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A FORWARD GENETICS APPROACH TO STUDY SEED AND SEEDLING DEVELOPMENT IN MAIZE

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Abstract

Embryogenesis, germination and early phases of seedling growth represent critical phases in the plant life cycle and are probably the most important events in determining the success of an annual plant.

A rapid and robust emergence positively influences the capacity of the plant to take advantage of the favourite environment and to compete with its neighbours. In the perspective of a more sustainable agriculture specific characters are envisaged for a crop seedling, such as the resistance to environmental critical abiotic as well as biotic factors. For these reasons key factors subtending plant developmental process and contributing to the achievement of a productive and robust plant have to be searched inside the genetic network that control embryo and seedling development.

Among the different aspects affecting seedling development the two that will be analysed in this study play an important role also in the interplay with the environment. Hormones are endogenous signals governing seedling growth and architecture establishment but at the same time are able to induce plant response to environmental stress. Wax deposition is required for determining a correct embryo and seedling development, and provides, beside that, a protective barrier that plants produce in their early developmental phases to defend themselves from pathogens as well as from variation in environmental abiotic components, such as temperature and water availability.

Here, we report the characterization of the mutants *lilliputian 1-1* (*lil1-1*) and *fused leaves 1-1* (*fdl1-1*), both ascribable to defective seedling (des) maize mutants.

lilliputian 1-1 (lil1-1) is a monogenic recessive mutant of maize, isolated from an active *Mutator* (*Mu*) stock and attributed to the insertion of a *Mutator1* element in the first exon of a the gene encoding the BR C-6 oxidase. The enzyme belongs to the superfamily of CYP85A proteins and catalyzes the final steps of brassinosteroid synthesis. *lil1-1* mutant exhibits a reproducible phenotype consisting of a large primary root, extremely reduced stature and crinkly leaves. Recently, another dwarf mutant of maize impaired in the same brassinosteroid C-6 oxidase and showing a very similar phenotype of lil1-1 has been characterized and the corresponding gene was termed *brasssinosteroid deficient 1* (*brd1*)

Allelism between the two mutant alleles has been demonstrated in this work. Moreover, it has been observed that the exogenous application of brassinolide to the lil1 mutant seedlings resulted in a partial recovery of the lil1-1 phenotype. This observation is in agreement to what previously observed for brd1-m in maize and other Br-deficient mutants in Arabidopsis, rice and tomato.

Differently from some of these mutants, i.e *det2* of Arabidopsis, *lil1* genotype does not influence the seed formation and development. It is evident that the comparison between homozygous lil1-1 mutant and Li11-1 wild-type seeds from the same segregating ear did not highlight any difference in weight. In addition, F2 progeny ears obtained from F1 heterozygous *Lil11/lil1-1* or homozygous *Lil1-1/Lil1-1* plants showed the same average kernel number and total kernel weight per ear and the average weight of single kernel.

BRs are also involved in the modulation of stress responses. Water loss assays and measurement of gas exchange demonstrated that *lil1-1* plants lost less water and maintained efficient gas exchange under drought stress for longer time than wild-type siblings.

Our hypothesis is that *lil1-1* mutant is more tolerant to drought stress because it is by default in a physiological water stress condition. A similar interpretation has been proposed to explain the behaviour of the *det2* mutant in Arabidopsis that is deficient in a steroid reductase. The *det2* mutant showed an enhanced resistance to general oxidative stress, correlated with a constitutive increase in superoxide dismutase (SOD) activity and increased transcript levels of the defence gene catalase (CAT).

To confirm this hypothesis, other studies must be performed, among them the expression analysis of genes involved in dehydration stress. However, the hypothesis is at the moment supported by the observation that lil1-1 mutant plants show phenotypic traits that are generally present in plant subjected to water stress, i.e. inhibition of lateral root growth, reduction in leaf area and plant growth, enlarged leaf thickness and increased stomatal density.

The *fdl1-1* mutant, previously isolated in our laboratory, allowed the identification and functional analysis of a novel maize MYB gene. The *fdl1-1* mutation was caused by an *Enhancer/Suppressor* (*En/Spm*) element insertion in the third exon of the sequence encoding ZmMYB94, a transcription factor of the R2R3-MYB subfamily.

In this work, proof of gene identity was obtained using an RNAi approach and by the analysis of the mutant cDNA sequence. The first experiment ascertained the lesion in the third

exon of the sequence encoding ZmMYB94. The second approach confirmed that the mutant transcript retains the *En/Spm* element.

The *fdl1-1* mutant phenotype is expressed at early stages of seedling development, from germination to the three-four leaves stage, causing a general delay in germination and seedling growth as well as phenotypic abnormalities. The main features of mutant plants are irregular coleoptile opening and the presence of regions of adhesion between the coleoptile and the first leaf and between the first and second leaves. A previous study showed that fusions could be attributable to the alterations in cuticle deposition and highlighted an irregular wax distribution on the mutant leaf surfaces. Phylogenetic analysis demonstrated that its closest Arabidopsis related genes, i.e. MYB30, MYB94 and MYB96 have all been implicated in the regulation of cuticular wax biosynthesis in Arabidopsis.

To gain insight into the role exerted by *ZmMYB94* a deeper characterization of cuticle components were therefore undertaken in this study by comparing mutant and wild-type tissues. We found a significant reduction of the amount of waxes in the mutant versus wild-type samples at earlier developmental stages. In particular, the production of C32 alcohols, which is the major compound of cuticular waxes in the maize seedling, resulted drastically reduced in the mutants and replaced by shorter chain alcohol (C26, C28 and C30) and alkane (C29).

On this basis, we speculate that ZmMYB94 specifically affects the activity of enzymes involved in the elongation of long chain wax molecules at the C30—C32 step.

In maize, some glossy mutants, i.e glossy 2 and glossy 4 show the same block in the long chain elongation. Thus, some of the subtending genes could be under the control of ZmMYB94. Contrary to *fdl1-1*, none of glossy mutant of maize so far characterized showed post-genital organ fusion. This difference could be due to a greater decrease (more than 90%) of epicuticular waxes observed in the *fdl1-1* mutant than in glossy mutants. It is also conceivable that ZmMYB94 affects directly or indirectly the expression of a set of genes involved in the biosynthesis of very-long-fatty acids and the failure of multiple activities has caused a worsening of the phenotype. Alternatively, ZmMYB94 could regulate also some genes involved in the biosynthesis of other cutin components. Although only minor changes in the cutin load were observed in the *fdl1-1* mutant, the affected components could be important for determining organ separation.

Recent studies strongly support the idea that cuticular wax accumulation contributes to drought resistance. However, it is still not known in crops how wax related genes are regulated

in response to drought. In our study, an increment of water loss in the mutant seedlings has been demonstrated and a correlation between the severity of the phenotype and the rate of water loss was revealed. Moreover, we found that the transcript level of *ZmMYB94* increased in plant under drought stress condition. Similarly to *AtMYB30*, *AtMYB94* and *AtMYB96*, which are considered positive regulators of wax biosynthesis during stress, it is conceivable that *ZmMYB94* stimulates the activity of genes involved in cuticular waxes biosynthesis thus contributing to increase drought tolerance in the early phases of maize seedling growth.

In conclusion, our study further indicate that the study of BR-related mutants and mutants impaired in cuticular waxes biosynthesis could be important for unravelling the molecular mechanisms underlying stress response in early developmental phases of cultivated plants and ultimately to identify new genetic tools of interest for their application in designing new breeding strategies.

Introduction

Embryogenesis, germination and early phases of seedling growth represent critical phases in the plant life cycle and are probably the most important events in determining the success of an annual plant. Embryogenesis can be seen as the first phase of a continuous process, only temporarily interrupted by dormancy (Vernoud et al., 2005). During the succeeding phase, the germination, the embryo becomes the seedling. Thus a proper embryo development is a prerequisite for a successful seedling emergence.

Germination and seedling emergence occur when environmental conditions for establishing a new plant generation are likely to be suitable. A rapid and robust emergence positively influences the capacity of the plant to take advantage of the favourite environment and to compete with its neighbors. From an agronomic point of view, synchrony in germination and seedling emergence is a favourite character for a cultivated plant since it will allow the optimization of the weed control practices. The possibility to predict and synchronize the time of emergence will reduce chemical use and will allow combining this strategy with more sustainable approaches, such as biological and physical weed control (Buhler et al., 2000). In the perspective of a more sustainable agriculture specific characters are envisaged for a crop seedling, such as the resistance to environmental critical abiotic as well as biotic factors. For these reasons key factors subtending plant developmental process and contributing to the achievement of a productive and robust plant have to be searched inside the genetic network that control embryo and seedling development.

There are evidences that heterosis is already determined at early developmental stages. Various studies on heterosis have shown that some traits related to this phenomenon are already evident during early phases of plant development, as clearly described for young maize roots (Hoecker et al., 2005, 2008). It has also reported, in Arabidopsis and in crops, that F1 seedlings are already larger than their parents (Meyer et al., 2004; Meyer et al., 2007). Greater cell number is the main determinant the larger size of organs in heterotic plants (Birchler et al., 2010). It is conceivable that the rate of cell division, which is defined very early during embryogenesis, is higher in the progeny than in parental lines. The level of heterosis is most probably posed very early and conditions the final organ size and numbers. On this basis the study of the mechanisms governing early phases of plant development, i.e. embryogenesis and seedling development, are

appealing not only for discerning the molecular and genetic network underlying these processes, but also for the identification of genetics tools that might be of interest in breeding programs.

Maize embryogenesis leads to the formation of two main structures, a well differentiated embryo axis and a storage organ, the scutellum. The mature embryo axis comprises at the two poles the embryonic primary root and the embryonic shoot, separated by the scutellar node. They are both enclosed in a protective structure, respectively the coleorriza for the root and the coleoptile for the shoot. The shoot stem comprises a first internode, called mesocotyl, that is located between the scutellar node and the coleoptilar node and five or six short internodes, depending on the genetic background, located above the coleoptilar node, with a leaf primordia attached to each node. Each leaf is rolled up inside those below it, thus forming a cone shape structure which encloses the shoot apical meristem (SAM). During embryogenesis the coleoptile develops as sheathing structure and envelops the stem tip and the embryonic seedling leaves (Randolph, 1936; Abbe and Stein, 1954).

The scutellum, a massive organ, in which mainly lipids and proteins are accumulated, is attached to the scutellar node. For its functional equivalence, it is considered to be the single cotyledone in the embryo of monocotyledons. The epidermis of the scutellum differentiates in two regions. On the side facing the endosperm a scutellar epithelium is produced, while on the side adjacent to the coleoptile, scutellum cell walls develop a heavy cuticle (Wolf et al., 1952).

Both coleoptile and the first set of seedling leaves primordia are initiated during maize embryogenesis at about 12-14 days after pollination (Randolph, 1936) but, as shown by morphological analysis (Abbe and Stein, 1954), they retain a different origin. The coleoptile arises as a ring of cells on the surface of the scutellum, whereas the first leaf is initiated at the basal face of the shoot apical meristem (SAM), where the coleoptilar ring closes, from the SAM cell population. This observation is also supported by the expression pattern analysis of different marker genes (Nardmann and Werr, 2009). For instances the knotted gene is specifically expressed early in development on the anterior side of the embryo in two groups of cells that will give rise to shoot and root meristems, whereas its expression has not been found in the coleoptile founder cells that are visible in the scutellum (Smith et al., 1995). Another example is the ZmWOX3A/B, whose expression is specifically confined to a ring of peripheral cells, marks the recruitment of cells to firm the P0 primordium (Nardmann et al., 2007).

In maize the coleoptile is the first organ that is produced when seed germination occurs. It appears as a cone shaped structure that elongates and pierce through the soil enclosing and thus

protecting the young leaves and the shoot apex, which comprises the shoot apical meristem (SAM), till they reach the aboveground. In this initial phase first leaf elongation keeps pace with that of coleoptile. Later on the coleoptile chases to elongate and opens at its apex. The first leaf continues to grow and emerges from a coleoptile gap that is initiated at the top and continues to grow laterally towards the grain. The second and following leaves appear subsequently in a sequential manner, soon after the coleoptile dyes.

Among the different aspects affecting seedling development the two that will be analyzed in this chapter play an important role also in the interplay with the environment. Hormones are endogenous signals governing seedling growth and architecture establishment but at the same time are able to induce plant response to environmental stress. Wax deposition is required for determining a correct embryo and seedling development, and provides, beside that, a protective barrier that plants produce in their early developmental phases to defence themselves from pathogens as well as from variation in environmental abiotic components, such as temperature and water availability. We will explore the genetic, biochemical and physiological factors implicated and highlight the most significant aspects that might be taken into consideration in future breeding programs.

Cuticular wax layer protects the plants against environmental stresses and work as a waterproof barrier.

In all higher plants, the outer surfaces of the aerial parts are covered by a thin and continuous layer of predominantly lipid material, known as cuticle, which provides a primary waterproof barrier and a protection against different environmental stresses (Post-Beittenmiller, 1996).

Cuticle synthesis starts in early stages of plant development and is co-regulated with plant growth to provide a constant wax and cutin deposition, required during stem elongation (Suh et al., 2005). Cuticle is involved in the regulation of epidermal permeability and nonstomatal water loss as well as protection against insects, pathogens, UV light and frost (Sieber et al., 2000). In addition, several studies have highlighted its functions in plant developmental processes, among them the prevention of postgenital organ fusion (Sieber et al., 2000). Many Arabidopsis transgenic plants with an impaired cutin load show organ fusion, suggesting the requirement of a proper cuticle to define organ boundaries (Sieber et al., 2000; Wellesen et al., 2001; Schnurr et al., 2004; Kurdyukov et al., 2006). Moreover changes in the cuticular wax component are often associated with morphological impairment of the epidermis, in particular of specialized cells

such as trichomes and stomata. In Arabidopsis, the overexpression of the SHN1 gene, a transcription factor involved in wax biosynthesis, leads to structural defects in the cuticle and causes changes in epidermal differentiation, including trichome formation and stomata number (Aharoni et al., 2004).

The cuticle is a complex structure with two main components, the cutin, made of interesterified hydroxy fatty acid, and the waxes, which include a variety of different acyl lipids. Due to different composition and reactivity to histochemical staining, the cuticle can be divided in two distinct layers. The first is the cuticular layer, which overlays the epidermis cell wall, and is composed of cutin, intracuticular waxes and polysaccharides. The second layer, the cuticle proper, which is less rich in polysaccarids, is made by cutin and intracuticular waxes. Waxes can also be deposited on the cutin surface as films or wax crystals, thus forming the outer layer of a plant tissue (Bernard and Joubes, 2013; Yeast and Rose, 2013).

Among different cuticle components, waxes and their biosynthesis have been extensively analysed in particular in the model plant Arabidopsis thaliana. Waxes are a heterogeneous mixture of very-long-chain fatty acids (VLCFAs) and their alkane, aldehyde, alcohol, ketone, and ester derivatives, which typically range from C24 to C32 in length (Samuels et al., 2008). Their biosynthesis begins with the esterification of C16 and C18 fatty acids, which are *de novo* synthetized in the plastid of epidermal cells, to Co-enzyme A (CoA) and subsequent transfer to the endoplasmic reticulum (ER). In the ER the multi-enzymatic fatty acid (FAE) elongase complex catalyzes successive reactions in which the acyl-chain is extended by two carbons per cycle. VLC-acyl- CoAs can be subsequently transformed into free VLCFAs and/or processed through two distinct pathways: an acyl reduction pathway, which gives rise to primary alcohols and wax esters, and a decarbonylation pathway, leading to the formation of aldehydes, alkanes, secondary alcohols and ketones (Bernard and Jubes, 2013)

Different species have distinct epicuticular waxes compositions and epicuticular wax composition can also differs among tissues within the same species as well as during development (Mariani and Wolters-Arts, 2000). As to young organs and leaves, alkanes are major component in Arabidopsis, representing up to 70% of the total waxes in rosette leaves, followed by aldheydes (14%), and 44 % in inflorescence stems, where the second components for importance are secondary alchools, which accumulate to 23 % (Bernard and Joubes, 2013). In young leaves of barley, rice, and maize, the major cuticular wax components were shown to be primary alcohols, aldehydes, and fatty acids, whereas alkanes comprise less than 15 % of the total wax amount (von Wettstein-Knowles 1971; Avato et al. 1982; Javelle et al. 2010; Mao et al.

