



CASS Project

Improving cassava yield to support smallholder farmers in Sub-Saharan Africa
www.cass-research.org

Metabolic engineering of carbon and nitrogen pathways to enhance cassava yield

AAP1 summary

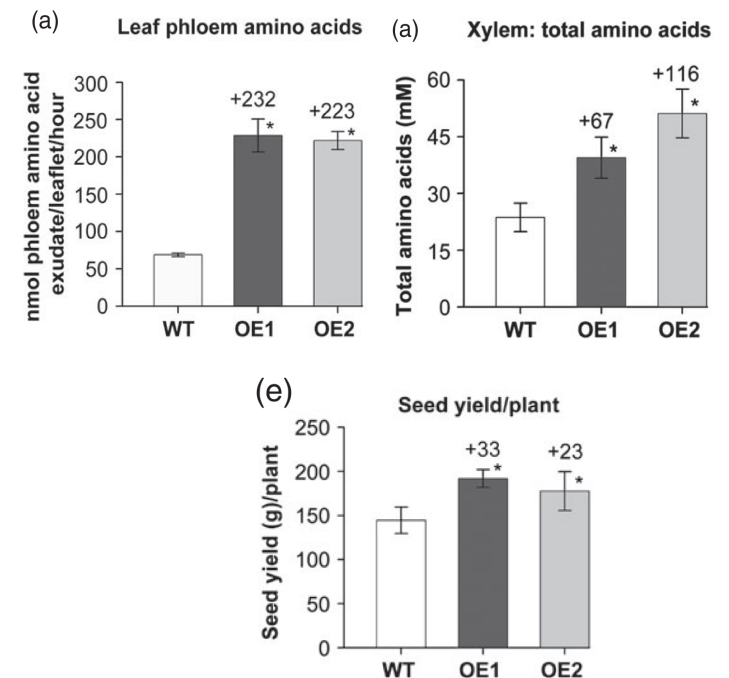
Laise Rosado-Souza
AG Fernie

Project Meeting
24.09.2025

Amino Acid Permease (AAP) from pea

(expression in phloem tissues, companion cells of leaves, stems and roots)

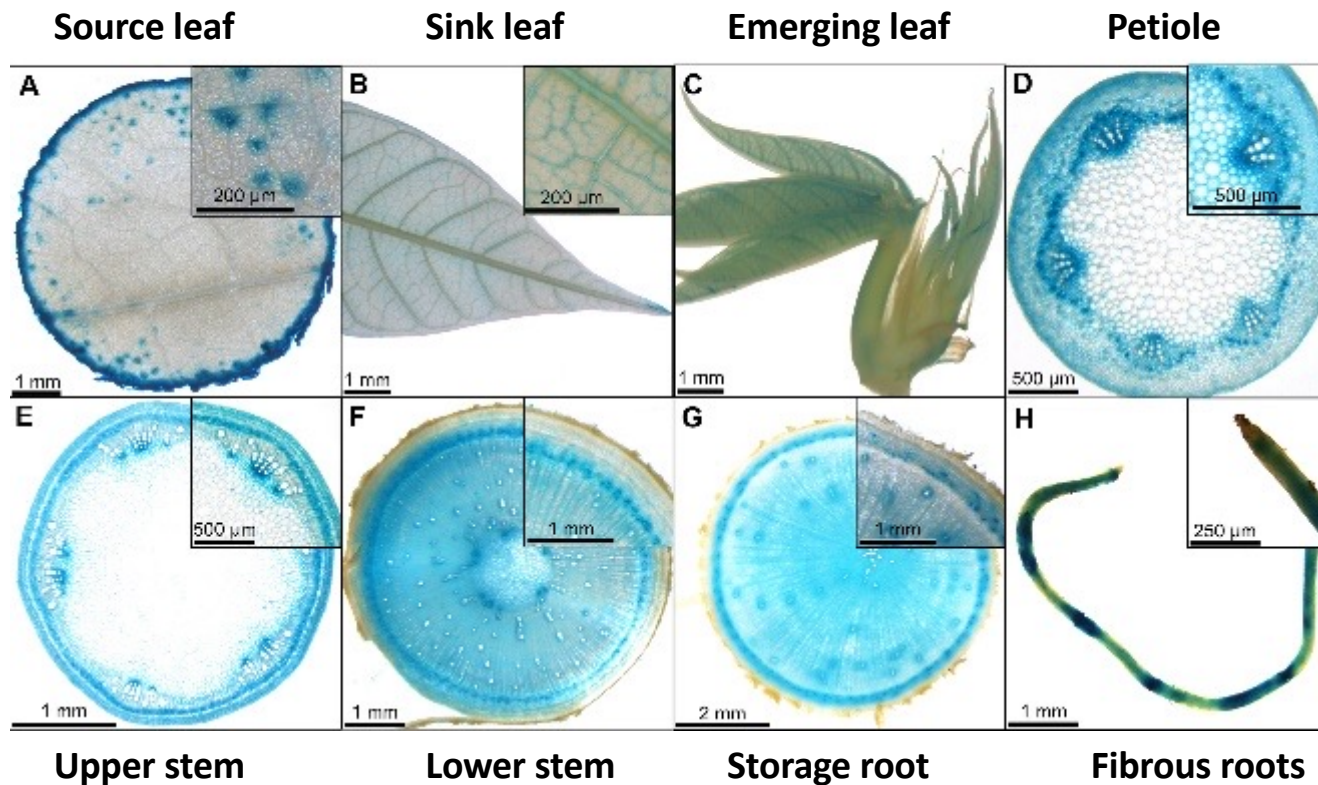
- Increase in biomass
- Increase in total free amino acid levels in the phloem exudate and in the xylem sap
- Increased yield per plant
- Under different N treatments



- Functional cassettes**

Promoter – *COMMELINA YELLOW MOTTLE VIRUS (CoYMV)*

5'-UTR, CDS, 3'-UTR-terminator – *Pisum Sativum AMINO ACID PERMEASE 1 (PsAAP1)*



✓ Strong promoter in vascular and reproductive tissues

✓ Active in the companion cells of leaves, stems and roots

Genotypes:

- WT 60444
- Vector control (6 lines)
- **AAP1 (6 lines)**
- mitoGS (6 lines)
- mitoGS+AAP1 (6 lines)

