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# The maize *vp*404* mutant is impaired in Moco-S biosynthesis.

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*to Deirdre, who inspired me*
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THE MAIZE *vp*404* MUTANT IMPAIRED in Moco BIOSYNTHESIS

# DESICCATION TOLERANCE


## *ABSTRACT*

In maize, desiccation tolerance is acquired by the embryo at a precise developmental stage, between 20 and 25 DAP (days after pollination) and is probably related to the maturation process characterized by the accumulation of storage products and LEA (late embryogenesis abundant) proteins, that have a protective role. In viviparous (vp) mutants, that are deficient in ABA synthesis or lack an active *vp1* factor, embryos do not express the normal set of maturation phase proteins; it is also conceivable that these embryos do not acquire the desiccation tolerance. To verify this, we applied premature desiccation to developing *vp* embryos about 25 DAP, and compared their germination capacity to embryos not subjected to such treatment.

When cultured immediately after their excision, immature embryos of all mutants tested germinated with a high frequency (95-100%) similarly to their wild type counterparts. On the other hand, if they were cultured following a premature dehydration treatment, only *vp1* and *vp10* maintained a partial desiccation tolerance while the other mutants lost it. These results seem to suggest that acquisition of desiccation tolerance requires the completion of the steps between carotenoid production and the later stages of ABA biosynthesis. They also suggests the presence of a link between embryo morphogenesis and desiccation tolerance which should be further investigated.

Another aspect we could consider is the possibility of a protective role of carotenoid against oxidative phenomena that take place even during desiccation, because the metabolism is still active.

# THE MAIZE *vp*404* MUTANT is IMPAIRED in Moco BIOSYNTHESIS


## *ABSTRACT*

Molybdenum is an important micronutrient for almost all organisms, since, in the form of Molybdenum cofactor, is a component of different enzymes. In plants there are four Moco enzymes, grouped in two families, Nitrate Reductase (NR) and Sulfite oxidase (SO), requiring the dioxoform, Xanthine Dehydrogenase (XDH) and Aldehyde Oxidase, with a third terminal sulfur ligand.

In maize the *vp10* and *vp15* genes have been isolated; they encode the orthologs of the Arabidopsis *Cnx1*, and *Cnx7* respectively, which catalyse the last steps of Moco-O biosynthesis. Moco-O is required for the activity of both Nitrate Reductase and Sulfite Oxidase.

Maize mutants defective in Moco-S are instead unknown, but Moco-S mutant plants have been described for Arabidopsis (ABA3), tomato (flacca) and tobacco (ABA1), which lack AO and XDH activities, but show normal NR enzymatic activity. These mutants show a wilty phenotype and are impaired in stress responses.

*vp*404* is a viviparous mutant with light green seedlings, reduced content of chlorophylls, carotenoids and ABA.Complementation tests with all viviparous mutants with green seedlings reported in literature showed that *vp*404* defines a new *vp* gene.

Moco enzyme activities analysis showed normal value for SO and an extremely reduced activity for both AO and XDH, suggesting that *vp*404* is likely a Moco-Sulfurase defective mutant. In *Arabidopsis* the ABA3 gene encodes the Moco-Sulfurase.

Blast analysis performed with the ABA3 sequence , gave two sequences with high Identity on chromosome 6. The two sequences, which are 30kb distal, encode the Moco-S N-terminal and the Moco-S C terminal domain respectively. However,since in all Eukaryotes analysed the two protein domains are encoded by the same gene, we refer to the gene in *Sorghum* as

a model, and we have reconstructedin silico the structure of a maize unique theoretical gene, in which the C terminal domain was correctly oriented and 30,000 bp between the two domains were arranged.
Specific primers were designed on the basis of the theoretical gene and the corresponding cDNA sequence was analysed by means of a RT-PCR approach. Preliminary data seem to confirm the proposed gene model, however gene isolation and sequence analysis will be necessary to confirm our hypothesis.

# CHAPTER 1

*SEED DORMANCY*

## 1.1 Introduction

Temporary delay of mature seed germination, a complex phenomenon classified under the term dormancy, is still not well understood (34, 36). There are different ways describing dormancy, reflecting ecological, evolutionary or simply agronomical point of view, but waiting for an optimal condition to germinate, for desiccated seeds, is a basic condition that has permitted the species survival among the environmental variation. By germinating only under a predictable subset of environmental conditions, plants do not need to adapt de novo in other traits expressed at later stages, provided those traits are adapted to the subset of environments determined by their germination phenology. (59). The plant diffusion through the continents as well as the local variations have selected different grades of dormancy, where the simple optimal condition (e.g. water and temperature) is flanked by new factors necessary for the germination.

The release from this block can happen, for these seeds, only under particular conditions, as disruption of seed coat (scarification), a period of time (after ripening), moist chilling (stratification) or after a burn (after burning) (39).

We do not know a priori what kind of history is related to each species, but the result of the complex interactions, between the seeds and their variable environment, is written in the dormancy of every single seed (15).

This is an enormous potential for species survival, but the standard agronomical practice, requiring an almost synchronic development of seeds cannot take advantage of the delayed germination and the reservoir of ungerminated viable seeds is only a loss for farmers.

It is obvious that the absence of dormancy is an undesirable phenomenon, and could be a serious problem having pre-harvest sprouting in cereals like rice, barley wheat and maize (16) or reduced longevity that can happen to

non-dormant seeds (26). Dormancy can be classified, in relation to the main constrain, in physical, physiological, embryo development related or in terms of tissue or seed structure involved in the process. Primary dormancy is acquired during seed maturation and the beginning of germination can take place when the environmental conditions are optimal. Imbibed seeds can acquire secondary dormancy if the conditions are unfavourable, because of temperature, light or nitrate. Furthermore there is the possibility that seeds, when the condition are suboptimal, switch to dormancy performing a seasonal cycle until a favourable condition to germinate or after a number of unfavourable seasons they die. From the secondary dormancy, where seeds do not germinate even if the environmental condition are optimal, to the primary dormancy, there are intermediate “steady state” with different rate of germination related to a slightly suboptimal condition (51).

Germination can be defined as the radicle emergence from the seed coat, when the plant leaves the quiescence and the embryo mobilizes the nutrients, starting cell division and elongations, with biochemical modification of the embryo and its surrounding tissues. It is difficult to define the signal inducing dormancy release and subsequent germination, also because it is not clear which is the process blocked during the dormancy acquisition. Seeds germinate after a burn, when the coat is damaged, but can germinate even if they are imbibed of chemical substance derived from the burned wood (as nitrogen oxides and butenolide) (38).

Gene expression, enzyme activity and hormones play an effective role in dry seed, showing a metabolic activity, with genes and subsequent proteins that remodel the biochemical structure of embryo during desiccation and preparing the molecules for the successive germination (23, 31).

In this contest is very difficult to discriminate qualitative from quantitative characters, or if the signal is acting alone or synergically.

## 1.2 Plant grow regulation of dormancy

### *1.2.1 Abscisic Acid*

During embryo maturation abscisic acid (ABA) seems to play an important role in dormancy induction (34, 64). Accumulation of ABA is correlated with dormancy induction whereas ABA deficient seeds because of mutation, or block of ABA activity with inhibitors, reduce dormancy. Overexpression instead leads to enhanced dormancy (82, 98). ABA exogenous supplied or from maternal tissue cannot restores ABA deficiency, suggesting that it has been produced by embryo or surrounded tissues (34, 35). Hormone balance is due to catabolism mediated by ABA 8'-hidroxylases encoded by the cytochrome P450 CYP707A family (106) and accumulation is followed by seed imbibition (1, 44).

Key enzymes involved in ABA metabolism are members of the 9-cis-epoxycarotenoid dioxygenase family (NCED) essential for hormone synthesis in both embryo and endosperm during dormancy induction (77). Aba Aldehyde Oxidase (AAO) catalyse the reaction converting Aba Aldehyde (AA) in ABA is another important enzyme. In *Arabidopsis* SDR1 and AAO3, expressed in localized cell type, such as stems and vascular tissue, overlap with NCED genes (70, 24). *vp10/13* and *vp15* define a class of maize viviparous mutants, accumulating chlorophyll but with lethal phenotype, defective in AAO, suggesting an essential role in ABA biosynthesis or signalling (108, 134).

Dormancy maintenance is related, other than NCED enzymes, to ZEP (zeaxanthin epoxydase). Whereas dormancy release is due to ABA catabolism (23, 88). ABA induces desiccation tolerance in the late stage of embryogenesis; scavenge active metabolite species (AOS) can possibly damage embryo tissues during the dehydration, (7, 13).

### *1.2.2.Giberellins*

Gibberellins (GA from Gibberellic Acid) are a family of tetracyclic diterpenes. They stimulate germination inducing hydrolytic enzymes that causes an increase of permeability of the endosperm tissue or seed coat, permitting the

mobilisation of storage reserve, stimulating cell expansions of embryo tissues (15). GA alone does not stimulate dormancy release in all species or in fully dormant *Arabidopsis* seeds (1, 15) and a decrease of ABA levels may be required before GA levels and sensitivity can increase (1, 60). Light promotes GA synthesis; light and GA promote the degradation of ABA in imbibing lettuce seeds (135).

Analysis of GA mutants suggesting that GA synthesis is essential for dormancy release; in *Arabidopsis* there is an increase of GA4 level before initial radicle protrusion related with GA-responsive gene induction (25, 105). Unlike dicots, cereals do not require GA for germination. This discrepancy may reflect differences in the structure and composition of these seeds, or an unknown source of redundancy in GA signaling. The balance between ABA/GA is still responsible for dormancy/germination equilibrium, but probably only for the dormancy breakdown (34).

### *1.2.3 Ethylene*

Ethylene promotes dormancy breaking interacting with ABA signaling. Resistant (*etr*) and insensitive (*ein*) ethylene, display-increased dormancy correlated with increased sensitivity to ABA in seed germination and increased ABA synthesis (25, 42). Treatment of wild-type seeds with the ethylene precursor 1-aminocyclopropane -1-carboxylic acid (ACC) result in decreased sensitivity to ABA (42). *etr1* and *ein2* mutants also overaccumulate GA, suggesting that ethylene could stimulate germination via antagonism of ABA signaling.

### *1.2.4 Brassinosteroids*

Brassinosteroids (BRs), known for their involvement in stem elongation, are involved in seed germination, even though mutants (biosynthesis and signaling) are not germination defective. Instead they are involved in ABA sensitivity, decreasing germination potential (27, 131).

### *1.2.5 Nitric Oxide, Nitrate, and Active Oxygen Species*

Nitrogen compounds, and Active Oxygen Species, promote dormancy release in many species. Enzymatic NO production in plants occurs primarily via

nitrate reductase (NR) from nitrite and nitrate (14) whose identity is still ambiguous (30). Seeds of NR-deficient plants accumulate nitrate and have reduced dormancy (1). Nitrogen-containing compounds may promote seed germination via effects on metabolism, oxidation state, or signaling (14). Nitrate and dormancy-relieving conditions induce the expression of some genes that encode enzymes in the pentose phosphate pathway, which may provide essential nutrients for germination (33). NO, nitrite, and nitrate may stimulate the pentose phosphate pathway, and therefore germination, by increasing oxidation of NADPH to NADP+, a limiting electron acceptor. Nitrate can also modify hormone levels by inducing the expression of enzymes that catalyze ABA deactivation and GA biosynthesis (33).