2012). In maize, where wax composition differs between juvenile and adult leaves (Lawson and Poethig, 1995), approximately 80% of the juvenile waxes are very-long-chain alcohols and aldehydes, whereas approximately 70% of the waxes produced throughout the life of a maize plant consist of esters (Bianchi et al., 1985).

Progresses have recently been made to reveal the molecular and genetic mechanisms underlying wax production and deposition. In some species like Arabidopsis, barley and maize, genes involved in wax synthesis were first detected through forward genetic approaches based on the altered surface appearance of their mutant stem/leaf. In Arabidopsis 89 *eceriferum* (literally ‘‘not carrying wax’’) mutants were first detected for their shiny appearance that defined at least 21 CER genes (Koornneef et al., 1989). In maize (*Zea mays*), at least 30 loci, namely the GLOSSY loci, have been found to affect the quantity and/or the composition of cuticular waxes on the surface of seedling leaves (Schnable et al., 1994) and in barley (*Hordeum vulgare*), about 80 independent cer loci had been defined (Lundqvist, 1985; Lundqvist and Lundqvist, 1988).

Molecular analysis performed on these mutants and related genes have assigned a function to their gene products, some of which are enzymes catalyzing major steps in fatty acid elongation and wax biosynthesis, while others are transporter or regulatory factors. An update list of the genes is provided in recent reviews (Lee and Shu, 2015; Yeats and Rose, 2013). Gene functional analysis has indicated that, at least in Arabidopsis, that total wax loads is mainly determined by regulatory mechanisms that act at the transcriptional level.

Total amount of cuticular waxes is modulated in response to changes in the environment; it is thus conceivable that waxes are actively involved in responding to environmental signals of biotic as well as abiotic nature. It has been documented that drought, salt or osmotic stress conditions, or exogenous application of abscisic acid (ABA) cause an increase of two fold in cuticular wax load in Arabidopsis (Kosma et al. 2009; Go et al. 2014). Several studies have also revealed a positive correlation between wax amounts and resistance to water stress in crop species. Cuticular wax deposition increased by approximately 1.5- to 2.5-fold by drought or water-deficiency stress in leaves of different plants including soybean (*Glycine max*), sesame (*Sesamum indicum*), sorghum, wheat (*Triticum aestivum*), maize, oats (*Avena sativa*), cotton (*Gossypium hirsutum*) (Shephered and Griffiths 2006; Kosma and Jenks 2007; Kim eta l., 2007a; Kim et al., 2007b).

Regarding wax involvement in plant/pathogen interaction, there are two interesting examples in Arabidopsis, showing how wax composition can influence the capability of the

pathogen to penetrate the cuticle barrier. One example is provided by the work of Weis et al. (2014), which showed that the enhanced resistance of cyp83A1 plants to *E. cruciferarum* was based primarily on the deficiency of long chain aldehydes in the cuticular waxes. The author suggested that these molecules seem therefore critical to initiate fungal development. A second example shows that AtMYB30 that positively regulates the accumulation of alkanes in cuticular waxes (Raffaele et al., 2008) is also a crucial regulator of the hypersensitive response (HR). It was shown that its overexpression can stimulate the production of VLCFAs and waxes, and proposed that these cuticle compounds could have a role in the HR processes consisting in a programmed cell-death limiting the propagation of pathogens (Mongrand et al., 2004).

Transcriptional regulators also mediate the interplay between wax biosynthesis and environmental factor. These regulatory genes contribute to wax regulation and modulate the interaction between waxes and abiotic stresses. WAX INDUCER1/SHINE1 (WIN1/ SHN1) is the first transcriptional regulator characterized. It is an AP2-EREBP-type transcription factor and activates cuticular wax biosynthesis by up-regulation of *KCS1*, *CER1*, and *CER2* genes. Overexpression of WIN1 conferred drought tolerance in Arabidopsis (Aharoni et al. 2004; Broun et al. 2004). In this case it was indeed demonstrated the WIN1/ SHN1 gene product directly activate the promoters of several cutin biosynthetic genes, indicating that the gene has a primary role in cutin regulation with a downstream effect on wax biosynthesis (Shi et al., 2011).

Other studies have shown that response to environmental variations is mediated by the activity of regulatory genes with their targets specifically involved in wax biosynthesis, among them some members of the MYB family. MYB96, whose direct targets are *KCS1, KCS2, KCS6*, *KCR1*, and *CER3*, up-regulates the biosynthesis under drought stress (Seo et al., 2011; Lee and Suh, 2013). Moreover plants ectopically expressing MYB96 showed enhanced drought tolerance in transgenic Arabidopsis. Also in these plants cuticular wax biosynthesis is up-regulated (Seo et al. 2011; Lee et al. 2014). Arabidopsis plants overexpressing MYB96 exhibited also increased freezing tolerance. In this case a lipid transfer protein, LTP3, was identified as one of the targets of MYB96, which is activated during plant response to both freezing and drought stress (Guo et al. 2013). Another example is constituted by MYB94, a transcription factor closely related to MYB96 that activates a set of distinct genes, i.e. *WSD1*, *CER2*, *FAR3*, and *ECR*, besides *KCS2/DAISY*, which is also regulated by MYB96 (Dubos et al., 2010). MYB94 contributes either independently or synergistically to MYB96 to the activation of wax biosynthesis under drought (Lee and Suh 2014).

Although regulatory mechanisms are less understood in crops, compared to the model species Arabidopsis, some regulatory factors, involved in wax biosynthesis and deposition, have been characterized. Three genes have been isolated in maize. OCL1, a transcription factor of the homeodomain-leucine zipper IV (HD-ZIP IV) family, is an epidermis-specific positive regulator of wax biosynthesis (Javelle et al., 2010). 14 target genes up- or down-regulated in transgenic maize plants overexpressing OCL1 have been detected that encode proteins involved in lipid metabolism as well as in other processes, such as defence, or in cuticle components biosynthesis. Moreover, OCL1 induce ZmWBC11a, an ortholog of AtWBC11 involved in the transport of wax and cutin molecules (Javelle et al., 2010). Another gene characterized in maize is GL3, encoding a MYB transcription factor. GL3 controls the level of aldehydes, which are the second most abundant wax compounds in maize leaves (Liu et al. 2012). Both genes are expressed in seedling leaves. In rice WR1 (OsWR1), a homolog of Arabidopsis WIN1/SHN1 activates cuticular wax biosynthesis through the up-regulation of OsLACS1 and OsFAE1-L genes by direct binding to DRE and GCC cis-elements in their gene promoters, respectively (Wang et al. 2012).

Despite these data, very little is known about the interactions between regulatory pathways and environmental stress in crop plants. Preliminary data have been obtained for the *fused leaves1* gene in maize showing that its transcript level is increased in drought stress condition (manuscript in preparation, this thesis). The comprehension of the molecular mechanisms underlying these processes in species of agronomical importance is advisable. Once their role is elucidated, transcription factors may be used as a tool to enhance stress tolerance in crop plants (Hussain et al. 2011).

Wax accumulation in young leaf tissues is an important physiologic process because leaves are the primary photosynthetic organs and are often severely affected by environmental stress (Jenks et al., 1995). Further investigation on the molecular mechanisms underlying the regulation of cuticular wax biosynthesis in crops species under stress conditions may lead to the identification of additional genes involved in critical aspects of plant adaptation to drought stress. These will allow to design new strategies to engineer of crop cuticular lipids in plants in order to better withstand environmental conditions that severely limit plant growth, especially in the early phases of development, and that will have a positive impact on plant productivity.

Plant growth and response to environmental cues are largely governed by phytohormones

It is well established that plant growth, comprising cell division and expansion, is regulated by specific plant hormones i.e. auxin [indole-3-acetic acid (IAA)], cytokinins (CKs), gibberellins

(GAs), brassinosteroids (BRs), and abscisic acid (ABA). It is also well know that cytokinins promote cell proliferation, while gibberellins stimulate cell elongation. Auxin and BRs are involved in both processes. After their discovery, the effect of these hormones on seedling growth had been demonstrated through different approaches, including in vivo assay and mutant phenotype analysis.

Auxin was the first hormone to be characterized in this context. The coleoptile unit, which includes the primary leaves and the coleoptiar node, is the main source of free IAA for the mesocotyl (Iino et al., 1980). Dependence of the apical growing region of the mesocotyl on the coleoptile unit as a source of free IAA is almost total. One-half or more of the supply of IAA comes from the coleoptile tip, the rest mainly from the primary leaves. Removal of the coleoptile tip results in inhibition of mesocotyl elongation (Iino et al., 1982). Elongations of maize coleoptile and pea internode were suppressed by application of auxin transport inhibitors (Haga et al., 1993). On the other hand application of IAA stimulates the hypocotyl of Arabidopsis elongation (Tanaka et al., 2003). The discovery of GAs, a large group of cyclic diterpene compounds soon revealed that, beside auxin, also this class of hormones is implicated in promoting the elongation of young stems during seedling growth (Phinney, 1983). It was soon evident that GAs promote cell elongation in the internodes, due to relaxation of the cell wall (Cosgrove and Sovonick-Dunford 1989). The action of BRs in promoting cell elongation was first observed in a ‘bean second internode test’ (Mitchell et al., 1970) and the structure of the stimulating molecule was years later and determined and named brassinolide (Grove et al., 1979).

Dwarf mutants have been isolated in difference species, such as maize, pea, tomato and Arabidopsis, and allowed the isolation of structural as well of regulatory genes. In particular mutants whose growth defects were rescued by the hormone exogenous administration have been instrumental for defining genes encoding enzymes involved in specific steps of the different pathways. On the other hand mutants exhibiting insensitivity the hormone treatment were diagnostic for genes involved either in the perception or in the transduction of the signal.

For example one important step in the comprehension of the role of GAs in promoting growth elongation derived from the cloning of the Arabidopsis GAI cDNA and its mutant allele *gai,* which produces GA-insensitivity (Peng at al. 1997). The product of GAI is member of the DELLA subgroup of the GRAS family of transcriptional regulators. DELLA proteins are located in the nucleus where act as repressors of genes involved in promoting plant growth (Bolle, 2004). It was shown that increase in GA level triggers DELLA degradation, via ubiquitination–

proteasome pathway, thus releasing the promoter of genes involved in growth from its inhibitory effect.

Gibberellins (GAs), a large group of cyclic diterpene compounds that promote stem elongation, and brassinosteroids (BRs), commonly occurring steroid hormones that regulate multiple aspects of plant growth and development, are two classes of hormones that alter plant architecture when aberrations occur in their biosynthesis or signalling pathways (Salas Fernandez et al., 2009; Clouse 2011).

For all these hormones the biosynthetic pathways as well as the genetic control underlying them along with the molecular mechanism related to their perception and responses have been extensively analysed. A comprehensive description of the data so far obtained and eventually of the models formulated on their bases is available in the literature (Hedden and Kamiya, 1997; Choi et al., 1996). A general view/theme emerging from all these studies is that these hormones do not act in isolation but are interrelated by synergistic or antagonistic cross talks so that they modulate each other's biosynthesis or responses. This aspect of hormone action was shown in particular for cell elongation, probably because it is experimentally easy to work with. Some examples of are provided here below.

For example GA and IAA interact in promoting stem elongation most probably via different mechanisms. A study reported that auxin increases GA biosynthesis (Ross et al., 2001) while another has more recently showed that GA is required for auxin transport (Willige et al., 2011). A **synergistic relationship** of brassinosteroids with auxin for hypocotyl elongation was demonstrated by simultaneous application of BL (the most biologically active BR) and IAA in Arabidopsis. BRs can induce, separately from auxin action, hypocotyl elongation in light-grown seedlings, but, at the same time, auxin can potentiate BR action on the same event.

Other physiological studies demonstrated additive effects of GAs and BRs on elongation growth of organs in pea, mung bean, and cucumber (Mandava and others 1981; Gregory and Mandava 1982; Katsumi, 1985). More recently it has been reported in Arabidopsis that BRs stimulate the GA biosynthesis. A study conducted by Unterholzner and co-authors (2015) has shown that BES1, a transcrition factor whose presence is regulated by BR, binds to a regulatory element in promoters of key genes of the GA biosynthesis, namely GA_{20} and GA_3, and control their expression. They also propose that degradation of the DELLA repressor, due to the increase of GAs, promote releases the repressive action of BES1 in the transcription of targets that act further downstream and that are required for plant growth and development.

In addition to a role in development, some phytohormones exert anti-stress effects on plants and are essential for the ability of plants to adapt to abiotic stresses by mediating a wide range of adaptive responses (Santner and Estelle, 2009; Argueso et al., 2009; Messing et al., 2010; Wang et al., 2009).

Several studies have demonstrated "anti-stress" effects of BRs, most of which have employed treatment of plants with exogenous application of BRs. They have proven the role of these hormones in a variety of environmental stresses, including high and low temperatures, drought, salinity and pathogen attack (Krishna et al., 2003; Divi and Krishna, 2009). *Brassica napus* and tomato seedlings grown in the presence of 24-epibrassinolide (EBR) are significantly more tolerant to a lethal heat treatment than are control seedlings grown in the absence of the compound (Dhaubhadel et al., 1999). The application of brassinosteroids to two susceptible varieties of sorghum resulted in alleviation of negative impact of osmotic stress on seed germination and seedling growth. Brassinosteroid application caused a decrease in the activities of the enzymes peroxidase and ascorbic acid oxidase. Moreover, they enhance the levels of soluble proteins and free proline, which are important manifestation of stress tolerance in many plant species (Wahid and Ghazanfar, 2006; Anuradha and Rao, 2001; Wahid, 2007) and promote the seedling growth under osmotic stress condition (Vardhini and Rao, 2003). Enhanced levels of soluble proteins and free proline were found also in rice (*Oryza sativa L.)* cultivar Super-Basmati foliar sprayed with BRs at five-leaf stage under drought stress condition. Exogenous application of Br also improved carbon assimilation and maintenance of tissue water status, improving seedling growth (Farooq et al., 2010).

Evidence of the role of BRs in **pathogen defence** has been found in potato, barley and cucumber plants, i.e. potato plants sprayed with BRs had a lower incidence of infection by *Phytophthora infestans* (Khripach et al., 1996). Moreover, wild-type tobacco treated with BL exhibited enhanced resistance to the viral pathogen tobacco mosaic virus (TMV), the bacterial pathogen *Pseudomonas syringae pv. tabaci* (Pst), and the fungal pathogen *Oidium sp*. In rice plants, BL enhances resistance to the fungal pathogen *Magnaporthe grisea* and the bacterial pathogen *Xanthomonas oryzae* (Nakashita et al., 2003). Application of the epibrassinolide (epiBL) to heads of 'Lux' barley reduced the severity of Fusarium head blight (FHB) caused by *Fusarium culmorum* by 86%. Growth of plants in soil amended with epiBL resulted in a 28 and 35% reduction in Fusarium seedling blight (FSB) symptoms on the Lux and 'Akashinriki' barley, respectively. Studies of gene expression showed also that pathogenesis-related genes are activated in plants as a result of growth in epiBL (Ali et al., 2013).

