GAGCAAGGTGAGATGACAGGAG |
| At2g01170scrFOR | GTTGCTGTGTTTTTCTGTGGC |
| At2g01170scrREV | GTGTTGATTATTATTCCATTCGC |
| At2g02000scr2f (GAD 3) | TCGGAGGCAGTGATGTTGGC |
| At2g02000scrFOR (GAD 3) | GGGCTGCCTTATGATAGACC |
| At2g02000scrREV (GAD 3) | GGCAATTATCCATCACGTTGCG |
| At2g02010scrFOR (GAD 4) | ATGGTTTTGTCTAAGACAGTTTCC |
| At2g02010scrREV (GAD 4) | CTCGAATCCAAGACGAATCAGC |
| At2g41190for | GAGGACAAGAACAATGATAAGG |
| At2g41190rev | GAGAGGTAGGAAATGATGCG |
| At3g17760scrFOR (GAD 5) | GCAAGGTACTTTGAGGTAGAGC |
| At3g17760scrREV (GAD 5) | CCAATACTTAGTGATATCCTCC |
| AtProT1 - for | GCCATTGCTGGTTTGATTTGTGCG |
| AtProT1 - rev | GCTTACAGGAGAAATAAACCTGCC |
| AtProT2 - for | TGAACAATTCGATCTCGAAGTCCC |
| AtProT2 - rev | GAGGACCCGTAAGCCAAATATCCG |
| AtProT3 - for | GATTTCTCACCATAGTCTGTCCCG |
| AtProT3 - rev | TGCCTGAATTTCTGGAAGCATCCC |
| GAD 1 F2 (At5g17330) | GGCAGAACAAGCGCAAAGCTG |
| GAD 1 R (At5g17330) | GGCCCAATCGGTAAGTTGG |
| GAD 1.b F2 (At5g17330) | ACAAGTGGCACTCCCTGCTCC |
| GAD 2 F (At1g65960) | CTTTGCAAATCGTGAATACCCG |
| GAD 2 R (At1g65960) | GTTCTGGTGTATCTTGCCGG |
| GAT 1 F (At1g08230) | CTATCTTCTTGCAACGCAGGC |
| GAT 1 hom F (At5g41800) | CCGGTTATTGTGATGTGGTGC |
| GAT 1 hom R (At5g41800) | TAAAAACTTTCCCTGAATCCG |
| GAT 1 R (At1g08230) | GCTACCATAGCAATCACCCC |
| ghbdh.2 for | TGCTGTGTATAGAGAGTTTCCTCC |
| ghbdh.2 rev | CATCACACCTAACAACCAAGCCCC |
| SSADH F | GCCCTGCTCTTGCTTCTGG |
| SSADH F (SALK) | GTCTCTGGTCACATCTAGATGGATTTCC |
| SSADH F .a | GGAGTTCCTCCGGTAATCTTTTAT |
| SSADH F .b | TGGAGTTCCTCCGGTAATCTT |
| SSADH R | CAGCTACATCCAGGTCTGC |
| SSADH R (SALK) | GTACCAGGGGGTATTCAATCTAGATTC |
| SSADH R .a | TCTTTTGGATAAAGAAATGCCTTC |
| SSADH R .b | GCACGAGAAACAAAAGCAGTC |

```
LB-F (Gabi-Kat)                  ATATTGACCATCATACTCATTGC
RB-F (Gabi-Kat)                  GTGGATTGATGTGATATCTCC
RB-RB (Gabi-Kat)                 CGCCAGGGTTTTCCCAGTCACGACG
GABA-T F (At3g22200)             CTTTCCCTTTTGGTGTCATTTTTA
GABA-T R (At3g22200)             GGCTAATCTGGTTGAGAACTCC
GHBDH for (At3g25530)            TCAGTATGTGAGAGTCCAGC
GHBDH rev (At3g25530)            AGAGTGTCAGAGCTAAGTCC
ghbdh.2 for (GK)                 CATCAATACTAATCAGCAAGC
ghbdh.2 rev (GK)                 GCGCATTGATAGCTCCCTGTG
SSADH F-2 (At1g79440)            TTGGAGCAGATTGACTGCTGG
SSADH R-2 (At1g79440)            CTTGGGAAGGTGTGGGAATGC

LB-F (WiscDSLox)                 AACGTCCGCAATGTGTTATTAAGTTGTC
450E1 (At2g19890) for            CTGCTCAAGAGTGGGCTGGGGC
450E1 (At2g19890) rev            GGACAGCATCATGAAGATGAGCG
453-456K22 (At2g02050) for       TCGTCTTAGCAATTTTGTTGCC
453-456K22 (At2g02050) rev       GTTCCAGAGAATCCCTGTCC

LBa1 (Syngenta)                  ATGGTTCACGTAGTGGGCCATC
ssadh-3 f1                       CTTTGTTCGATTGAAGTTTGGG
ssadh-3 r1                       CATGATGTCAACATAAAGCATTCC
```