## 1.3 Molecular biology of seed dormancy

### *1.3.1 Loci encoding dormancy regulating proteins*

There are numerous loci that encode dormancy regulating proteins (12), including standard signaling proteins as the G proteins, transcriptional regulators, phosphatases and kinases regulating transcription factors activity. A variety of mutants, which have major defects in seed maturation, result in nondormancy (34, 35). These loci encode transcriptional regulator but because they are a part of the regulatory hierarchy, many of their effects are indirect and, owing to their pleiotropic effect, this mutants do not produce viable desiccation tolerant seed. Probably mutants do not reach the stage where many of these genes are expressed.

### *1.3.2 Transcriptional and post-transcriptional regulators*

Studies of *ABI*-like orthologs genes in dicots, conifer and cereal (34, 64) show the importance of these genes in dormancy regulation. Studies on cereal cultivars with different degrees of dormancy and their wild relatives show some correlation between the depth of dormancy and the high expression of the *vp1* (*ABI3*) gene from oats (62) and wheat (97). Wheat and Barley *vp1* gene appear to defect in splicing, such that the non-properly functional protein

is produced, and then these species are predisposed to preharvest sprouting (88). Further studies (including QTL) re required to demonstrate the role of these genes in controlling seed dormancy.

Correlations in expression of “dormancy genes” are observed for factors that antagonize each other as well as for those that promote the same process. Although transcriptional profiling studies of dormancy cycling in the highly dormant Cvi ecotype of *Arabidopsis* show a preponderance of ABA-responsive element (ABRE)- containing genes correlated with dormancy, *Arabidopsis* differs from cereals in that none of the *ABI* transcription factors are included in this set and *ABI4* actually appears in the after ripening set (11, 33).

Researchers have identified mutations resulting in increased dormancy and/or ABA hypersensitivity at germination that disrupt RNA capping miRNA biosynthesis; mRNA splicing, export, and degradation; and degradation of polyadenylated RNA (72).

Transitions between different states of dormancy are active processes that involve changes in gene expression even in dry after-ripening seeds. The expression of dormancy-associated genes correlates quantitatively with depth of dormancy. Comparison of dormant versus non-dormant transcriptomes and proteomes indicates regulation at multiple levels (e.g. transcript accumulation, translation, protein stability, and modifications affecting activity).

### *1.3.3 LEA proteins*

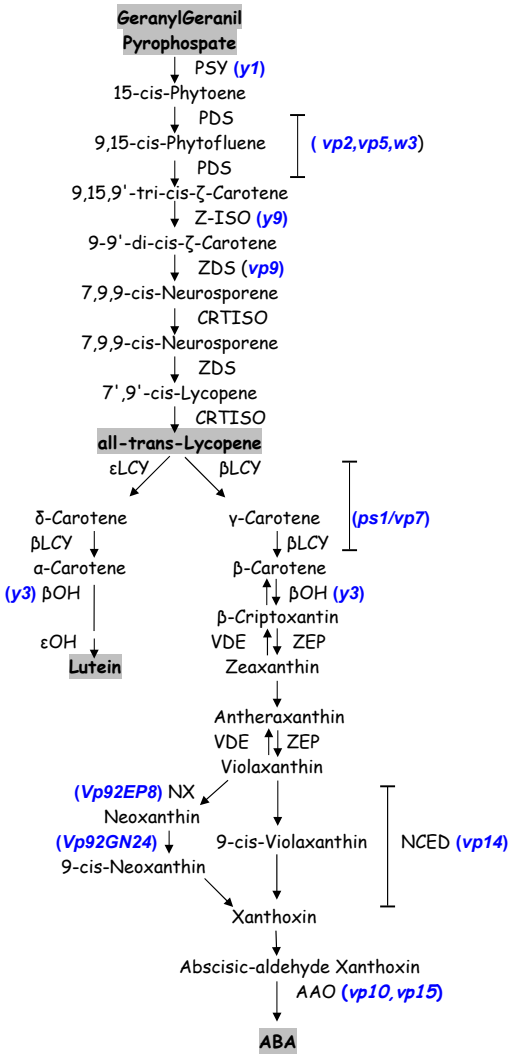
Another important category of molecules related to protection of desiccating tissues is the late embryogenesis abundant (LEA) proteins (13), even though their transcript level does not seem correlate with dormancy (11, 33).

Combination of LEA and sugars form an intracellular “glassy state” that protects organelles from desiccation damage. In addition the pigments of seed coat (testa), generally phenolic compounds, crossed linked to wall components by oxidation, can generate a thick cell layer, which results is a mechanical resistance and reduced permeability, with reduction of degradation phenomena inside the seed.

## 1.4 Maize viviparous mutants

In maize vivipary, the precocious germination of the seed while still attached to the ear is the diagnostic phenotype of mutations impaired in the biosynthesis or the sensitivity of the embryo to Abscisic Acid (ABA)(Fig. 1.1) Because of their easily detectable phenotype, these mutants have been recognised for a long time. Eight non-allelic mutants were reported by Mangelsdorf (84) and were later characterized genetically (114).
The list of viviparous mutants includes 15 genes: *vp1, vp2, vp5, vp7, vp8, vp9, vp10, vp12, vp14, vp15, al1, y9, w3, rea* and *dek33* (87), grouped into three classes. Mutants *vp1* and possibly *rea*, referred to as response mutants (class 1), define genes not impaired in ABA biosynthesis. Class 2 includes mutants with reduced or suppressed carotenoid accumulation in both endosperm and vegetative tissues (*vp2, vp5, vp7, vp9, vp12, al1, y9* and *w3*) as a result of a mutational block in early biosynthetic steps before the branching point that separates ABA and carotenoid biosynthesis (127). Class 3 includes *vp8* and *vp14*, which affect later steps of the biosynthesis (122), as well as *vp10* and *vp15* that regulate the synthesis of the molybdenum cofactor required for the last step in ABA biosynthesis (108, 134). The relationship between *dek33* and ABA has not yet been investigated.

Fig.1.1 ABA pathway. Enzymes are in uppercase and the viviparous impaired in related steps are between brackets.



# CHAPTER 2

*SEED DESICCATION*

## 2.1 Introduction

Following morphogenesis, developing seeds enter a maturation stage, where the seed reorganizes metabolism and starts to accumulate reserves (starch, protein and oil). After this phase the seed enters a period of "maturation drying" (66). In recent studies use of desiccation was preferred, more precise because related to a major loss of water, in preparation to a quiescence (65). Modern genomics in the last period has analysed in detail the stage of desiccation showing a complex dynamics of gene expression and metabolic reactions (116, 101). This is in fact a demarcation line, between the end of seed development, and the beginning of germination, depending on how long is the quiescence period. The borders are one of the most interesting objects in biology, and spacing from limit of different stages transitions (e.g. transition between desiccation and germination), to cell membranes up to ecotones. The borders are particularly rich because of in a small space there are both object belonging to the two compartments, special entity strictly related at the border but not confined there, and finally regulatory mechanisms controlling the exchanges between the compartments.

## 2.2 Seed storage compounds

The orthodox seeds (113) are those that tolerate desiccation and can be stored for a long period of time termed after-ripening period, that is species specific, depending on moisture, amount of seed storage molecules, seed coats and temperature (85).
In this period the seed maintains low metabolic activity, there is a decrease of germination inhibitors and a series of structure (e.g. membrane) alterations

that predispose the seed to germination takes place (34). Among the molecules stored and/or synthesized, there are di- and oligo-saccharides (8), storage proteins (139), late embryogenesis abundant (LEA) proteins (125) and antioxidants (7).
Metabolism requires oxygen but the seed structure does not permit a quantitative flux because of the absence of stomata, sometimes because a thick coats, or the accumulation of oil.

## 2.3 Dehydration process

### *2.3.1 Energy demand*

Anatomical change, owing to the accumulation of seed storage and the increase of density prevent oxygen penetration (18) and energy remains a limiting factor (141, 43). The picture, then, is an oxygen demand to sustain metabolism versus a low flux of gas, some resource could come from photosynthetic activity, but only for green seeds. This condition increases of intensity because desiccation leads to a glassy state of seed matrix, which slows the oxygen flux (22, 115). It is not clear if energy demand is due principally to dehydration process or to other metabolic reactions, and the lowered level of oxygen even tough limits metabolism level, could protect the cell structure and proteins from damage (18). It is still difficult to understand what is the energy source sustaining dehydration process.

### *2.3.2 Genes transcription*

During the transition from reserve accumulation to desiccation stage, there is a considerable changes in gene expression. Analysis of transcripts in *Arabidopsis thaliana* at the stage of seed desiccation (4) indicates that the expression of 6963 genes (30% of genome), have a significant change (Fig.2.1). Only 21% of these genes begins during the late stage of reserve accumulation and continues without change of trend during desiccation.
Of these genes, 43% are upregulated, including heat shock and Late Embryogenesis Abundant (LEA) genes, as well as Maturation (MAT) genes

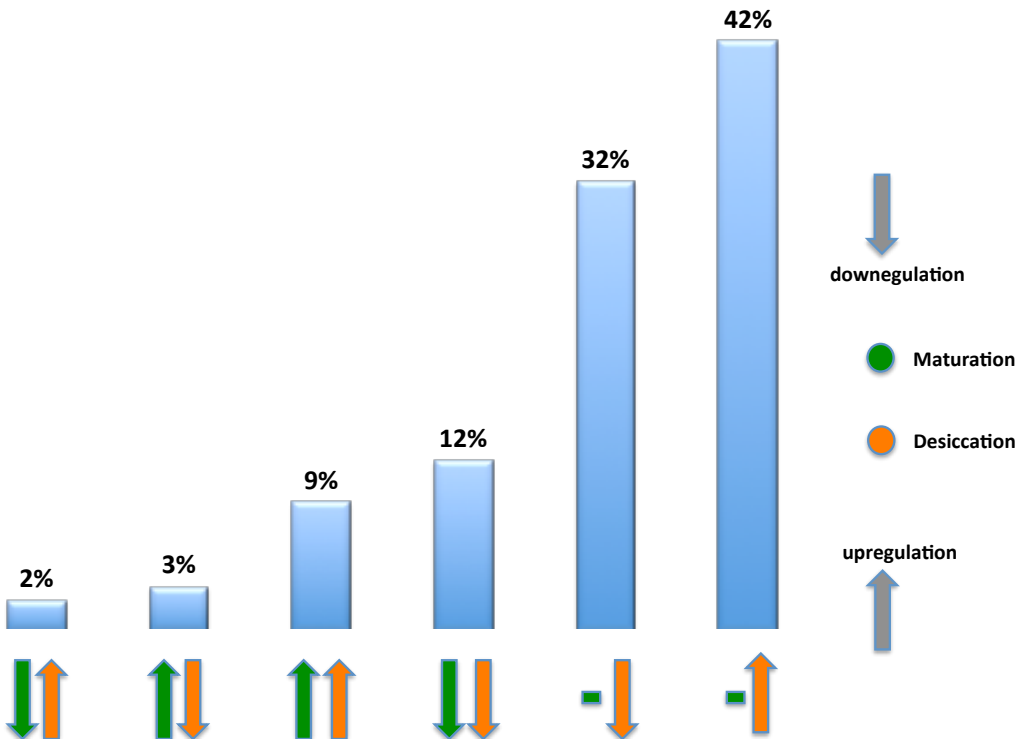
(143). The suppression of the latter genes, mediated by RY/Sph element repression system during desiccation, continues in the early seed germination phase by the VAL1/HSI2 family repressors (46), indicating a probable link between these two stages.
Expression changes of a consistent number of the 6963 genes is associated with desiccation, and the expression of genes related to germination declines during desiccation period, such as some genes in TCA cycle, glycolysis, cell wall metabolism, synthesis and transport of nucleotides and amino acids. These genes are down-regulated during desiccation and up-regulated during germination (59).
Many of the processes occurring during desiccation, probably serve for germination. This hypothesis is supported by the observations that genes encoding ribosomal proteins, translation factors (99, 23), proteases, peptidases, proteins related to the proteasome or associated with energy metabolism, DNA repair and lipids degradation (114), are up-regulated during seed desiccation.
Transcription modification, between late maturation and germination in the embryo, indicating that some germination programs begin in the desiccating seeds (131).

