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Ministero dell'Università e della Ricerca

Italiadomani
PIANO NAZIONALE DI RIPRESA E RESILIENZA

Study and design of a plant-based biotechnology platform for the production of biopharmaceuticals for human therapy

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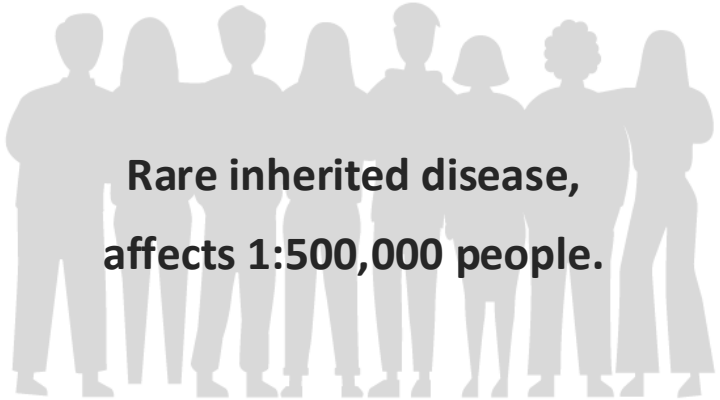
Università degli Studi di Urbino
23 maggio 2024

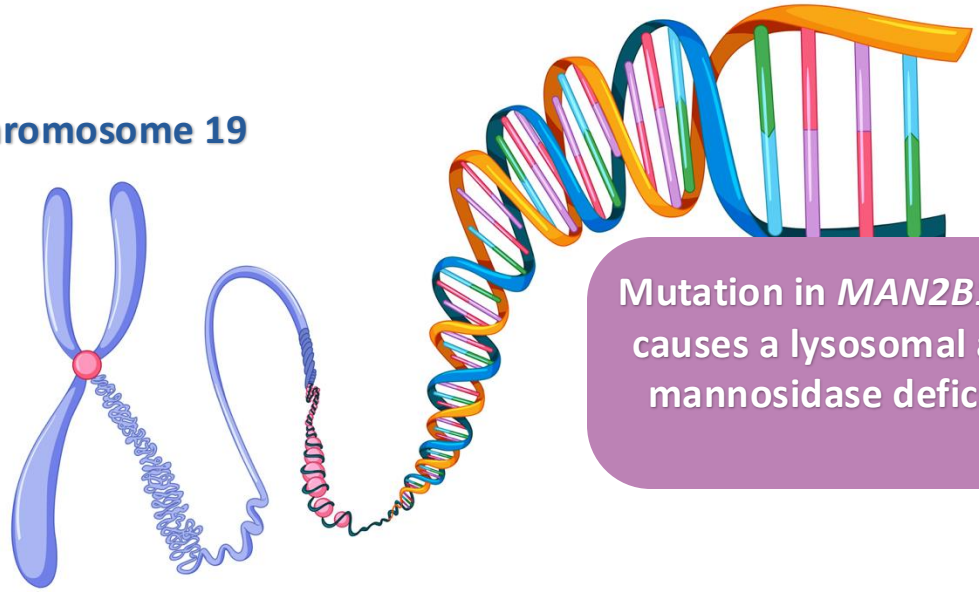
Alpha-mannosidosis



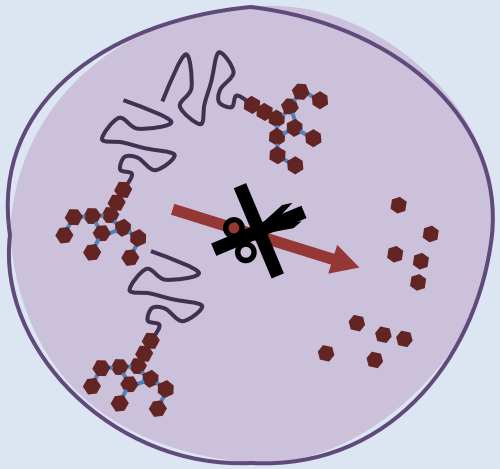
Rare inherited disease,

affects 1:500,000 people.

Chromosome 19



Mutation in *MAN2B1* gene, causes a lysosomal alpha-mannosidase deficiency



Lysosome

Alfa-mannosidase is an exoglycosidase that cleaves alpha-linked mannose residues during the degradation of the N-linked glycans of glycoproteins.

Oligosaccharides progressive deposition in human cells.