Further studies are required to unravel the mechanism of phytohormones action in alleviating the impact of biotic and abiotic stress in model plants as well as in crops. The information arising from these studies may be of help to design gene manipulation approaches as well for potential use of such compounds in agriculture and horticulture. However in the application of these findings, one aspect has substantially to be taken into consideration. There is a competition between plant immunity, which is stimulated by hormones, and plant growth for resource allocation. Mutant plants with constitutively activated defence response often have stunted growth and retarded development (Heidel et al. 2004). The induction of immune responses needs reprogramming of transcription: priority is given to genes involved in defence over growth related function. This transition is controlled by a limited number of transcription factors. One of them, *TL1*-binding transcription factor1, or TBF1, regulate the expression of endoplasmic-reticulum-resident (ER) genes required for antimicrobial protein secretion. TBF1 controls not only immune response genes but also genes involved in growth and development; in particular it turns on multiple defences and inhibits primary growth and development. Knockout mutants in this gene exhibit partial suppression of growth inhibition associated with defence activation, and transcriptional profiling of these mutants showed a general promotion of growth-related genes and repression of defence-related genes involved in immune responses induced by salicylic acid (SA) and by microbe-associated molecular pattern, elf18 (Pajerowska-Mukhtar et al., 2012). SA and IAA are hormones known to control stress and defence responses as well as plant growth. Transcriptome analysis of a number of Arabidopsis F_1 hybrids conducted by Groszmann and co-workers (2015) show altered expression of genes of the SA biosynthesis pathway and auxin (IAA) biosynthesis pathway. IAA-targeted gene activity increased, whereas SA concentration and target gene responses were reduced and these changes correlate with greater leaf cell numbers and increased leaf cell size respectively.

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Chapter 1

Abstract

In the last decade, important advances have been made in elucidating the metabolism and signaling pathways of brassinosteroids (BRs). Among these, castasterone (CS) and brassinolide (BL) are considered the active molecules of BRs in the plant kingdom. BRs control several traits of agronomic importance, such as plant growth, photosynthesis, architecture, and flowering time. A number of mutants with lesions in BR biosynthesis and perception/signal transduction have been isolated in both model plants and crops. In *Zea mays*, two BR-deficient mutants were recently characterized, namely nana plant1 (na1) and brd1-m1.

Here, we report the characterization of an allele of *brd1-m1*, referred to *as lilliputian 1-1* (lil1-1), a monogenic recessive mutant of maize, isolated from an active *Mutator* (*Mu*) stock and attributed to the insertion of a *Mutator1* element in the first exon of a the gene encoding the BR C-6 oxidase. The enzyme belongs to the superfamily of CYP85A proteins and catalyzes the final step of brassinosteroid synthesis. *lil1-1* mutant exhibits a reproducible phenotype consisting of a large primary root, extremely reduced stature and crinkly leaves.

Allelism between the two mutants has been demonstrated in this work. Moreover, it has been observed that the exogenous application of brassinolide to the lil1 mutant seedlings resulted in a partial recovery of the lil1-1 phenotype. This observation is in agreement to what previously observed for *brd1-m* in maize and other Br-deficient mutants in Arabidopsis, rice and tomato.

On the contrary to some of these mutants, i.e *det2* of Arabidopsis, lil1 genotype does not influences the seed formation and development. It is evident that the comparison between homozygous lil1-1 mutant and Li11-1 wild-type seeds from the same segregating ear did not highlight any different in weight. In addition, F2 progeny ears obtained from F1 heterozygous *Lil11/lil1-1* or homozygous *Lil1-1/Lil1-1* plants showed the same average kernel number and total kernel weight per ear and the average weight of single kernel.

BRs are also involved in the modulation of stress responses. Water loss assays and measurement of gas exchange demonstrated that *lil1-1* plants loss less water and maintain efficient gas exchange under drought stress for longer time than wild-type siblings.

Our hypothesis is that *lil1-1* mutant is more tolerant to drought stress because it is by default in a physiological water stress condition. A similar interpretation has been proposed to explain the behaviour of the *det2* mutant in Arabidopsis that is deficient in a steroid reductase. The *det2* mutant showed an enhanced resistance to general oxidative stress, correlated with a constitutive increase in superoxide dismutase (SOD) activity and increased transcript levels of the defence gene catalase (CAT)

To confirm this hypothesis, other studies must be performed, among them the expression analysis of genes involved in dehydration stress. However, the hypothesis is at the moment supported by the observation that lil1-1 mutant plants shows phenotypic traits that are generally present in plant subjected to water stress, i.e. inhibition of lateral root growth, reduction in leaf area and plant growth, enlarged leaf thickness and increased stomatal density.

In conclusion, our study further indicate that the study of BR-related mutants could be important for unravelling the molecular mechanisms underlying stress response in cultivated plants and ultimately to design new breeding strategies.. A final challenge would be a proper modulation of the endogenous brassinosteroids levels that lead to improve plant resistance to different environmental stress without impairing the plant growth.

Introduction

In the last decade, important advances have been made in elucidating the metabolism (biosynthesis and catabolism) and signalling pathways of sterols and brassinosteroids (BRs) as well as their importance for plant growth and development, in both model plants and crops (Schaller, 2004; Choe, 2006; Clouse, 2011; Williams, 2011). To date, more than 50 BRs have been identified from the entire plant kingdom (reviewed in Fujioka, 1999; reviewed in Bajguz and Tretyn, 2003). Among these, castasterone (CS) and brassinolide (BL) have been frequently identified in plant materials and are considered the most important BRs in the plant kingdom. The biosynthesis of these BRs in plants has been extensively investigated by molecular genetic analyses of BR-deficient mutants (reviewed in Sakurai, 1999; reviewed in Bishop and Yokota, 2001; reviewed in Fujioka and Yokota, 2003). As a result, two parallel pathways, namely, the early and late C-6 oxidation pathway for C28-BRs, have been fully established. In the early C-6 oxidation pathway castasterone is the final product, while in the late C-6 oxidation pathway CS is converted to BL. Consequently, CS is now considered a direct biosynthetic precursor of BL (Suzuki et al., 1993, 1995; Noguchi et al., 2000; Kim et al., 2003).

BRs control several traits of agronomic importance, such as plant growth, photosynthesis, architecture, and flowering time. Sterols and BRs are also capable of increasing plant tolerance/resistance to a wide range of biotic and abiotic stresses, such as drought, salinity, heat, cold, virus infection, and pathogen attack (Divi and Krishna, 2009). BR has also been reported to accelerate the biosynthesis of ethylene (Shi et al., 2006) and guide the distribution of IAA (Li et al., 2005).

A number of mutants with lesions in BR biosynthesis and perception/signal transduction have been isolated in different species as *Arabidopsis thaliana* (Choe et al., 2000), *Pisum sativum* (Nomura et al., 1997, 1999; Schultz et al., 2001), *Lycopersicon esculentum* (Bishop et al., 1999) and *Oryza sativa* (Yamamuro et al., 2000). In *Zea mays,* two Br-deficient mutants were recently characterized; *nana plant1* (*na1*), carries a loss-of-function mutation in a DET2 homolog – a gene in the BR biosynthesis pathway encoding for a 5 α-steroid reductase (Hartwig et al., 2011) and *brd1-m1*, impaired in one of the genes involved in the last steps of brassinosteroid biosynthesis that encodes for a brassinosteroid C-6 oxidase (brC-6 oxidase) (Makarevitch et al., 2012). Both are severely compromised in height, floral development and overall plant architecture (Hartwig et al., 2011; Makarevitch et al., 2012).

Here, we report the characterization of an allele of *brd1-m1,* referred to as *lilliputian 1-1 (lil1-1),* a monogenic recessive mutant of maize, isolated from an active *Mutator* (*Mu*) stock and attributed to the insertion of a *Mutator1* element in the first exon of a the gene encoding the BR C-6 oxidase.

The enzyme belongs to the superfamily of CYP85A proteins and has been classified, through phylogenetic analysis, as cytochrome *P450 CYP85A1* C-6 oxidase (Makarevitch et al., 2012). It catalyzes the final step of brassinosteroid synthesis involving the C-6 oxidation of 6-deoxocastasterone (6-DeoxoCS) to castasterone (CS) and in some cases the further conversion of CS to brassinolide (BL). Whereas dicots, such as pea (Jager et al., 2007) tomato (Bishop et al., 1999) and Arabidopsis (Shimada et al., 2001) appear to have two different brC-6 oxidase genes, in the maize genome, similarly to that of rice, only one gene coding for brC-6 oxidase is present (Hong et al., 2002; Mori et al., 2002).

This *lil1-1* mutant exhibits a reproducible phenotype consisting of a large primary root (Fig 1B), extremely reduced stature (Fig.1A) and crinkly leaves (Fig.1C). Histological analyses revealed that this mutation is due to a disruption at the level of microtubule organization: mutant cells of the primary root and of the leaves have a prolonged mitotic activity and defects in the division plane alignment (Dolfini et al., 1999).

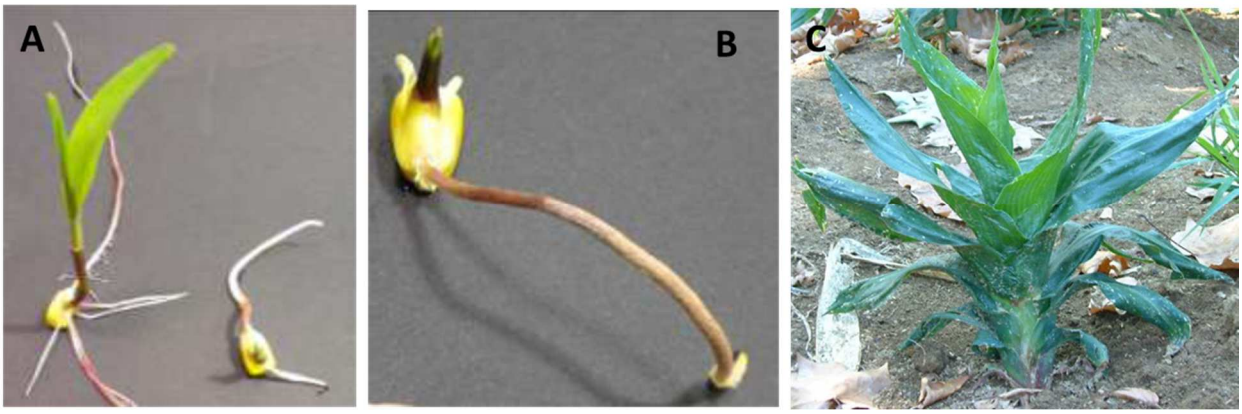

Fig. 1*: Comparison between wild-type (left) and mutant seedlings (right) 8 day after germination (A). Mutant seedling 8 days after germination showing the enlarged primary root (B). lil1-1 plant at maturity. Note the extremely reduced stature and the crinkly leaves (C).*

Mutant analysis conducted in this study revealed that lack of active BR molecules in maize is associated with increased stomatal density, probably responsible for the higher stomatal conductance and transpiration rate. However, water use efficiency (WUE) is significantly higher in lil1-1 seedlings during drought stress.

Materials and Methods

Plant materials

The maize dwarf mutant here analysed, referred to as *lilliputian1-1* (*lil1-1*) was originally isolated from the selfed progeny of the *Mutator* stock outcrossed to an unrelated stock. The dwarf mutant was introgressed three times into B73 and one in A188, H99 and $Rscm_2$ inbred lines. Due to the seedling lethality or impaired inflorescence development, the *lil1-1* mutant was maintained as heterozygote.

Allelism test

Pollen of a given *+/lil1-1* plant, whose heterozygous condition was ascertained by PCR analysis, was applied to the silks of plants *+/brd1*, whose heterozygous condition was ascertain by selfing. For each F1 200 seeds were germinated to score the phenotypes. About one-quarter of mutant seedling is expected in case of lack of complementation (allelism) of the two mutants. Statistical analysis was conducted using the chi-square test.

Plant genotyping

DNA from plant was extracted with a technique based on ethanol precipitation for concentrating and de-salting nucleic acids preparations in aqueous solution. The basic procedure is that salt and ethanol are added to the aqueous solution, which forces the precipitation of nucleic acids out of solution. After precipitation, the nucleic acids can then be separated from the rest of the solution by centrifugation. The pellet is washed in cold 70% ethanol then after a further centrifugation step the ethanol is removed, and the nucleic acid pellet is allowed to dry before being resuspended in clean aqueous buffer (Chabi Sika et al., 2015).

For PCR analysis, 50 ng of genomic DNA was subjected to 35 cycles of amplification with 1.25 U of GoTaq® Flexi DNA Polymerase (Promega), 1 X Colourless GoTaq®Flexi Buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.2 µM primers and 1 M betaine. Annealing temperatures were between 56 and 60 °C (optimized for each primer pair) and an extension time of 60 s. Plant were genotyped with a two set of primers by PCR analysis (lil1F-lil3R, lil1F-Mu53s Table 1). lil1F-lil3R set is specific for the wild-type allele and lil1F-Mu53s for the mutant allele (Fig.2)

The PCR product was run on a 1% agarose gel stained with ethidium bromide

Name	Sequence (5'-3')	Temperature
lil1F	GGGAAAACAACACCGACTT	58°C
Lil3R	GCATGAAGGCGTCGATCT	56°C
Mu53s	GGATTCGACGAAATAGAGGC	60°C

***Table 1**. lil1 oligonucleotide primers used in this study.*

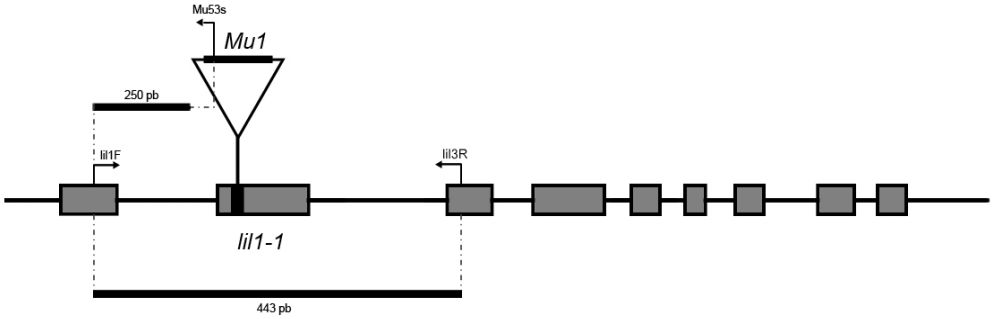

***Fig.2** The predicted coding sequence of the maize lil1 gene (GRMZM2G103773) and the specific primer sets. The Mutator1 insertion is located in the second exon.*

Exogenous application of brassinolide

Three different treatments were applied in this experiment: distilled water supplemented with 10^{-7} BL, 10^{-8} BL and distilled water. 200 seeds from a segregating ear were used for each treatment.

Seeds obtained from selfed *+/lil1-1* B73 heterozygous plants were soaked in distilled or in water supplemented with 10^{-7} BL and 10^{-8} BL for 24 hours and succeeding germinated in glass boxes on wet filter paper imbibed either with the different solutions in the dark at 25 °C. To keep the seeds moisturized, they were covered with one more layer of water-saturated paper towel.

Measurements of root, mesocotyl and coleoptile elongation and seedling stature were obtained every three days, starting from 3 days of culture. Data were analysed using one-way analysis of variance (ANOVA) to detect differences between wild-type and lil1-1 seedlings.