### *2.3.3 Level of metabolites*

Another important change in the same transition period, is in the level of some metabolites, such as sugars, tocopherols, flavonoids and intermediate of TCA cycle, amino acids and fatty acids (10, 107).
Sugar, belonging to Raffinose-family oligosaccharides (RFO), seems related to the membrane stabilization during dehydration (57) and provide substrate for energy production during germination.
Flavonoids are chemical barrier against infections (110) and contribute to decrease permeability to solutes, limiting damage in the early germination (112), and Tocopherols are antioxidants.
Amino acids, instead, probably support translational process, since their number decrease during early germination stage (33), whereas about 15% of the oil synthesized is degraded during desiccation period. Oil degradation

probably supports energy production during desiccation (9) as well as early germination (33).
Contribution of every single process to the germination, integration of transcriptome, proteome and metabolome, such as the energy sources fuelling seed desiccation (4) are still unclear.

Fig. 2.1 Distribution of genes whose expression is altered during seed desiccation. Rows indicate down- and up-regulation; colors represent maturation stage (green, left side) and desiccation stage (orange, right side). (Angelovici R. *et al* 2010 modif.).



# CHAPTER 3

*DESICCATION TOLERANCE IN MAIZE*

## 3.1 Results and discussion

In maize, desiccation tolerance is acquired by the embryo at a precise developmental stage between 20 and 25 DAP (days after pollination) and is probably related to the maturation process characterized by the accumulation of storage products and LEA (late embryogenesis abundant) proteins with a protective role. Viviparous mutant embryos that are deficient in ABA synthesis or lack an active *vp1* factor do not express the normal set of maturation phase proteins and do not acquire desiccation tolerance. To verify this, we applied premature desiccation to developing *vp* embryos at about 25 DAP, and compared their germination capacity to sibling embryos not subjected to such treatment. Germination was determined after 10 days of culture.

The test was conducted on the mutants in our collection (tab.3.1), either on mutant seeds by selecting and germinating those mutants arrested in an early phase of germination (incomplete germination), as well as on corresponding normal sibs, and by comparing the germination rate of immature *vp* embryos, before or after artificially induced desiccation.

When cultured immediately after their excision, immature embryos of all mutants tested germinated with a high frequency (95-100%) like their wild type counterparts (data not shown). On the other hand, if they were cultured following a premature dehydration treatment, most of them didn't germinate. Only *vp1* and *vp10* maintained a partial desiccation tolerance. They showed a germination frequency of 77% and 78% respectively. Also *vp5* showed a minimal germination (13%), consisting of primary root protrusion without a shoot., whereas the other mutants seemed to have lost their germination capacity (Tab. 3.2, Fig. 3.1).

The viviparous 1 (*vp1*) mutant, which has an ABA insensitive phenotype, encodes a complex transcription factor, is a major regulator in seed

development, activating embryo maturation and repressing germination. *vp10/13* and *vp15* define a class of maize viviparous mutants accumulating chlorophyll but with lethal phenotype, defective in AAO, enzyme catalysing the last step of ABA biosynthesis; *vp 5* is involved in the early steps of ABA biosynthesis, where the hormone pathway has steps in common with carotenoids biosynthesis, and *vp5* mutant is albino.

These results suggest that the acquisition of desiccation tolerance requires the completion of the steps between carotenoid production and the late stage of ABA biosynthesis.

Another aspect we could consider is the possibility of a protective role of carotenoid against oxidative phenomena that take place even during desiccation, because the metabolism is still active. Further analysis should be done on total protein extract, performing a bi-dimensional electrophoresis, in order to separate proteins in classes, comparing green and albino mutant with their wt siblings.

Tab. 3.1 Mutants Origin and phenotype description.

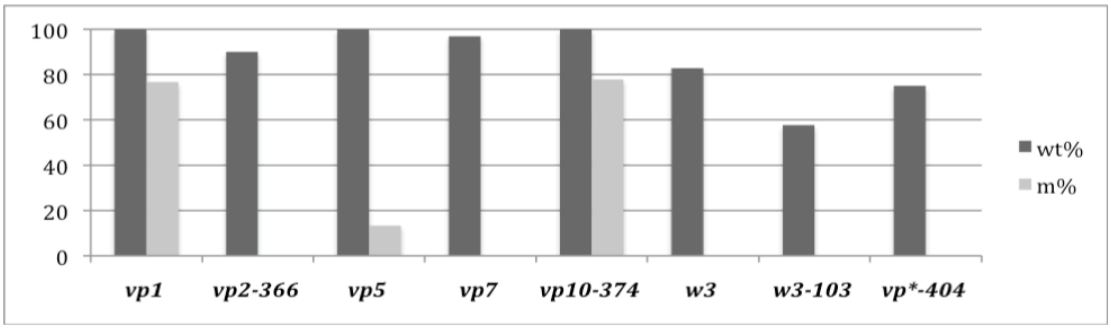
| *Mutant* | *Origin* | *note on Phenotype* |
|---|---|---|
| | | |
| *vp1* | Ac stock | _ |
| *vp2-366* | EMS to seeds | In.ger.[a], reduced leaf margin |
| *vp5* | Ac stock | reduced carotenoids content |
| *vp7* | Ac stock | reduced carotenoids content |
| *vp10-374* | EMS to seeds | Enrolled leaves, In.ger., callus[b] |
| *w3* | Ac stock | reduced carotenoids content |
| *w3-103* | d2 stock | In.ger., distorted leaves |
| *vp*-404* | EMS to seeds | Enroled leaves, In.ger. |

a. Incomplete germination.
b. Tendency of immature embryos to yield callus.

Table 3.2 Germination percentage of different viviparous (vp) and corresponding wild-type (WT) embryos following premature dessication

| DAP | | WT | | vp | |
|---|---|---|---|---|---|
| | Mutant | n° seeds | % germin. | n° seeds | % germin. |
| | | | | | |
| 28 | *vp1* | 31 | 100 | 30 | 77 |
| 25 | *vp2-366* | 10 | 90 | 9 | 0 |
| 24-26 | *vp5* | 30 | 100 | 30 | 13 |
| 24 | *vp7* | 32 | 97 | 30 | 0 |
| 26 | *vp10-374* | 24 | 100 | 18 | 78 |
| 26 | *w3* | 29 | 83 | 38 | 0 |
| 24 | *w3-103* | 33 | 58 | 17 | 0 |
| 25 | *vp*404* | 20 | 75 | 8 | 0 |

Figure 3.1. Effect of premature desiccation on germination of wild-type (wt) and viviparous sib embryos (m) following premature desiccation.




## 3.3 Material and methods.

### *3.3.1 Origin of the mutants*

Mutants of reference genes were obtained from the Maize Genetics Stock Center except *vp1*, a gift from Dr. D. Robertson. The origin of the mutants analyzed here is showed in Tab.3.1. They were originally isolated in different genetic backgrounds and were outcrossed once to B73 and W64A inbred lines to obtain vigorous F2 ears as a source of material for the embryo rescue experiments.

### *3.3.2 Embryo rescue*

Immature F2 ears were harvested at 25 Days After Pollination (DAP), treated with Na hypochlorite: $dH_2O$ (1:1 v/v) solution for 30 min and then rinsed in sterile water. Mutant embryos on segregating ears are recognizable because of incipient vivipary or pale yellow seed (for albino mutants). The *vp* and normal sibling embryos were excised and transferred to plant cell culture vessels or in petri dishes, in sterile ambient under laminar flux hood.

### *3.3.3 Premature desiccation*

Excised *vp* and normal siblings were transferred to plant cell culture vessels (Phytatray Sigma) on basal MS medium containing 2% sucrose solidified with 0.8% agar, or in petri dishes if subjected to desiccation. Embryos were maintained in a growth chamber at 25°C with a 14/10 h light/dark photoperiod. Seedling elongation (from the first node to the tip of the third leaf) was determined after 8 days of culture. For the drying treatment, embryos were laid between two disks of blotting paper within a sterile Petri dish and incubated in an oven at 35°C for 48 h. At the end of treatment the dishes, which was added silica gel, were sealed with Parafilm and conserved at 5°C. Samples were stored for 2 months until the start of the germination test on MS medium. For this purpose, embryos were maintained in a growth chamber at 25°C with 14/10 h light/dark photoperiod. After 10 days of culture, germination and seedling elongation were determined. The parameters employed to define germination were protrusion of the primary root and/or shoot

# CHAPTER 4

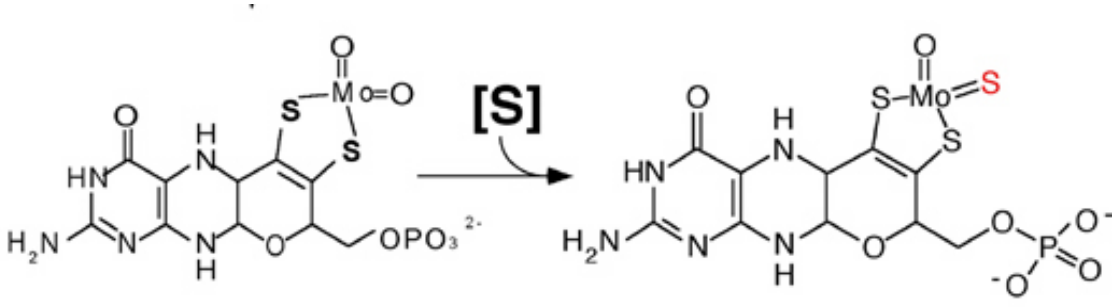
*MOLYBDENUM COFACTOR*

## 4.1 Introduction

Molybdenum is an important micronutrient for almost all organism. It is generally present as a part of enzymes in the form of Molybdenum cofactor, covalently bonded to the dithiolate moiety of tricyclic pterin, molybdopterin (MPT) (110). The exception is Nitrogenase, with an iron-Mo cofactor (FeMoco) (2). Pterin positions molybdenum in the active center, where probably participates in the electron transfer.
The reaction catalysed by Molybdo-enzyme are involved in the important cycles of Carbon, Nitrogen and Sulfur (55).
In plant there are four Moco enzymes, grouped in two families (54, 68) (Fig. 4.1): Nitrate Reductase (NR) and Sulfite oxidase (SO), which have Mo covalently bond to a strictly conserved cysteine (Fig.4.1a), Xanthine Dehydrogenase (XDH) and Aldehyde Oxidase, belong to a second class (41, 124), with a third terminal sulphur ligand (Fig 4.1b).

Fig. 4.1 Molybdenum cofactor: **a**. In enzymes of Nitrate Reductase (NR) and Sulfite Oxidase (SO) family. **b**. In enzymes of the Aldehyde Oxidase (AO) and Xanthine Dehydrogenase (XDH).