Mental retardation and hearing loss from early childhood

Respiratory disfunction

Musculoskeletal abnormalities

Alpha-mannosidase

N-glycosylated, disulphide bridges, zinc binding
homodimer formation

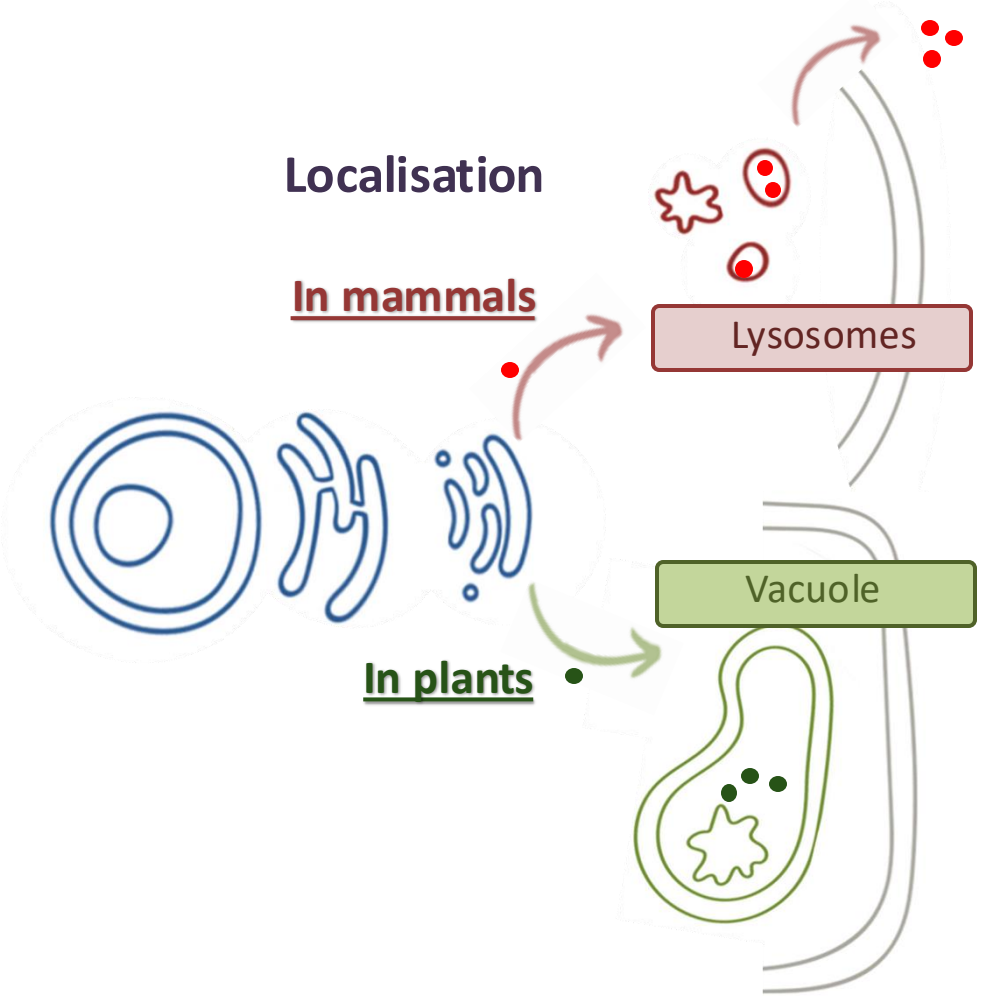
Several proteolytic fragments with different molecular weights ranging from **15** to **70 kDa**.

An enzyme diffused in different kingdoms

Localisation

In mammals

In plants



A small fraction of the alpha-mannosidase precursor is secreted in the extracellular space

Strategy for therapy?

Alpha-mannosidosis therapies

Current approaches:

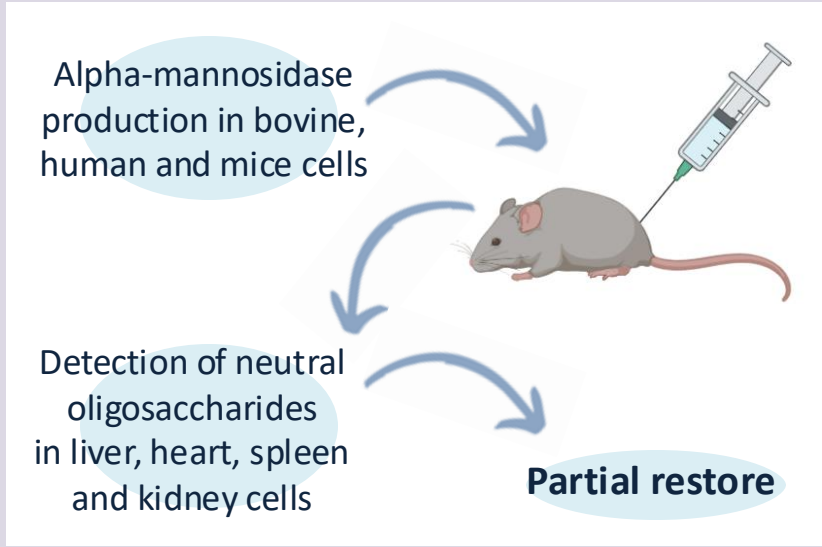
Enzyme replacement therapy (**ERT**)

Recombinant protein production, purification and intravenous injection

Bone marrow transplantation (**BMT**)

Normal donor stem cells release the normal enzyme, which is then widely distributed by host cells upon absorption.

In mice, a corrective effect was observed in those affected by alpha-mannosidosis



Roces *et al.* (2004)

To date, **there is not** a system for the production of recombinant alpha-mannosidase available on a large scale.