Full nutrition substrate

~3 months old plants

10L pot





AAP1_4349



AAP1_4353



AAP1_4354



AAP1_4355



AAP1_4358



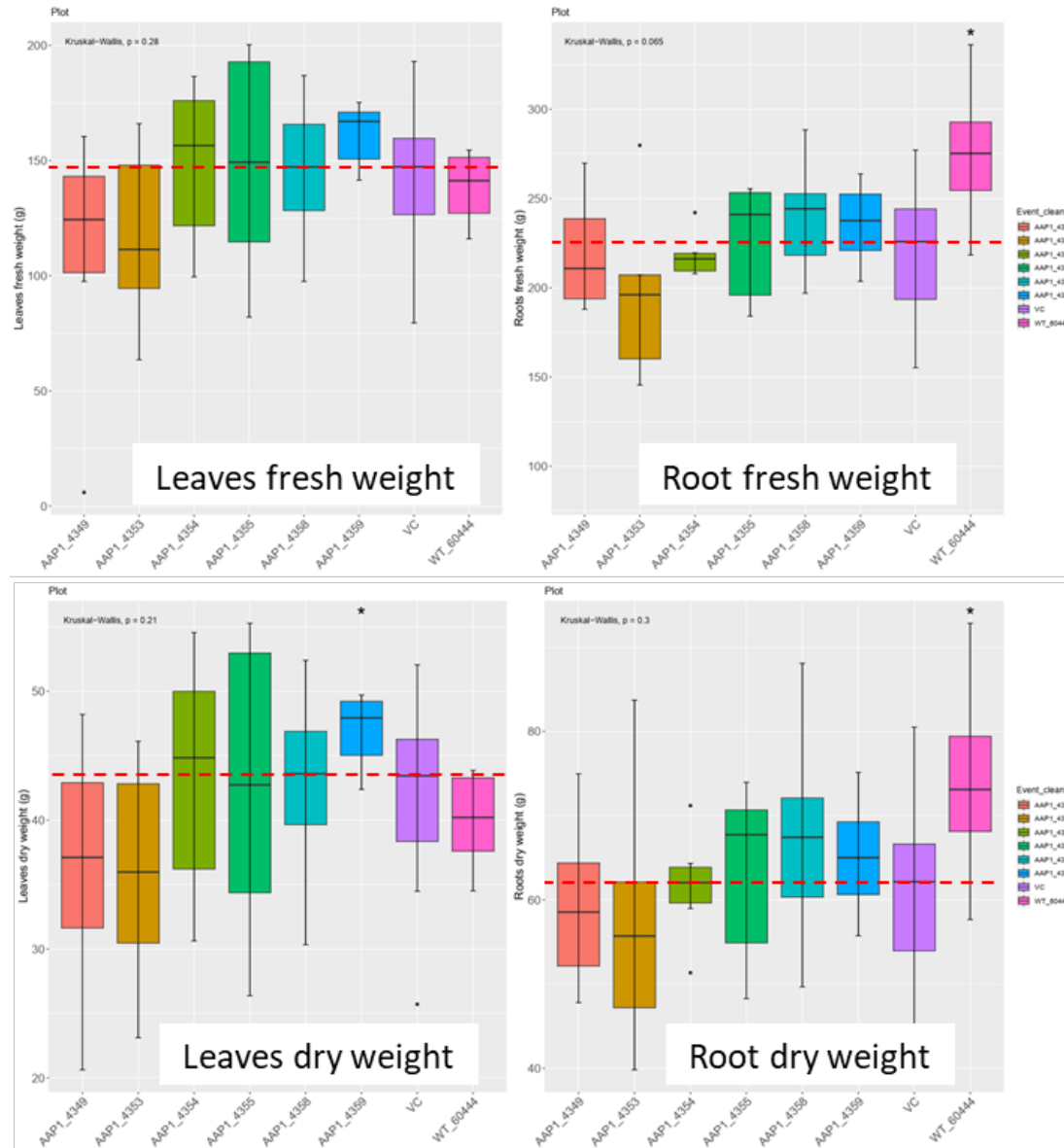
AAP1_4359



VC= vector control
or
EV= empty vector

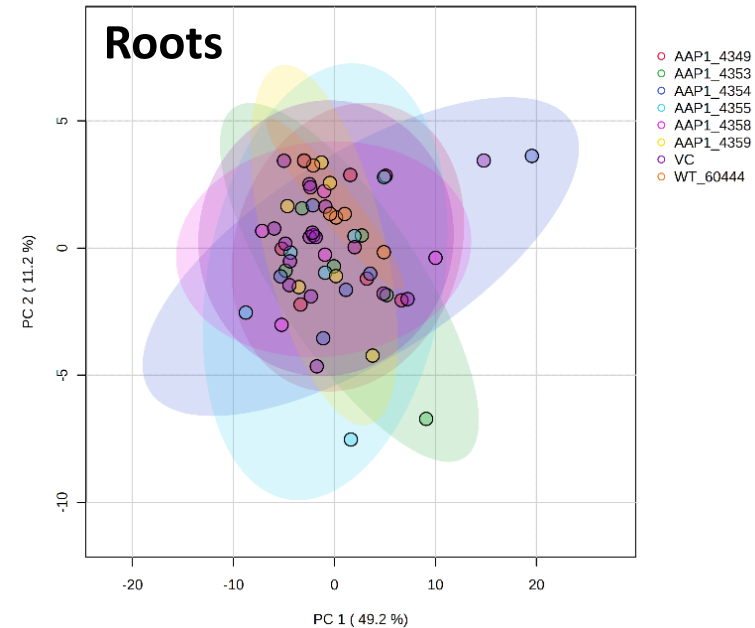
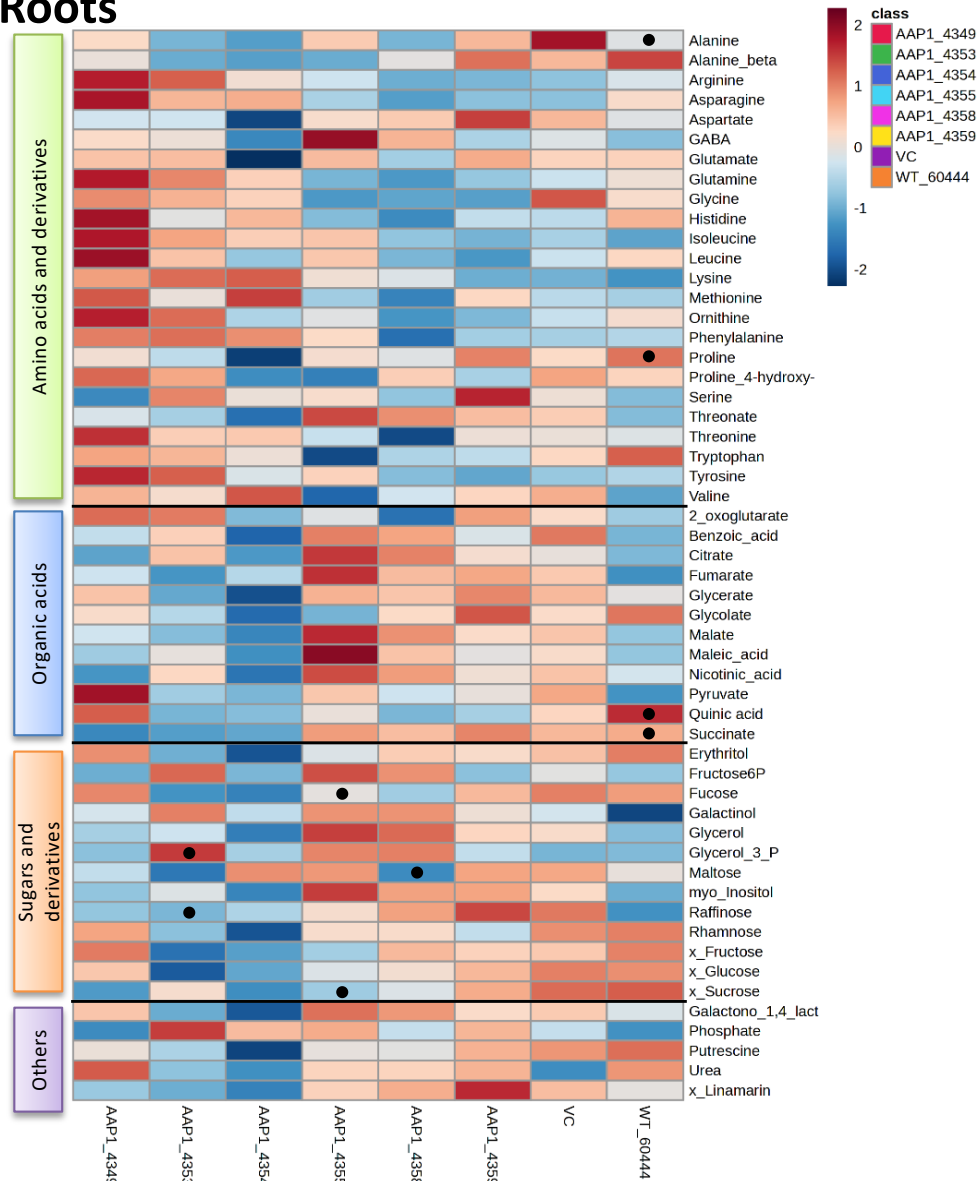


WT
Wild type



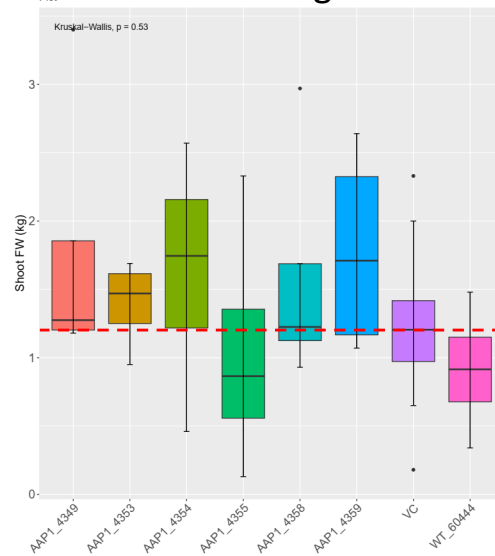
- No difference between VC and AAP1 lines
- Penalty in root (fresh and dry) weight and harvest index (HI) compared to WT
- AAP1_4359 higher leave dry weight compared to VC

Roots

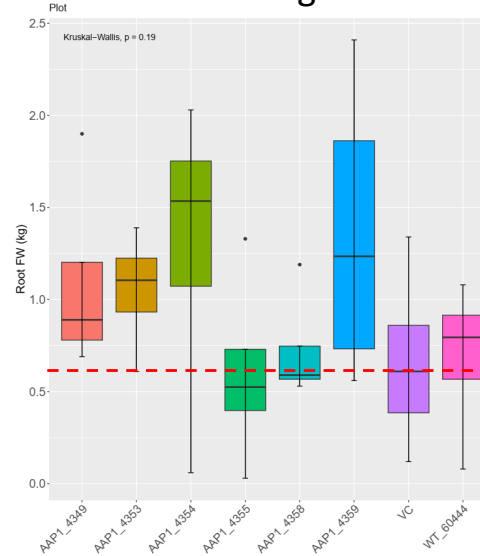


- Metabolically, AAP1 is very similar to VC for leaves and root tissues

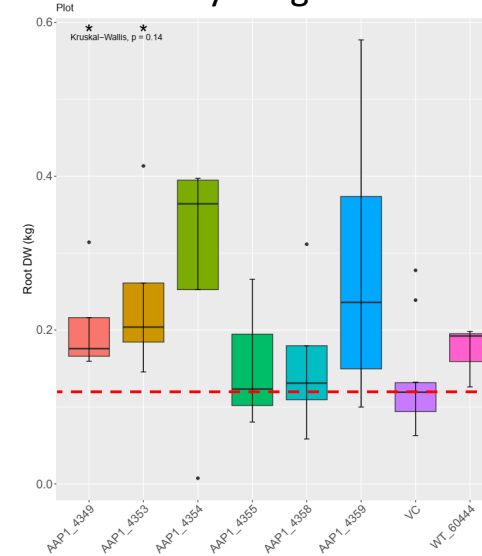
Shoot fresh weight



Root fresh weight

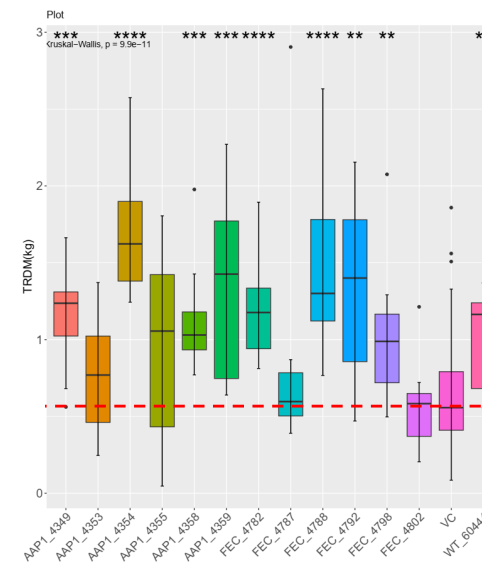
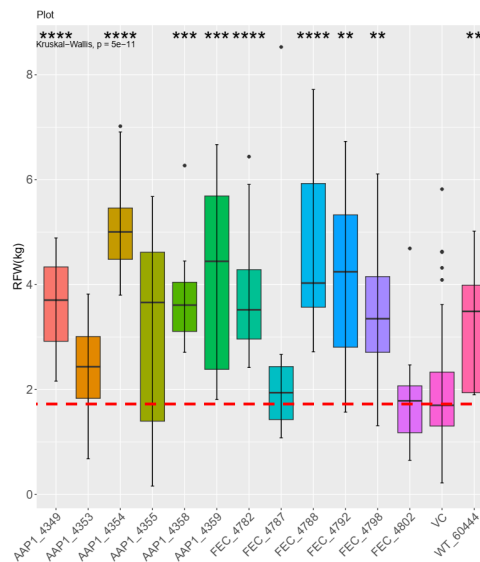
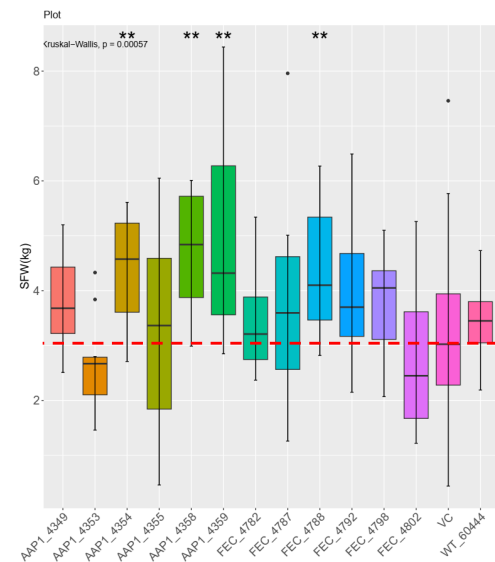


Root dry weight



Intermediate Harvest (3 months)

- AAP1_4349
- AAP1_4353



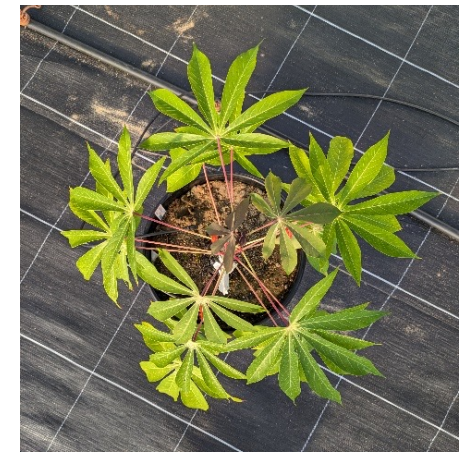
Final Harvest (9 months)

- AAP1_4349
- AAP1_4354
- AAP1_4358
- AAP1_4359

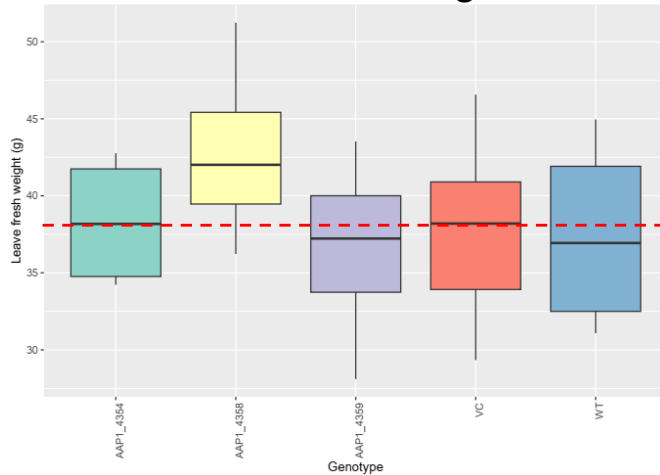
Genotypes:

- WT 60444
- Vector control (3 lines)
- AAP1 (3 lines)
 - AAP1_4354
 - AAP1_4358
 - AAP1_4359

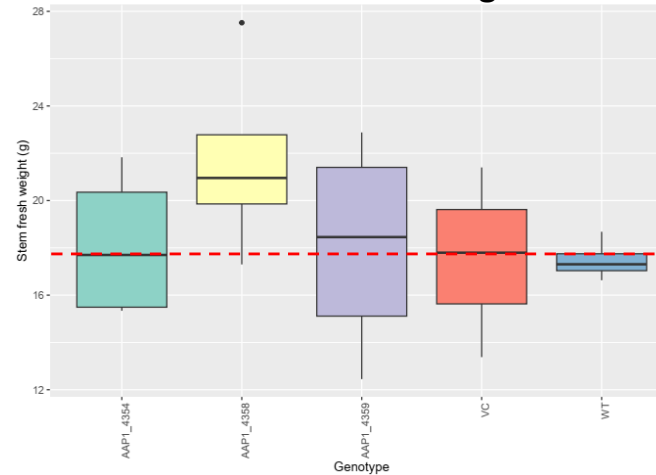
Low nutrition soil
(local sandy soil from A horizon)
~3 months old plants
10L pot