## 6.4.2. Cloning

```
Primer                    Sequence
AtGHBDH.1-GFP for          CACCATGGAAGTAGGGTTTCTGGGTT
AtGHBDH.1-GFP rev          TTCGCGGGAGAATTTCACAGC
AtGHBDH.2-GFP for          CACCATGCCTTTGGTTTCATATATCTTTTGC
AtGHBDH.2-GFP rev          AGCTTCTCGGGATTTTGCAGCTT
```

## 6.4.3. RT-PCR

```
Primer                    Sequence
At2g01170 RT for.a         GAGTGTGGTGTACGGATGGTTC
At2g01170 RT rev.b         CCACAGAAAAACACAGCAACCGC
At2g01170FOR               GTTGCTGTGTTTTTCTGTGGC
At2g01170REV               GTGTTGATTATTATTCCATTCGC
At2g02000f (GAD 3)         TCGGAGGCAGTGATGTTGGC
At2g02000FOR (GAD 3)       GGGCTGCCTTATGATAGACC
At2g02000REV (GAD 3)       GGCAATTATCCATCACGTTGCG
At2g02010FOR (GAD 4)       ATGGTTTTGTCTAAGACAGTTTCC
```

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| | |
|---|---|
| At2g02010REV (GAD 4) | CTCGAATCCAAGACGAATCAGC |
| At2g41190for | GAGGACAAGAACAATGATAAGG |
| At2g41190rev | GAGAGGTAGGAAATGATGCG |
| At3g17760FOR (GAD 5) | GCAAGGTACTTTGAGGTAGAGC |
| At3g17760REV (GAD 5) | CCAATACTTAGTGATATCCTCC |
| GABA-T F | ATGGTCGTTATCAACAGTCTC |
| GABA-T R | CTTCTTGTGCTGAGCCTTGAG |
| GABA-T RT for.b | CATGGATTTACTTATTCTGGTC |
| GABA-T RT rev.a | AAATCAGCACTCTGCCATCCAGC |
| GAD 1pro F | CTTGAAAGATAGCAGCTGTC |
| GAD 1pro R | CTCGTCTTCTTCCTGTCGGCG |
| GAD 2pro F | TCTCAAGGACCATAGTTTCC |
| GAD 2pro R | TTCATCTTCTTCCTCTCCTTC |
| GHBDH-1 for (RT-PCR) | TAGGAAGTAGGGTTTCTGGG |
| GHBDH-1 rev (RT-PCR) | CTACGAACCGACCACCCTTCCCG |
| GHBDH1 RT for.a | CAGACCATCATCACTAAGCAG |
| GHBDH1 RT rev.b | CAAATACCGAAAGAGCAGCAC |
| GHBDH-2 for (RT-PCR) | CTGCAATCTACTACTCCCTCTACC |
| GHBDH-2 rev (RT-PCR) | CTCGTTCGCAGCGGCTGCAATCGG |
| SSADH for (RT-PCR) | ATGGACGCACAAAGCGTTTCTG |
| SSADH rev (RT-PCR) | GCACCAACAACACCGACAGG |
| SSADH RT for.a | ATGGTAATAGGAGCAGCAGCG |
| SSADH RT rev.a | CTCTCCAGCAGTCAATCTGCTCC |

