Potential use of Heavy Metal Atpases (HMA) mutants to reduce cadmium translocation from root to leaf in tobacco.

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Metals in plants

- Essential: iron (Fe), zinc (Zn), manganese (Mn), copper (Cu) are used as co-factors in enzymes
- Non-essential: cadmium (Cd), lead (Pb), mercury (Hg)….can enter the plant using the same transporters
- Different mechanisms of detoxification exists:
  - Transport to major storage organs/tissues
  - Sub-cellular compartmentalization
  - Chelation
  - Efflux from the plant

Verbruggen et al., 2009
Current Opinion in Plant Biology

Cadmium in hyperaccumulators
GMO attempts to reduce cadmium in tobacco leaf

- Dorlhaç et al., 1998: plants expressing a human metallothionein gene
- Korenov et al., 2009: plants expressing AtCAX2 gene

![Cd distribution chart](chart.png)

- Cd distribution (% total Cd): 
  - Root: 70%
  - Stem: 15-25%
  - Midrib: 15-25%
  - Leaf: 15-25%

![Cd concentration chart](chart2.png)

- Cd µg/g dry wt:
  - TOP: 35SControl, 35SCAX2: 1-2 µg/g, 35SCAX2-14B: 2-3 µg/g
  - MID: 35SCAX2: 1-2 µg/g, 35SCAX2-14B: 2-3 µg/g
  - BOTTOM: 35SCAX2: 1-2 µg/g, 35SCAX2-14B: 2-3 µg/g
Non-GMO approach: HMA mutants to reduce Cd in tobacco

HMA P-type ATPases are the major mechanism for root-to-shoot Cd translocation in *Arabidopsis thaliana*

Wong et al., 2009
Two HMA genes identified in tobacco

- Blast search with AtHMA4 protein: one contig identified in tobacco, corresponding to exons 4 to 8 in A. thaliana
- Cloning and sequencing on *N. tabacum* and its ancestors
  - *NtHMAα* from *N. sylvestris*
  - *NtHMAβ* from *N. tomentosiformis*
- BAC library (CNRGV-Toulouse) to get the full length sequences
HMA expression in tobacco

Transcripts accumulation

- NtHMAα
- NtHMAβ

F Leaf  | Root
---|---
C     | a
C     | b
Zinc and Cadmium effects on transcripts accumulation in roots

For Zinc (Zn):
- 0 µM of Zn: No significant change.
- 3, 10, 30, 100 µM of Zn: Significant increase in transcripts accumulation.

For Cadmium (Cd):
- 0 µM of Cd: No significant change.
- 3, 10, 30, 100 µM of Cd: Significant increase in transcripts accumulation.

Both graphs show the accumulation of transcripts for NtHMAα and NtHMAβ proteins.
HMA genes silencing by miRNA

<table>
<thead>
<tr>
<th>Targets</th>
<th>pGreen</th>
<th>NtHMAα</th>
<th>NtHMAβ</th>
<th>NtHMA α NtHMA β</th>
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<tbody>
<tr>
<td>exon</td>
<td>-</td>
<td>6</td>
<td>11</td>
<td>9</td>
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</tbody>
</table>

qPCR

- Alpha
- Beta
Cadmium in aerial parts of miRNA lines

In vitro culture with Cd 1µM

<table>
<thead>
<tr>
<th>Targets</th>
<th>Cd (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGreen</td>
<td>a</td>
</tr>
<tr>
<td>NtHMAα</td>
<td>b</td>
</tr>
<tr>
<td>NtHMAβ</td>
<td>b</td>
</tr>
<tr>
<td>NtHMAαβ</td>
<td>c</td>
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</tbody>
</table>
Zinc and Iron in aerial parts of amiRNA lines

- Best results with both copies silenced
- Strong impact on Zinc with both copies
In vitro assessment of Cd and Zn in aerial parts with different Cd treatment

- Best results with both copies silenced
- Strong impact on Zinc with both copies
Identification of NtHMA mutants

M1 seeds → Self-pollination → M1 plants → Chemical agent

M2 seeds → Seeds collection → DNA preparation

Pooling plates → Mutated nucleotide
DNA GENE CIBLE
Wild type nucleotide

M2 plants

(Science et Vie, 2002)
Mutation screening on 4000 M2 families

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Silent</th>
<th>Missens</th>
<th>Nonsens</th>
<th>Intron</th>
<th>Splicing</th>
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<tbody>
<tr>
<td>NtHMAα</td>
<td>42</td>
<td>11</td>
<td>28</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NtHMAβ</td>
<td>29</td>
<td>5</td>
<td>21</td>
<td>1</td>
<td>2</td>
<td>0</td>
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</tbody>
</table>