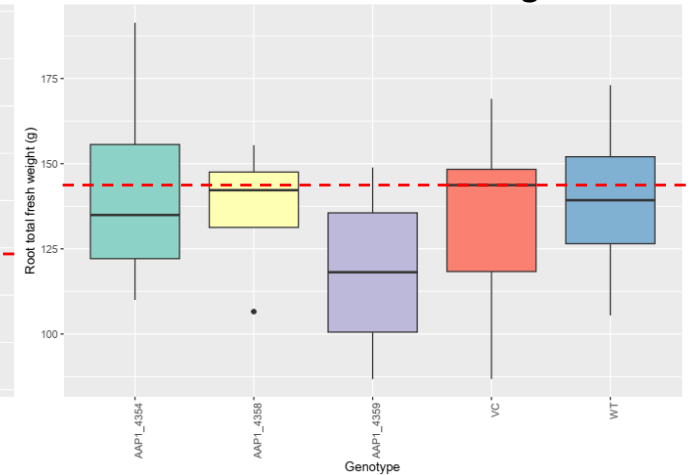
Leaves fresh weight



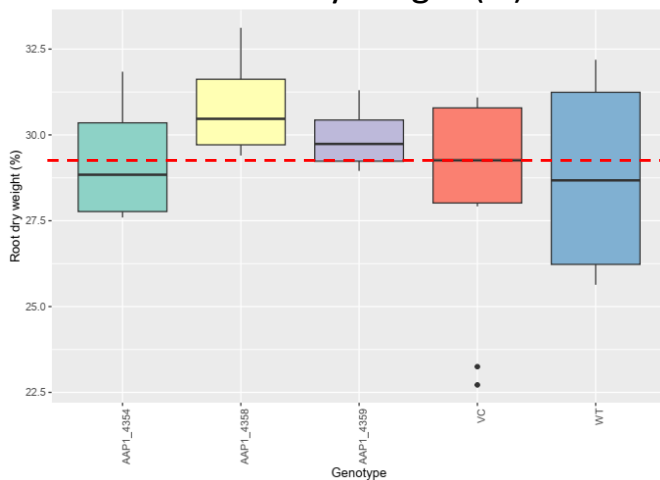
Root fresh weight



Root fresh weight

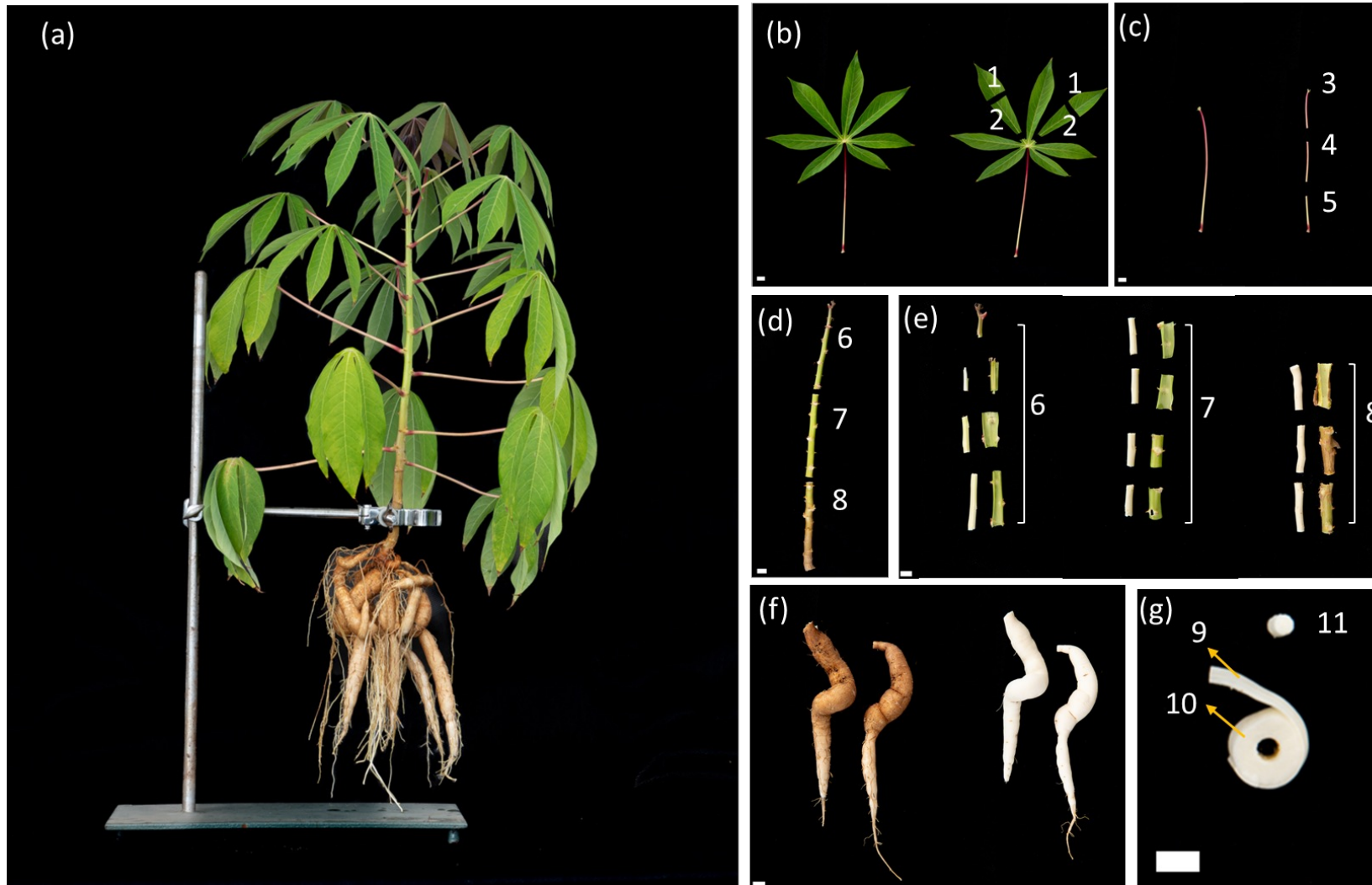


Root dry weight (%)



No significant difference:

- Height
 - Stem diameter
 - Leaves, stem root fresh weight
 - Root dry weight
- AAP1 lines and VC similar to WT under nutrient-limited conditions



Samples processed, extracted and selected samples currently running

Phloem exudate collection

Genotypes:

- WT 60444
- Vector control (VC_4220)
- AAP1 (3 lines)
 - AAP1_4354
 - AAP1_4358
 - AAP1_4359
- Optimal conditions
- 6 biological replicates



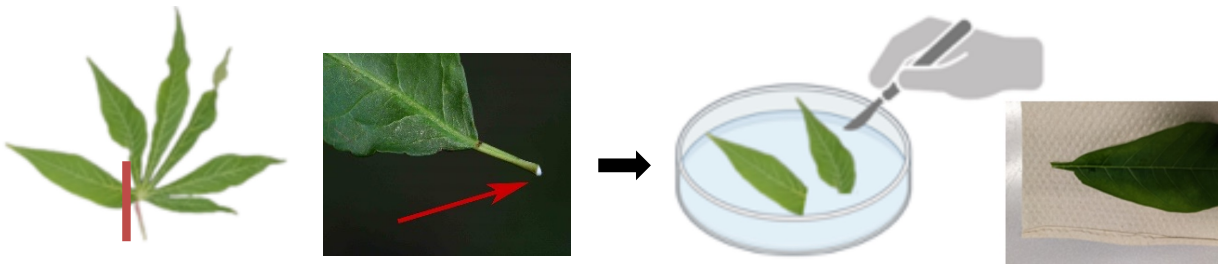
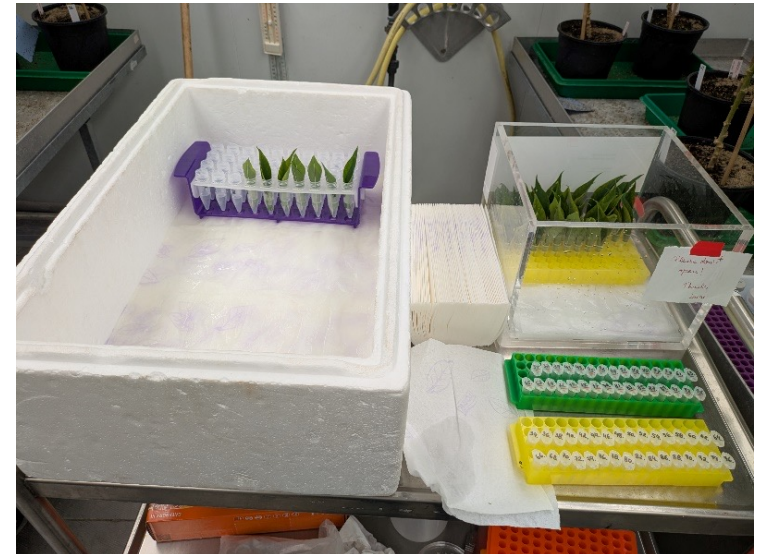
Positive samples: 10 mM (K)EDTA in 10 mM HEPES

Negative samples: 10 mM HEPES (without EDTA)

Phloem exudate collection

Genotypes:

- WT 60444
- Vector control (VC_4220)
- AAP1 (3 lines)
 - AAP1_4354
 - AAP1_4358
 - AAP1_4359
- Optimal conditions
- 6 biological replicates



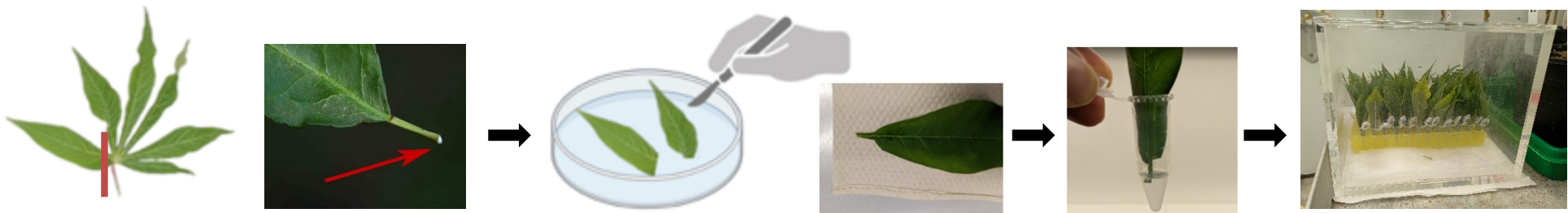
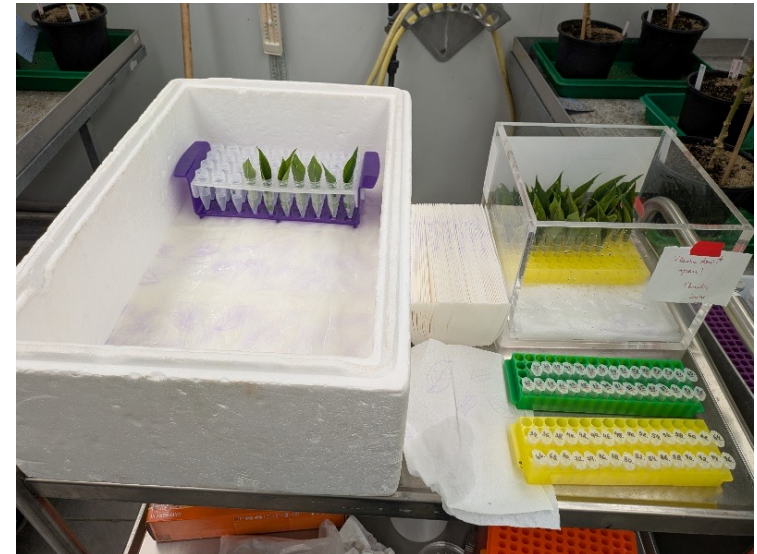
Positive samples: 10 mM (K)EDTA in 10 mM HEPES

Negative samples: 10 mM HEPES (without EDTA)

Phloem exudate collection

Genotypes:

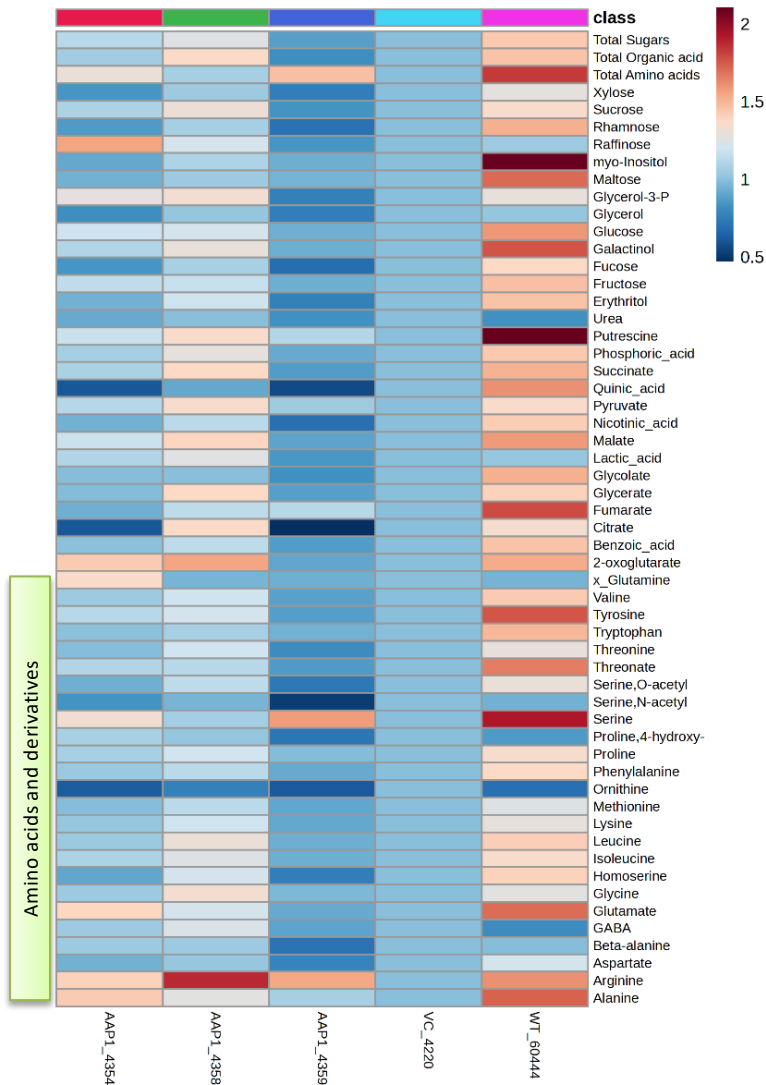
- WT 60444
- Vector control (VC_4220)
- AAP1 (3 lines)
 - AAP1_4354
 - AAP1_4358
 - AAP1_4359
- Optimal conditions
- 6 biological replicates



6-8h incubation

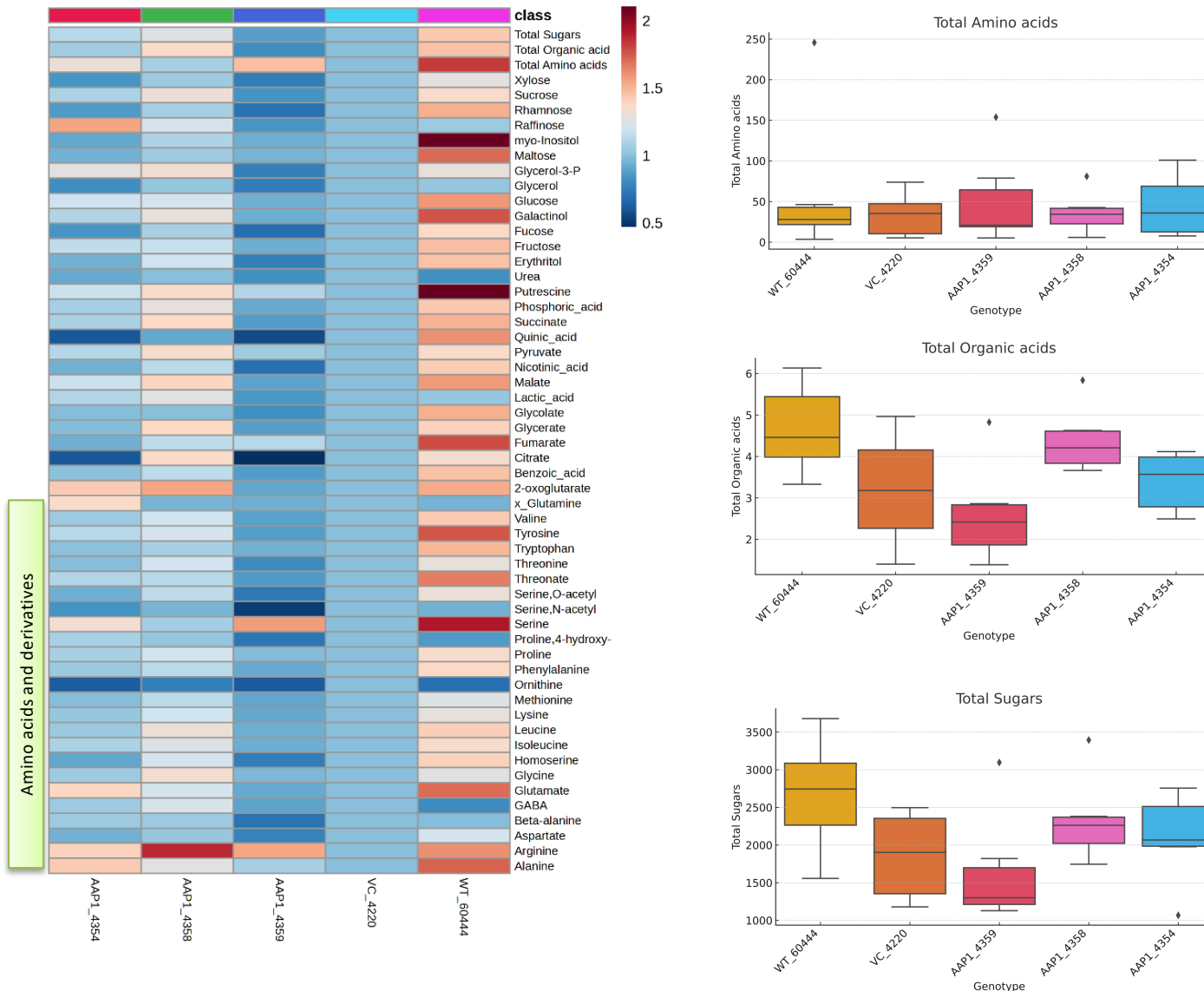
Positive samples: 10 mM (K)EDTA in 10 mM HEPES

Negative samples: 10 mM HEPES (without EDTA)



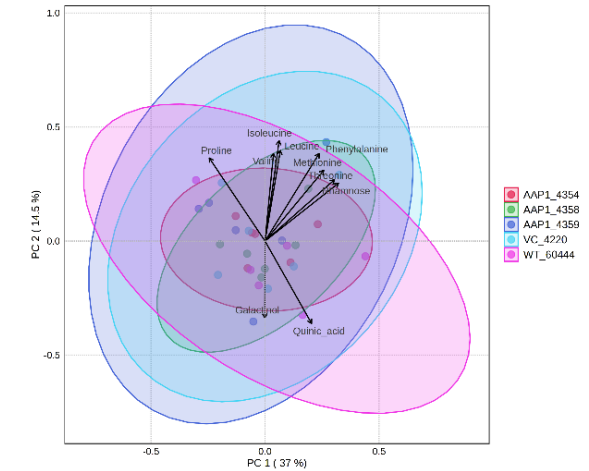
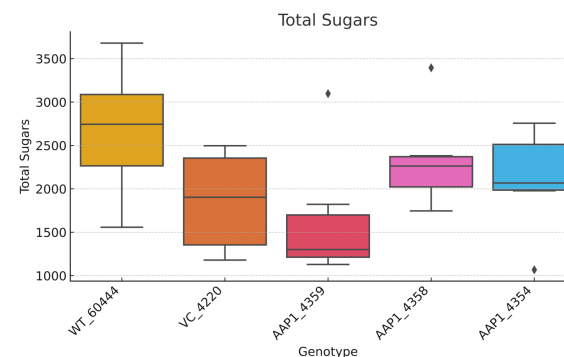
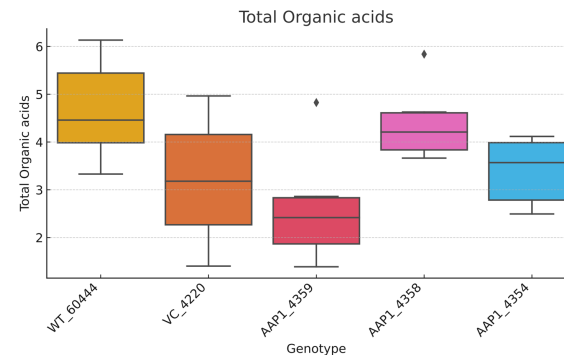
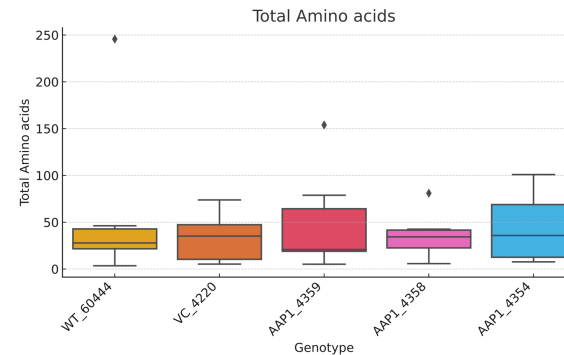
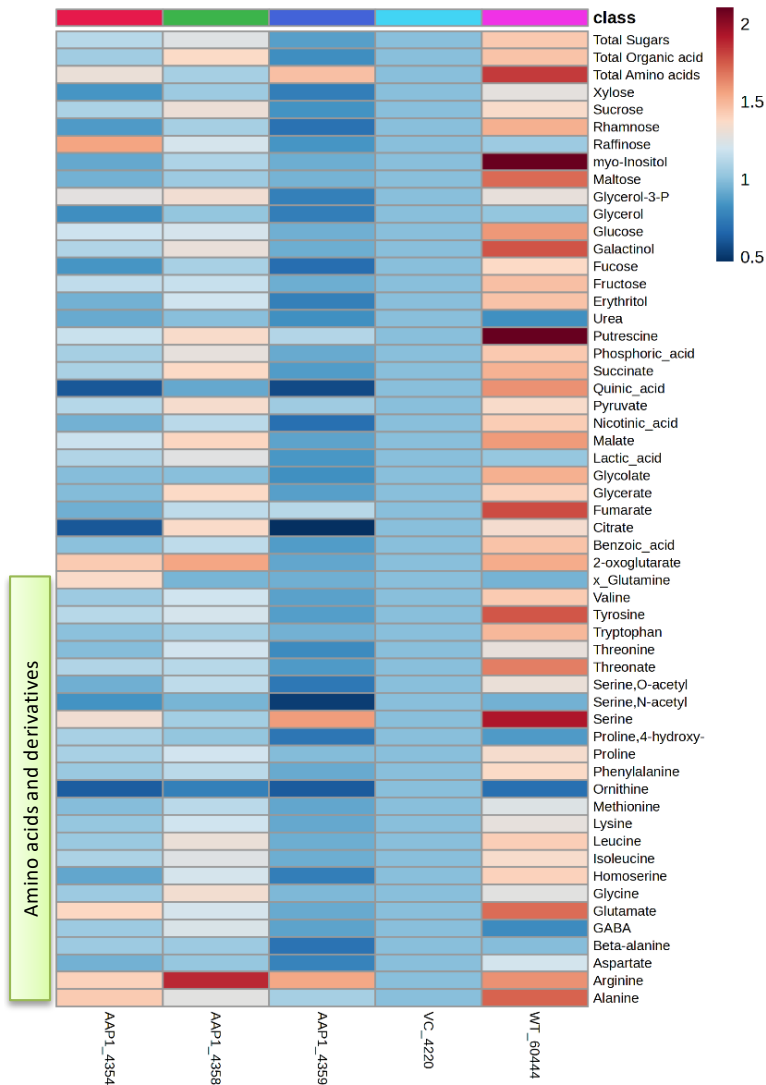
Positive samples only

Kruskal-Wallis test, comparison to VC, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$



Positive samples only

Kruskal-Wallis test, comparison to VC, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$



- Big variation in the data
- Not completely inactivation of enzymes during the collection process
- Eg. Linamarin

Positive samples only

Kruskal-Wallis test, comparison to VC, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

Polytunnel, Germany 2022

Leaves and roots – Done!