## 6.4.4. Sequencing

```
gaba-t 1              TACTTTATTCTCTCTCTAGGG
gaba-t 1.2            AGGGGATATGTGTGTGAACG
gaba-t 2              AGTTTTTCCTACTATCCCTCC
gaba-t 3              AATAAAAACACCAACTAGGCC
gaba-t 4              CTACTGCACCAGTTACAACC
gaba-t 5              TTTATTTAATCTTTTCGAGTTCC
gaba-t 5.2            GATTGTTTGAGAGGGAGAGC
gaba-t 6              CCAAATTGACGTGGATCAGG
gaba-t 7              GGTTAGGATATAATGATCTCC
gaba-t 8              GTGACGTTTTTCTTACAGGG
gaba-t 9              AGAAGGAAGAATACCTAAGGC
gaba-t 10             ATCAGATGCCAACGATACCC
gaba-t 11             ATAAACTTTTTCTTCTCGGGC
gaba-t 12             AGGCGAAACGGAAGAGGAG
gaba-t 13             TGGTTCAGCTATAAAAGCACC
gaba-t 14             GCTCGGGACAATGTTTGGC
gaba-t 15             TGGCTCATAAGAATGGCTCC
gaba-t 16             ATGTCGCCAAAGTTGCCCC
gaba-t 17             TCAGTCCCAAGAATCAAACC
gaba-t 18             GAAAGCATTGAAGGCAACGG
gaba-t 19             CATCGTCAGATGTTGTCCC
gaba-t 20             GAAGGGATAAGCCATATCGC


ghbdh.1 1             GAATTGTAGGGAAGAACACTGC
ghbdh.1 2             GTTGATTCCAGCAGGCACTG
ghbdh.1 3             CCATCATCGCTGAAGAAAGGC
ghbdh.1 4             GTGGCGAAATCCTGTTCGTTC
ghbdh.1 5             TTACTGCTTAGTGATGATGGAC
ghbdh.1 6             ATCCTTGTGCTGCTCTTTCGG
ghbdh.1 7             TATCTGCTCCAAAACACGGCC
ghbdh.1 8             GGACAAGTTGGAAACGGAGC
ghbdh.1 9             TGTCAGCCAATACAAGCCCC
ghbdh.1 10            CTTCTCTGCTGTGATTGAAGC
ghbdh.1 11            ACATAGATTTGAGTATCTCAGCC
ghbdh.1 12            GTGAAGAGAAGCAAGAGGCGG


ghbdh.2 1             ATCTTCCTTTGACCGACTTCG
ghbdh.2 2             GAGCACAGAGTTAGACAATCC
ghbdh.2 3             TAATAAGCTTGAGGAGAGATGG
ghbdh.2 4             GAGTATCGTACATAGTGAGGC
ghbdh.2 5             AACTGACAGATTTTTACTGAGG
ghbdh.2 6             TTGGGGTAGTCTTATCTGAGG
ghbdh.2 7             GATTCCCATACCCAGAAACCC
```

```
ghbdh.2 8          GCTTGCTGATCCTGAAAGTGC
ghbdh.2 9          GGCTCCATTCTTTCCACATGC
ghbdh.2 10         TATCAGATTATTTTGTCTTCGC
ghbdh.2 11         TCACCTAAAATGTCAAGTGCC
ghbdh.2 12         ATCCAAATGTACTTGTCGAGG
ghbdh.2 13         ACACAAACCTTCTGCTGATGC
ghbdh.2 14         CGTTGACTACAATAAATACGGC
ghbdh.2 15         AAGTGGAAACGGCAACGAGC
ghbdh.2 16         GCACCACAAGAAACCTCCCC


At2g01170 1        TTCGTAAGTGGTGATGGTAGG
At2g01170 2        GCAGCCCTTGCTAGSCCSGCC
At2g01170 3        ATCTGAAGCCTTATGCAGAAGC
At2g01170 4        GTTTGTGTATCAAGGAGTAGGC
At2g01170 5        GGACTCTCAAAAATATTCATGTCC
At2g01170 6        ATCTCTCGTCTGTCTCCGCC
At2g01170 7        TAGACCAGAGTGACAGTGCCG
At2g01170 8        CTCTTCTCAACAGCCTCCCC
At2g01170 9        CAGAGACAAAAGAGAGATTGGG
At2g01170 10       GTGCTTAGGCGTTGTTGCGG
At2g01170 11       ATCAAGTCCTAAATGCAGACAG
At2g01170 12       GGCAGTTGCTGGTTTGGTGGC
At2g01170 13       CTTAAATGGGCTTCGTCGAGGC
At2g01170 14       AAGTTGAAATGTGGGTGTTATC
At2g01170 15       TTATTGTAAAGACTGTCTGTGTC
At2g01170 16       GAGAGAAGAAGATGGAGGGAGG


At2g41190 1        TCGTAATTTTCCTTCCACC
At2g41190 2        AAATGTGACACCTATGAG
At2g41190 3        ACCCATAGTTTATCCCCTG
At2g41190 4        ACCATTAAGGAGCAAATC
At2g41190 5        TTCTAAGAACAGCACTCG
At2g41190 6        TTATCAGCCATGGATTGGT
At2g41190 7        TTGATAGCAGTAAGCGTG
At2g41190 8        CATAACATTGCAGAAACC
At2g41190 9        TGATATTGGAGAAGCAGC
At2g41190 10       TCGTATAAGAATCAGTCG
At2g41190 11       TTCCTTGTAACCCATCTC
At2g41190 12       TGATTGCTCTTGTTCTCCG
At2g41190 13       ACTTGTCGTCGATCAAAAG
At2g41190 14       TTCTAAATCTAAGTGTCTCGC
At2g41190 15       TAACACGATTACATTTACACC
At2g41190 16       TTTTGGAGACCGTGACTTGTG
```