Plant molecular farming* could represent a good strategy for the scale-up production of the alpha-mannosidase recombinant protein

Plant molecular farming*: the production of proteins or other metabolites valuable to medicine or industry in plants traditionally used in an agricultural setting

Plants as bioreactors

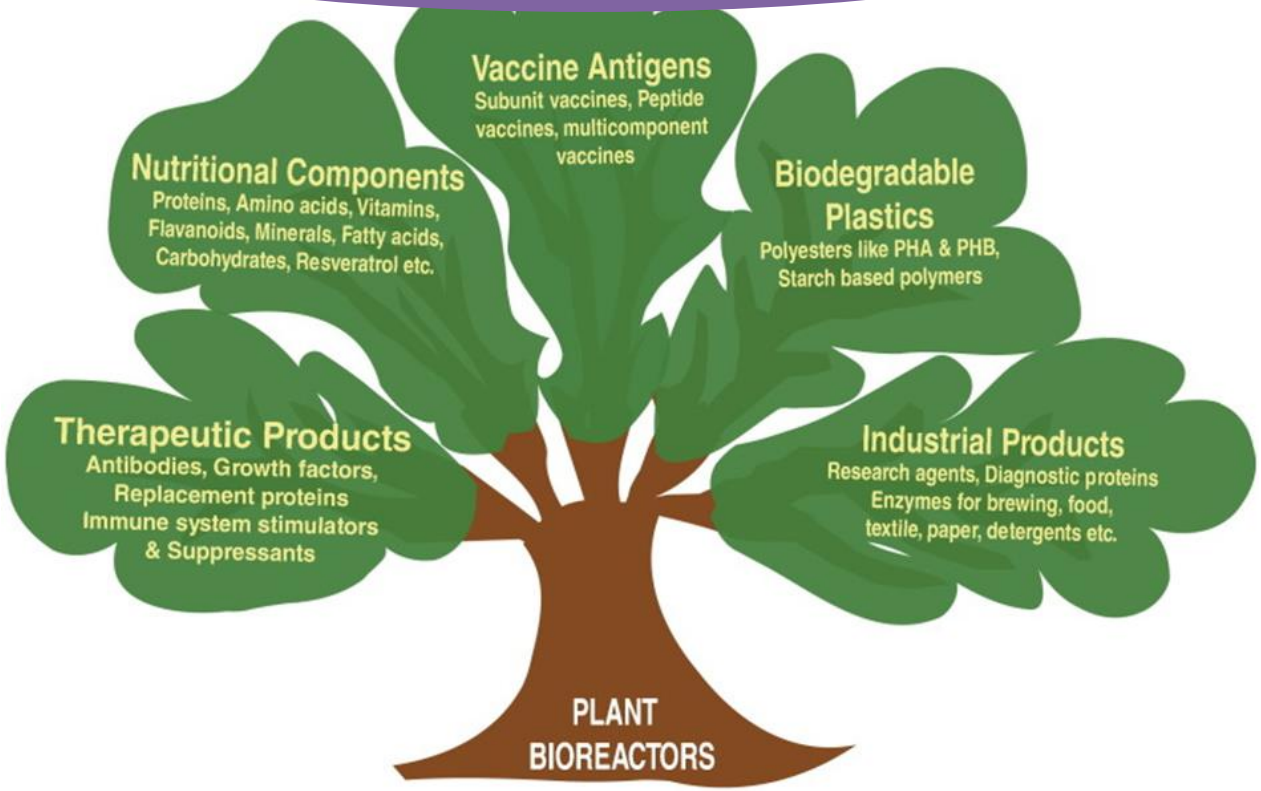
PROs

- Low risk of contamination with human pathogens
- Maximum scale-up possibility
- Complex proteins production
- Optimized culture protocols

CONs

- Different glycosylation patterns
- Lack regulatory approval

Several molecules have already been produced in plants:



Aim of the project

Alpha-mannosidase production in plants for a therapeutic approach

Production of stable transformants for *MAN2B1* gene of *Nicotiana tabacum* plants

Study of different culture systems to optimise alpha-mannosidase protein expression

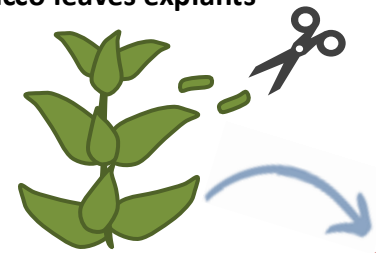
Alpha-mannosidase quantification strategies and enzyme activity assays

Tobacco transformation

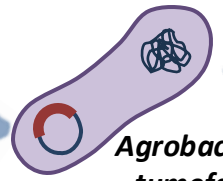
Agrobacterium tumefaciens mediated transformation