Polytunnel, Germany 2024

11 tissues – selected samples running

High light Phytotron, Germany 2024/25

Phloem sap – Done!

Field Taiwan, July2022_Intermediate Harvest

5 tissues – extracted, GC running

Field Taiwan, Dec 2022_Final Harvest

2 tissues – extracted, run completed, data analysis

CFT, Nigeria 2024/25

No WT or vector control, not completely dry samples - stopped

- Total protein in different tissues
- Verify precise amino acid composition in different tissues and phloem sap
- Additional experiment
 - Sink leaves before storage root formation for amino acid and linamarin quantification
 - Naturally expelled sugary solution
 - Apoplastic fluid from the leaves

MPIMP

AG Fernie

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Green team

Adm team
International/
Travel office

CASS project

Germany:

FAU Erlangen
RPTU Kaiserslautern
Forschungszentrum Jülich

Nigeria:

IITA Ibadan
NRCRI Umudike

Switzerland: ETH Zürich

Taiwan: NCHU

Finland: University of Helsinki

UK: University of Cambridge
University of Oxford

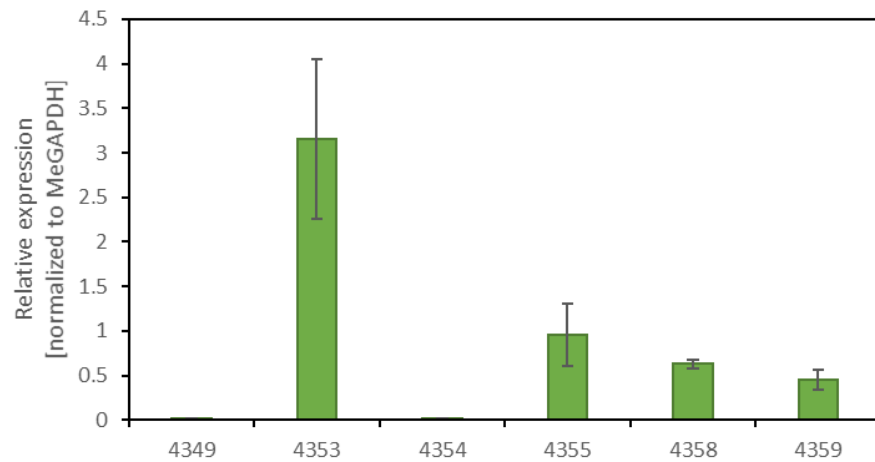
USA: BTI



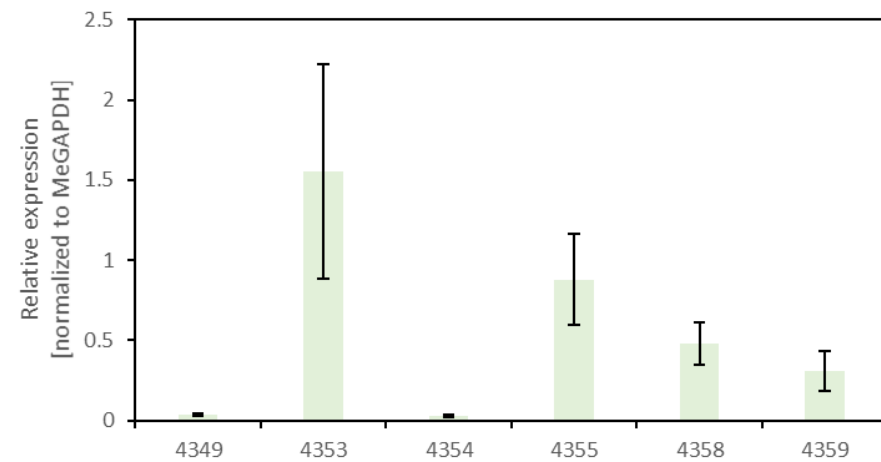
... and you for your attention!



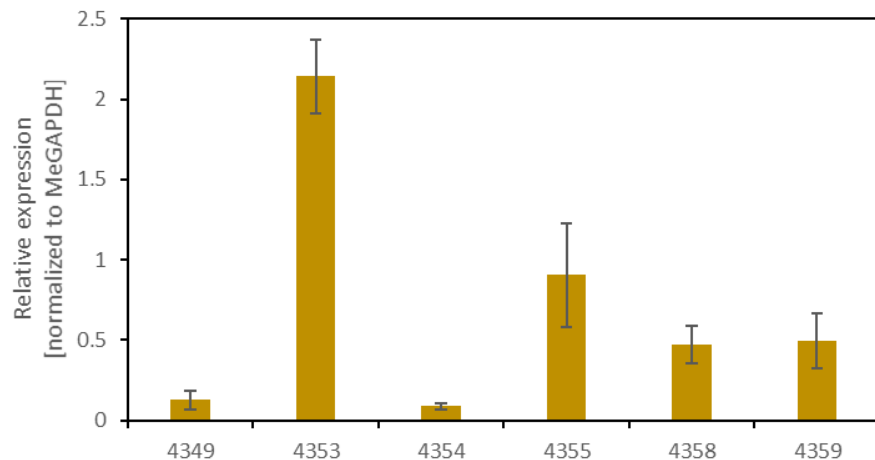
AAP1 - Transgene expression in leaves



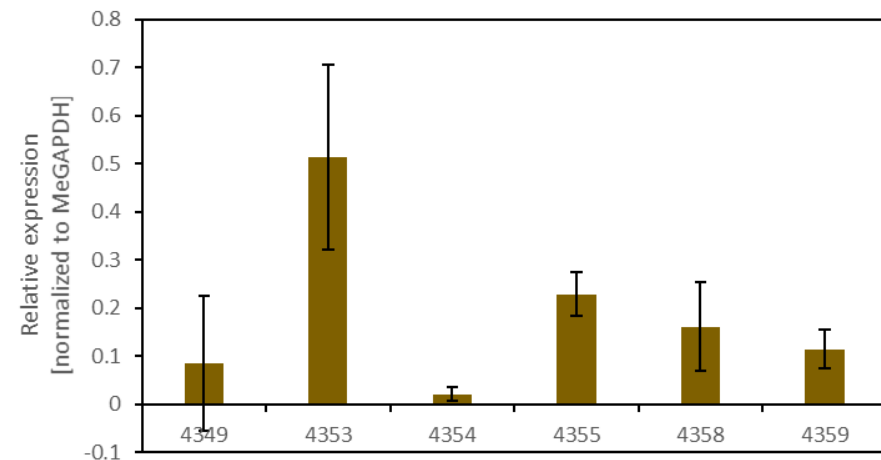
AAP1 - Transgene expression in petioles

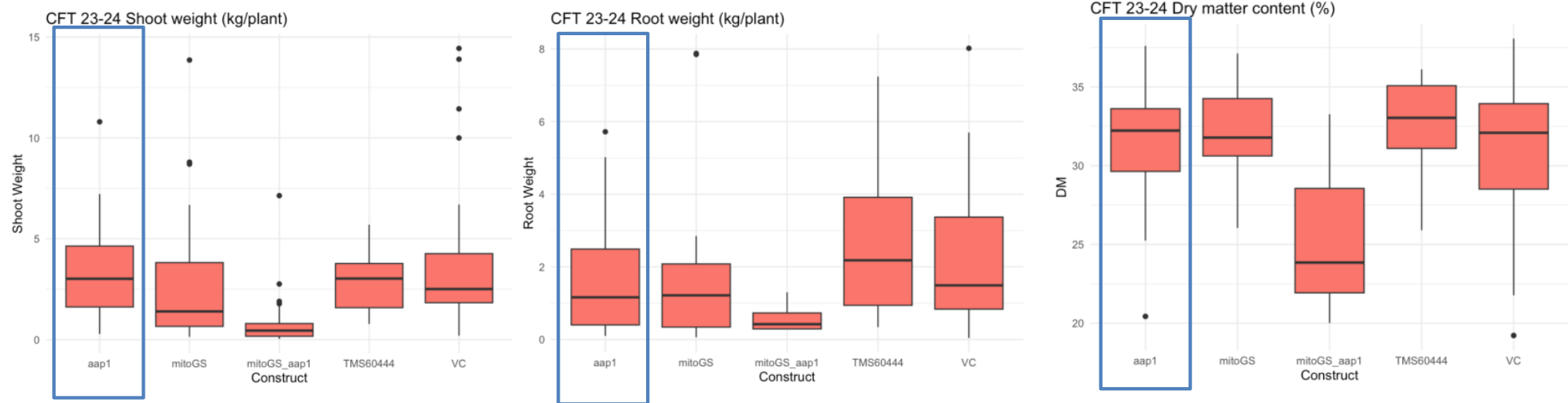


AAP1 - Transgene expression in lower stem



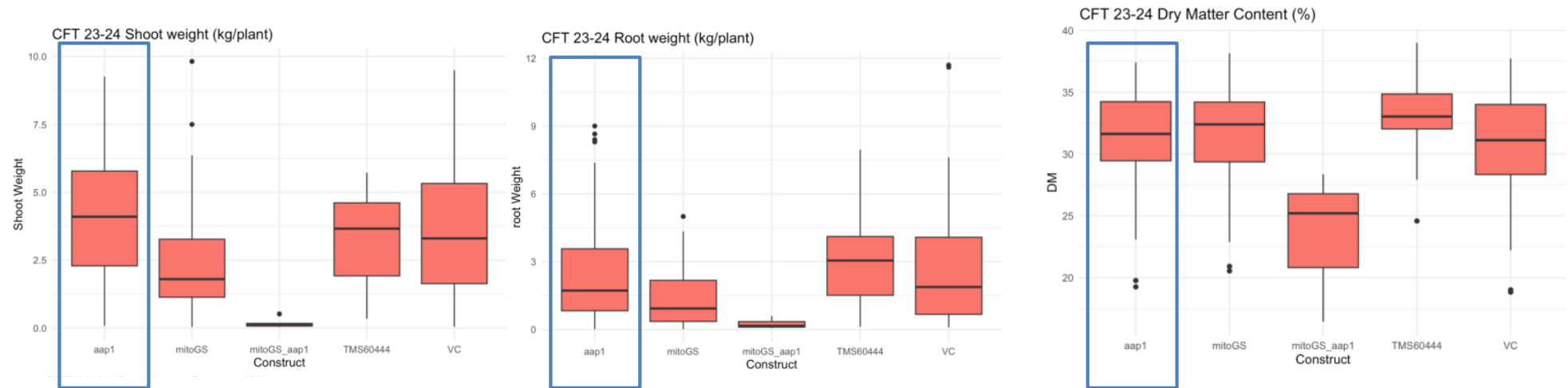
AAP1 - Transgene expression in storage root





Construct	Shoot weight (kg)	Root weight (kg)	Root number	Dry matter %
aap1	3.4 (2.07)	1.8 (1.63)	2.8 (1.66)	31.3 (3.66)
TMS60444	3.0 (1.47)	2.8 (2.61)	4.2 (2.86)	32.3 (4.04)
Vector control	3.4 (2.79)	2.3 (1.93)	3.6 (2.88)	30.9 (4.41)