**a** **b**



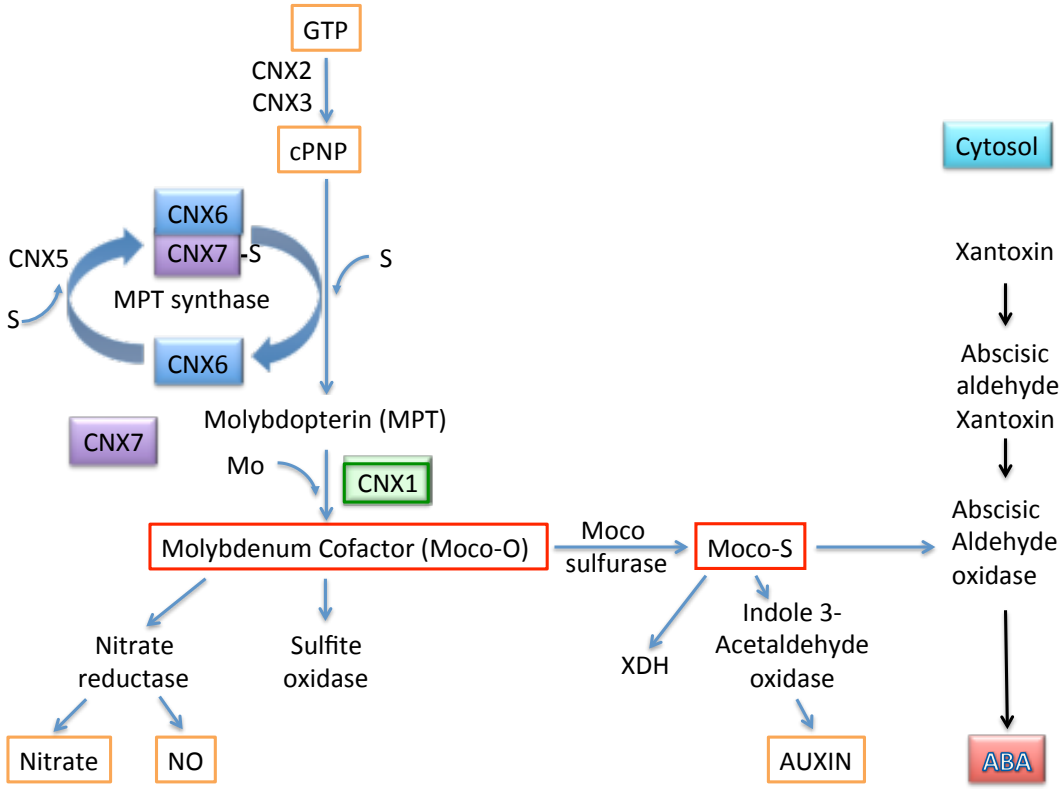
Plants defective in Moco pathway loss essential function, such as the reduction of oxidized nitrogen molecules, and the production of active Aldehyde Oxidase, key enzymes in the last step of ABA biosynthesis. Moco related mutants are described in numerous species, such as *Arabidopsis* (20, 29), Barley (68), tobacco (91), but its synthesis was best studied in *Nicotiana Plumbaginifolia*, where mutations in six different loci (cnx) corresponding to six different proteins (CNX) give a phenotype with crinkled leaves, chlorosis and stunted growth (40). In human Moco deficiency lead a severe genetic disease (61). The cofactor is common to all Moco enzymes (67, 99, 100) except NR containing FeMoco. The attempts to reconstitute the cofactor in vitro have to take in account the oxigen sensitive nature of Moco (89, 90, 142).

## 4.2 Moco biosynthesis

### *4.2.1 the first model*

The first model of Moco biosynthesis came from *E. coli*, with four specific genes in the *mol* operon (110). Moco is synthesized by a conserved pathway (fig. 4.2), in eukaryotes; six genes have been identified in plants (92), fungi (5, 93, 137, 138) and humans (112, 129, 130). The name *cnx* of the genes derives from **C**ofactor for **N**itrate reductase **X**anthine dehydrogenase; for human, instead the genes are named MOCS, from **MO**lybdenum **C**ofactor **S**ynthesis.

Fig. 4.2 Moco pathway. Showing intermediates steps known in eukaryote.




### *4.2.2 From GTP to cPMP*

The first step starts from GTP (49) and generates cyclic pyranopterin monophosphate (cPMP). In *A. thaliana* this step is catalysed by CNX2 and CNX3, whose action was demonstrated using functional complementation in *E. coli*, thus showing the functional conservation between bacterial and plant (57). CNX2 belongs to the family of S-adenosyl methionine (SAM)-dependent radical enzymes, but nothing is known about CNX3 and its homologs. CNX2 should have a [4Fe-4S] cluster similar to MOCS1A (48), and because of this it is a possible candidate for mitochondrial import as this is the major site of eukaryotic Fe-S-cluster synthesis (81). The reaction product of CNX2 and CNX3 is cPMP, (originally named precursor Z).

### *4.2.3 Incorporating sulfur to cMPT*

In the second step Molybdopterin ditiolate is formed incorporating two sulphurs into cPMP a modification catalyzed by MTP synthase, an heterotetrameric complex of two small and two large subunits that in plant is encoded by *cnx6* (23,8 kDA) and cnx7 (10,5 kDA). Biochemical studies demonstrated that CNX6 can replace the function of MoaE (the bacterial counterpart). CNX7 are conserved only in C-terminal region including a double glycine motif and the sulphur as thiocarboxilate (46, 79). This C terminus in bacteria is inserted into the large subunits to form the active site (117).

Other proteins, instead catalyze the sulfur transfer to CNX7. In bacteria the protein transferring sulfur is MoeB, in plant, the homolog is CNX5, with which, as for CNX7, no functional reconstitution of MoeB was achieved (103), probably this transfer is not conserved between eukaryotes and prokaryotes.

### *4.2.4 Mo insertion*

When MTP is synthesized Mo can be coordinated. The mechanism of this insertion is well studied in prokaryotes but poorly understood in eukaryotes. The modular structure of the proteins seem to indicate a functional cooperation between their domains, such as product-substrate channelling. Functional complementation of bacteria mutants to clone the corresponding plant *cnx1* shows only a partial reconstitution of function, indicating differences in metal insertion.

### *4.2.5 CNX1*

Cristal structures of CNX1G domain in complex with MPT confirmed binding of MPT (73). The covalent bound to the C4' carbon of MPT, forming adenylylated MPT (73) and $Mg^{2+}$ and ATP-dependent in vitro synthesis of MTP AMP using purified CNX1G was demonstrated (83).

In this structure there was the identification of copper bound to the MPT ditiolate sulfurs (73), but the function of this MPT ligand is still unkown.

Studies based on transcriptional regulation of the *E. coli mod* operon showed that Cnx1 can bind molybdate in an MPTdependent manner (74), it was found also (120) a molybdate-dependent hydrolysis of MPT-AMP Cleavage of AMP

was coupled to the metal exchange reaction where bound copper was released and Mo was transferred to MPT, thus yielding mature Moco. Probably during MPT-AMP hydrolysis adenylylated molybdate is formed as a reaction intermediate, which is subsequently used as the Mo source for Moco formation and CNX1 is essential for stabilizing the newly formed Moco (75).
CNX1, binding actin filaments, can anchoring to cellular structures like the cytoskeleton, it might help organize and stabilize such a biosynthetic machinery (121).

### *4.2.6 Additional modifications*

Additional modifications are needed before the cofactor insertion in the appropriate Apoenzyme. NR/SO family enzymes provide sulfur from a conserved cysteine to covalently bond a Mo, whereas XDH/AO family it must be added in a final maturation step. In vitro this sulfur can be lost in a reversible reaction; in vivo Moco Sulfurase (MOCS) is necessary for S insertion, described for *Drosophila* (143), humans (76) and ABA3 in *Arabidopsis* (80). In plant biochemical data are available for ABA3 (17).
The N terminus of ABA3 shares significant homologies to the bacterial cysteine sulfurases SufS, IscS, and NifS (17), and in literature is called MocoN terminal NifS like protein. *trans*-sulfuration, pyridoxal phosphate-dependent, by ABA3-bound persulfide, transfers S from a free cystein to Mo (51). The C-terminal domain of ABA3 mediate the contact between XDH/AO and the *trans*-sulfurase domain of ABA3 (3, 17), with this final sulfuration ABA3 could control the amount of functional XDH/AO molecules in the cell and it could be a control of cellular levels of hormones in case of plant AO. In fact, transcription of the *aba3* gene is inducible by drought and salt stress in plants (17) as well as by ABA (148).

## 4.3 Moco Enzymes

### *4.3.1 Nitrate Reductase (NR)*

NR is a cytoplasmic enzyme with a mass of about 200 kDa with a dimeric structure; each monomer contains three regions associated with the Mo center: the Fe-heme of the cytochrome *b*5 domain, and a C-terminal domain associated with a FAD cofactor. The Mo containing domain can be further subdivided into the N-terminal Moco-binding and C-terminal dimerization domains.

NR occurs in three different forms: NADH-specific forms are frequently present in higher plants and algae, NADPH-specific forms are unique to fungi, and NAD(P)Hb ispecific forms are found in all above-mentioned organisms. The catalytic cycle of NR can be divided into three parts: a reductive halfreaction in which NAD(P)H reduces FAD, electron transfer via the intermediate cytochrome *b*5 domain, and an oxidative halfreaction in which the Mo center transfers its electrons onto nitrate, thereby forming nitrite and hydroxide/water (126). The primary structure of NR is conserved among plants, algae, and fungi whereas they are completely different from bacterial NR regarding sequence similarity and structural composition (132).

### *4.3.2 Sulfite Oxidase (SO)*

SO is highly conserved within the plant kingdom: The SO gene is found in higher plants, algae and mosses, and the encoded protein seems to be highly conserved plant SO. Animal SO contain a heme domain (28), therefore among eukaryotes, plant SO possesses only one redox center. Oxygen is the terminal electron acceptor for plant SO and becomes reduced to hydrogen peroxide (47). of plant SO is localized in Peroxisoma (21, 100), whereas animal SO is found in the mitochondria (15), and mainly in liver. Plant SO has a sulphite-detoxifying function, protecting the cell against a surplus of sulphite derived from $SO_2$ in the atmosphere (50) or coming from the decomposition of amino acids that contain sulphur. The compartmentalization of sulphur assimilation and sulphite oxidation in different organelles allows plants to co-regulate these opposing metabolic demands. It can be divided into the N-

terminal Moco domain and C-terminal domain. Comparison to the catalytic domain of NR shows the common fold of enzymes of the NR/SO family with some differences in the boundaries of the enzymes and very important changes within the active site that are believed to determine substrate specificity and catalytic properties (37).

### *4.3.3 Xanthine Dehydrogenase (XDH)*

Plant XDH is homodimeric 300 kDa enzyme. In *Arabidopsis,* where two differentially regulated XDH genes have been found and one of the two, AtXDH1, responded to stresses such as drought, cold, and salinity, and to natural senescence (52). Plant XDH plays a role in important cellular processes like plant-pathogen interactions, such as between phytopathogenic fungi and legumes and cereals (94, 95). In this process requiring the formation of reactive oxygen species, XDH was supposed to be able to produce superoxide anions (52). The native enzyme was also shown to produce superoxide radicals, but not $H_2O_2$ in response to water stress (149).

### *4.3.4 Aldehyde Oxidase (AO)*

AO is a cytoplasmic enzyme with an apparent molecular mass of 300 kDa. The prostetic groups are FAD, iron, and Moco (4:1:1) (71).
The redox-active iron is incorporated in the form of [2Fe-2S] localized on the N-terminal. In *Arabidopsis* there are four AO genes in different chromosomes (123). The encoded enzyme isoforms have relatively substrate specificity for several aldehydes including abscisic-aldehyde, indole-3-aldehyde, indole-3-acetaldehyde, naphthylaldehyde, and benzaldehyde. *Arabidopsis* AO3 catalyses the conversion of abscisic-aldehyde to ABA (124), the last step in ABA pathway. IAA (auxin) is another important member of the aldehyde oxidase family and the characteristics of these two pathways give to this family an important adaptive role in response to environmental stresses.