## 6.4.5. Mapping

### 6.4.5.1. SSLP-Marker

SSLPs (simple sequence length polymorphisms) are short stretches of simple sequences, that differ in the repeat number between *Arabidopsis* ecotypes and can therefore be used to distinguish between them [17].

| Marker | Size (Col-0) | Size (Ler) | Primer 1 | Primer 2 |
|---|---|---|---|---|
| NGA 63 | 111 | 89 | ACCCAAGTGATCGCCACC | AACCAAGGCACAGAAGCG |
| NGA 392 | 170 | 162 | GGTGTTAAATGCGGTGTTC | TTGAATAATTTGTAGCCATG |
| NGA 280 | 105 | 85 | GGCTCCATAAAAAGTGCACC | CTCCAGTTGGAAGCTAAAGGG |
| GENEA | 209 | 205 | ACATAACCACAAATAGGGGTGC | ACCATGCATAGCTTAAACTTCTTG |
| NGA 111 | 128 | 162 | TGTTTTTTAGGACAAATGGCG | CTCCAGTTGGAAGCTAAAGGG |
| NGA 1145 | 213 | 217 | GCACATACCCACAACCAGAA | CCTTCACATCCAAAACCCAC |
| NGA 1126 | 191 | 199 | GCACAGTCCAAGTCACAACC | CGCTACGCTTTTCGGTAAAG |
| CZSOD 2 | 183 | 187 | GAATCTCAATATGTGTCAAC | GCATTACTCCGGTGTCGTC |
| NGA361 | 114 | 120 | ACATATCAATATATTAAAGTAGC | AAAGAGATGAGAATTTGGAC |
| NGA 168 | 151/150 | 135/130 | GAGGACATGTATAGGAGCCTCG | TCGTCTACTGCACTGCCG |
| NGA 172 | 162 | 136 | CATCCGAATGCCATTGTTC | AGCTGCTTCCTTATAGCGTCC |
| NGA126 | 107/110 | 85/89 | CTCTGTCACTCTTTTCCTCTGG | CATGCAATTTGCATCTGAGG |
| GAPAB | 142 | 150 | TCCTGAGAATTCAGTGAAACCC | CACCATGGCTTCGGTTACTT |
| NGA 6 | 143 | 123/128 | ATGGAGAAGCTTACACTGATC | TGGATTTCTTCCTCTCTTCAC |
| NGA12 | 247 | 234 | TGATGCTCTCTGAAACAAGAGC | AATGTTGTCCTCCCCTCCTC |
| NGA8 | 154 | 198 | TGGCTTTCGTTTATAAACATCC | GAGGGCAAATCTTTATTTCGG |
| NGA1111 | 148 | 154 | AGTTCCAGATTGAGCTTTGAGC | GGGTTCGGTTACAATCGTGT |
| DET1.2 | 138 | 136 | GGTGAAAATGGAGGAGACGA | TTCAAACACCAATATCAGGCC |
| NGA1139 | 114 | 118 | TTTTTCCTTGTGTTGCATTCC | TAGCCGGATGAGTTGGTACC |
| NGA1107 | 150 | 140 | CGACGAATCGACAGAATTAGG | GCGAAAAAACAAAAAAATCCA |
| NGA 249 | 125 | 115 | GGATCCCTAACTGTAAAATCCC | TACCGTCAATTTCATCGCC |
| CA72 | 124 | 110 | CCCAGTCTAACCACGACCAC | AATCCCAGTAACCAAACACACA |
| NGA 151 | 150 | 120 | CAGTCTAAAAGCGAGAGTATGATG | GTTTTGGGAAGTTTTGCTGG |
| NGA106 | 157 | 123 | TGCCCCATTTTGTTCTTCTC | GTTATGGAGTTTCTAGGGCACG |
| CDPK9 | 106 | 104 | TCAATCATTGTCCAAAACTTGG | GAAACTGACTTGGAGAAGGCA |
| NGA 139 | 174 | 132 | GGTTTCGTTTCACTATCCAGG | AGAGCTACCAGATCCGATGG |
| SO262 | 220/231 | 300/>250 | ATCATCTTCCCATGGTTTTT | TTGCTTTTTGGTTATATTCGGA |
| NGA 76 | 145 | 159 | AGGCATGGGAGACATTTACG | GGAGAAAATGTCACTCTCCACC |
| NGA129 | 177 | 179 | CACACTGAAGATGGTCTTGAGG | TCAGGAGGAACTAAAGTGAGGG |
| CIW 10 | 140 | 130 | CCACATTTTCCTTCTTTCATA | CAACATTTAGCAAATCAACTT |

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## 6.4.5.2.    CAPS-Marker

CAPS (cleaved amplified polymorphic sites) are DNA fragments amplified by PCR and subsequently digested with specific restriction endonucleases. The number of recognitions sites varies for each marker between *Arabidopsis* ecotypes and the resulting fragment pattern can therefore be used to distinguish between them [99].