De Marchis *et al*., 2011

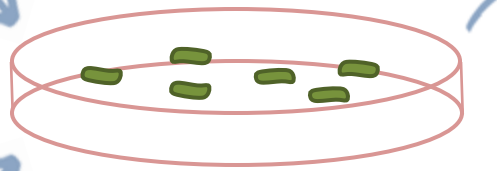
Tobacco leaves explants



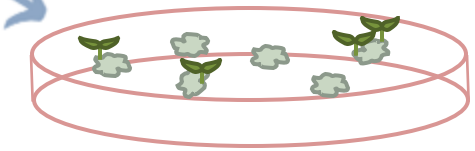
Construct: pDHA.(SP)MAN2B1



Agrobacterium tumefaciens



Co-cultivation



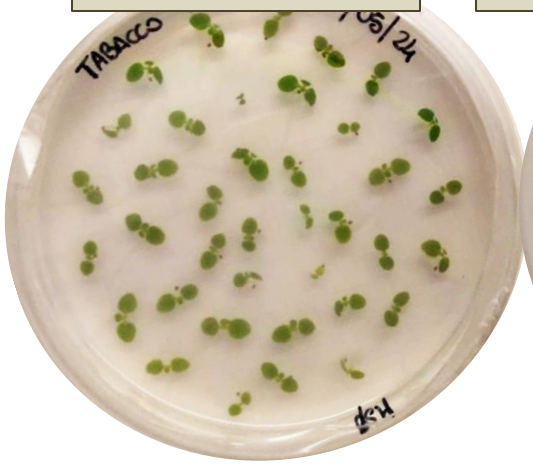
Callus induction and regeneration

Transformants selection

Seeds collection

Seed sterilization

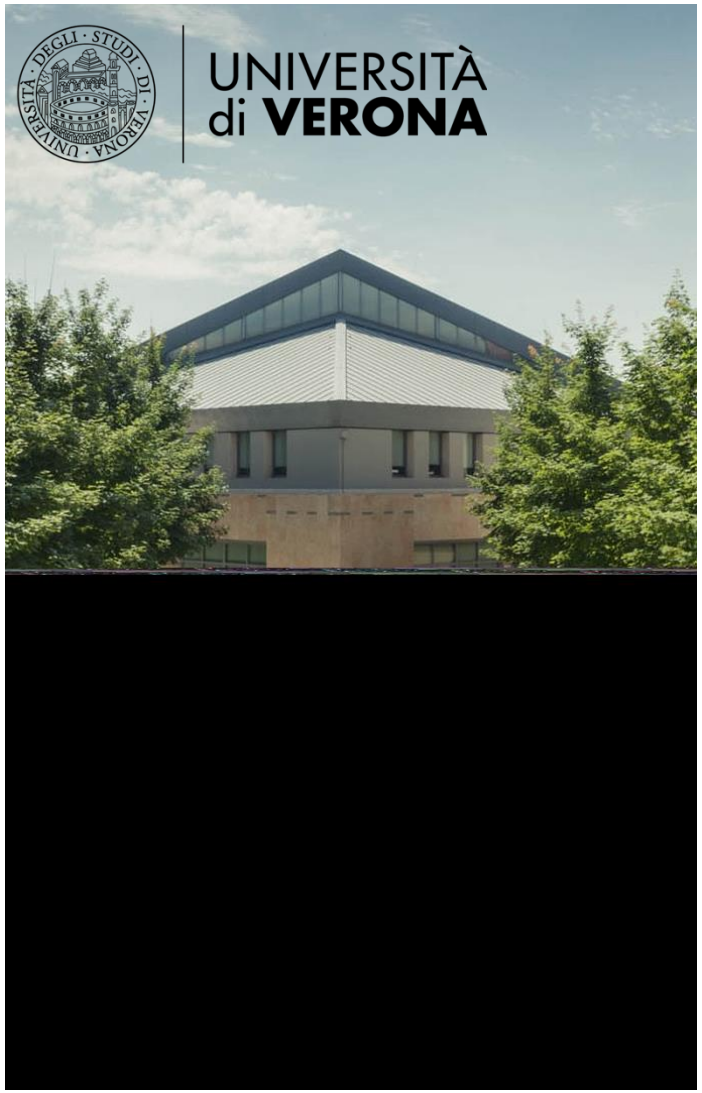
Tobacco wt



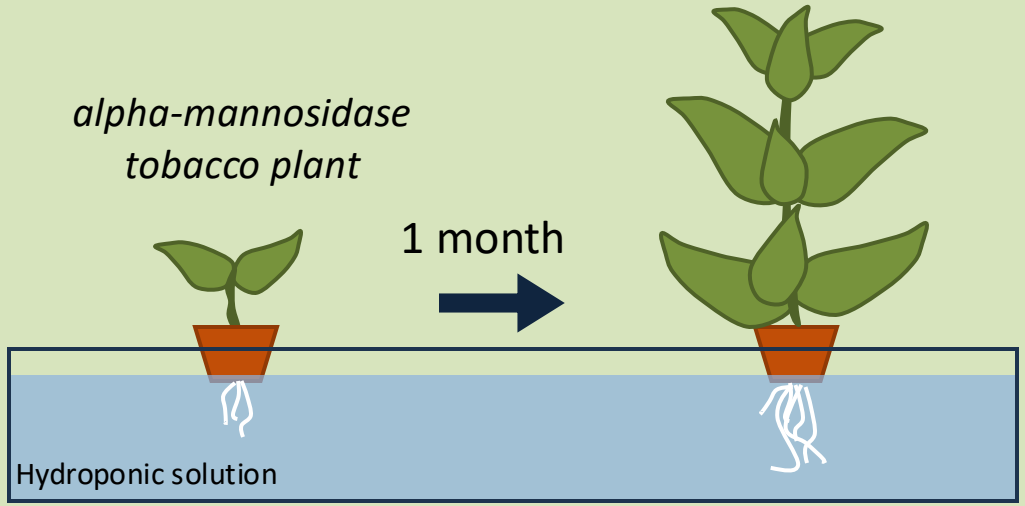
Tobacco alpha-mannosidase

Shoots transfer

Tobacco hydroponic culture



Hydroponics is the technique of growing plants using a water-based nutrient solution rather than soil