# CHAPTER 5

## *THE MAIZE vp*404 MUTANT IS IMPAIRED IN Moco BIOSYNTHESIS*

### 5.1 Introduction

In maize vivipary, the precocious germination of the seed while still attached to the ear is the diagnostic phenotype of mutations impaired in the biosynthesis or the sensitivity of the embryo to Abscisic Acid (ABA).
The list of viviparous mutants includes 15 genes: *vp1, vp2, vp5, vp7, vp8, vp9, vp10, vp12, vp14, vp15, al1, y9, w3, rea* and *dek33* (87), grouped into three classes. Mutants *vp1* and possibly *rea*, referred to as response mutants (class 1), define genes not impaired in ABA biosynthesis. Class 2 includes mutants with reduced or suppressed carotenoid accumulation in both endosperm and vegetative tissues (*vp2, vp5, vp7, vp9, vp12, al1, y9* and *w3*) as a result of a mutational block in early biosynthetic steps before the branching point that separates ABA and carotenoid biosynthesis (127). Class 3 includes *vp8* and *vp14*, which affect later steps of the biosynthesis (122), as well as *vp10* and *vp15* that are involved in the synthesis of the molybdenum cofactor required for the last step in ABA biosynthesis (108, 134).
Mutants impaired in Moco biosynthesis have been isolated in various species (40, 118, 144). In *Nicothiana plumbaginifolia* six Moco mutants have been studied, suggesting that these mutations are in *CNX* genes (40) and extensive physiological and genetic analysis of Moco biosynthesis were performed in Tobacco and *Arabidopsis*.
Enzymes requiring the molybdenum cofactor are divided in two groups, enzymes with a dioxo-molibdenum (NR and SO) and enzymes with monoxo-molybdenum, where an oxigen is substitute by sulphur (AO and XDH)(53).
Maize mutants with defects in Moco-O are *vp10* and *vp15*.
The *viviparous10* (*vp10/13*) mutant has strong precocious germination and accumulates chlorophyll but has a lethal phenotype since it is unable to break the pericarp and cannot be rescued through tissue culture (108). The mutant

develops five-six leaves that became necrotic after 2-3 weeks. vp10 was isolated through transposon tagging,it encodes the ortholog of *Cnx1*, which catalizes the last step of Moco-O biosynthesis, necessary to activate Nitrate Reductase and Sulphite Oxidase.

*viviparous15* (*vp15*) (134), isolated from the UniformMu transposon tagging population, exhibits precocious germination and early lethal phenotype. *vp15* encodes the small subunit of molybdopterin (MPT) synthase and shows reduced activity of enzymes requiring Moco. The mutant plant is smaller than wild type, develops spindly leaves and after the emergence of the second true leaf (in soil), becomes necrotic. When grown in agar medium it develops one or two more leaves before the necrosis.

When the mutant is exposed to decreasing values of relative humidity the stomata conductivity is not altered while transpiration is enhanced in association with a rapid decrease in seedling weight. Addition of ABA ($10^{-7}$molL$^{-1}$) to the MS medium restores wild-type values. When immature embryos (25 DAP) are grown on solidified MS+ABA medium the mutants elongate more than the wild-type siblings, showing less sensitivity to the hormone. (Dalla Vecchia N, Rascio N. data unpublished).

Maize mutants defective in Moco-S are unknown, but the Moco-S mutant plants have been described in Arabidopsis (ABA3) (116) tomato (flacca) (86) and tobacco (ABA1) (78), which lack AO and XDH activity, but show normal NR enzymatic activity. Because the activity of AO and XDH could be restored in vitro with sulfide/dithionite, it was proposed that these plants are defective in the sulfuration step of Moco pathway. These mutants show a wilty phenotype and are impaired in stress responses.

In this study we focus to the *vp*404*, a novel viviparous mutant characterized by a precocious germination phenotype, light green seedlings, reduced content of chlorophylls, carotenoids and ABA. Complementation tests with all viviparous mutants with green seedlings reported in literature showed that *vp*404* defines a new *vp* gene that might be related to the Moco-Sulfurase activity.

## 5.2 Results

### *5.2.1 vp*404 phenotype*

*vp*404* is a viviparous mutant characterized by a strong precocious germination phenotype (Fig. 5.1)

Fig. 5.1 self-pollinated ear segregating for *vp*404* at 35 DAP.

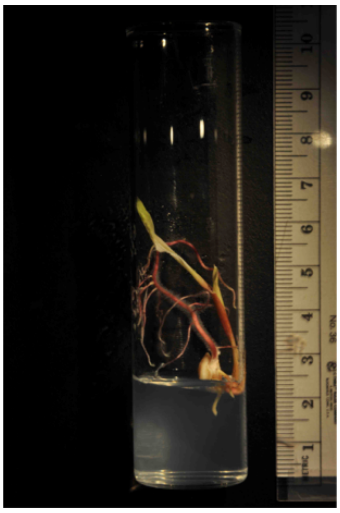
Seedlings are light green (5.2a), with reduced chlorophylls and carotenoids content and lower ABA level in both embryo and seedling tissues when compared to wild type (data not shown). Mutant leaves are rolled and frequently seedling does not grow upward (Fig. 5.2b) and almost 20% of the mutants grown from immature embryo on MS agar, showed defect in the direction of roots and shoots growth (Fig 5.3)

Fig. 5.2 a. wild type (left side), compared with the mutant, (right side), at 15 DAG (days after germination) grown in plastic box (phytatray SIGMA), at 100% RH. Mutant shows stunted growth, light green colour, and some necrosis at the apex of the leaf. b seedling with rolled leaves growing horizontally.

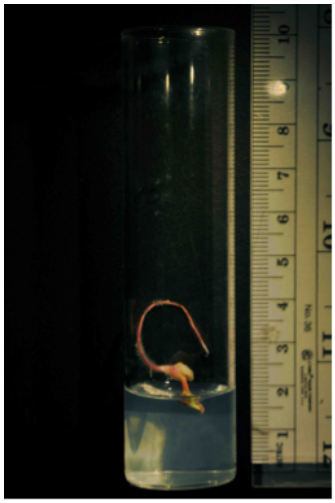
a

b

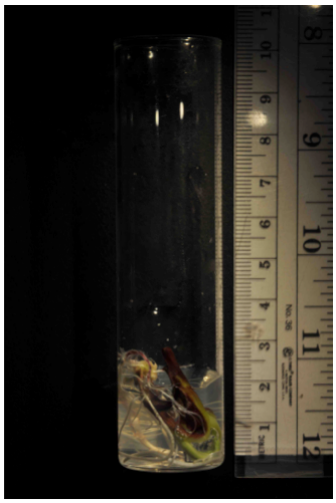
Fig. 5.3 Mutant embryos grown in MS agar, after 15 DAG (days after germination). a, b. Roots grown upward. c. Shoot and root grown downward.



a



b



c

### *5.2.2* Genetic and biochemical analysis

We performed complementation test with all viviparous mutants with green seedlings reported in literature (*vp1, vp8, vp10, vp14, vp15*); the result was that all mutants complement (Tab. 5.1a) with *vp*404,* showing that it probably defines a new *vp* gene.

Because of the analogies between the phenotype of this mutant and the *vp10* (108) and *vp15* (134), impaired in the Molybdenum Cofactor (Moco) pathway we crossed the *vp*404* mutant with TB-10L and 5L, uncovering *vp10* and *vp15* respectively. These two mutations affect genes encoding the CNX1 and CNX7 proteins respectively. The results of TB-A translocation confirm those obtained with complementation test (Tab.5.1b).

Tab 5.1 results of complementation tests with all viviparous mutants with green seedlings and TB-A translocations uncovering *vp10* and *vp15*, the two mutants showing analogies with *vp*404* (candidate alleles). Each test was performed crossing 5 or 5 to10 individuals.

| | ***vp*404*** | ***vp1*** | ***vp8*** | ***vp14*** | ***vp10*** | ***vp15*** |
|---|---|---|---|---|---|---|
| **a** | **comp. test 5-10 crosses heterozygous individuals** | compl. | compl. | compl. | compl. | compl. |
| **b** | **TB-A trans. 5 crosses** | - | - | - | negative | negative |

Four other *Zmcnx* genes encoding CNX proteins are involved in MoCo biosynthesis. To verify the possibility that *vp*404* carries a mutation in one of these genes, we analyzed the activity of sulphite oxidase (SO) (Tab. 5.2), an

enzyme requiring Moco dioxigenate, the first form of Moco, the product of the early steps of Moco pathway,.

Furthermore we analyse the activity of Xanthine Dehydrogenase (XDH) and Aldehyde Oxidase (AO) (Fig. 5.4), enzymes requiring the sulfurate form of Moco, to verify if the mutation is involved Moco-Sulfurase, a key enzyme in the last step of Moco pathway (Fig 5.5).

Tab. 5.2 SO activity on Fresh Weight, and on total proteins. Measures are obtained as a mean of three replicate per every sample. The three groups wt/mut derived from three different ears.

| ENZYME ACTIVITY | | PHENOTYPE |
|---|---|---|
| mmol $min^{-1}\mu g^{-1}$ FW | mmol $min^{-1}\mu g^{-1}$ (Tot. prot.) | |
| 0,448 | 0,1942 | wt |
| 0,140 | 0,1302 | *vp*404* |
| 0,124 | 0,2201 | *vp*404* |
| 0,172 | 0,1286 | wt |
| 0,284 | 0,2166 | *vp*404* |
| 0,196 | 0,1485 | *vp*404* |
| 0,272 | 0,1830 | wt |
| 0,188 | 0,1945 | *vp*404* |

Fig. 5.4 NATIVE gel electrophoresis analysis of AO and XDH. *vp7* is the positive control, with normal enzyme activity and vp15 the negative control, because this mutant is impaired in *cnx7* gene.

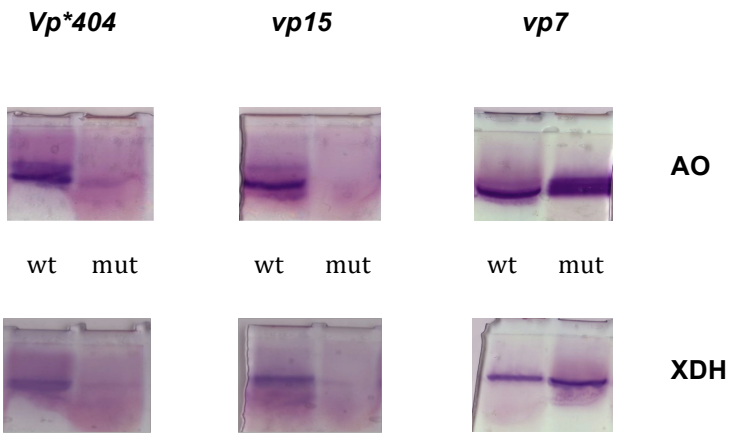
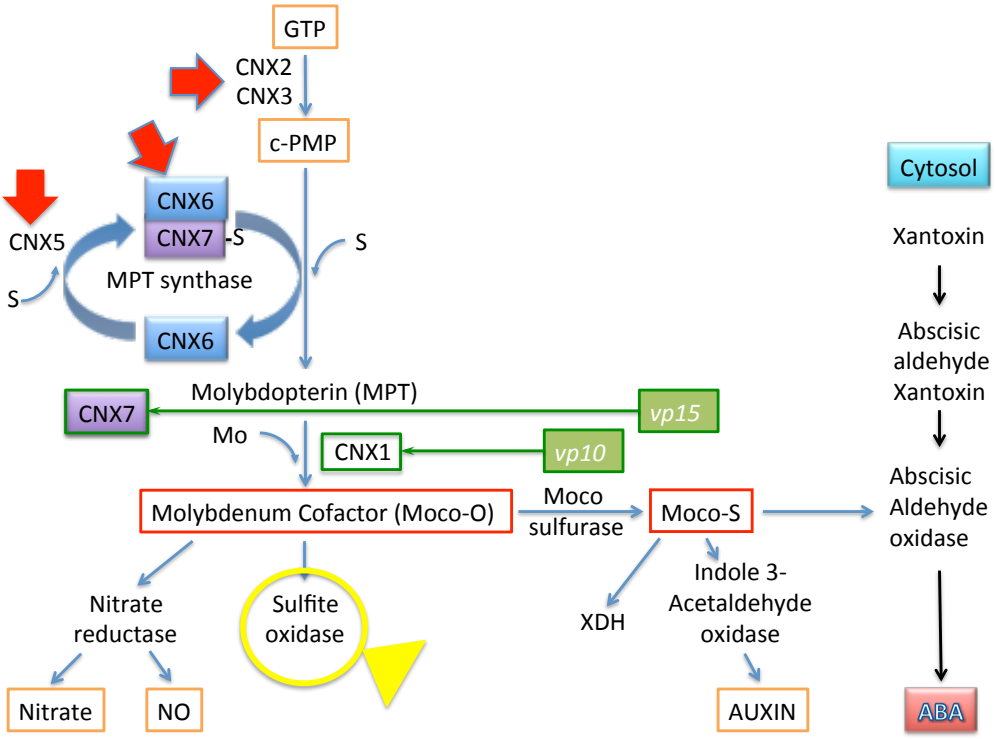