### 6.4.5.2.1.    Line 4.D

| Gene | Primer A | Primer B | Enzyme | Col | Ler |
|---|---|---|---|---|---|
| At4g00895 | GAAAGGCCTGCAATTGTTT | AATTCTTTGTGGAAGCTAAATCA | XbaI | 808 | 275 533 |
| At4g01750 | GACCCTTCACCAACAACGAC | GCCAATCTACTCATTGCGAGT | XbaI | 233 346 | 579 |
| At4g02000 | TCATACAAGAAAGGTCGGACA | GAGGTTTCAGAATCCCTCCA | HindIII | 409 591 | 1000 |
| At4g08390 | TCAGCAATCTTGGAGCAGAA | TGAATAATCACTTCCGCAATTT | XbaI | 590 | 198 392 |

### 6.4.5.2.2.    Line 17.J

| Gene | Primer A | Primer B | Enzyme | Col | Ler |
|---|---|---|---|---|---|
| At5g39600 | CCTGACAATTGGGATTCGAT | TGATGGAATCTTCTTATATCTTCACG | MseI | 59 332 | 391 |
| At5g44110 | AACCAGCAGTTTTACTCCATGA | TGCAGTTTTCTTACGATGTTCAA | SnaBI | 533 | 265 268 |
| At5g45280 | CAAAGTTCGGTATTGCGATG | TTCATGTAACCAAACATTTTAAATTCC | DraI | 21 361 | 21 119 242 |
| At5g47050 | TCCATCATCATCATCATGTCC | AACTGAGATTTATGCGGCTGA | HindIII | 92 127 | 219 |

### 6.4.5.2.3.    Line 21.H

| Gene | Primer A | Primer B | Enzyme | Col | Ler |
|---|---|---|---|---|---|
| At2g28970 | GGCACTAAAAATTTGTTATGTAGGTTT | GGGATCACCTCGGTGTCAAT | NcoI | 708 | 40 668 |
| At2g39725 | TCTCGTTGAATCAGATACTGTCA | GAGTTGTGGCGGAACTCTGT | XhoI | 535 | 47 488 |
| At2g47485 | CGCATAGTGAGCAGAGATCG | ACTCCTTCGACGCCAAAC | HindIII | 127 202 | 329 |
| At2g41910 | GGTTCATACGGTTCGGTGAG | TTCTCGAGAGACTCAGCGTTT | SacII | 45 61 | 106 |