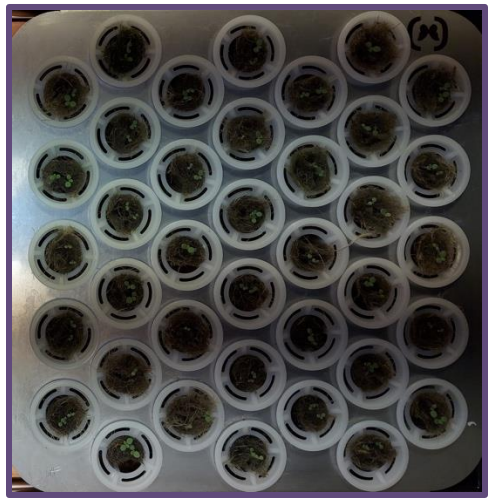


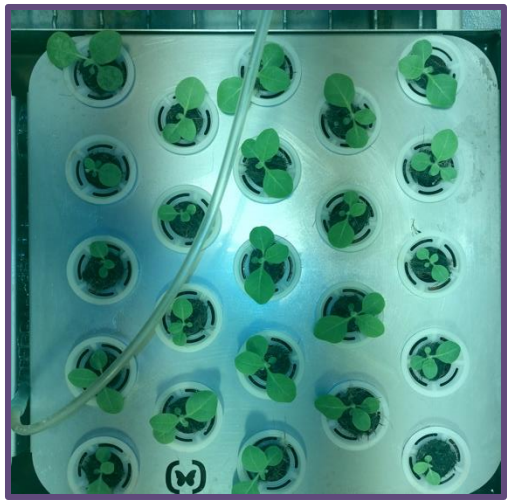
Hydroponic solution parameters adjusted every day:
pH 5.5
EC (Electrical conductivity)2.4 mS/cm

Fixed parameters:
Photoperiod 12h light/12h dark
Light intensity 250 µmol*$m^{-2}s^{-1}$

Tobacco hydroponic culture: *sampling (1)*

After germination they are transferred to plugs





Before cutting

After 6 weeks (from seeds) plants are cut and collected following different strategies

4 Plants

All leaves sampled together (P_1,P_2,P_3,P_4)

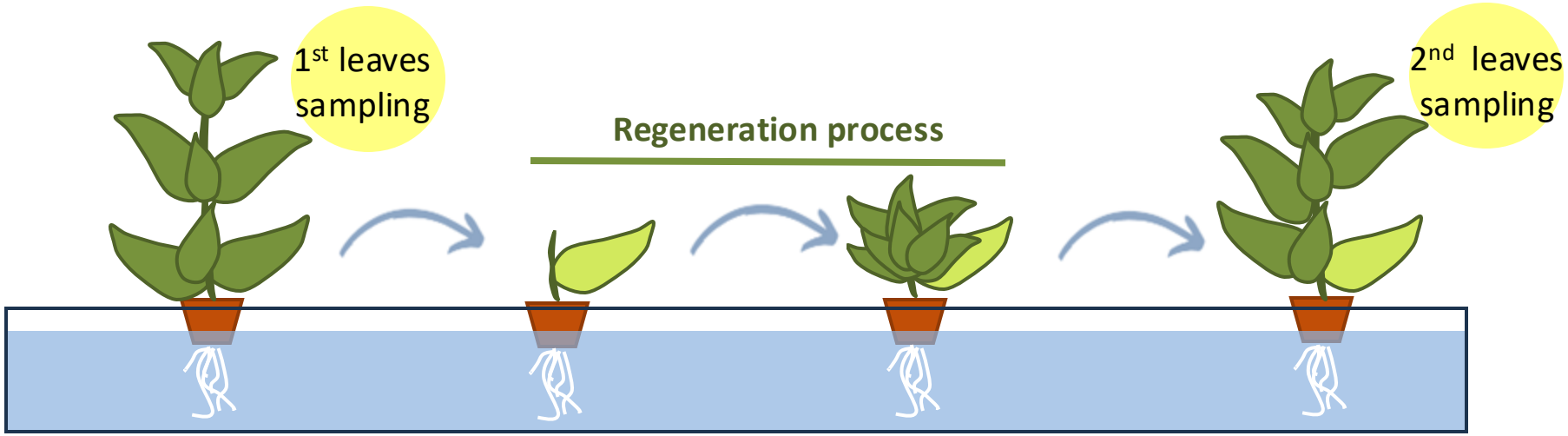
3 Plants

Leaves sampled at **different positions** Bottom(B), Medium(M) and Top(T)

(+ 4 plants for dry weight)

(2 batches)

Tobacco hydroponic culture: *sampling (2)*



After cutting they need 3 weeks to grow to the same size (around 30cm)

After cutting

Regeneration after 4 days

Why to work on regenerated shoots?

Time-saving

Waste reduction

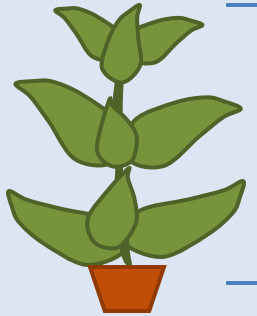
Different protein expression?