Microscopy analyses

For shoot apical meristem (SAM) images, homozygous lil1-1 and wild-type plants from the same segregating progeny were grown in a growth chamber under standard conditions (25°C, with 16 h-light photoperiod for 2 weeks), and shoot apices were dissected from two-week old seedlings. Shoot tissues were immediately fixed in 3% gluteraldehyde in phosphate-buffered saline (PBS) (130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO_4) for 24 hours. The fixed material was placed in 70% ethanol and embedded in LR White resin. Semi-thin sections (2 μm) were applied to poly-lysine coated slides, stained with toluidine blue O [1% toluidine blue 1% sodium tetraborate (1:1, v/v)] and imaged under light microscope (Ortholux, Leitz, Germany).

Leaf blade samples were fixed with 3% glutaraldehyde in phosphate-buffered saline (PBS) and embedded in 5% agar. Sections of 30 μm were cut with a microtome.

Gametophytic selection

To test if the function of *lil1* gene is required during male gametophytic development, the distribution of lil1-1 kernels in the selfed ears of a heterozygous +/lil1-1 mutant was determined by dividing each ear into three sectors (apical, central, basal) of equal length. If the mutant is not undergoing gametophytic selection, a random mutant distribution should be observed in all sectors, whilst a significant deviation from the random distribution is expected in the basal sector as a result of gametophytic selection against the mutant during pollen development, pollen germination or pollen tube growth. For this analysis, data from three ears were combined and the heterogeneity was performed.

Plant growth condition

Mutant and wild-type plants used for leaf gas exchange analysis, water loss assays, relative leaf water content and analysis of stomata density were grown in equal amounts of same type of soil in a growth chamber at 25°C, with 16 h-light photoperiod. After 14 days, seedlings at the same developmental stage were selected. All measurements were performed on fourth leaves of both mutant and wild-type samples.

Drought treatment and water loss assays

To study plant transpiration during drought, lil1-1 and wild-type plants were subjected to drought by withholding irrigation for 9 days. To measure the rates of water loss from both mutant and wild-type plants, decreases in fresh weight were recorded every 3 days as a function of time and the percentages of decreases were expressed as percentage of water loss.

For water loss assay the fourth detached leaves were collected from mutant and wild-type seedlings, placed on a shit of Wattman paper and exposed to open air at room temperature. Their weight was measured every ten minutes for two hours. The water loss at each interval was expressed as fresh weight decrease (%).

To measure gas exchange of plants under drought stress condition, seedlings were grown in well-watered condition for 14 days. When the fourth leaf was emerging thirty plants per genotype were assigned to three irrigation regimens, i.e. 72 and 96 hours of drought and relative control. Drought was achieved by withholding irrigation.

Leaf gas exchange analysis

Measurements of gas exchange were made with a portable open system infra-red gas analyzer (CIRAS-2, PP Systems, Hitchin, Herts, UK) on fourth leaves of 10 well irrigated mutant and wild-type plants. These measurements were made from 12.00 a.m. to 17:00 p.m. on 1.7 cm2 leaf areas with a 300 ml min−1 air flow rate, at a photosynthetic photon flux density (PPFD) of 1000 μmol m−2 s−1. The ambient temperature was 35 to 38°C and the vapour pressure deficit (VPD) was similar during the whole experiment. Mutant and wild-type leaves were measured each three time in random order when gas exchange parameters had become stable to minimize differences during the day. Water use efficiency (WUE) was calculated as ratio between net photosynthesis (A) and transpiration rate (E).

Leaf gas exchange analyses were conducted on both well-watered and drought-stressed plants.

Statistical analysis

All the data were subjected to statistical analysis and the means were tested by one-way analysis of variance (ANOVA) at 5% level of significance. All the statistical analyses were conducted using the statistical package SPSS 21.0.

Analysis of stomatal density and stomatal index

To measure stomatal density and stomatal index, leaves of the same age and from the same relative position were sampled from wild-type and mutant plants. A leaf surface imprint method was used. Briefly, a drop of glue was applied to a glass slide, and both the adaxial and abaxial side of a sampled leaf was pressed on the glue for ~30 s. The leaf was removed and the imprint on the glass slide was observed under a light microscope. For statistical analysis of stomatal density, five leaf areas were sampled for each plant and for each side and seven plants were sampled for the wild-type and the mutant. Stomatal index (si) was determined as [number of stomata/(number of epidermal cells + number of stomata) × 100; (Salisbury 1927)].

Results

The lil1 gene encodes for a brassinosteroid C-6 oxidase

The genetic analysis conducted in our laboratory indicated that *lil1* encodes for a brassinosteroid C-6 oxidase that catalyzes the final step of brassinosteroid synthesis (GRMZM2G103773). Recently, another dwarf mutant of maize impaired in the same brassinosteroid C-6 oxidase has been characterized and the corresponding gene was termed *brasssinosteroid deficient 1* (*brd1).* Both mutants exhibit a reproducible phenotype consisting of a large primary root, extremely reduced stature and crinkly leaves (Fig.3).

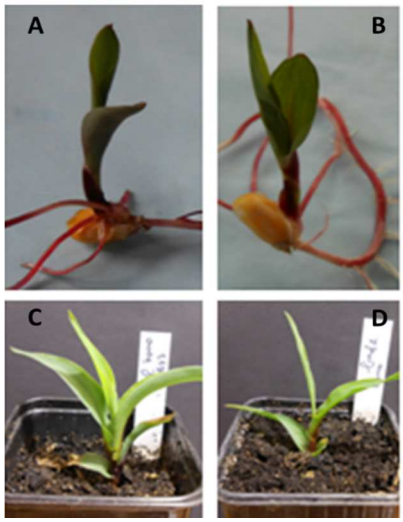

Fig. 3*. Representative lil1-1 (A,C) and brd1-m (B,D) mutant seedlings.*

To understand if the two mutants are allelic, heterozygous *+/lil1* plants (whose genotype was confirmed by PCR analysis) were crossed as female with heterozygous *+/brd1* pollen donor (whose genotype was confirmed by selfing). The results of the screening of the progeny indicate that, as reported in Table 2, the two mutants did not complement in the F1. Since lack of complementation between two mutant alleles is generally considered as genetic evidence of allelism, we concluded that *lil1-1* and *brd1* mutants are impaired in the same gene.

Code	**Cross**	**Progeny scoring**		
		wild-type	**mutant**	**X^2**
C 849bis-27/C 850T-1	+/*lil1-1*^{c} X +/*brd1*^{cs}	188	56	0.546 NS
C 848bis-13/C 850T-2	+/*lil1-1*^{c} X +/*brd1*^{cs}	187	59	0.136 NS
C 855(1)/C 850bis-5	+/*lil1-1*^{c} X +/*brd1*^{cs}	76	24	0.053 NS

Table 2*. Allelism test. Heterozygous +/lil1 plants were crossed as female with heterozygous +/brd1 pollen donor. The symbol c means that the heterozygous constitution was ascertained by PCR while the symbol C means that the heterozygous constitution was "confirmed by selfing".*

To analyse the response of *lil1-1* mutant plants to exogenous BL, mutant and wild-type siblings were grown in the dark in presence of either 10^{-7} or 10^{-8} M BL solution. The effects of brassinolide treatment were evaluated for root elongation and seedling height. More than 100 seedlings for each treatment were measured at succeeding development stages. When germinated and grown in the dark, *lil1-1* plants exhibited no etiolation response, with complete absence of mesocotyl, coleoptile and internode elongation (Fig. 6 A).

Elongation of both mutant and wild-type roots was increased by BL, with the maximum increase at 10^{-8} M BL (Table 3). Both mutants and wild-types BL treated roots were two-fold longer then their control, revealing the same effect of brassinolide on lil1-1 and wild-type root elongation (Fig.4 A ,B).

Mesocotyl and coleoptile elongation was not affected by exogenous application of 10^{-7} M and 10^{-8} M brassinolide in both mutant and wild-type seedlings (data not shown), whereas both treatments positively influenced whole plant height, as measured from scutellar node to leaf tip (Table 4). The maximum increase was observed at 10^{-8} M (Fig.5 A) in both mutant and wild-type seedlings, and this increase was more pronounced in lil1-1 samples (Fig.5 A, B). The average plant height was always statistically significant between the two genotypes, in control as well as in treated plants, however the relative elongation was much higher in the mutant than in wild-type seedlings and the difference in the whole plant height between wild-type and mutant seedling was reduced by nearly 50% in BL treated seedlings (Fig.6 B).

Overall, these results indicated that lil1-1 phenotype could be partially rescued by exogenous application of brassinolide. This experiment and the allelism test between *brd1-m* and *lil1-1* confirmed that *lil1* encodes for a brassinosteroid C-6 oxidase.

Genotype	Time [days]	Treatment		
		Control	10^{-7}	10^{-8}
Lil1-1/-	T1=3	3.68 ±2.76	6.14 ± 3.14	3.99 ± 2.47
	T2=6	9.82 ± 6.93	19.01 ± 6.94	18.72 ± 7.65
	T3=9	10.55 ± 6.79	20.68 ± 9.39	23.67 ± 7,10
lil1-1/lil1	T1=3	2.62 ± 2.31	4.63 ± 2.85	3.81 ± 2.50
	T2=6	6.94 ± 4.77	12.72 ± 6.32	13.75 ± 4.33
	T3=9	7.14 ± 4.85	15.38 ± 7.40	17.38 ± 7.42

***Table 3**. Measurements of root elongation after 3 (T1) 6 (T2) and 9 (T3) days fallowing exogenous application of brassinolide at 10^{-7} and 10^{-8} concentration. Data of root measurements are averaged and ±SE calculated.*

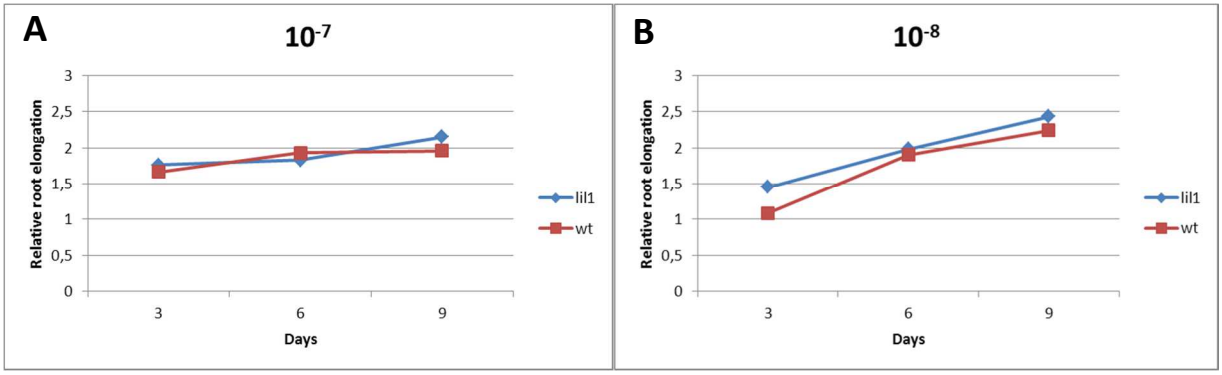

Fig. 4. *Time course of primary root relative elongation for wild-type (wt) and mutant (lil1) seedlings. Seeds were incubated either with (A) 10^{-7} or (B) 10^{-8} BL solution or in water for 24 hours and then transferred to filter paper and seedlings watered with the same solution for 3 days (T1), 6 days (T2) and 9 days (T3).*

Genotype	Time [days]	Treatment		
		Control	10^{-7}	10^{-8}
Lil1-1/-	T1=3	2.47 ± 1.42	3.84 ± 2.13	2.63 ± 1.38
	T2=6	9.28 ± 3.69	12.21 ± 3.91	15.02 ± 3.67
	T3=9	12.02 ± 3.58	16.15 ± 5.21	20.79 ± 5.30
lil1-1/lil1	T1=3	0.47 ± 0.33	0.94 ± 1.15	0.81 ± 0.44
	T2=6	1.93 ± 1.35	4.19 ± 1.97	4.93 ± 1.67
	T3=9	2.38 ± 1.29	6.29 ± 2.24	7.49 ± 2.68

***Table 4**. Measurements of seedling height after 3 (T1) 6 (T2) and 9 (T3) days fallowing exogenous application of brassinolide at 10^{-7} and 10^{-8} concentration. Data of seedling height measurements are averaged and ±SE calculated.*

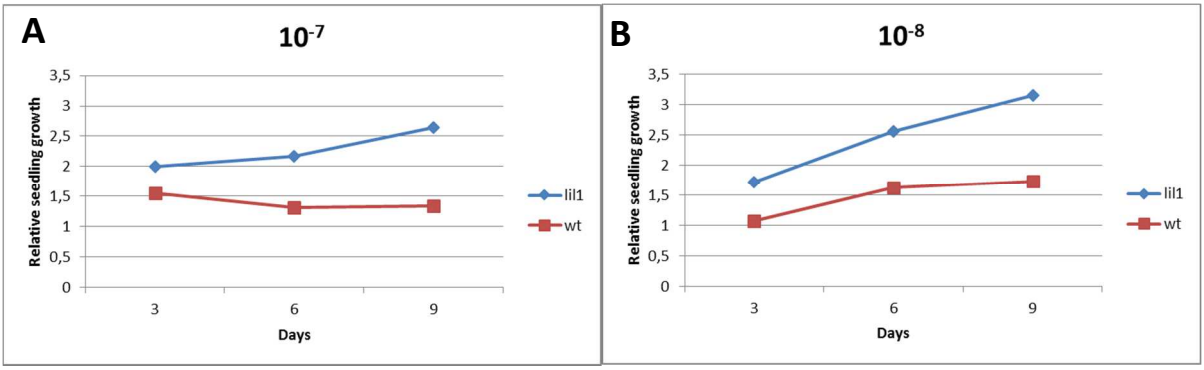

Fig. 5. *Time course of relative seedling growth for wild-type (wt) and mutant (lil1) seedlings. Seeds were incubated either with (A) 10^{-7} or (B) 10^{-8} BL solution or in water for 24 hours and then transferred to filter paper and seedlings watered with the same solution for 3 days (T1), 6 days (T2) and 9 days (T3).*

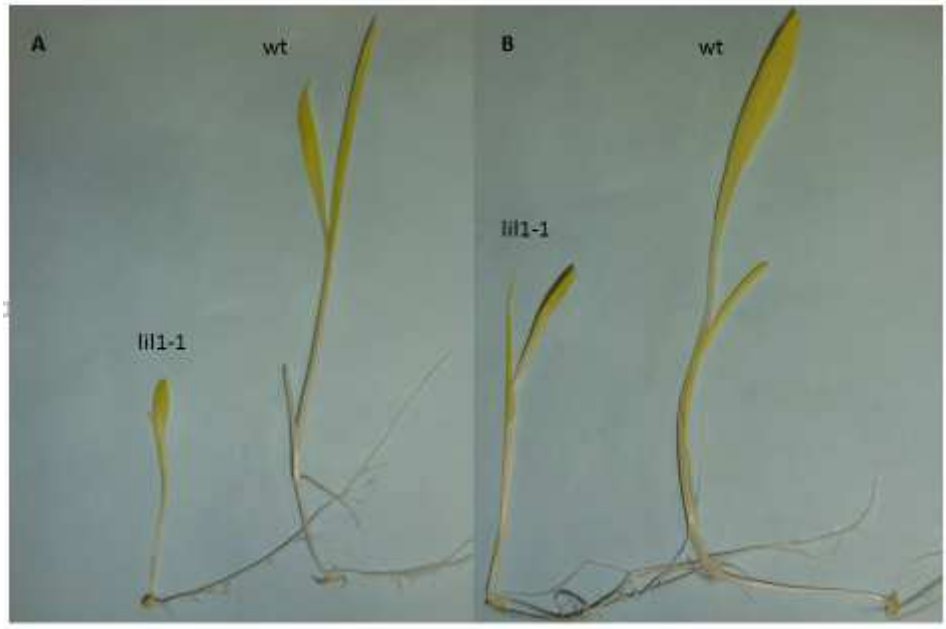

Fig. 6. *Representative lil1-1 and wt seedlings before (A) and after (B) exogenous application of brassinolide at* 10^{-8}*. The difference in seedling elongation between mutant and wild-type seedling is reduced by nearly 50%.*

Mutant characterization

The dwarf phenotype of lilliputian 1-1, as well as the tick primary root, is easily detectable in the first stage of plant growth (Fig.1 A, B). At maturity, this mutant shows delayed flowering, reduced stature, caused by reduced internode length, and curly leaves (Fig.1 C) (Dolfini et al., 1999).