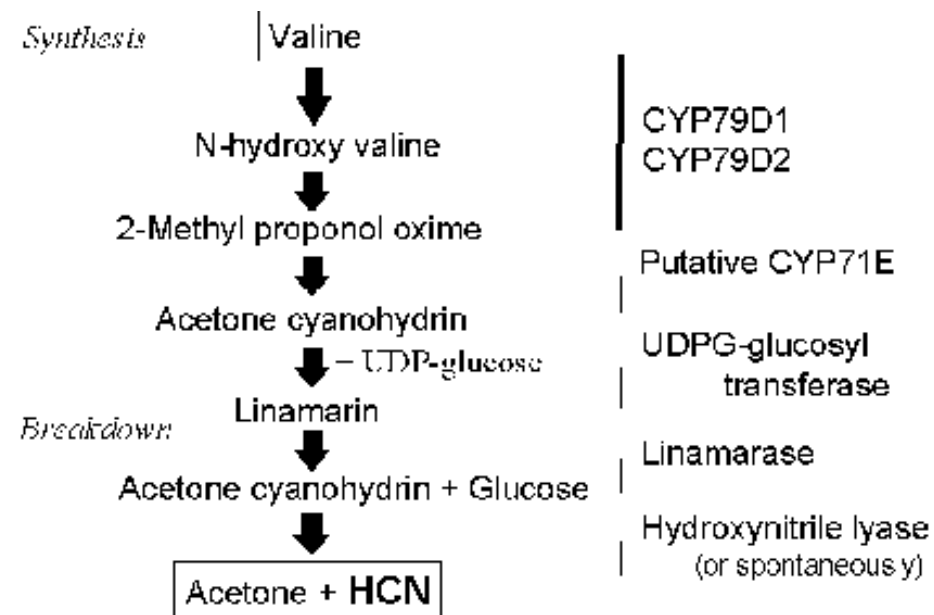
- Substantial incidence of root rot
- AAP1 vigorous aboveground growth

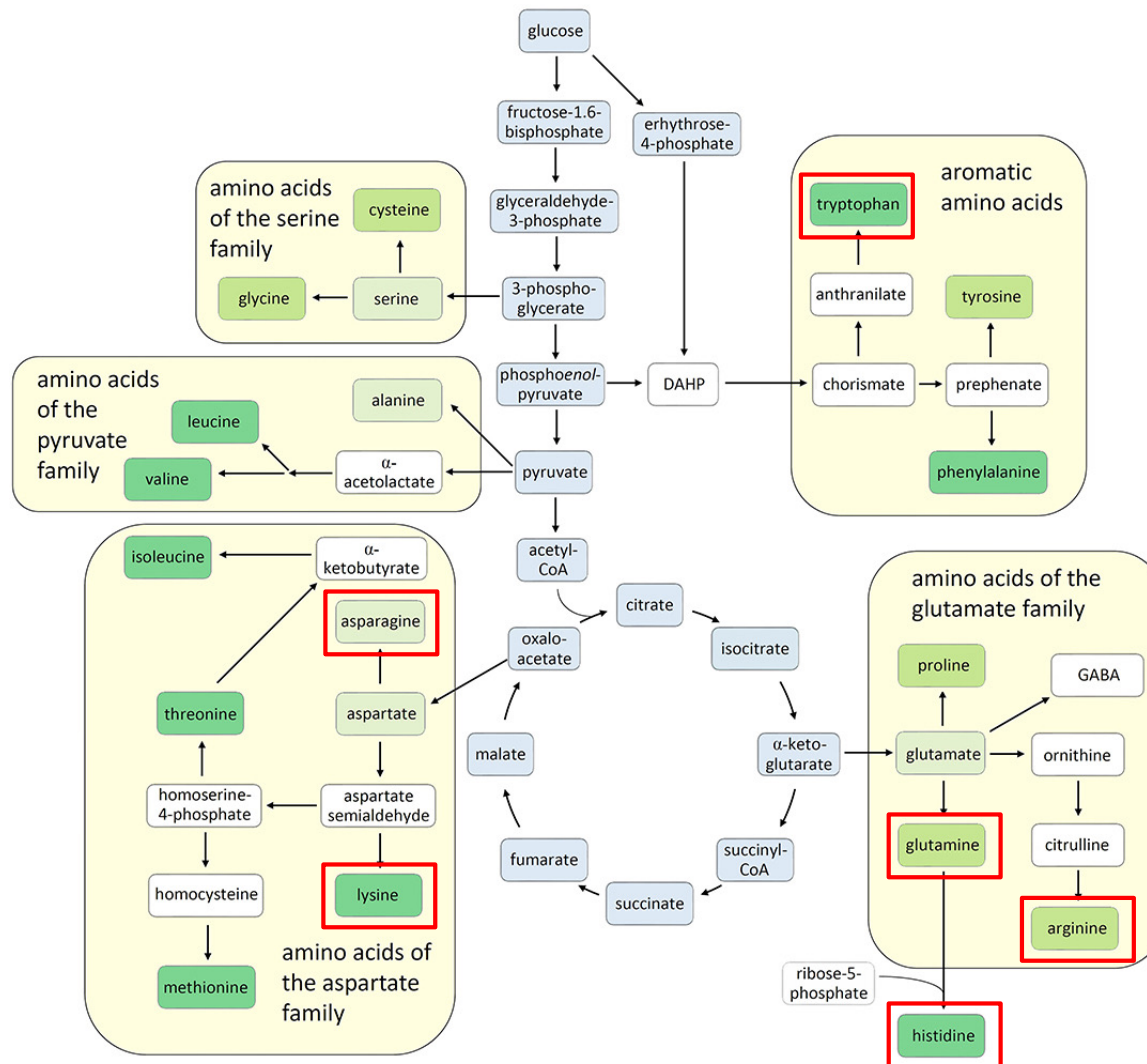


Construct	Shoot weight (kg)	Root weight (kg)	Root number	Dry matter %
AAP1	4.5 (3.2)	2.7 (2.4)	6.8 (4.2)	29.9 (5.2)
TMS60444	4.3 (3.5)	4.4 (4.6)	9.4 (5.5)	33.2 (2.7)
Vector_control	3.9 (3)	3.2 (3.8)	7.4 (4.2)	29.5 (4.6)

- AAP1 displayed robust shoot and root growth

Linamarin biosynthesis





Nitrogen-rich amino acids

Among the 20 amino acids constituting proteins, six amino acids contain more than one nitrogen atom in each molecule. They were **tryptophan (W)**, **asparagine (N)**, **glutamine (Q)**, **lysine (K)**, **histidine (H)**, and **arginine (R)**.



127
WT_6



106
WT_5



117
VC_4218_6



118
VC_4220_6



125
VC_4239_6

Supplemental info AAP

- Functional cassettes**

Promoter – *COMMELINA YELLOW MOTTLE VIRUS (CoYMV)*

strong promoter in vascular and reproductive tissues (Medberry et al., 1992)
and active in the companion cells of leaves, stems and roots (Matsuda et al., 2002).

5'-UTR – *Pisum Sativum AMINO ACID PERMEASE 1 (PsAAP1)*

CDS – *Pisum Sativum AMINO ACID PERMEASE 1 (PsAAP1)*

3'-UTR-terminator – *Pisum Sativum AMINO ACID PERMEASE 1 (PsAAP1)*

- Selectable marker cassette**

Promoter – *Agrobacterium tumefaciens NOPALINE SYNTHASE (AtuNOS)*

CDS – *HYGROMYCIN PHOSPHOTRANSFERASE2 (Hpt2)*

Terminator – *CAULIFLOWER MOSAIC VIRUS 35S*

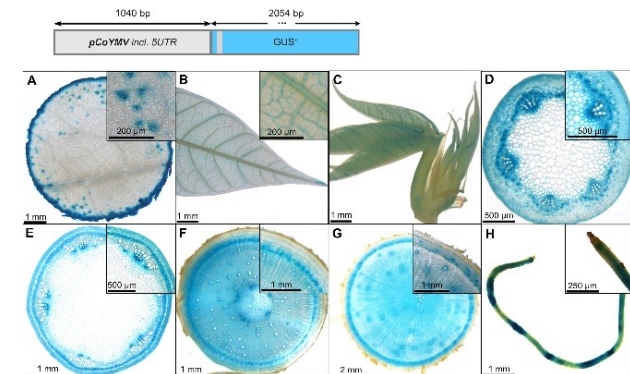
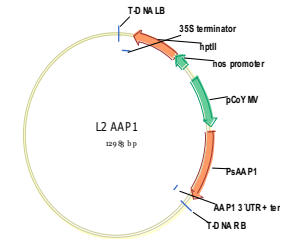


Figure 6 Representative GUS staining pattern of four *pCoYMV* promoter-reporter lines. **(A)** Source leaf (Inlay = Close-up), **(B)** Sink leaf (Inlay = Close-up), **(C)** Emerging leaves, **(D)** Petiole cross-section (Inlay = Close-up), **(E)** Upper stem cross-section (Inlay = Close-up), **(F)** Lower stem cross-section (Inlay = Close-up), **(G)** Storage root cross-section (Inlay = Close-up), **(H)** Fibrous roots (Inlay = Root tip).

Amino Acid Permease (AAP)

Multigene family

Organ-specific expression

Low transport selectivity

Arabidopsis: AAP1 is seed-specific

***Vicia faba*:** AAP1 is expressed in embryonic storage parenchyma cells

Pea: AAP1 and AAP2 is expressed in seed coats
epidermal transfer cells
storage parenchyma cells
vegetative organs

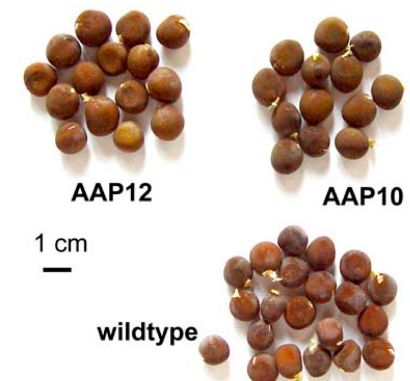
AAP1 from *Vicia faba* - broad bean, fava bean

Overexpression in seeds of pea (*Pisum sativum*) and *Vicia narbonensis*

- Increase in seed total nitrogen
- Increase in sink strength for amino acids (more [14C]-glutamine uptake)
- Stimulation of nitrogen root uptake (application of [¹⁵N]-labeled ammonia to the roots)
- No change in seed yield per plant



Vicia faba

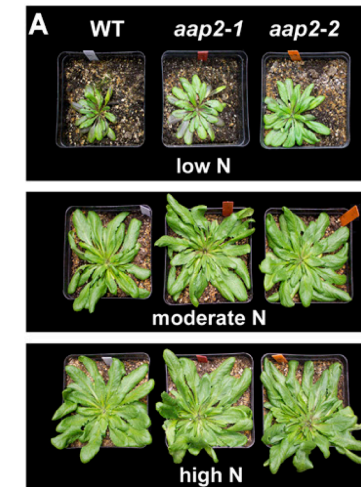


Vicia narbonensis
AAP1ox seeds
growth chamber

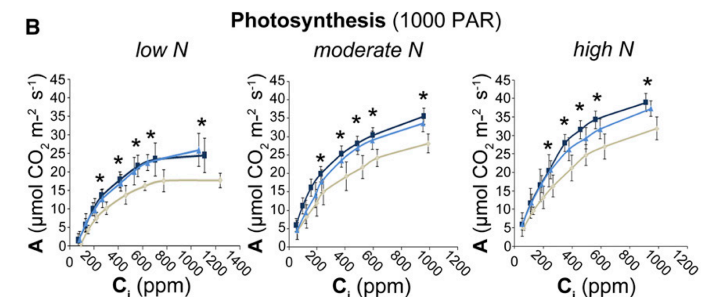
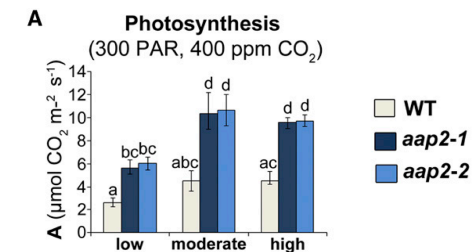
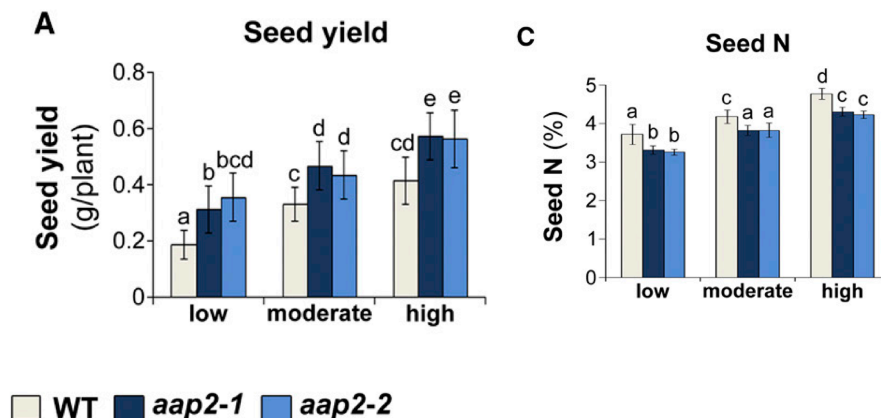
AAP1 in Arabidopsis

The *aap2* plants outperformed wild-type plants with respect to growth, seed yield and carbon storage pools, and nitrogen use efficiency in both high and deficient nitrogen environments. (Perchlik and Tegeder 2018 Plant Physiol)

But reduced seed protein levels (Zhang et al., 2010)



Perchlik et al.