## 6.5. Vector Maps

The fragments were first cloned into pDONR207, and then the inserts were recombined using LR-Clonase into the binary vector pGWB5.
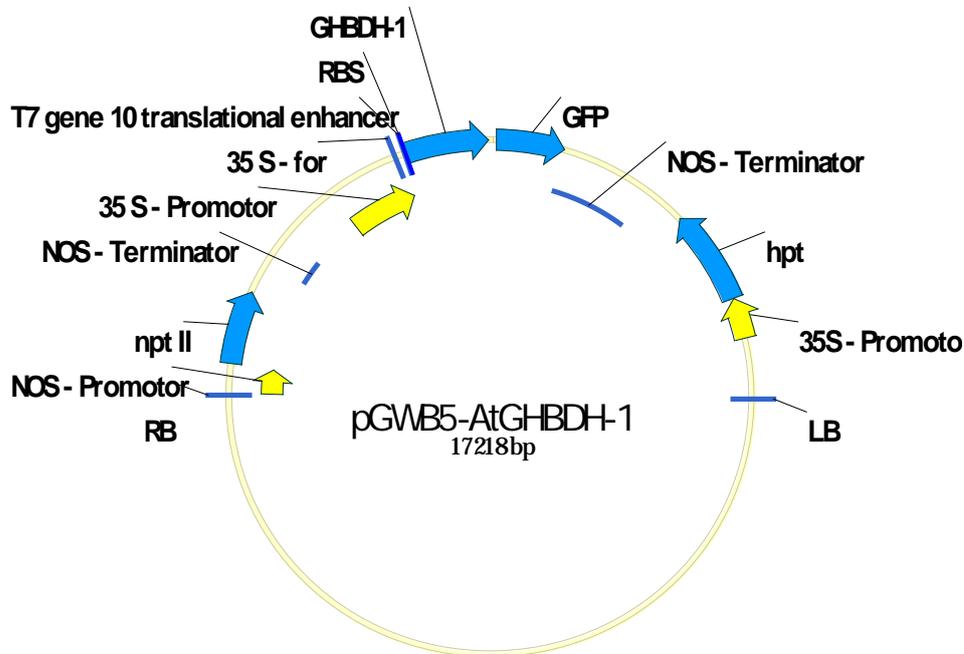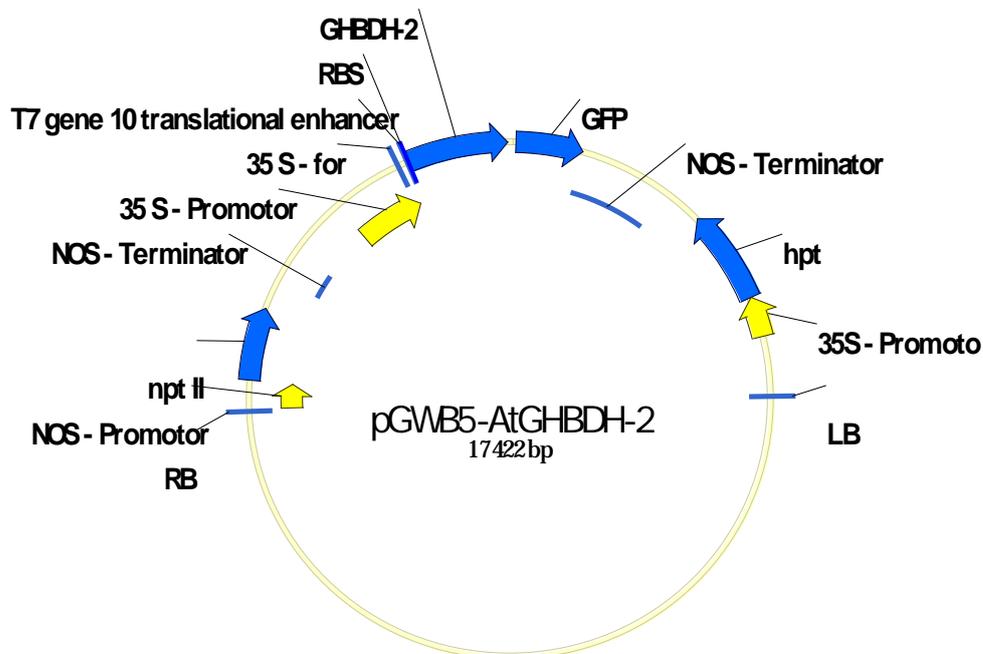


**Figure 44: Map of the AtGHBDH-1-GFP fusion construct**



**Figure 45: Map of the AtGHBDH-2-GFP fusion construct**

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# Abbreviations

| | |
|---|---|
| α-C-Atom | first carbon atom following the highest functional group |
| % | Percent |
| °C | Temperature (in Grad Celsius) |
| μ | micro- |
| ½ MS | half strength MS medium |
| A | Absorption |
| AHL | N-acyl-homoserine lactone |
| ATP | Adenosintriphosphate |
| bd· | distilled twice |
| bp | base pairs |
| BSA | bovine serum albumin |
| C | carbon |
| c | Concentration |
| ca. | circa |
| $Ca^{2+}$ | Calcium |
| CaM | Calmodulin |
| CBL | Calcineurin B-like Proteins |
| cDNA | copy DNA |
| CDPK | $Ca^{2+}$-dependent protein kinase |
| cGMP | cyclic Guanidine monophosphate |
| dATP | 2-Desoxyadenosintriphosphate |
| DEPC | Diethylpyrocarbonat |
| DMSO | Dimethylsulfoxyd |
| DNA | Desoxyribonucleic acid |
| dNTP | 2-Desoxynukleosidtriphosphat |
| DTT | 1,4-Dithiothreitol |
| EDTA | Ethylendiamine tetraacetate |
| $F_2$ | second generation from crossings |
| FW | Fresh weight |
| g | Gramm |
| GABA | γ-amino butyric acid |
| GABA-T | GABA transaminase |
| GAD | Glutamatate decarboxylase |
| GDH | Glutamate dehydrogenase |
| GHB | γ-Hydroxybutyric acid |
| GHBDH | γ- Hydroxybutyric acid dehydrogenase |
| HPLC-$H_2O$ | pure water |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| conc. | concentrated |
| L | Liter |
| LB | T-DNA Left border |
| LB | Luria/Bertani (Medium für Bakterienkultur) |
| M | Molar (mol/l) |
| m | milli- |