Fig. 5.5 Moco pathway. Red arrows indicate the five genes involved in the first part of Moco biosynthesis, which product is the dioxigenate form of cofactor needed for SO activity (in the yellow ring). Figure also shows *vp10* (*cnx1*) and *vp15* (*cnx7*) genes.



Analysis of enzyme was performed on 15 DAG wild-type and mutant seedlings frozen at -80 following the Eilers method (30) for sulfite oxidase (SO) and the Mendel and Müller method (91) for Xanthine Dehydrogenase (XDH) and Aldehyde Oxidase (AO). The measure of absorbance for SO analysis was performed on three replicates per every sample. We detected high SO activity in the mutant vp404 seedlings, even higher than the wt. The same method used to analyse the activity of vp10 (108) and vp15 (134) showed almost zero values. Analysis on XDH and AO, was performed using *vp15* as negative control, and *vp7* as positive control, the former because of the mutant lack of AO and XDH activity, the latter because is a viviparous mutant with normal enzymes activity (Fig 5.5). Enzyme activity was absent or almost undetectable. Because of this we pointed the attention on the Moco-Sulfurase enzyme, producing Moco-S needed for the AO and XDH activity.

### *5.2.3 Moco-Sulfurase gene*

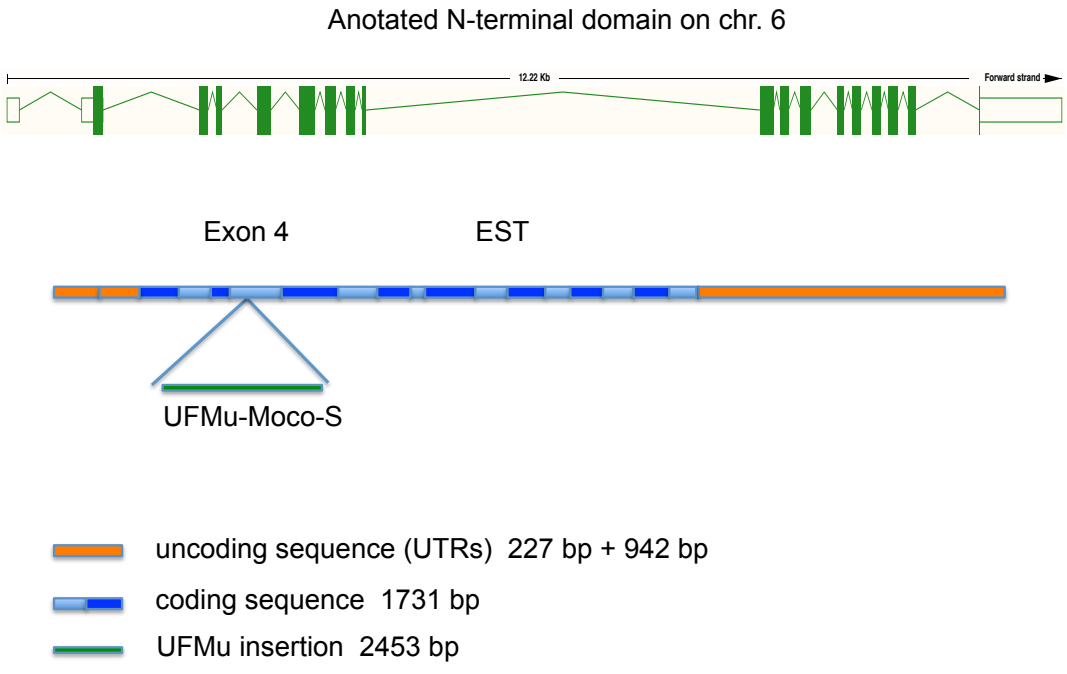
We started the analysis using the protein sequence of *Arabidopsis* ABA3 coding for Moco-Sulfurase. Blast analysis was performed on the maize sequence available from the database (Maizesequence.org). Sequence identity was found on different chromosomes. On chromosome 2 the sequence (GRMZM2G037875_P02) showed a low identity (~30%) when was compared with ABA3 and the sequence seemed to code for C terminal of the Moco-Sulfurase. On chromosome 9 instead, a sequence (GRMZM2G313162_P01) with higher ID (60%), seemed to code for the N terminal Moco-Sulfurase.

On chromosome 6 we obtained a value around 60% related to a sequence (GRMZM2G061821_P01) coding for Moco-S N terminal and with, the same Identity, to a sequence (GRMZM2G048092_P01) located 30kb downstream, coding for Moco-S C terminal, which is the second protein domain.

In the UniformMu collection available at the UFMu database (http://uniformmu.uf-genome.org/ ) we found an insertion in the N terminal domain, in the exon 4 (Fig. 5.6) of UFMu-05371 plants.

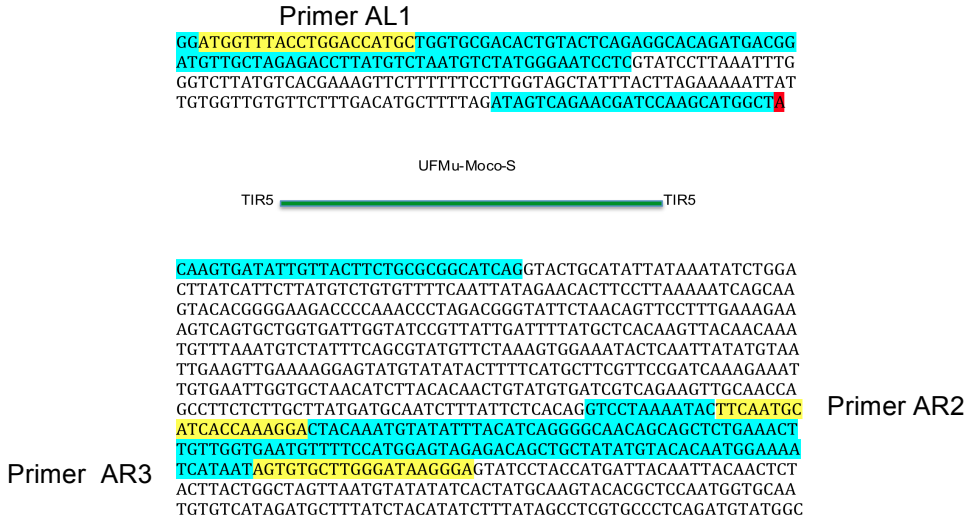
We developed a PCR based assay to genotype the insertion, designing primers upstream and downstream the transposon and using TIR5 as a primer linking the extremities of the insertion (Fig. 5.7, 5.9)

Fig. 5.6 Moco-S N terminal with the UFMu-Moco-S insertion.



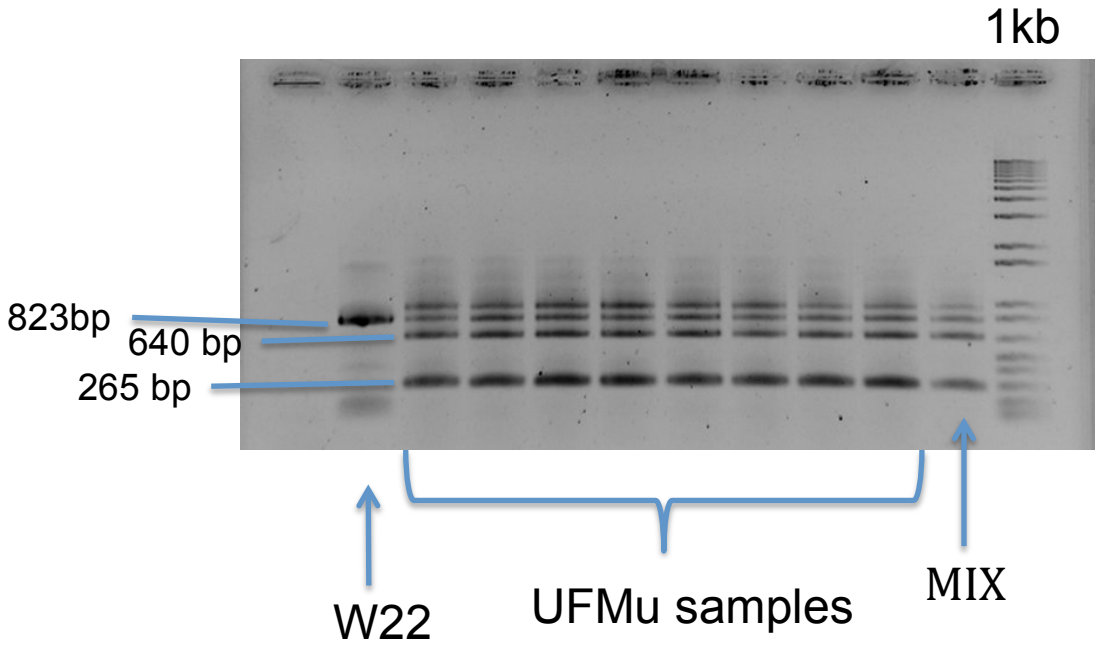

**Expressed sequences and Insertion point of transposon in Moco-S gene on Chromosome 6 in Maize.**

Fig. 5.7 the figure represents a part of the annotated N-terminal Moco-S gene surrounding the UFMu-Moco-S insertion (green bar). The primers are in yellow, TIR5s are primers on the insertion junction sequence, and the nucleotide highlighted in red is the transposon insertion point. The exon sequences.




We prepared a mix of the eight individuals, amplifying with the three primers, and obtained three bands. This result indicated that in our samples there were both mutant and wt sequences. We used the mix as a positive control for mutants. Analysis of parent genotypes revealed that all the eight plants in the experiments were heterozygous, showing the expected bands (Fig 5.8), 823bp, between the primers AL1 (upstream) and AR3 (downstream) the Mu insertion, 265bp and 640 from AL1 to TIR5 and 640 from TIR5 to AR3.
We tested another primer, R2, coupled with L1 on W22, obtaining the expected result (data not showed). The sequence analysis of the 265bp and 640bp, fragments (AL1/TIR5 and TIR5/AR3) confirmed that the primers amplified the region in the Moco-S N-terminal annotated sequence, and that the UFMu insertion is in the exon 4.
Enzyme analysis of AO and XDH, requiring Moco-S, showed normal activity in mutant and wt genotype, confirming that the transposon insertion does not have any detectable effect on phenotype.