In this work, to test the effect of the mutation into different genetic backgrounds, the mutation was introgressed in four inbred lines, i.e. B73, $Rscm_2$, H99 and A188 and root growth, mesocotyl and coleoptile elongation and seedling stature were evaluated. These phenotypic traits were studied in both mutant and wild-type seedlings 10 days after germination (Table 5).

In all tested backgrounds, *lil1-1* seedlings did not show mesocotyl elongation and showed a reduction of 75% in coleoptile elongation and 70% in whole stature if compared to the wild-type siblings. Some variability among the different lines was found only for root growth. In B73 and $Rcsm_2$ the difference between mutant and wild-type is lower (ranging from 30-35%) than in H99 and A188 (ranging from 45-50%). However, the mutant phenotype seems not to be influenced by genetic background.

Inbred	↑	**Average seedling height (cm)**		**Average mesocotyl lenght (cm)**		**Average coleoptile lenght (cm)**		**Average root lenght (cm)**	
		M	**wt**	**m**	**wt**	**m**	**wt**	**m**	**wt**
B73	3	3.700	13.025	0.040	0.937	0.660	2.595	11.525	16.075
$Rscm_2$	1	3.400	11.845	0.000	0.995	0.774	2.835	8.750	13.725
H99	1	5.340	18.930	0.000	0.595	0.595	2.575	12.605	23.805
A188	1	4.272	15.200	0.000	0.445	0.622	2.080	12.706	22.145

***Table 5**. Comparison between mutant and wild-type seedling height, mesocotyl length, coleoptile length and root length in 4 different genetics background. ↑: number of introgression.*

Root gravitropism was also evaluated in mutant and wild-type seedlings in the same genetic background (Table 6). All mutant samples analysed showed altered root gravitropic curvature. In B73 and $Rcsm_2$, about 40% of mutant revealed root parallel to the surface (Fig.7 B)

and 60% of mutant root grew upward (Fig.7 A). In H99 background, 50% of mutant roots were parallel to the surface and 50% upward. In A188 most of the mutants have roots growing upward (about 70%). Positive gravitropic response (Fig.7 C) was instead observed in all wild-type seedlings analysed.

Inbred	**↑**	**Seedling phenotype**	**Seedling number**	**Gravitropic response**		
				N-U	**N**	**P**
B73	3	wt	92	0	0	92
		m	38	22	16	0
Rscm$_2$	1	wt	132	0	0	132
		m	35	23	12	0
H99	1	wt	73	0	0	73
		m	30	15	15	0
A188	1	wt	39	0	0	39
		m	18	13	5	0

Table 6*. Seedlings were germinated on wet filter paper and root phenotypes were detected after 10 days of germination. Mutant roots grew either parallel to the surface (N) or upward (N-U). Positive (P) gravitropic response was instead observed in all wild-type seedlings analysed. F_2 segregating populations used in this study were obtained after introgressing the mutation in different genetic backgrounds. ↑: number of introgression.*

Thus, both gravitropism and elongation of roots are altered in lil1-1 seedlings.

We also used light microscopy to study the internal structure of the shoot apex (Fig.8 A, B) and of the leaf blade (Fig.8 C, D) of wild-type (Fig.8 A, C) and mutant (Fig.8 B, D) samples. The shoot apex showed no significant differences between wild-type and mutant plants, beside internode compression (Fig.8 B). Conversely, altered shape was observed in the mutant leaf section. Supernumerary cell layers were present in the mutant section in the region between the leaf vessels and the epidermis (Fig. 8 D).

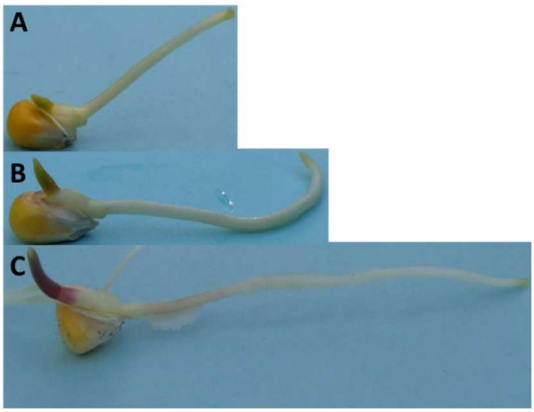

Fig. 7*. Reduced elongation and altered gravitropic response are evident in mutant roots at early stage of development. About 50% of mutant root grew parallel to the surface (B) and 50% grew upward (A). All wild-type root showed a positive gravitropic response (C).*

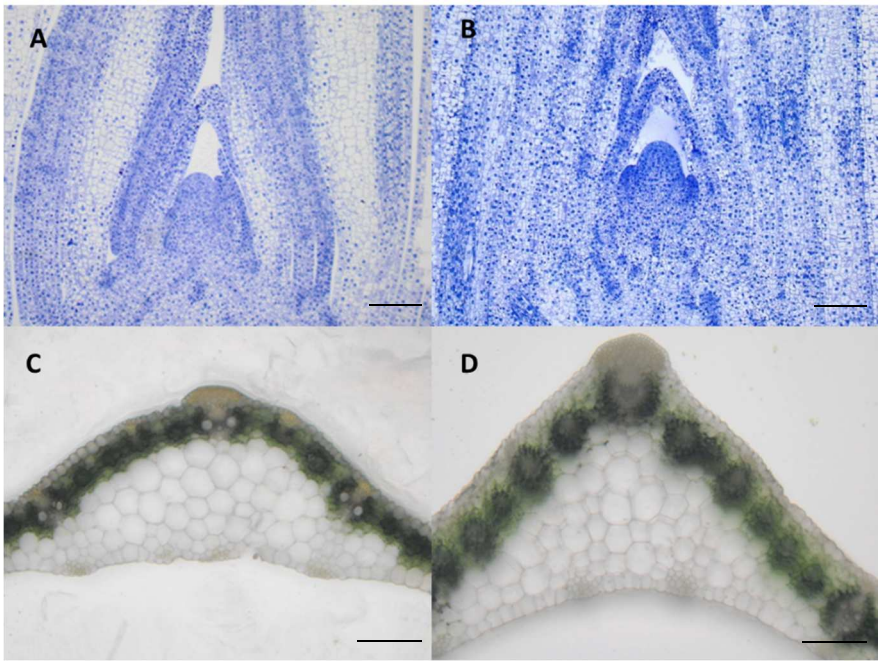

Fig. 8. *Longitudinal sections of wild-type (A) and mutant (B) shoot apex have been stained with toluidine blue and observed under light microscopy. Scale bars represent 100 µm. Longitudinal agar-embedded section of wild-type (C) and mutant (D) leaf blade. Scale bars represent 50 µm.*

The *lil1* genotype does not affect seed production and seed size

To study the effect of the *lil1* genotype on seed and ear development, F_2 progenies were obtained from F_1 heterozygous and homozygous plants and analyzed for kernel number and weight. F_1 progenies derived from introgressing the mutation in the B73 line, for one, two and three generations and in the A188, H99, $Rscm_2$ lines for one generation. The F_1 plants were selfed.

For the F_2 ears obtained from self-pollinated plants, the segregating or non-segregating constitution was determined by phenotypic scoring of 20 seedlings per ear, which were germinated on filter paper. Self-pollinated segregating and non-segregating ears were compared for the average kernel number per ear, the average kernel weight per ear and the average weight of single kernel (Table 7). Statistical analyses were conducted by using IBM® SPSS statistics version 21 and differences between the two constitutions were found also in ears from plants introgressed in B73 and A188 for one generation. The average kernel number per ear and the average kernel weight per ear resulted higher in non segregating ears, whereas no difference in the average weight of single kernel was detected.

In addition, kernels from segregating ears were individually weighed and subsequently germinated to ascertain their mutant or wild-type genotype. Also in this experiment, no differences were detected between *lil1-1* and wild-type seeds, which showed the same average weight in every background analysed (Table 8).

Overall, these results indicated that homozygous or heterozygous wild-type constitution of the F_1 plants does not affect ear development and the *lil1* gene product is not required for seed development.

To test if the function of *lil1* gene is required during male gametophytic development, the distribution of *lil1-1* kernels in the selfed ears of a heterozygous *+/lil1-1* mutant was determined by dividing each ear into three sectors (apical, central, basal) of equal length. The distribution in basal versus central and apical portion of the ear, are reported in Table 9. Data from the three ears analysed showed the expected one-quarter segregation in the F_2 generation, and the heterogeneity χ^2 test indicates that the mutant distribution in the basal sector was as expected. These results suggest that *lil1* gene function is not required in these mutants for male gametophyte development.

		F_2 average number of kernels per ear			F_2 average whole ear kernel weight (gr)			F_2 single seed weight (mg)		
Inbred line	↑	N	segregating ears	not segregating ears	N	segregating ears	not segregating ears	N	segregating ears	not segregating ears
B73	1	14	294 (7)	391 (7)	14	85.379* (7)	110.515* (7)	14	290.994 (7)	287.993 (7)
	2	24	439 (12)	495 (12)	24	103.357 (12)	116.781 (12)	24	243.733 (12)	236.245 (12)
	3	32	567 (16)	591(16)	32	117.720 (17)	133.128 (15)	29	208.480 (13)	228.120 (16)
A188	1	8	424* (4)	527* (4)	8	119.351* (4)	147.523* (4)	8	284.003 (4)	280.085 (4)
H99	1	12	205 (6)	208 (6)	12	68.514 (6)	66.928 (6)	12	336.604 (6)	325.062 (6)

Table 7. *Analysis of ears obtained through controlled self-pollination of F_1 plants. Measurements of kernel number, kernel weight per ear and kernel weight were conducted in different genetic background. ↑: number of introgression.*

		Average seed weight from segregating ears (mg)		
Inbred line	↑	N	*lil1/lil1*	*Lil1/-*
B73	1	139	292.407 (41)	295.929 (98)
	2	233	245.836 (58)	248.243 (175)
	3	130	210.363 (38)	217.435 (92)
A188	1	157	286.388 (48)	284.731(109)
H99	1	103	299.980 (30)	288.837 (73)
Rscm$_2$	1	167	201.629 (35)	202.508 (132)

Table 8. *Average kernel weight from ears obtained through controlled self-pollination of F_1 plants ↑: number of introgression.*

F_1 Genotype	**Kernels number**	**Segregation** (%)	**Distribution in different ear sectors (%)**			**Heterogeneity χ2 test**	***df***	**P**
			Apical	**median**	**basal**			
+/lil1-1 (B73 2^)	1492	24.06	26.91	21.11	25.63	4.978	2	0.10 - 0.05 0

Table 9. *Distribution of lil1-1 kernels in ears obtained by selfing three heterozygous +/lil1-1 plants.*

Effect of the *lil1-1* mutation on leaf conductance and the control of water loss.

To assess the effect of the *lil1-1* mutation on leaf development and physiology, gas exchange measurements were conducted on fourth leaves of mutants and wild-type seedlings with CIRAS2 portable photosynthesis system (Table 10). Stomatal conductance and transpiration rate were higher in the mutant (about 15% and 12% respectively) compared to the wild-type. Instead, the net photosynthesis rate of these two genotypes followed the same trend, despite the higher intercellular CO_2 concentration in the mutant. As expected, water use efficiency (WUE) was significantly higher in wild-type seedlings. All the differences resulted statistically significant ($p<0,001$ one-way ANOVA). These results suggest that lil1-1 leaves are altered in stomata formation and/or functionality.

Genotype	**Transpiration rate (E) (mmol $H_2O/m^2/s$)**	**Stomatal conductance (Gs) (mmol/m^2/s)**	**Intercellular CO_2 (Ci) (μ mol/mol)**	**Net photosynthesis (A) (μmol$CO_2/m^2/s$)**	**Water use efficiency (WUE) (μmol CO_2/mmol H_20)**
Lil1/-	3.48 ± 0.09	161 ± 3.57	36.81 ± 4.65	25.11 ± 0.52[a]	7.28 ± 0.18
lil1-1/lil1-1	3.96 ± 0.10	190 ± 5.77	62.24 ± 3.90	26.09 ± 0.86[a]	6.59 ± 0.16

Table 10. *Gas exchange traits in wild-type and mutant lil1 seedlings. Values in the table are mean ± SE. Values followed by the same letter within columns are not significantly different according to the ANOVA test (P < 0.05).*

Stomatal density and distribution were therefore analysed in both adaxial and abaxial fourth leaves surface in mutant and in wild-type seedlings by light microscopy (Fig.9). In both wild-type and mutant leaves, the stomatal density (number of stomata per square millimetre) resulted higher in abaxial surface (Fig 9.B, D), compared to the adaxial surface (Fig.9 A, C). However, in lil1-1 young leaves have been found supernumerary stomata unevenly distributed on both surfaces in comparison with the wild-type. Because stomatal density may be affected by alterations in epidermal cell size and/or changes in the ratio of pavement cells per stomata, stomatal index (si) were determined (Table 11). The cell arrangement is clearly altered in the leaf

epidermis of *lil1-1* and the stomata index is equal to that of the wild-type. Thus, the increased stomatal density, which was probably due to the higher number of cell divisions generally observed in the mutant tissue (table 11), may be responsible for the higher stomatal conductance and transpiration rate.

Genotype	**Leaf surface**	**Epidermal cell (number mm^2)**	**Stomata (number mm^2)**	**Stomatal index**	**Ratio of stomata (adaxial/ abaxial)**
Lil1/-	Adaxial	293±25	37±6	11.27±1.25	0.59
	Abaxial	287±44	61±9	17.55±1.39	
lil1-1/lil1-1	Adaxial	549±79	45±10	7.71±1.81	0.61
	Abaxial	432±70	76±13	15.03±1.92	

Table 11. *Quantification of cell number and stomata in leaf epidermis. Values are mean ± SD of 5 replicates taken from seven different fourth leaves. Stomatal index is the ratio of stomata to epidermal cells. Each value between lil1 and wild-type has a very significant statistical difference (P< 0.01)*

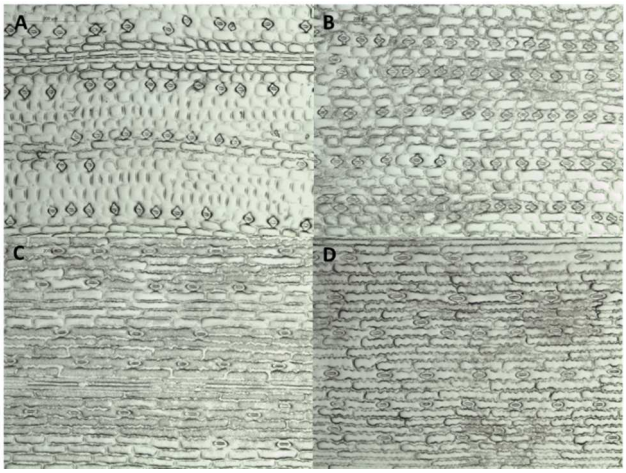

Fig. 9*. Micrographs of the adaxial (A,C) and abaxial (B,D) epidermis of lil1-1 (A,B) and wild-type (C,D) fourth leaves.*

A water loss assay was also performed to study the impact of altered stomata formation in mutant leaves in response to drought stress. Fourth leaves from mutant and wild-type seedlings

were excised and weighed over time to measure the amount of water loss. Unexpectedly, the rate of water loss, expressed as percentage of fresh weight decrease, revealed that mutant lost less water than wild-type leaves (Fig.10 A).