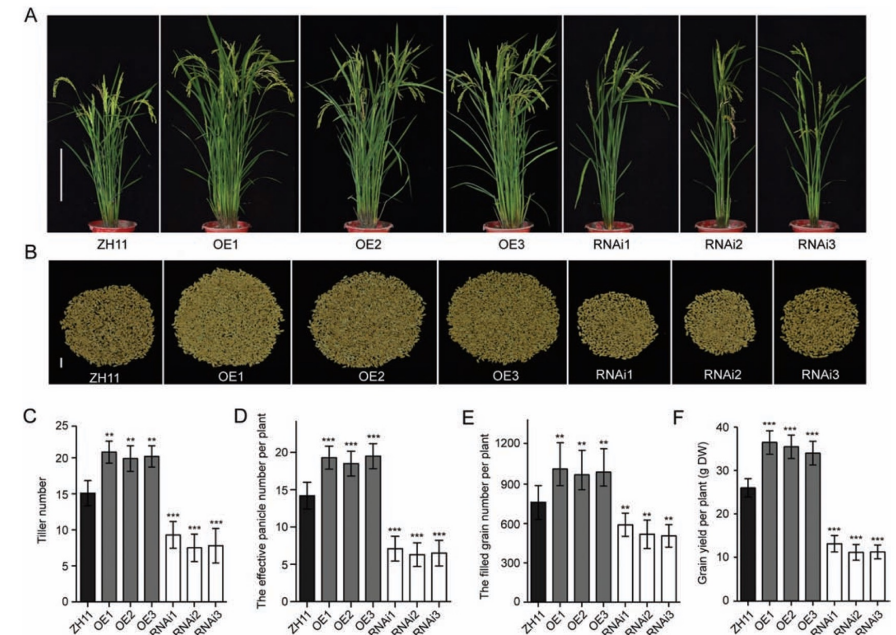


AAP1 in *Oryza sativa*

OsAAP1 gene is highly expressed in rice axillary buds, leaves, and young panicles, and that the *OsAAP1* protein is localized to both the plasma membrane and the nuclear membrane.

OE lines exhibited increased filled grain numbers as a result of enhanced tillering,

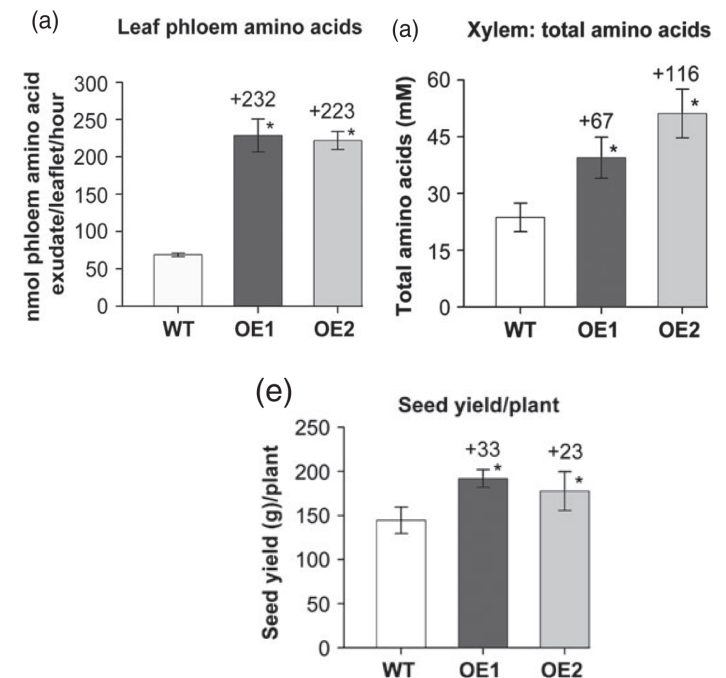
RNAi and CRISPR (*Osaap1*) knockout lines showed the opposite phenotype. In addition, *OsAAP1*-OE lines had higher concentrations of neutral and acidic amino acids, but lower concentrations of basic amino acids in the straw.



AAP1 from pea (*Pisum sativum*)

Simultaneous overexpression in pea targeting amino acids source-sink partitioning via
import into the seed (cotyledon epidermis)
and phloem loading (sieve element-companion cell)

- Increase in biomass
- Increase in seed and leaves total nitrogen
- Increase in total free amino acid levels in the phloem exudate and in the xylem sap
- Increase in photosynthetic rate
- Increased yield per plant
- Under different N treatments



Supplemental info mitoGS

- Functional cassettes

- ***mitoGS***

Promoter – *Solanum lycopersicum* RIBULOSE BIPHOSPHATE CARBOXYLASE SMALL SUBUNIT 2 (***pSIRBCS2***)

5'-UTR – *Solanum lycopersicum* RIBULOSE BIPHOSPHATE CARBOXYLASE SMALL SUBUNIT 2 (*pSIRBCS2*)

5'-Targeting signal – *Saccharomyces cerevisiae* CYTOCHROME C OXIDASE 4 (***ScCOX4***)

5'-CDS – *Solanum lycopersicum* GLUTAMINE SYNTHASE CYTOSOLIC (*SIGScyt.*)

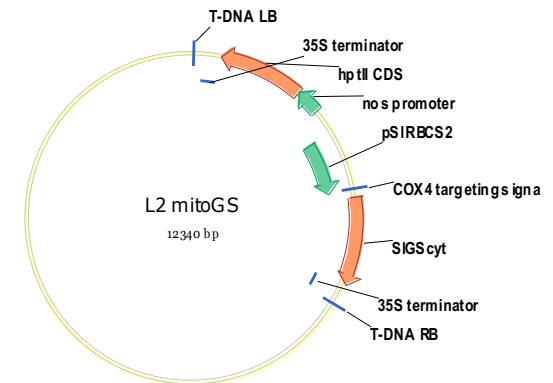
3'-UTR-terminator – CAULIFLOWER MOSAIC VIRUS 35S

- Selectable marker cassette

Promoter – *Agrobacterium tumefaciens* NOPALINE SYNTHASE (*AtuNOS*)

CDS – **HYGROMYCIN** PHOSPHOTRANSFERASE2 (*Hpt2*)

Terminator – CAULIFLOWER MOSAIC VIRUS 35S



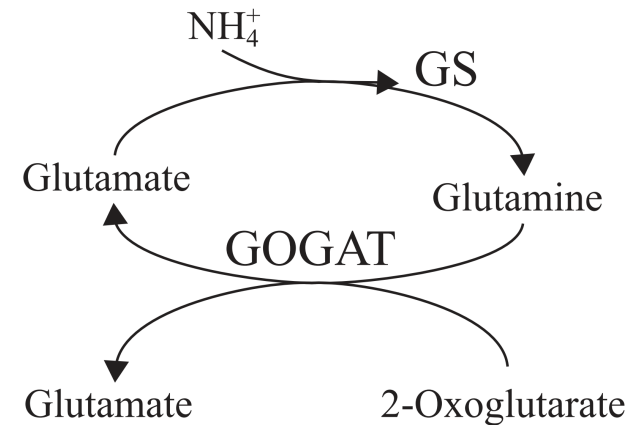
Although no dedicated promoter-GUS lines were created for the promoters of *SIRBCS2* and *AtRBCS1A*, they appeared very active in **source leaf tissues** in transcript studies. In addition, *pSIRBCS2* also appeared to be specific for this tissue.

GS/GOGAT cycle

Glutamine synthetase (GS)

Glutamate synthetase (GOGAT)

Incorporation of inorganic nitrogen
into organic molecules



GS1: cytosol

encoded by multiple genes

primary NH_4^+ assimilation, N remobilization and translocation

GS2: plastid

generally encoded by a single gene

reassimilation of photorespiratory NH_4^+ , and NH_4^+ from nitrite reduction

Increasing expression of GS is in general beneficial, associated with N use and stress tolerance

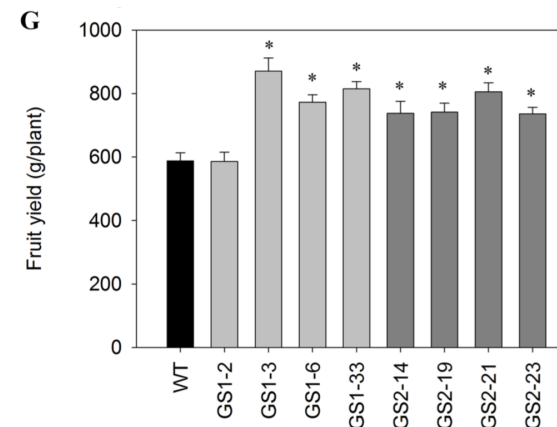
Glutamine synthetase (GS) Flux balance analysis (FBA) model Tomato plants

Increase expression
GS1 (cytosolic) or *GS2* (plastidic) +

Leaf mesophyll-specific promoter
(ribulose-bisphosphate carboxylase, *RbcS*) +

Mitochondrial transit peptide COX4ts
(for mitochondrial localization)