| | |
|---|---|
| mA | Milliampere |
| MES | 4-Morpholinoethan-sulfonic acid |
| min | Minutes |
| MOPS | 4-morpholinopropan-sulfonic acid |
| mRNA | messenger RNA |
| MS-Medium | Murashige & Skoog-Medium |
| n | nano |
| N | nitrogen |
| NAD(H) | Nicotine amide adenine dinucleotide (reduced) |
| NADP(H) | Nicotine amide adenine dinucleotidephosphate (reduced) |
| nm | Nanometer |
| OD | Optical density |
| p | Pico |
| PCR | polymerase-chain reaction |
| pI | Isoelectric point |
| QS | Quorum sensing |
| RB | T-DNA right border |
| RNA | Ribonucleic acid |
| rpm | rounds per minute |
| rRNA | ribosomal RNA |
| RT | Reverse Transcription |
| SAP | Shrimps Alkaline Phosphatase |
| SDS | sodium dodecylsulfate |
| SSA | Succinic semialdehyde |
| SSADH | Succinic semialdehyde dehydrogenase |
| Taq | DNA polymerase (from *Thermophilus aquaticus*) |
| TCA | Citric acid cycle |
| T-DNA | Transfer-DNA |
| Tris | Tris-(hydroxymethyl)-aminomethane |
| UV | ultraviolet |
| w/v | Weight per volume |
| WT | Wild type |

# Publications

Ludewig, F., Hüser, A., Fromm, H., Beauclair, L., and Bouche, N.
*Mutants of GABA transaminase (POP2) suppress the severe phenotype of succinic semialdehyde dehydrogenase (ssadh) mutants in arabidopsis.*
PLoS ONE, 2008. **3**:e3383.

# Conference Abstracts

**Analysis of GABA-shunt metabolites in *Arabidopsis thaliana***
19th International Conference on Arabidopsis Research,
July 23 – 27, 2008 – Montréal, Canada
Poster presentation, ICAR 1008

**Do GABA-shunt intermediates act as signaling molecules in *Arabidopsis thaliana?***
Botanikertagung
September 3-7, 2007 - Hamburg, Germany
Poster presentation

**Analysis of metabolite contents in Arabidopsis GABA shunt mutants**
2nd International Symposium on Plant Neurobiology
May 22-27, 2006 - Beijing, China
Poster presentation
Invited talk at the Normal University of He'nan, Xinxiang, China

**Gamma Aminobutyric Acid (GABA) Metabolism in Plants: Analysis of knock-out Mutants**
2nd International Symposium on Plant Neurobiology
May 22-27, 2006 - Beijing, China

**Arabidopsis knock-out mutants of GABA metabolism and their response to different growth conditions**
1st International Symposium on Plant Neurobiology
May 17-20, 2005 - Florence, Italy

**γ -aminobutyric acid (GABA) metabolism in plants**
Botanikertagung
September , 2004 - Braunschweig, Germany
Poster presentation

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# Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit − einschließlich Tabellen, Karten und Abbildungen −, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie − abgesehen von unten angegebenen Teilpublikationen − noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. U.-I. Flügge betreut worden.

Köln, den 28.02.2009