Moreover, we investigated whether whole plant transpiration was also reduced during drought. Both wild-type and *lil1-1* plants were grown and watered for 14 days and then subjected to drought stress by terminating irrigation. Wild-type and *lil1-1* plants showing similar developmental stages and similar number of leaves were specifically selected for drought treatments (Fig 10 C). After 7 days of drought treatment, wt plants showed severe wilting and leaf chlorosis. In contrast, *lil1-1* plants were turgid and leaves remained green (Fig 10 D). Moreover soil water content in pots of *lil1-1* plants decreased more slowly during drought stress than those of wt plants (Fig 10 B), consistent with plant phenotypes.

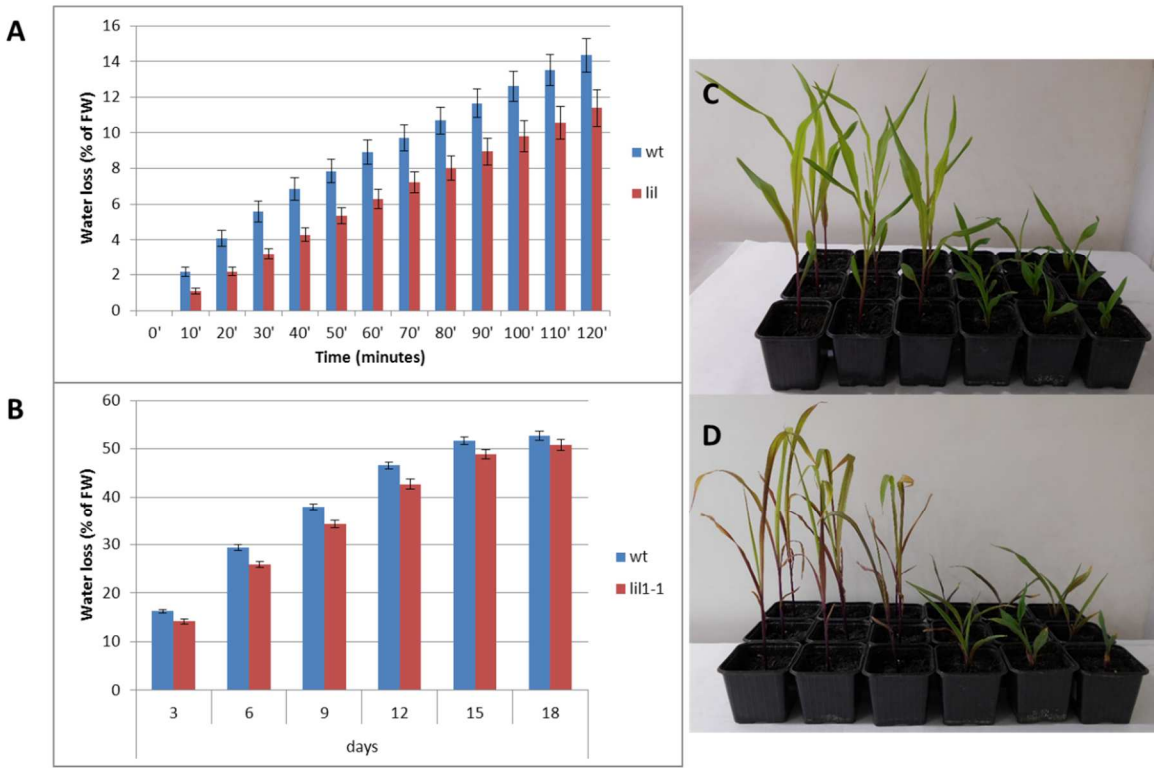

***Fig. 10**. Water loss assay. (A) Time course of water loss from excised fourth leaves, expressed as a percentage of the initial fresh weight at indicated intervals. Each point indicates the mean of 12 measurements ± SE. (B) Water was withheld from mutant and wild-type plants for 18 days and weighed every three days. Each point represent the means of 20 measurements. Bars indicate ± SE of the mean. Representative wild-type (left) and mutant (right) plants before (C) and 9 days after (D) the cessation of irrigation.*

Another gas analysis experiment was conducted on fourth leaves of mutant and wild-type seedlings grown in soil. In this case, plants were previously deprived of water for 72 and 96 hours. Plants stressed for 72 hours exhibited signs of slight drought stress (mild wilting), plants stressed for 96 hours exhibited signs of severe drought stress (strong wilting) in the drought group and no stress sign for the control plants.

Stomatal conductance (Gs) and transpiration rate (E) in both genotypes decreased noticeably compared to well-irrigated plants. However, very low levels were found only in the wild-type, especially in plant deprived of water for 96 hours, indicating an almost complete stomatal closure (Fig 11 A, B). At first (72 hours) the decreases in Gs caused a reduction in intercellular CO_2 concentration (Ci), but after 96 hours without water wild-type leaves showed a drastic increase in intercellular CO_2 concentration (Fig 11 C). In fact, contrary to mutant seedlings, wild-types were unable to maintain positive photosynthesis during drought stress (Fig.11 D) Thus, water use efficiency (WUE) was significantly higher in *lil1-1* seedlings deprived of water for 96 hours (Fig. 11 E).

All the differences resulted statistically significant ($p<0,001$ one-way ANOVA). Pooling all data together, good correspondence was found between An and Gs and between Ci and A_n. In conclusion, the reduction in gas exchange attributes under drought stress was greater in wild-type than in *lil1-1* plants.

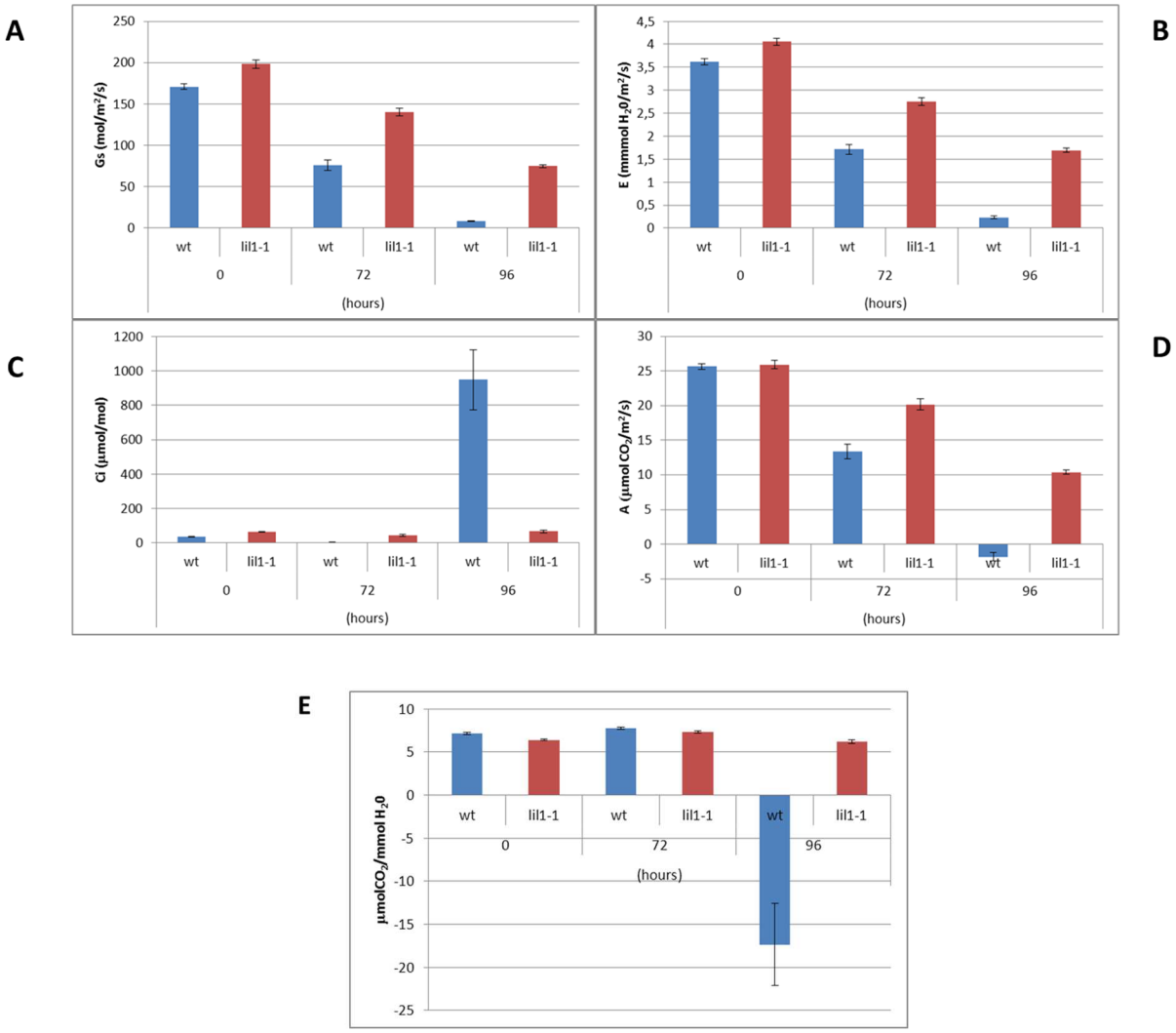

***Fig. 11**. Mutant and wild-type plant were deprived of water for 0, 72, 94 hours. The physiological variables stomatal conductance (A), transpiration rate (B), intercellular CO_2 (C) and net photosynthesis rate (D) are directly retrieved from CIRAS-II measurements. Five samples in two biological replicate were averaged and bars indicate ±SE of the mean.*

Genotype	Drought treatment	Transpiration rate (E) (mmol $H_2O/m^2/s$)	Stomatal conductance (Gs) ($mol/m^2/s$)	Intercellular CO2 (Ci) (µmol/mol)	Net photosynthesis (A) (µmol$CO_2/m^2/s$)	Water use efficiency (WUE) (µmol CO_2/mmol H_20)
Lil1/-	72 h	$1.71^a \pm 0.11$	$0.076^b \pm 6.69$	3.78 ± 1.36	13.39 ± 1.09	$7.77^c \pm 0.16$
	96 h	0.23 ± 0.04	0.009 ± 1.36	948.50 ± 174.10	-1.8 ± 0.61	-17.37 ± 4.77
lil1-1/lil1-1	72 h	2.75 ± 0.09	0.140 ± 4.82	44.07 ± 4.95	20.16 ± 0.79	$7.31^c \pm 0.14$
	96 h	$1.69^a \pm 0.04$	$0.075^b \pm 1.56$	67.22 ± 6.92	10.39 ± 0.28	6.17 ± 0.21

***Table 12**. Gas exchange traits in wild-type and mutant lil1 seedlings after 72 and 96 hours without irrigation. Values in the table are mean ± SE. Values followed by the same letter within columns are not significantly different according to the ANOVA test (P < 0.05).*

Discussion

Several plant hormones are known to be involved in controlling plant growth and development, including gibberellin (GA), brassinosteroids (BRs), and auxin (Hong et al., 2003; Multani et al., 2003; Ueguchi-Tanaka et al., 2005). Brs are steroidal plant hormones whose involvement has been well documented in several plant species, such as Arabidopsis, rice and tomato.

This study describes the characterization of a BR-deficient mutant of maize, called *lilliputian1-1*, which is ascribable to the maize gene *brd1*. As other mutant plants with inactive brC-6 oxidases (Bishop et al., 1999; Schultz et al., 2001; Shimada et al., 2001; Hong et al., 2002; Mori et al., 2002), *lil1-1* exhibit severe dwarfism due to almost suppressed internodes, reduction in both leaf sheath and blade size and absence of etiolation response (Fig.1). The phenotypic abnormalities of maize *lil1-1* mutants are similar to those described for *brd1-m1* mutants of maize. Allelism tests conducted in our laboratory suggested that *brd1* and *lil1-1* mutant, both impaired in brC-6 oxidases, are alleles at a single locus.

After exogenous application of brassinolide, the difference in the whole plant height between wild-type and *lil1-1* seedling was reduced by nearly 50%, indicating a partial recovery of the mutant phenotype (Fig.6). A partial recovery of the phenotype when treated with exogenous brassinolide was observed also in *brd1-m1* mutant of maize, in *brd1* mutants of rice (Hong et al., 2002; Mori et al., 2002) and *extreme dwarf* mutant in tomato. Treatment of the tomato mutant with BL effectively restored the dwarf phenotype but the length of hypocotyl did not reach that of the wild-type tomato (Bishop et al., 1999). Similarly, *lil1-1* mutant showed a relative seedling elongation much higher than that of the wild-type seedlings, but the average plant height remained statistically significant between the two genotypes (Table 4).

Another phenotypic aspect shared between the two brC-6 oxidases maize mutants is the unusual morphology of the leaf. Both displayed a leaf blade tissue with a ‘‘wave’’ patterns and microscopically analysis showed a disrupted epidermal cell organization and reduced elongation (Makarevitch et al., 2012). In lil1-1 leaves a supernumerary cell layers in the region between the leaf vessels and the epidermis was found (Fig.8)

In *lil1-1*, abnormalities in cell organization are present also in the root: mutant cells of the primary root have a prolonged mitotic activity and defects in the division plane alignment (Dolfini et al., 1999).

In this study, we show that lack brC-6 oxidases influenced gravitropic response. Differently from wild-type seeds placed in the horizontal position to germinate that form a primary root exhibiting a downward curvature, thus indicating that they are sensing the gravitropic force, lil1-1 seeds in the same position, exhibit roots that grow either parallel to the surface or in upward orientation (Fig.7).

The presence of brassinosteroids (BRs) was demonstrated in Arabidopsis, maize (*Zea mays*), pea (*Pisum sativum*) and tomato (*Lycopersicon esculentum*) roots (Kim et al., 2000; Yokota et al., 2001; Bancos et al., 2002; Shimada et al., 2003). Moreover, genes involved in BR biosynthesis (Bancos et al., 2002) and genes involved in BR signaling (Friedrichsen et al., 2000) are expressed in roots, suggesting that BRs are important regulatory substances in roots. Enzymes of the families CYP85 and CYP90 catalyze different oxidative steps of brassinosteroids biosynthesis and the distribution of their transcripts in Arabidopsis, pea and tomato root suggests that this organ actively participate in BR synthesis (Bancos et al., 2002). Moreover, BRI1, the putative brassinolide receptor, is expressed at high levels in the meristem, shoot, and hypocotyl, as well as in the root of Arabidopsis seedlings (Friedrichsen et al., 2000). Seong-Ki Kim et al. (2000) demonstrated that BRs are also involved in the regulation in maize primary roots, increasing the indole-3-acetic acid (IAA)-induced gravitropic response.