Fig 5.8 PCR products obtained from the amplification of heterozygous UFMu individuals. W22 is the reference wt. **a** Each lane indicated as UFMu represents the single individuals. MIX is mutants positive control **b** Mu insertion in exon 4 on annotate N-terminal domain.




823bp = wt band AL1/AR3

640bp = Mu band AL1/TIR5

265bp = Mu band TIR5/AR3

**b**

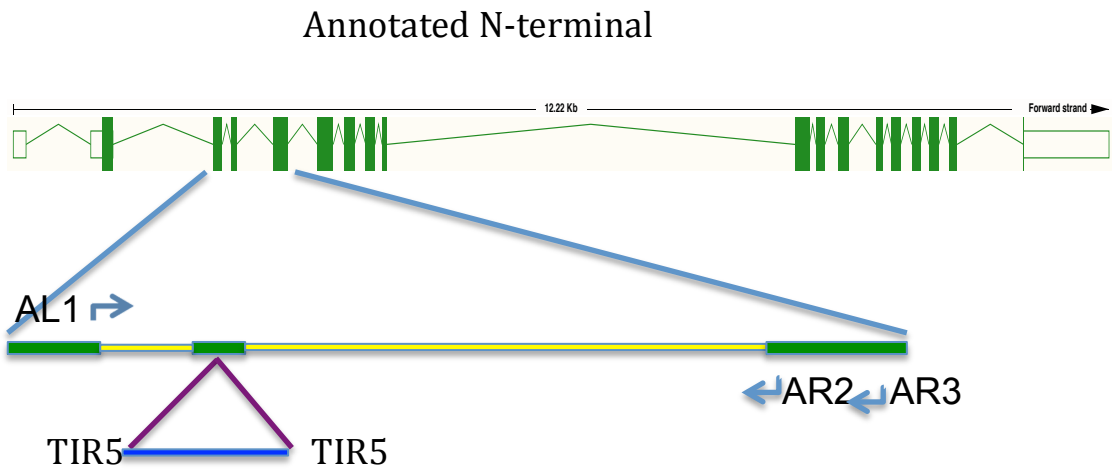
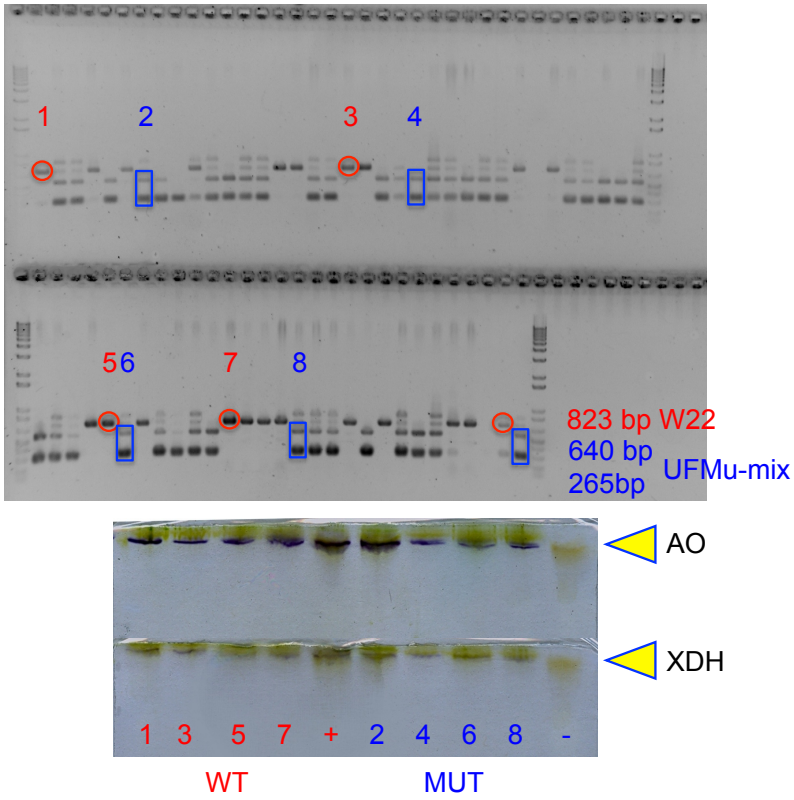


Fig 5.9 Segregation in F2 ears. Enzyme analysis reveals instead normal enzymatic activity in mutant and wt.



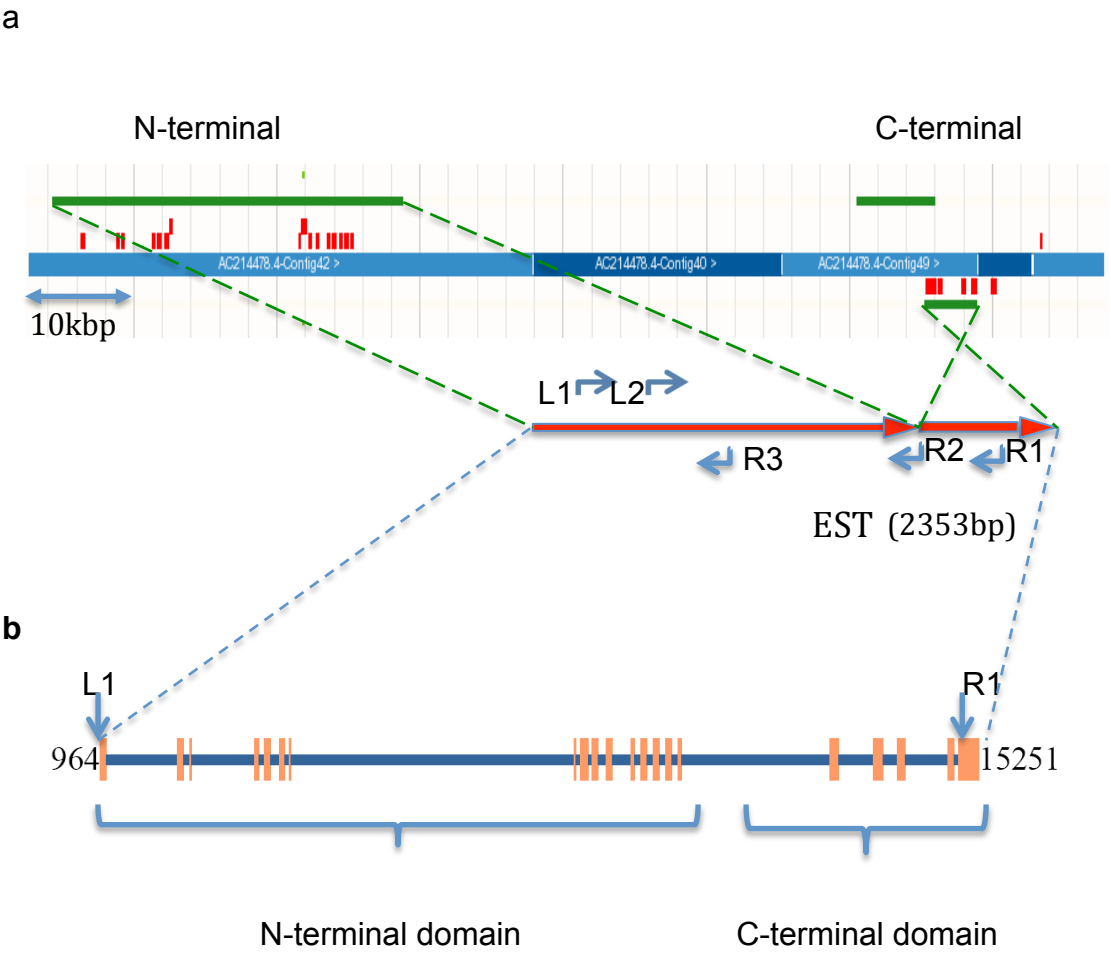

823bp w22= wt band

640bp = Mu band L1/TIR5

265bp = Mu band TIR5/R3

We began with the hypothesis that the two protein domains were encoded by the same gene, as normally happens in Eukaryotes, and using *Sorghum* as a model, we made in silico the structure of a theoretical gene, by inverting sequences in the C terminal domain and making some corrections on bioinformatics arrangements on the 30,000 bp between the two domains, (Fig. 5.11). This figure indicates that the Reverse primers (R1, R2) would have been on the same gene to produce the expected fragments, otherwise we could have obtained only the amplification between L1, L2 and R3 in the annotated N-terminal domain.

5.10 **a** BAC region corresponding to N-terminal + fragment of MOSC-C (C-terminal domain) in Maize (annotated sequences). **b** Gene model assembled from maize genomic sequence, based on the closest *Sorghum* gene and EST analysis. L1 and R1 primers locations are indicated.

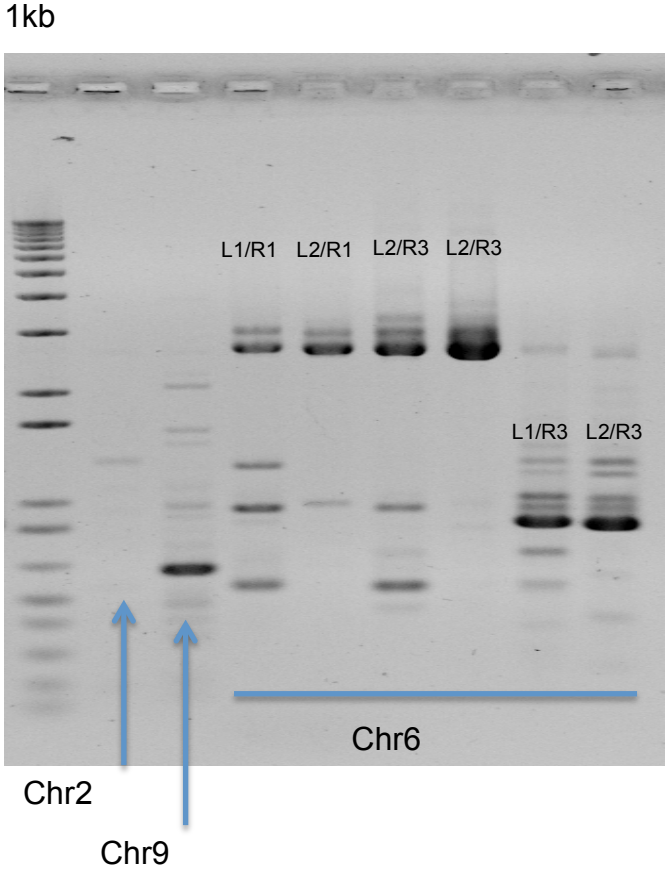



### *5.2.4 Expression analysis*

On the basis of the gene model sequences we used L1, L2 and R3 primers for RT-PCR on N terminal domain sequence and R2, R1 primers on C-terminal. We extracted RNA from W22 young adult leaves, and performed RT-PCR (Fig. 5.11). The primers in the N-terminal (L1, L2/R3) gave us a longer sequence (~ 850bp) than we expected (614bp L1/R3, 597bp L2/R3), the other four couples of primers amplified fragments longer than we expected on the theoretical gene, around 2.8kbp instead of 2.4kbp. The two

amplification products L1/R3 and L2/R3 were obtained using primer that could amplify in the annotated N-terminal cDNA sequence that would have given a product of 850bp. The other four amplifications were obtained by linking the two assembled sequences in the gene model , in the theoretical provisionally chromosome. In the same gel there are two additional lanes, one showing a PCR product from chromosome 2 and the other from chromosome 9. We designed the primers on the coding region of EST: on chr2 primer L exon1, primer R exon 8; on chr9 primer L exon1, primer R exon12.
The expected bands for chromosome 2 was1390bp and for chromosome 9 1578bp. There are two bands corresponding to this length, but the intensity is low. Further analysis should be done to clarify this preliminary observation.