(2 batches)

Protein extraction and quantification

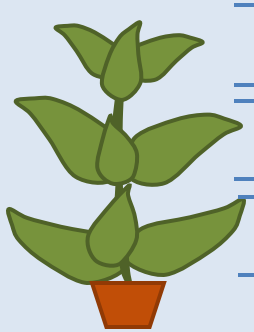
Samples legenda

Full plant



All leaves sampled together **P(n)**

Plant divided into sections:

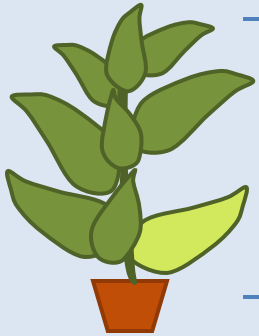


Top; **P(n)T**

Medium; **P(n)M**

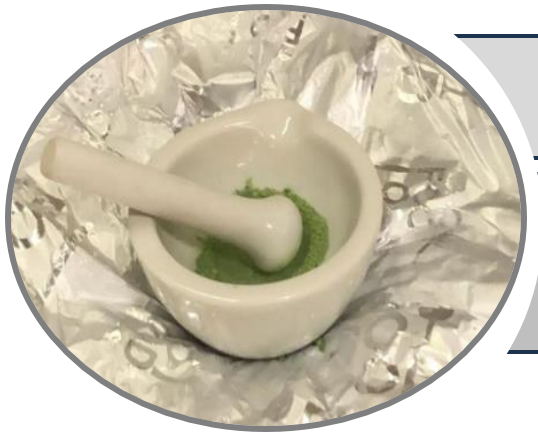
Bottom; **P(n)B**

Full regenerated plant:



All leaves sampled together **P(n)rig**

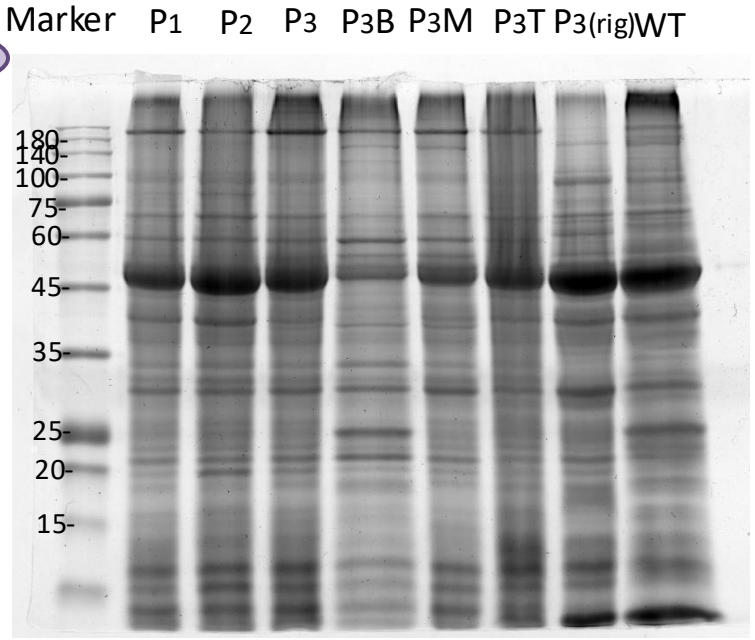
Total soluble protein extraction



Leaves are **grinded** with liquid nitrogen and homogenated

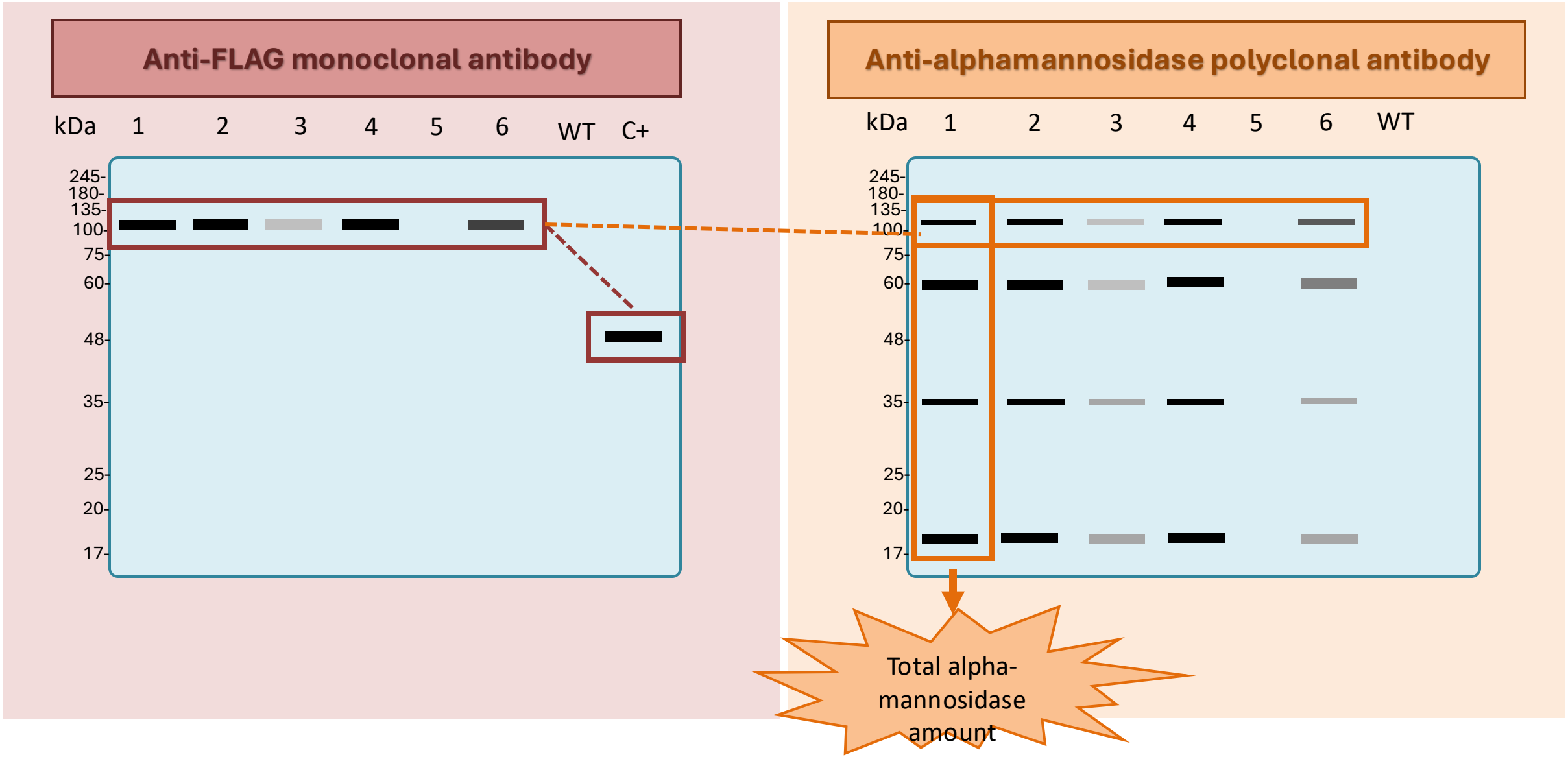
Different extraction systems were tested: best results by using Homogenation Buffer + **2% B-mercaptoethanol**