BRs have crucial function also in seed development, but the molecular mechanisms remain unclear. However, recent studies demonstrated that BR regulated seed size, shape and number by transcriptionally modulating specific seed developmental pathways (Jiang and Lin, 2013). In Arabidopsis, seeds of the BR-deficient mutant *det2* and the BR insensitive mutant *bri1–5* were smaller compared to wild-type seeds. Furthermore, exogenous application of BR partially rescued seed size and weight of *det2*, confirming BR positive regulation of seed size/mass. Moreover, *dwf5* and *shk1-D* produce aberrantly shaped in Arabidopsis seeds. Similarly, rice BR deficient mutant *brd2* (Hong et al., 2005) and *d61* (Morinaka et al., 2006) exhibit shortened and smaller grains. The ectopic overexpression of a BR-biosynthetic gene DWF4 in Arabidopsis transgenic plants results in increased seed yield due to a greater total seed number (Choe et al., 2001). In rice, the manipulation of the BR pathway in the stems, leaves, and roots enhances the grain yield due to more tillers and seeds and higher seed weight, probably due to the increase angle of leaf blades of the transgenic plants (Wu et al., 2008).

Our study did not reveal any influence of the *lil1* genotype on the seed formation and development.

It is evident that the comparison between homozygous *lil1-1* mutant and *Li11-1* wild-type seeds from the same segregating ear did not highlight any different in weight (Table 8). In addition, F_2 progeny ears obtained from F_1 heterozygous *Lil11/lil1-1* or homozygous *Lil1-1/Lil1-1* plants showed the same average kernel number and total kernel weight per ear and the average weight of single kernel (Table 7).

Finally, BRs are involved in the modulation of stress responses (Clouse and Sasse 1998, Bishop and Koncz, 2002).

In our study, water loss assays both on whole plants and detached leaves demonstrated that wild-type plants lost more water compared to mutants (Fig. 10 A, B). Thus, we examined the behaviour of *lil1-1* mutant plants when grown in water stress condition. After 96 hours of drought stress, wild-type leaves completely closed stomata to conserve water, impairing net photosynthesis rate. Conversely, mutant plants showed positively net photosynthesis rate and higher stomatal conductance values compared to the wild-type, indicating stomata opening (Fig 11). Moreover, wild-type plants showed wilting and chlorosis of leaves, while *lil1-1* plants were turgid with green leaves, in accordance to the observations of the water loss assay on whole plants (Fig 10 D). These data suggest that *lil1-1* plants are more resistant to drought stress and more efficient in water use during water stress.

A similar phenomenon was also observed in BR-deficient *det2* mutant, which showed an enhanced resistance to general oxidative stress. Goda et al. (2002) demonstrated that *ATPA2* and *ATP24a* genes encoding peroxidases were constitutively upregulated in the *det2* mutant. Furthermore, some oxidative stress-related genes, i.e. the cold and drought stress response genes *COR47* and *COR78* and the heat stress-related genes *HSP83, HSP70, HSF3, Hsc70-3 and Hsc70-G3* have been identified by microarray analysis of either BR-deficient or BR-treated plants (Mussig et al., 2002). Therefore, a possible explanation for the fact that the *det2* mutant exhibited an enhanced oxidative stress resistance is that the long-term BR deficiency in the *det2* mutant results in a constant in vivo physiological stress condition that, in turn, activates the constitutive expression of some defence genes and, consequently, the activities of related enzymes. If this is the case, it is conceivable that endogenous BRs in wild-type plants somehow act to repress the transcription or post-transcription activities of the defence genes to ensure the normal growth and development of plants (Shuqing et al., 2005).

DREB1A/CBF3 is a transcription factor that specifically interacts with the DRE (dehydration response element) and induces the expression of stress tolerance genes, thus conferring improved tolerance to drought, salt loading and freezing. The overexpression of the cDNA encoding DREB1A/CBF3 results in constitutive expression of the COR genes at normal growth temperature (Gilmour et al. 2000, S.J. Gilmour, A. Sebolt unpublished results) and also in severe growth retardation under normal growing conditions (Mie et al., 1999). *bri1-9*, a brassinosteroid-insensitive mutants of Arabidopsis, showed constitutively high expression of stress-inducible genes under normal conditions. Thus, this mutant showed increased cold tolerance compared with both wild-type and BRI1-overexpressing transgenic plants, despite its severe growth retardation. Moreover, endogenous expression of both stress-inducible genes as well as genes encoding transcription factors that drive the expression of stress-inducible genes were maintained at higher levels in *bri1-9* than either in wild-type or in BRI1 overexpressing plants. This suggests that the *bri1-9* mutant could always be alert to stresses that might be exerted at any times by constitutive activation of subsets of defence (Kim et al., 2010).

Our hypothesis to explain the better performance of *lil1-1* plants in water stress condition is that *lil1-1* plants are by default in a physiological water stress condition. *lil1-1* mutant plants showed phenotypic traits that are generally observed in plant subjected to water stress, i.e. inhibition of lateral root growth (data not shown; Xiong et al., 2006), reduction in leaf area and plant growth (Xu and Zhou, 2005; Monclus et al., 2006; Aguirrezabal et al., 2006), enlarged leaf thickness and increased stomatal density (Galmés et al., 2007).

In order to determine whether these phenomena occur also in *lilliputan1-1* mutants, the expression of genes involved in dehydration stress must be studied, comparing mutant and wild-type plants both in normal and drought stress condition.

The study of BR-related mutants could be important for agriculture. A final challenge would be a proper modulation of the endogenous brassinosteroids levels that lead to improve plant resistance to different environmental stress without impairing the plant growth.

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Chapter 2

Abstract

The MYB family of proteins, present in all eukaryotes, comprises different members, all characterized by the presence of a highly conserved domain: the MYB domain. Depending on the number of MYB repeats family members have been assigned to different classes. In plants, the R2R3-type MYB class of genes is the most abundant. They are involved in the control of many aspects of plant secondary metabolism, as well as determining the identity and fate of plant cells. 157 genes encoding R2R3-MYB proteins have been identified in the maize genome, but functional studies have been performed for only a small group of genes that are involved in the control of phenylpropanoid metabolic pathway.

The *fdl1-1* mutant, previously isolated in our laboratory, allowed the identification and functional analysis of a novel maize MYB gene. The *fdl1-1* mutation was caused by an *Enhancer/Suppressor* (*En/Spm*) element insertion in the third exon of the sequence encoding ZmMYB94, a transcription factor of the R2R3-MYB subfamily.

In this work, proof of gene identity was obtained using an RNAi approach and by the analysis of the mutant cDNA sequence. The first experiment ascertained the lesion in the third exon of the sequence encoding ZmMYB94. The second approach confirmed that the mutant transcript retains the *En/Spm* element.

The fdl1-1 mutant phenotype is expressed at early stages of seedling development, from germination to the three-four leaves stage, causing a general delay in germination and seedling growth as well as phenotypic abnormalities. The main features of mutant plants are irregular coleoptile opening and the presence of regions of adhesion between the coleoptile and the first leaf and between the first and second leaves. A previous study showed that fusions could be attributable to the alterations in cuticle deposition and highlighted an irregular wax distribution on the mutant leaf surfaces. Phylogenetic analysis demonstrated that its closest Arabidopsis related genes, i.e. MYB30, MYB94 and MYB96 have all been implicated in the regulation of cuticular wax biosynthesis in Arabidopsis.

To gain insight into the role exerted by *ZmMYB94* a deeper characterization of cuticle components were therefore undertaken in this study by comparing mutant and wild-type tissues.

We found a significant reduction of the amount of waxes in the mutant versus wild-type samples at earlier developmental stages. In particular, the production of C32 alcohols, which is the major compound of cuticular waxes in the maize seedling, resulted drastically reduced in the mutants and replaced by shorter chain alcohol (C26, C28 and C30) and alkane (C29).

On this basis, we speculate that ZmMYB94 specifically affects the activity of enzymes involved in the elongation of long chain wax molecules at the C30—C32 step.

In maize, some glossy mutants, i.e glossy 2 and glossy 4 show the same block in the long chain elongation. Thus, some of the subtending genes could be under the control of ZmMYB94. Contrary to fdl1-1, none of glossy mutant of maize so far characterized showed post-genital organ fusion. This difference could be due to a greater decrease (more than 90%) of epicuticular waxes observed in the *fdl1-1* mutant than in glossy mutants. It is also conceivable that ZmMYB94 affects directly or indirectly the expression of a set of genes involved in the biosynthesis of very-long-fatty acids and the failure of multiple activities has caused a worsening of the phenotype. Alternatively, ZmMYB94 could regulate also some genes involved in the biosynthesis of other cutin components. Although only minor changes in the cutin load were observed in the *fdl1-1* mutant, the affected components could be important for determining organ separation.

Recent studies strongly support the idea that cuticular wax accumulation contributes to drought resistance. However, it is still not known how wax related genes are regulated in response to drought. In our study, an increment of water loss in the mutant seedlings has been demonstrated and a correlation between the severity of the phenotype and the rate of water loss was revealed. In addition, we found that the transcript level of *ZmMYB94* increased in plant under drought stress condition. Similarly to AtMYB30, AtMYB94 and AtMYB96, which are considered positive regulators of wax biosynthesis during stress, it is conceivable that ZmMYB94 stimulates the activity of genes involved in cuticular waxes biosynthesis thus contributing to increase drought tolerance in the early phases of maize seedling growth.

Introduction

Regulation of gene expression at the level of transcription controls many crucial biological processes. A number of different factors are required for the process of transcription, including factors required for chromatin remodeling and DNA unwinding, as well as proteins of the pre-initiation complex and the RNA polymerase II complex. The transcription factors are proteins that recognize DNA in a sequence-specific manner and that regulate the frequency of initiation of transcription upon binding to specific sites in the promoter of target genes. Transcription factors—, which can be activators, repressors, or both—display a modular structure. Based on similarities in one of the modules, namely the DNA-binding domain, transcription factors have been classified into families (Pabo and Sauer, 1992). MYB family proteins possess a conserved DNA-binding domain (DBD), which is homologous to the DBD of animal c-Myb (Klempnauer et al., 1982) and is composed of up to three imperfect repeats (named sequentially as R1, R2 and R3, respectively) (Dubos et al., 2010). The R2R3 sub-class of MYB factors contains two repeats and is the most common type in plants (Du et al., 2013). 157 genes encoding R2R3-MYB proteins have been identified in the maize genome and classified into 37 subgroups, according to their structure and phylogenetic relationships (Du et al., 2013). However, functional studies have been performed for only a few maize myb genes that are involved in the control of phenylpropanoid metabolic pathway (Paz-Ares et al., 1987; Marocco et al., 1989; Grotewold et al., 1991; Heine et al., 2007; Fornalé et al., 2006). In Arabidopsis, up to 126 members belong to the R2R3- type subfamily (Yanhui et al., 2006) and the roles of many of this proteins have been demonstrated in a variety of development process, such as development of meristem, flower and seeds (Schmitz et al., 2002; Zhang et al., 2007; Petroni et al., 2008), cell cycle control (Araki et al., 2004) and stomatal closure (Liang et al., 2005). Some Arabidopsis MYB members also regulate plant responses to biotic and abiotic stress conditions (Abe et al., 2003; Raffaele et al., 2008; Van der Ent et al., 2008).

The *fdl1-1* mutant was previously isolated and characterized in our laboratory. Its mutant phenotype is expressed at early stages of seedling development, from germination to the three-four leaves stage, causing a general delay in germination and seedling growth as well as phenotypic abnormalities (Fig.12). The main features of mutant plants are irregular coleoptile opening and the presence of regions of adhesion between the coleoptile and the first leaf and between the first and second leaves. Previous study suggested that the *fdl1-1* mutation was caused by an *Enhancer/Suppressor* (*En/Spm*) element insertion, which was located in the third

exon of the sequence encoding ZmMYB94, a transcription factor of the R2R3-MYB subfamily (La Rocca et al., 2015).

Moreover, the spatial and temporal expression profile of the *ZmMYB94* gene, as determined by quantitative RT-PCR, was shown to perfectly overlap the pattern of mutant phenotypic expression. High expression was observed in the embryo, in the seedling coleoptile and in the first two leaves, whereas RNA level decreases at the third leaf stage (La Rocca et al., 2015).

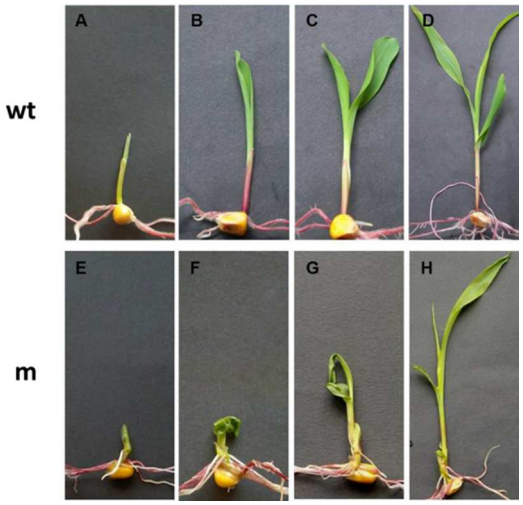

***Fig. 12**. Representative wild-type (A-D) and mutant (E-H) seedlings at succeeding stages of development: coleoptile (A,E), first leaf (B,F), second leaf (C,G) and third leaf (D,H).*

Preliminary phylogenetic reconstructions considering 104 unambiguously alignable amino acid residues from the highly conserved MYB domains of all annotated R2R3-MYB proteins from *Zea mays*, *Oryza sativa*, *Brachypodium distachyon*, *Arabidopsis thaliana* and *Vitis vinifera* indicate that ZmMYB94 falls within a well-supported clade containing representatives of all species considered, but did not allow confident assignment of an exact phylogenetic placement. Consideration of only sequences within this clade allowed a subsequent phylogenetic reconstruction including 166 aligned amino acid sites which, while failing to unambiguously resolve all relationships within the MYB sub-clade, provided strong support for co-orthology of ZmMyb94 and ZmMyb70 with functionally uncharacterized *Brachypodium* and Rice genes and as part of a monocot-specific gene family expansion. These analyses recover no support for direct orthology between ZmMyb94 or ZmMyb70 and any dicot homolog. Indeed, the most closely related dicot MYBs were *Vitis vinifera* MYB30, an additional uncharacterized *Vitis vinifera* MYB and a clade of recently duplicated Arabidopsis MYBs (AtMYB30/94/96) (La Rocca et al., 2015). The absence of direct dicot orthologs of ZmMYB94 notwithstanding, it is interesting to note that MYB30, MYB94 and MYB96 have all been implicated in the regulation of cuticular wax biosynthesis in Arabidopsis (Shepherd et al., 2006; Raffaele et al., 2008; Seo et al., 2011; Lee et al., 2014).

AtMYB30, AtMYB94 and AtMYB96 were characterized as positive regulators of wax biosynthesis (Raffaele et al., 2008; Seo et al., 2011). AtMYB94 and AtMYB96 transcription factors, closely located in the phylogenetic tree (Lee et al., 2015), provided evidence for their

function in ABA dependent regulation of wax synthesis during water stress (Seo et al., 2011; Lee et al., 2015), while AtMYB30 during biotic stress (Raffaele et al., 2008).

Deeper analysis of the fdl1-1 mutant leaves was performed by SEM analysis and evidenced an alteration in epicuticular wax deposition, which was less homogenous on the mutant than in the wild-type surfaces. Indeed, mutant leaf surfaces exhibited a patchy distribution of epicuticular waxes, with some areas evenly covered and others completely devoid. This trait, similarly to the other fdl1-1 traits, was confined to the first two leaves, since later leaves show a regular distribution of epicuticular waxes that was indistinguishable from that of wild-type leaves. This leads to the hypothesis that phenotypic alterations observed in the mutant seedlings may be attributable to e defect in the wax biosynthetic pathway (La Rocca et al., 2015).