Fig. 5.11 Expression analysis using designed primers showed in fig. 5.11. L2/R3 danno prodotto alto peso molecolare (5 e 6 lane) a basso peso nel 6,



### *5.2.5 Cross between vp*404 and UFMu-Moco-S*

We performed the allelism test between F2 plants heterozygous for vp404 and the UFmu-Moco-S (obtained from Maize stock center (http://www.maizegdb.org/stock.php) individuals carrying the transposon insertion in the exon 4 Moco-Sulfurase N-terminal annotated gene, located on the long arm of chromosome 6.

The heterozygous constitution of +/*vp*404* individuals was confirmed through selfing these plants, that produced segregating ears, whereas UFMu-Moco-S ears obtained from selfing showed only wt kernels. This result was unexpected since the analysis of the parent genotype, revealed that all the eight plants employed in the experiments were heterozygous for the insertion. PCR analysis of their genomic DNA showed the expected bands (Fig 5.8a): 823bp, between the primers 1L (upstream) and R 3 (downstream) the Mu insertion, 265bp and 640 from L1 to TIR5 and 640 from TIR5 to R3 (Fig. 5.8b). As control, we tested the second couple of primers, from L1 to R2, on W22 (data not showed), obtaining the expected result. The sequence analysis confirmed this result. The Mu insertion does not have any evident phenotype.

## 5.3 Discussion

### *5.3.1 Moco-Sulfurase*

Molybdenum cofactor (Moco) sulfurases catalyse the insertion of a terminal sulphur ligand into the molybdenum cofactor, thereby converting the oxo form of Moco to a sulfurylated form.

Plant Moco sulfurase genes have a multidomains structure, comprising a N-terminal NifS-like catalytic protein domain, and a C-terminal domain.

The beta-barrel C-terminal domain of Moco sulfurase (MOSC domain) has a beta-barrel structure similar to that of the beta-barrel domain in pyruvate kinase and contains a highly conserved cysteine residue required for activity. MOSC domains are found in several diverse metal-sulphur cluster biosynthesis proteins from both eukaryotes and prokaryotes. MOSC domains occur as either standalone forms, such as the YiiM protein from *E.coli* or

fused to other domains, such as a NifS-like catalytic domain in Moco sulfurase (the N-terminal Moco-Sulfurase). The MOSC domain is predicted to be a sulphur-carrier domain that receives sulphur abstracted from pyridoxal phosphate-dependent NifS-like enzymes, on its conserved cysteine, and delivers it for the formation of diverse sulphur-metal clusters. The MOSC domain contains several patches of hydrophobic residues and an absolutely conserved cysteine residue situated closer to the C-terminal end of the domain.
Recent studies (147) seems indicate that the C-terminal domain is capable of transferring sulfur to AO and XDH proteins, either in a low molecular form (S) or in the higher molecular form as sulfurated Moco.

### *5.3.2 Mu insertion and Moco gene.*

We blasted the Arabidopsis thaliana ABA3 protein sequence on the maize sequence available maizesequence.org and we obtained higher Identity on two regions on chromosome 6, a region on chromosome 9 and lower Identity in chromosome 2.
The Blast analysis showed for the first region on Chromosome 6 an ID value of about 60% for the sequence coding for Moco-sulfurase N-terminal domain, and the same Identity for a second region located 30kb downstream, putatively coding for MOSC, the C-terminal domain. We referred to the structure of the *Sorghum* gene as a model and reconstructed in silico a gene model structure with a unique sequence comprising both the N and -C terminal domains. To validate this model, we designed the primers for RT-PCR, that we tested using RNA extracted from W22 young adult leaves. Both primers combination amplifying in the N-terminal and linking the N-terminal to the C-terminal region gave us longer sequences than we expected on the theoretical gene. We thus need to revisit this structure, cloning and sequencing the gene, because the annotated N-terminal sequence seems match better than the theoretical one.
Genotype analysis of the UFMu-05371 plants showed that all the plants were heterozygous for an insertion in the N-terminal Moco-S domain and that the insertion was in the exon 4. Heterozygous plants were selfed and 62 seedlings, randomly chosen, from germinated seeds of four ears obtained

from selfing the UFMu plants, were genotyped. Insertion presence was confirmed. However these ears do not show any mutant phenotype.
From the same samples we choose four homozygous mutants and four wild-types to verify AO and XDH enzyme activities. All the samples showed normal activity, confirming definitively that there is not any evident mutant phenotype. One possible explanation is that that a truncated but likely functional protein could be made by the mutant plants.
A second hypothesis we may take into consideration is that the Chr2 and Chr9 genes could express the two Moco-Sulfurase domains from two transcripts. The two domains could interact following a model of bacteria *Rhodobacter capsulatus* (102). To evaluate this possibility, we designed primers for the sequence coding for MOSC C-terminal on chromosome 2 and Moco-S N-terminal on chromosome 9, to verifying the expression of these two candidates. The expected band for chromosome 2 was1390bp and for chromosome 9 1578bp two bands of the corresponding length were produced however their intensity appeared very low.
Data supporting this hypothesis is that the N-terminal sequence on Chr9 has higher ID to *At*ABA3 than the corresponding sequence on the complete Chr6 ABA3 like gene. Further analysis should be performed to better evaluate this hypothesis and to exclude that the two domains do not represent pseudo-genes. If we need to design new primers or if this domains

## 5.4 Material and methods

### *5.4.1 Complementation test*

To test the allelic relationship between *vp*404* mutant in the collection and the green *vp* mutants reported in the literature (class a), we crossed *vp*404* with all mutants of the collection belonging to class a. To perform this test, pollen of 5–10 +/*vp*404* plants, whose heterozygous condition was ascertained by selfing, was applied to the silks of plants representing the selfed progeny of +/vp parents heterozygous for each of the reference mutants. More specifically, *vp*404* were tested against vp1, vp8, vp10, vp14, vp15. The

resulting ears were then scored for visual evidence of segregation for vivipary.

### *5.4.2 TB-A translocation*

From the translocation stock, 3-4 selects plants with approximately 25 % pollen abortion. We applied pollen from each selected plant to silks of an appropriate tester plant that is carrying one or more recessive genes beyond the breakpoint of the translocation involved. We pollinate silks of each of 4-5 *vp*404* plants. Testcross and *vp*404* plants having segregating ears were examined.

### *5.4.3 Embryo rescue*

Immature F2 ears 25 Days After Pollination (DAP), were left in a Na hypochlorite: $dH_2O$ (1:1 v/v) solution for 30 min and then rinsed in sterile water. Mutant embryos on segregating ears are recognizable because of incipient vivipary or pale yellow seed (for albino mutants). The *vp* and normal sibling embryos were excised and transferred to plant cell culture vessels or in petri dishes, in sterile ambient under laminar flux hood.

### *5.4.4 Genomic DNA extraction*

Leaf tissue in a 2 mL eppendorf , Add Urea DNA extraction buffer (800 $\mu$L for 10÷20 mg maize leaf tissue), add 2 steel bead, and put 1 min in the bead beater. Centrifuge 5 min to remove the foam, add equal volume (≈ 800 $\mu$L) of phenol: chloroform: IAA (25:24:1) and mix vigorously for 15 min. Centrifuge 5 min to separate the phases, transfer aqueous phase to a new 1,5 mL conical tube. Add 1/10 volume 3M NaOAc pH 5. Add 0.7 volume Isopropanol, invert tubes several times. Spin or hook out DNA and wash with 70% EtOH, Draw the ethanol solution using the micropipette, and resuspend DNA in 50 $\mu$L TE pH 8.0.

### *5.4.5 Total RNA extraction*

Total RNA was extracted from liquid nitrogen ground tissues using the TRIZOL protocol followed by a DNAse I treatment (Invitrogen). The RNA preparations were reverse transcribed using the SUPERSCRIPT III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

### *5.4.6 PCR*

Primers for MuMoco-S N-terminal insertion in Chr. 6 annotated gene sequences:

TIR5,

AL1 5’ –ATGGTTTACCTGGACCATGC-3’, AR2 5’ –TCCTTTGGTGATGCATTGAA-3’,

AR3 5’ – TCCCTTATCCCAAGCACACT-3’.

35 cycles.

### *5.4.7 RT-PCR*

Primers for cDNA:

Chr 2. L 5’-CATCTCTTTGAGCGGGAGTG-3’ R 5’-TCTCAAATTAAATATCCACGCTGA-3’

Chr 9. L 5’-GCGAGCTCTTACGCATCTTC-3’ R 5’-ATAACTGCGATGAGCCACAC-3’

Chr 6 .L1 5’-ATGGGCCAGAGCAAGG-3’, L2 5’-GGAGTTTTTGGAGCAGTTCG-3’;
R1 5’-GTACATTTGTTTTCTATGTATTTGTGTC-3’, R2 5’-AGGCTTCAAGGCCTTATGTG-3’,
R3 5’-ACGATTAAGGCACCAAGACC-3’.

35 cycles

### *5.4.8 SO assay*

Activity of SO was determined using a steady-state kinetic assay, as described by Eilers et al. (31). The protein was extracted with an SO extraction buffer (0.1 M HEPES, 1 mM EDTA, 5% glycerol, 1 mM Na2MoO4, 1 mM phenylmethylsulphonylfluoride pH 7.3) in a ratio of 4 ml buffer to 1 g tissue FW. Protein extracts were cleared prior to loading by centrifugation at 15 800 g for 30 min at 4°C. For each assay, 100 ll extract was mixed with 900 ll assay buffer (20 mM Tris–acetic acid pH 8.0, 0.1 mM EDTA, 395 µM potassium ferricyanide, 400 lM sodium sulphite). Activity was measured by following the change in absorbance of ferricyanide at 420 nm using a Beckman DU-40 spectrophotometer (Fullerton, CA, USA) with a 1-cm light path. The ferricyanide extinction coefficient used was 1020 $M^{-}1$ $cm^{-}1$, and 2 mol ferricyanide were assumed to be reduced by 1 mol sulphite. Protein concentration was determined with the same methods as above.

### *5.4.9 AO and XDH assay*

MoCo-dependent enzyme activities were measured from 0.1–0.2 g leaf tissue stored at -80°C. Aldehyde oxidase and XDH were measured by native PAGE

assays essentially as described by Walker-Simmons et al. (1989) for AO, and by Mendel and Müller (91) for XDH. The frozen tissues were ground in liquid nitrogen and thawed in XDH extraction buffer (0.1 M potassium phosphate pH 7.5; 1 mM EDTA; 10 mM 2-mercaptoethanol; 1% TritonX- 100) at a ratio of 2 ml extraction buffer to 1 g tissue FW. Protein extracts were cleared prior to loading by centrifugation at 15 800 g for 30 min at 4°C. The extracts were loaded based on equal fresh weight of starting samples and the proteins were separated in two identical gels by native PAGE at 4°C. After electrophoresis, each gel was equilibrated in 0.1 M sodium pyrophosphate pH 8.0, and then stained for AO [0.1 M Tris–HCl pH 8.5; 1 mM indole-3-aldehyde; 1 mM Thiazolyl blue tetrazolium bromide (MTT); 0.1 mM phenazine methosulfate] or XDH (0.1 M sodium pyrophosphate pH 8.0; 2 mM hypoxanthine; 1 mM MTT; 0.1 mM phenazine methosulfate) at room temperature in the dark. Gels were destained with water prior to drying and digital imaging. The protein concentrations of extracts were determined using optical device Biotek tak 3$^{TM}$ wit Gen5 $^{TM}$ software.

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