Early flowering and fruit set
Faster growth rate
Increased fruit yield





- [Enzymes and Nitrogen Cycling](#)
- John A. Berges, Margaret R. Mulholland, in [Nitrogen in the Marine Environment \(Second Edition\)](#), 2008
- **2.2.2.3 Enzyme regulation and interpretation of activity assays**
- GOGAT activity does not appear to be as heavily regulated as GS activity, or perhaps it is just less studied (Florencio *et al.*, 1987; Muro-Pastor *et al.*, 2005). However, GOGAT does appear to be regulated by light and metabolite sensing systems in higher plants (Suzuki and Knaff, 2005)
- Functions of macronutrients☆
- Malcolm J. Hawkesford, ... Philip J. White, in *Marschner's Mineral Nutrition of Plants (Fourth Edition)*, 2023
- 6.1.4.2 Ammonium assimilation
- Ammonium is a central intermediate in plant N metabolism. As well as uptake from the soil by roots, ammonium is constantly generated in plant tissues by processes such as nitrate reduction, photorespiration, lignin biosynthesis, senescence-induced N remobilization, and N₂ fixation in legumes (Joy, 1988). Irrespective of the source of ammonium, or the organ in which it is assimilated (roots, root nodules, and leaves), the key enzymes involved are glutamine synthetase (GS) and glutamate synthase (GOGAT; glutamine-oxoglutarate aminotransferase). Both enzymes are present in roots, in chloroplasts, and in N₂-fixing microorganisms, and assimilation of most if not all ammonium derived from ammonium uptake, N₂ fixation, nitrate reduction, and photorespiration is mediated by the GS-GOGAT pathway. In this pathway the amino acid glutamate acts as the acceptor for ammonium, and the amide glutamine is formed (Fig. 6.9). The net reaction is:
Sign in to download full-size image
- Figure 6.9. Model of ammonium assimilation via the glutamine synthetase-glutamate synthase cycle. Pathways at low (1) and high (2) ammonium supply.
- (6.2) $\text{Glutamate} + 2\text{-oxoglutarate} + \text{NH}_4^+ \rightarrow \text{glutamine} + \text{H}^+$
- GS exists in multiple enzyme forms located in the cytosol and in plastids (Bernard *et al.*, 2008). Cytosolic GS assimilates ammonium into glutamine for transport and distribution throughout the plant. During leaf senescence, cytosolic GS fulfills a key function in the assimilation and recycling of ammonium generated in various catabolic processes (Masclaux-Daubresse *et al.*, 2010). This role is particularly important after anthesis and during grain development and filling in cereals, when N is remobilized to the reproductive sinks (Thomsen *et al.*, 2014). Several isoenzymes of the cytosolic GS1 gene family are abundantly expressed in roots and can be classified into high-affinity or low-affinity subtypes differing in V_{max} values (Ishiyama *et al.*, 2004). Some isoenzymes play a redundant role under low ammonium supply and others dominate under high external ammonium supply (Konishi *et al.*, 2018). This dynamic regulation may contribute to the homeostatic control of glutamine synthesis in roots. In arabidopsis shoots, separate isoforms of cytosolic GS play distinct roles for seed yield structure and for coping with ammonium toxicity (Guan *et al.*, 2015; 2016). The same is the case in maize, where the Gln1–3 and Gln1–4 genes affect kernel number and kernel size, respectively, with a specific impact on grain production (Martin *et al.*, 2006).
- In chloroplasts, light-stimulated nitrate reduction and enhanced ammonium assimilation are coordinated through the import of 2-oxoglutarate from the stroma and export of glutamate from the stroma of chloroplasts into the cytoplasm, thus preventing high ammonium concentrations. Chloroplast GS is activated by high pH and high concentrations of Mg and ATP, and all three factors are increased in the chloroplast stroma upon illumination. In cytosol and chloroplasts, GS is also subject to posttranslational regulation by phosphorylation and subsequent interactions with proteins (Finnemann and Schjoerring, 2000; Lima *et al.*, 2006).
- The other enzyme involved in ammonium assimilation, glutamate synthase (GOGAT), catalyzes the transfer of the amide group (–NH₂) from glutamine to 2-oxoglutarate, which is a product of the tricarboxylic acid (TCA) cycle. The conversion of glutamine to glutamate takes place in plastids that have two isoforms of GOGAT. One form accepts electrons from reduced ferredoxin (from photosystem I), the other from NADPH from respiration. The ferredoxin-linked GOGAT isoform dominates in leaves, particularly in the chloroplasts of phloem companion cells in leaf veins (Masclaux-Daubresse *et al.*, 2007), whereas the NADPH isoform is prevalent in roots (Tabuchi *et al.*, 2007). Both forms contain an Fe-S cluster that transfers electrons during the reductive synthesis of two glutamate molecules from one 2-oxoglutarate and one glutamine molecule. One of the two molecules of glutamate produced is required for the maintenance of the ammonium assimilation cycle and the other can be transported from the sites of assimilation and utilized elsewhere for the biosynthesis of proteins. When the ammonium supply is high, both glutamate molecules can act as ammonium acceptors, and one molecule of glutamine leaves the cycle (Fig. 6.9). Two Fd-GOGAT genes (GLU1 and GLU2) have been characterized in higher plants, with GLU1 playing a major role in the assimilation of ammonium derived from photorespiration, whereas the GLU2 gene may play a major role in the primary N assimilation in roots (Suzuki and Knaff, 2005).

GS1 and GS2

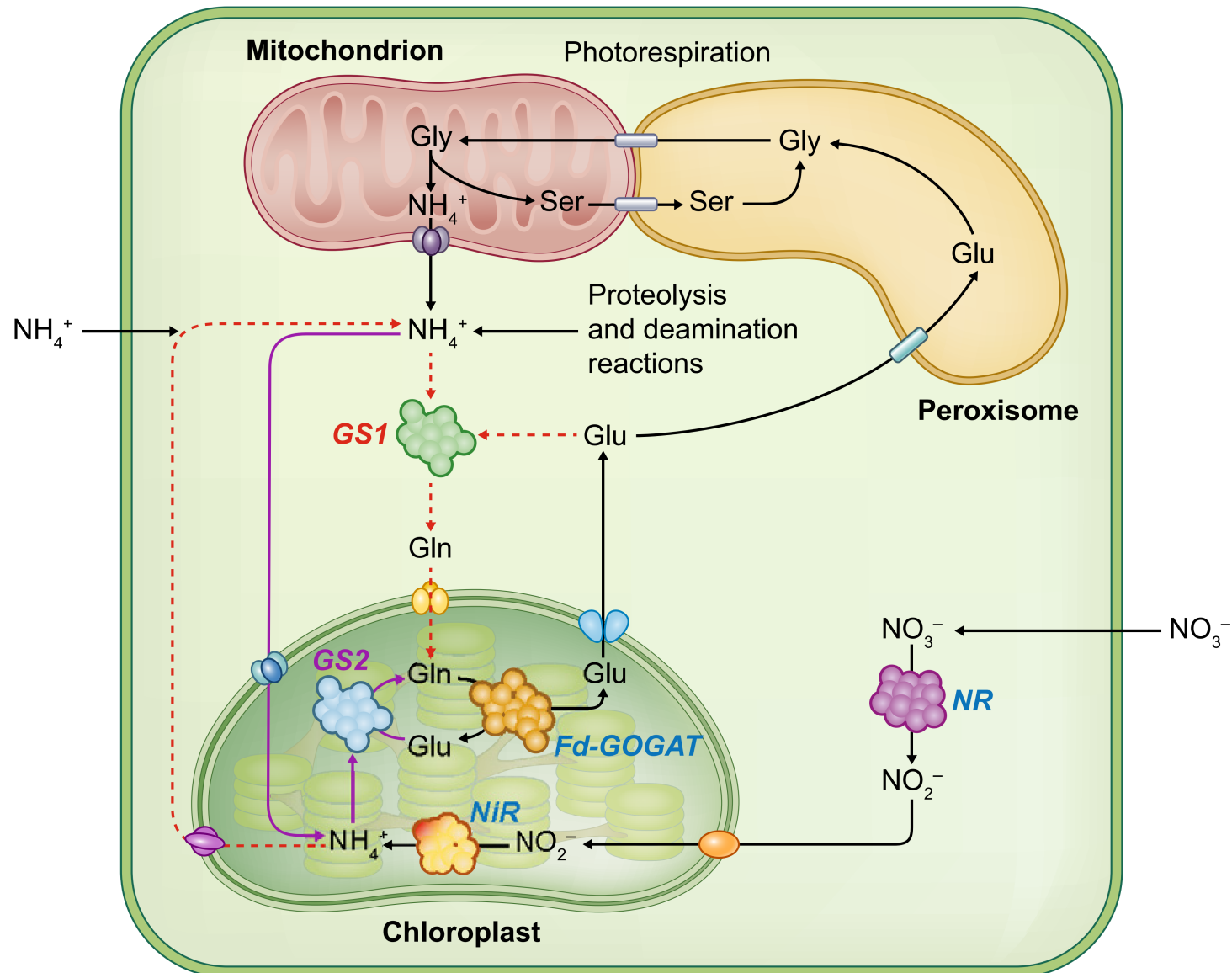


Table 1 Catalogue of mutant and transgenic plant phenotypes engineered for higher or lower GS2 expression.

Species	Transgenic plant/mutant	Growth conditions	Phenotype	Reference
<i>Hordeum vulgare</i> cv Maris Mink	KO (azide)	Control conditions NPC	Lethal Similar to WT	Wallsgrave <i>et al.</i> (1987) Blackwell <i>et al.</i> (1988)
<i>H. vulgare</i> cv Maris Mink	KO (GS2) × KO (Fd-GOGAT) (azide)	Control conditions NPC	Lethal Similar to WT	Blackwell <i>et al.</i> (1988)
<i>Nicotiana tabacum</i>	Overexpression 35S:NtGS2 Co-suppression 35S:NtGS2	High-intensity light	Increased tolerance Increased sensitivity	Kozaki & Takeba (1996)
<i>Oryza sativa</i> cv Kinuhikari	Overexpression 35S:OsGS2	Salinity Cold stress	Increased tolerance Increased tolerance	Hoshida <i>et al.</i> (2000)
<i>N. tabacum</i> line SR1	Co-suppression 35S:OsGS2 Overexpression rbcS:NtGS2	Salinity Control conditions	Increased sensitivity Increased growth	Migge <i>et al.</i> (2000)
<i>Lotus japonicus</i>	KO (EMS)	Control conditions NPC	Lethal Similar to WT	Orea <i>et al.</i> (2002)
<i>Brassica napus</i>	Reduced expression 35S: antisense BnGS2	Control conditions	Similar to WT	Husted <i>et al.</i> (2002)
<i>N. tabacum</i> line SR1	Co-suppression 35S:PsGS2	Control conditions	Reduced growth and chlorosis	Oliveira <i>et al.</i> (2002)
<i>Arabidopsis thaliana</i> Col-0	Overexpression 35S:DvGS2 ¹	Control conditions Low N Phosphinothricin	Increased growth Increased growth Enhanced resistance	Zhu <i>et al.</i> (2014)
<i>O. sativa</i> cv Zhongua 11	Co-overexpression pOsAct1:PsGS1 + pZmUbi1:PsGS2	Phosphinothricin	Enhanced resistance	Sun <i>et al.</i> (2005a)
<i>O. sativa</i> cv. Zhongua 11	Co-overexpression pOsAct1:PsGS1 + pZmUbi1:PsGS2	N deficiency	Enhanced growth	Sun <i>et al.</i> (2005b)
<i>Triticum aestivum</i>	Co-overexpression pOsAct1:PsGS1 + pZmUbi1:PsGS2	Phosphinothricin	Enhanced resistance	Huang <i>et al.</i> (2005)
<i>L. japonicus</i>	KO (EMS)	Drought in NPC	Increased sensitivity	Díaz <i>et al.</i> (2010)
<i>O. sativa</i> cv Zhongua 11	Co-suppression 35S:OsGS2	Control conditions	Reduced growth and chlorosis	Cai <i>et al.</i> (2010)
<i>N. tabacum</i> cv Xanthi	Overexpression rbcS:AtGS2 Co-overexpression rbcS:AtGS2 + rbcS:Dof1;7	Low-N condition	Increased growth Increased growth relative to rbcS:AtGS2	Wang <i>et al.</i> (2013)
<i>T. aestivum</i> cv Ji5265	Expression of GS2 allele from Xiaoyan 54 pTaGS2-2Ab:TaGS2-2Ab	High-N field trial Low-N field trial	Increased growth and yield Increased growth and yield	Hu <i>et al.</i> (2018)
<i>O. sativa</i> cv Nipponbare	Co-overexpression with GS1;1 pOsAct1:OsGS2 + pOsAct2 OsGS1;1	Osmotic stress Salinity Drought Phosphinothricin	Increased tolerance Increased tolerance Increased tolerance Enhanced resistance	James <i>et al.</i> (2018b)
<i>N. tabacum</i> cv K326	Overexpression SP: TaGS2	Control conditions N starvation	Similar to WT Similar to WT	Wei <i>et al.</i> (2018)
<i>A. thaliana</i> Col-0	Knocked-out (T-DNA)	Normal air Salinity NPC	Reduced growth and chlorosis Increased tolerance Reduced growth	Ferreira <i>et al.</i> (2019)
<i>N. tabacum</i> cv K326	Overexpression SP: TaGS2	Drought	Increased tolerance	Yu <i>et al.</i> (2020)
<i>A. thaliana</i> Col-0	Co-suppressed 35S:AtGS2 Knocked-out (T-DNA)	Control conditions Control conditions Ammonium stress	Reduced growth, no chlorosis Reduced growth, no chlorosis Increased tolerance	Hachiya <i>et al.</i> (2021)

At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Dv, *Dunaliella viridis*; EMS, ethyl methanesulfonate mutagenesis; KO, knockout; NPC, non-photorespiratory condition; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Ps, *Pisum sativum*; SP, super promoter; Ta, *Triticum aestivum*; WT, wild-type; Zm, *Zea mays*.

¹DvGS2 is a homologue of the *GLN2* gene from the green algae *Chlamydomonas reinhardtii* and thus corresponds to a different evolutionary lineage than GS2 from seed plants (Valderrama-Martín *et al.*, 2022).