SDS-Page



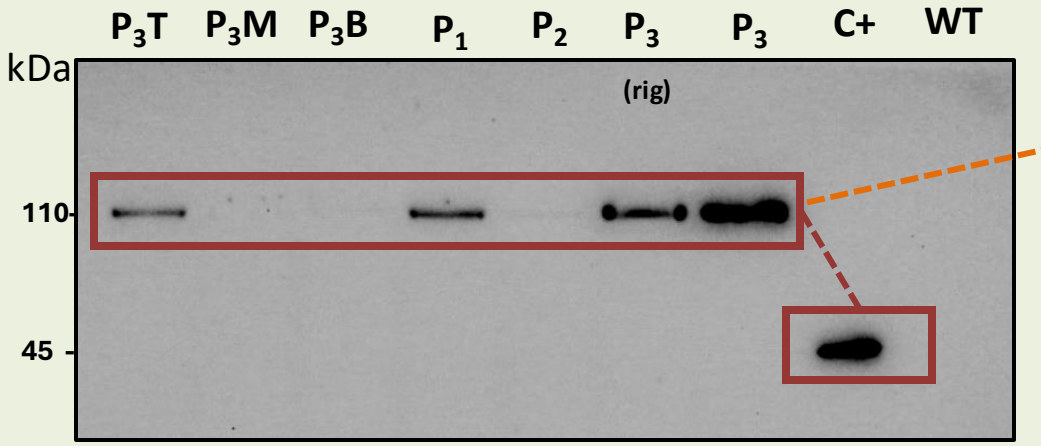
Protein quantification strategy

Western Blot analysis



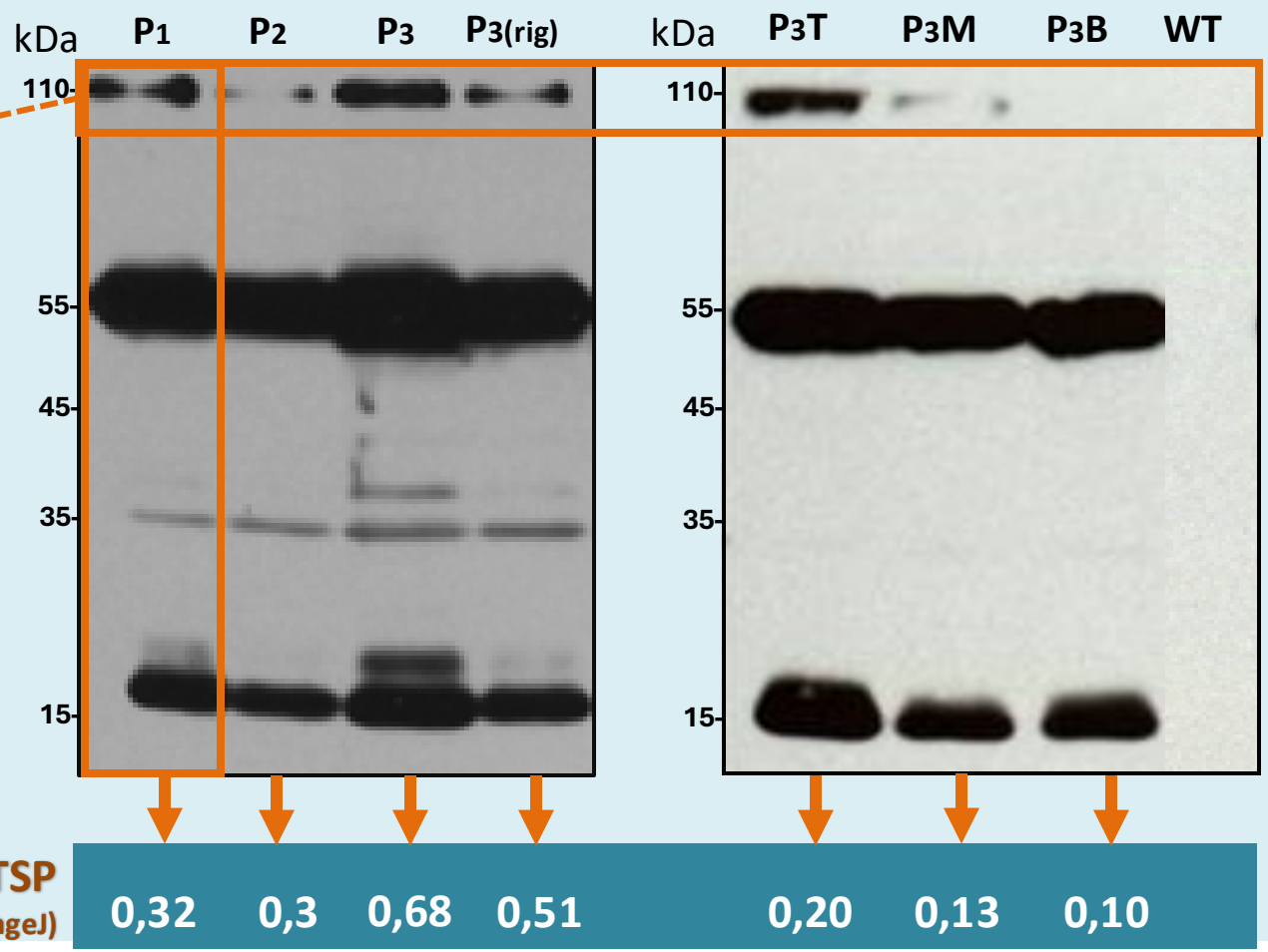
Protein quantification

Anti-FLAG monoclonal antibody



Legenda:
P(n)- all leaves of the plant are sampled together
P(n)T – leaves sampled at the top level of the plant
P(n)M – leaves sampled at the medium level of the plant
P(n)B – leaves sampled at the bottom level of the plant
P(n) rig – all leaves of the regenerated plant sampled together

Anti-alphamannosidase polyclonal antibody



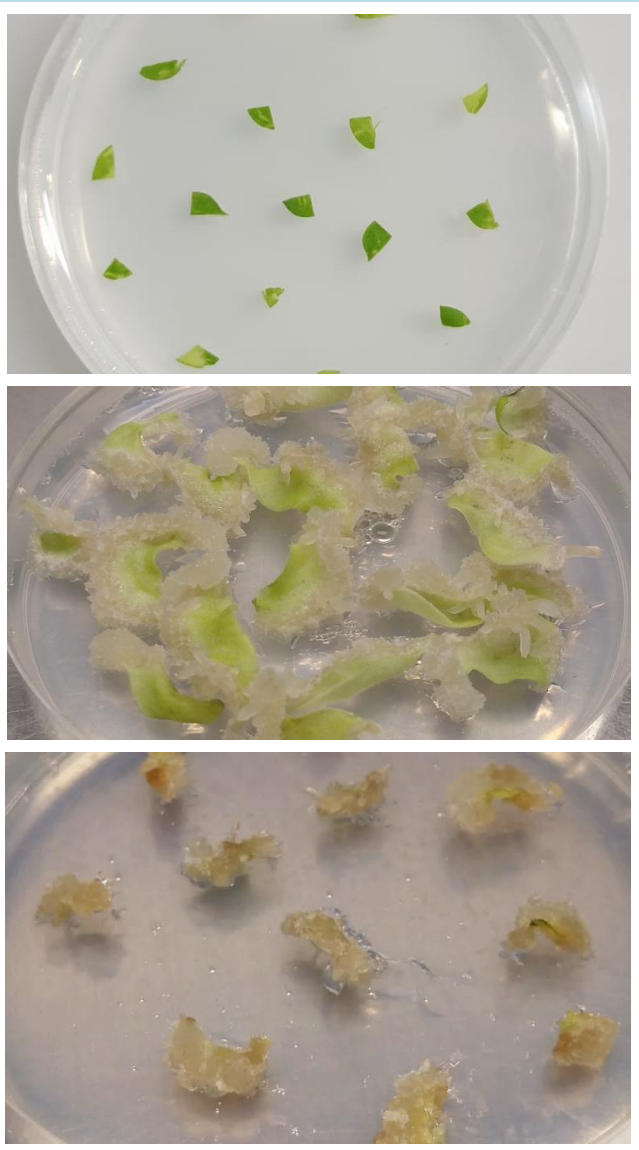
%alpha-mann/TSP
(estimated values through ImageJ)

0,32	0,3	0,68	0,51	0,20	0,13	0,10

Future goals

- Test other extraction methods to optimize the alpha-mannosidase quantity in all samples
- Repeat the analysis on the remaining samples and subsequent batches
- Proceed with the extraction of total soluble proteins from tobacco calli and compare the content between the two culture systems
- Study the enzymatic activity of alpha-mannosidase using a special kit, supporting the WB quantification

CALLUS CULTURE (MS0+ 0,5 mg/L 2,4D + 0,1 KIN)



Sample collection still in progress

Thanks for your attention!

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