**NtHMAα**
- WT: 410 bp
- Mutant: 283 bp
- Total: 16.5 kb

**NtHMAβ**
- WT: 386 bp
- Mutant: 283 bp
- Total: 16.5 kb
Cleaning and pyramiding mutants

\[ \text{NtHMA}_\alpha \text{ BC1} \]

\[ \text{NtHMA}_\beta \text{ BC2} \]

\[ \text{NtHMA}_\alpha \text{ BC2S1} \]

\[ \text{NtHMA}_\alpha \text{ BC2S2} \]

\[ \text{NtHMA}_\beta \text{ BC2} \]

\[ \text{NtHMA}_\beta \text{ BC2S1} \]

\[ \text{NtHMA}_\beta \text{ BC2S2} \]

\[ \text{NtHMA}_\alpha \ \text{F1} \]

\[ \text{NtHMA}_\alpha \ \text{F2} \]

\[ \text{NtHMA}_\alpha \ \text{F3} \]

\[ \text{NtHMA}_\beta \ \text{F1} \]

\[ \text{NtHMA}_\beta \ \text{F2} \]

\[ \text{NtHMA}_\beta \ \text{F3} \]
In vitro assessment of cadmium transport in *NtHMA*<sub>α</sub> BC2S2 mutants

- Significant impact on Cd for E200K and W78*
- No significant impact on Zn, Fe, Pb (not shown)

\[ \mu g \text{Cd/g of dry matter in aerial parts} \]

\[ 1 \mu M \text{Cd} \]

\( \alpha = \text{mutant in } NtHMA\alpha \)

\( \alpha = \text{wild type in } NtHMA\alpha \)
« Hydroponic » assessment of cadmium transport in F2 double mutants

\[ \text{Cd} \mu g/g \text{ dm} \]

\[ \text{Zn} \mu g/g \text{ dm} \]

\( \sigma = \text{mutant in } \text{NtHMA}\alpha \)

\( \alpha = \text{wild type in } \text{NtHMA}\alpha \)

\( \beta = \text{mutant in } \text{NtHMA}\beta \)

\( \beta = \text{wild type in } \text{NtHMA}\beta \)
Potential limitations with double HMA mutants

- Zinc is strongly affected in miRNA NtHMAα NtHMAβ plants:
  - Impact on development, growth and fertility

- This phenotype is also observed in some F2 double mutants:
  - Example of F2 (NtHMAα W78* NtHMAβ R42*)

Expected segregation with 2 independant genes in 354 plants from a F2 population:

<table>
<thead>
<tr>
<th>Genotype</th>
<th>αα/ββ</th>
<th>αα/ββ</th>
<th>αα/ββ</th>
<th>αα/ββ</th>
<th>αα/ββ</th>
<th>αα/ββ</th>
<th>αα/ββ</th>
<th>αα/ββ</th>
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<tbody>
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<td>Observed</td>
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<td>25</td>
<td>20</td>
<td>17</td>
<td>61</td>
<td>41</td>
<td>42</td>
<td>45</td>
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<tr>
<td>Expected</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>44</td>
<td>44</td>
<td>44</td>
<td>88</td>
</tr>
<tr>
<td>P</td>
<td>0.0078 &lt;0.01</td>
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<td></td>
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</tr>
</tbody>
</table>

Hussein et al, 2004

A. thaliana
hma2hma4
Conclusion and perspectives

- It is possible to decrease cadmium content in tobacco leaf with *NtHMA* mutants.
- Double mutants show a dramatic reduction of cadmium in aerial parts.
- Severe mutations lead to an impact on zinc, with consequences on morphology and fertility.

- The best combination of mutations in *NtHMA*α and *NtHMA*β must be defined to obtain the strongest Cd decrease in aerial parts, without affecting plant growth.
- In vitro and hydroponic results must be confirmed into the field.
  - Some F3 populations are currently evaluated in the field in Bergerac
The team

Victor Hermand (RNAi experiments and functionnal analysis of NtHMA)

Pierre Berthomieu
Françoise Gosti

And the team of the Tobacco Institute of Bergerac

François Dorlhac de Borne, Julien Cotucheau, Christophe Decorps, Céline Sentenac and the others...
Thank you for your attention!