Cuticular waxes are complex mixtures of very long chain fatty acids (VLCFAs) and their derivatives (reviewed in Tulloch, 1976). The chemical composition of these waxes varies among different species. For example, the cuticular waxes found on the leaves of Arabidopsis are mainly composed of alkanes, alcohols, and fatty acids (Jenks et al., 1995). In contrast, the waxes found on maize seedling leaves are primarily composed of alcohols, aldehydes, and esters (Bianchi et al., 1985). In maize, two distinct pathways for cuticular wax biosynthesis have been set forth (Bianchi et al., 1985). One pathway would be responsible for wax synthesis in the first five or six juvenile leaves, whereas the other would produce waxes during the whole life cycle of the maize plant. Approximately 80% of the juvenile waxes are very-long-chain alcohols and aldehydes (in particular C32) whereas approximately 70% of the waxes produced throughout the life of a maize plant consist of esters. These ontogenetic differences in wax composition lead to different phenotypes of the maize leaves; juvenile leaves of wild-type maize plants have a glaucous surface appearance, whereas all leaves appearing later in plant development have a glossy surface (Bianchi et al., 1985).

In this study, we confirmed that the mutant phenotype is ascribable to the insertion of the *Enhancer/Suppressor* mutator (*En/Spm*) in the third exon of the sequence encoding ZmMYB94, a transcription factor involved in epicuticular waxes biosynthesis, required to establish correct organ morphogenesis and the formation of boundaries between organs during maize embryo and seedling development. Moreover, the expression analysis suggests that ZmMYB94 could regulate the transcription of cuticular wax biosynthetic genes under drought stress.

Materials and Methods

Analysis of RNAi phenotype

The six progenies analysed in this study were obtained from crosses between the inbred line A188, used as female, and a hemizygous transgenic plant as pollen donor (A188 x S190.1B, A188 x S186.1B, A188 x S183.1 A188 x S185.3) and one progeny resulted from a reciprocal cross (S186.1A x A188).

Transgenic seeds, kindly provided by Dr. Peter Rogowsky RDP, ENS de Lyon, Lione, Fr., were obtained with the amplification of 481 bp *ZmMYB94* from genomic DNA of genotype B73 with primers attB1-FDL-RNAi and attB2-FDL-RNAi recombined in inverted orientation and under the control of the constitutive rice *Actin* promoter into a derivative of the integrative plasmid pSB11 (Ishida et al., 1996). This plasmid contained a Basta resistance cassette and a GFP cassette, to yield plasmid L1258. Transformation of genotype A188 was performed as described previously (Pouvreau et al., 2011). To evaluate the molecular efficiency of the RNAi construct, *ZmMYB94* expression was assayed in the third leaf of primary transformants by quantitative RT-PCR in technical duplicate.

Wild-type and mutant maize seeds were germinated in a growth chamber at 23 °C on wet filter paper. Seeds were kept in the dark for 4-5 days and then transferred to a 14h-light photoperiod and photon fluence of 70 µmol m^{-2} s^{-1}. The six T_1 progenies were visually scored for mutant phenotypes and genotyped with a single set of primers by PCR analysis (Bar1 – Bar2).

For PCR analysis, 50 ng of genomic DNA was subjected to 35 cycles of amplification with 1.25 U of GoTaq® Flexi DNA Polymerase (Promega), 1 X Colourless GoTaq®Flexi Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM primers and 1 M betaine. Annealing temperatures were between 48 and 60 °C (optimized for each primer pair, as listed in Table 13) and an extension time of 60 s.

Name	Sequence (5'-3')	Temperature
Bar1	GCGGTCTGCACCATCGTCAACCACTACATC	60°C
Bar2	ACGTCATGCCAGTTCCCGTGCTTGAA	60°C

Table 13.. *Oligonucleotide primers used to analyse T_1 progenies.*

Transcript analysis

Total RNA was extracted with Bio-Rad "Aurum Total RNA Mini Kit". For each sample, total RNA was obtained from two biological replicates to assess the repeatability of the data. All RNA samples were digested with DNAse I (Invitrogen) prior to synthesizing cDNA. First-strand cDNA was synthesized using "SuperScript III First-Strand Synthesis System for RT-PCR", following the manufacturer's protocol. cDNAs were diluted 100 fold and the quality of the cDNA was checked by means of Tub6-105 (forward primer) and Tub6-513 (reverse primer), designed on the Tubulin gene, which were used as internal control.

fdl1-1 mutant transcript

To verify the presence of the *En/Spm* element insertion in the mutant transcript, cDNAs were amplified by using one primer sets specific for the mutant allele (Spm3-F-AW-R; Table 14). Primers were designed using the Primer3 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Name	Sequence (5'-3')	Temperature
Spm3-F	TGACGGCTAAGAGTGTCGG	58°C
AW-R	CCACACAACATGCAACTTGC	58°C

Table 14. *Oligonucleotide primers used to analyse fdl1-1 mutant cDNA.*

PCR products were electrophoresed on agarose gels and purified with the Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare), according to the instructions of the manufacturer, and sequenced in both orientations at the CRIBI Biotechnology Center (BMR Genomics, University of Padova, Padova, Italy, http://www.bmr-genomics.it).

Expression of *ZmMYB94* in drought stress condition

2 µL of each cDNA were used in a final volume of 20 µL containing 10 µL of iQ SYBR Green Supermix and 0.25 µM of each primer. The primers used were AW-F and AW-R, designed on *fdl1* gene. Amplifications were carried out using an iCycler thermocycler equipped with the MyiQ detection system (Bio-Rad, Milano, Italy) in 96-well optical reaction plates sealed with optical tapes (Bio-Rad). The reaction conditions were as follows: 96 °C for 30 s, 40 cycles 96 °C for 30s, 58°C for 30s, 72 °C for 30s.

Primers used are listed in Table 15. Data are presented as the mean of two biological replicates and three technical replicates. The absence of aspecifically-amplified products was checked with derivative melting curves, obtained by progressive heating at 0.3 °C every 15 s. Raw data were collected and analyzed with the iQ5 software (Bio-Rad, Milano, Italy) with the following parameters: baseline from the 2nd to the 10th cycle and threshold calculated automatically by the software for every reaction. Differences in gene expression were calculated by the comparative delta–delta CT method (Schmittgen and Livak, 2008) with a dedicated Microsoft Excel macro created by Bio-Rad. All quantifications were normalized to the housekeeping gene ORP. Expression of the targeted gene is presented as the expression level in the stressed plant relative to expression of the same gene in plants irrigated.

Name	Sequence (5'-3')	Temperature
Tub6-105	AATGTGGCAACCAGATTGGC	54°C
Tub6-513	ATACCAGATCCAGTACCACC	54°C
AW-F	TAGCTGTTCAGATCGGTCG	58°C
AW-R	CCACACAACATGCAACTTGC	58°C
RP1-L	GAGGGCTGTACATTCTGGGA	58°C
ORP1-R	TCCTGATCAGTCACGCTGTC	58°C

Table 15. *Oligonucleotide primers used to study the expression of ZmMYB94 during drought stress.*

Light and electron microscope

Heterozygous +/*fdl* plants were grown in field and were self-pollinated. Mutant and wild-type kernels from segregating ears were collected at different days after pollination (DAP).

Immature seeds were dissected from cobs at 25, 28 and 32 DAP both for wild-type and mutant samples. Samples were immediately fixed in freshly prepared 4% p-formaldehyde in phosphate-buffered saline (PBS) (130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO_4) for 24 hours. The fixed material was placed in 70% ethanol and stored at 4°C until processed. The samples were embedded in Paraplast Plus. Microtome sections (8 μm thick) were applied to poly-lysine coated slides and de-paraffinized in xylene, dehydrated trough a graded ethanol series and stained with toluidine blue O [1% toluidine blue 1% sodium tetraborate (1:1, v/v)] and imaged under light microscope (Ortholux, Leitz, Germany).

For the analysis of epicuticular waxes, leaf pieces of wild-type, *fdl1-1* mutant and RNAi transformed plants, were dried and processed according to La Rocca et al. (2014). The specimen surfaces were examined with the same scanning electron microscope.

Cuticle analysis

The cuticle analyses were performed at the University of Bordeaux, (Laboratoire de Biogenese membranaire) with the supervision of Dr. Frédéric Domergue.

For both wax and cutin analysis, seedlings belonging to the same F_2 progeny were allowed to germinate on wet filter paper and harvested at four succeeding developmental stages. Data was collected for each stage and the same analysis were conducted both on four wild-type and mutant samples and for the comparison the means were calculated.

Wax analysis

Before extracting the waxes, fresh weight is measured.

The sample are immersed 30 seconds in 5 ml of chloroform containing 50 μg C23 alcohol and 20 μg C25 alkane. Extracts are dried under a gentle stream of nitrogen and dissolved into 150 μg of N,O-bis(trimethylsilyl)trifluoroacetamide:trimethylchlorosilane (BSTFA-TMCS; 99:1). Free hydroxyl groups are derivatized at 110 °C for 30 minutes and surplus BSTFA-TMCS is evaporated under nitrogen. Samples are dissolved in hexane for analysis by gas-chromatography.

Quantitative analysis are performed using an Agilent 6850 gas chromatograph equipped with an HP-5MS column and an Agilent 5975 mass spectrometric detector with helium as carrier gas. The initial temperature of 50 °C is held for 1 minute, increased at 50 °C/min to

200°C, held for 1 minute at 200°C, increased again at 10 °C/min to 320 °C and held for 20 minutes at 320 °C. Quantification is based on peak areas of the respective internal standard.

Cutin analysis

After wax extraction, leaf tissues are immersed in hot isopropanol for 30 minutes at 85 °C. After cooling, samples are extensively delipidated by extracting the soluble lipids successively for 24 hours with $CHCl_3$:CH_3OH (2:1), $CHCl_3$:CH_3OH (1:1) $CHCl_3$:CH_3OH (1:2) and CH_3OH, all performed at room temperature on a wheel rotating at 40 rpm. Samples are dried in a fume hood at room temperature for 2 days and then in a desiccator for another 2 days.

The dried residues are weighed and 10 to 30 mg of each samples is depolymerized by transmethylation at 85°C for 3 hours using 1M sulfuric acid in methanol containing 2% dimethoxypropane as well as 5 µg of pentadecanol (C15:0-OH) as internal standards. After cooling, 1 ml of NaCl (2.5%) is added and the released fatty acyl chains are extracted with 2.2 ml MTBE. Extracts are washed with 1 ml of saline solution (200mM NaCl and 200mM Tris, pH 8.0), dried under a gentle stream of nitrogen and dissolved in 150 µl of BSTFA-TMCS. Free hydroxyl groups are derivatized at 110°C for 30 minutes, surplus BSTFA-TMCS is evaporated under nitrogen and samples are dissolved in heptanes/toluene (1:1) for analysis using GC-MS under the conditions described above. Quantification is based on peak areas, which are derived from total ion content, using the respective internal standards (C17:0, ω-pentalactone or C15:0-OH).

Plant growth

For the water loss assay and the analysis of *fdl1* transcript under drought stress, plants of the B73 inbred line were grown in a growth chamber with a 16h illumination period (100Wm-2) at 25 °C for 14 days and regularly watered.

Water-loss assay and drought stress administration

For the water loss assay, three detached leaves were collected from mutant and wild-type seedlings at three succeeding stages of development: coleoptile/first leaf, second leaf and third leaf. Five samples for each stage were analysed. Samples were placed on a shit of Wattman paper and exposed to open air at room temperature. Their weight was measured every ten minutes for two hours.

Two replicates were performed for each stage analysed. The water loss at each interval was expressed as fresh weight decrease (%). Data were analysed using one-way analysis of variance (ANOVA).

For drought treatment, plants were subjected to progressive drought by withholding water. At the last time point leaves were severely wilted. Drought-stressed leaves from three different plants were harvested at 0, 24, 48 and 72 hours from the beginning of the treatment.

Results

The *fdl1* gene corresponds to *ZmMYB94*

The *fdl1* gene was previously detected in our laboratory through co-segregation analysis, which indicated that the fdl1-1 mutant allele was caused by the insertion of an *Enhancer*/*Suppressor mutator* (*En/Spm*). The insertion was located in the third intron of the *ZmMYB94* sequence (GRMZM2G056407) putatively encoding a transcription factor of the R2R3-MYB subfamily.

To verify the results obtained two strategies have been adopted. The first one was aimed at analysing the mutant transcripts and the second implies an RNAi approach and subsequent analysis of the mutant phenotypes.

For the mRNA analysis, mutant cDNAs were amplified by using two primer sets specific for the mutant allele (Consfdl2-F - Spm1-R, Spm3-F - AW-R; Fig. 13 A) and produced amplicons of the same size as those obtained from genomic DNA. In particular, two products, of 309bp and 625bp respectively, which are specific for the mutant allele (Fig.13 B, C), were found in all mutant cDNA analysed. Thus, sequence analysis showed that the fdl1-1 mutant transcript retains the *En/Spm* element and is predicted to encode a non-functional protein carrying a frameshift mutation at amino acid position 278 and a premature stop codon at position 298.

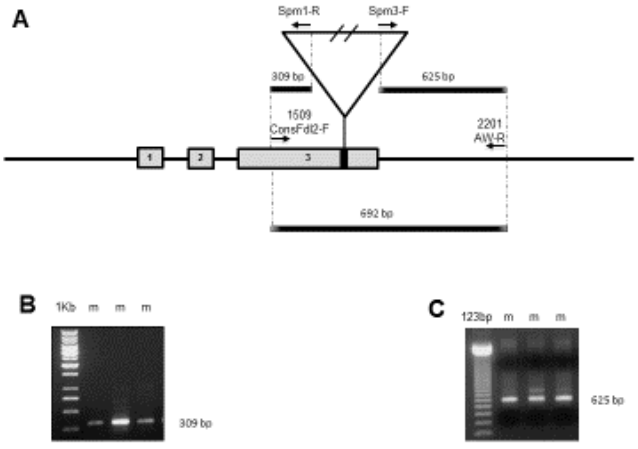

Fig. 13. *Analysis of the mutant cDNA. (A) Schematic representation of the fdl1 gene with indicated the ConsFdl2-F/Spm1-R and Spm3-F/AW-R primer sets, specific for the mutant allele, used in the analysis. The triangle corresponds to the spm insertion. PCR products from the mutant alleles (B, C). (m: mutant; 123 bp: 123 bp DNA ladder; 1kb: 1 kb DNA ladder).*

In the second strategy, proof of gene identity was obtained using an alternative approach consisting in an RNAi experiment, in which *ZmMYB94* as well as the closely related paralog *ZmMYB70* (GRMZM2G139284) were targeted (see Material and Methods). Six independent transformants were obtained as described in material and methods. The T_1 progenies were genotyped by PCR with a set of primer specific for the Basta resistance cassette, inserted in the plasmid of transformant seeds (Fig 14 B).

The T_1 progenies showed 50% segregation of transgenic individuals, as expected for single locus insertion. Transformed individual were visually scored for mutant phenotypes (Fig 14 A). Although the mutant phenotype was very mild and rather sensitive to environmental conditions, transgenic seedlings of two transformants grown at 23 °C showed some typical fdl1 mutant traits consisting of curly leaves and regions of adhesions between the first two leaves (Fig.15). In these two transformants the relative expression level of *ZmMYB94* in the third leaf was 4% and 5% respectively of that observed in wild-type (Data not shown).

A

genotype	No of seeds	% Germination	Samples genotyped	Wild type	Fdl-like	Samples with L1258-FDL-RNAi	Fdl-like
A 188 x S 190.1B	50	82%	49	18	0	31	0
A 188 x S 186.1B	59	86,4%	53	36	0	17	0
A 188 x S 183.1	19	100%	14	6	0	8	0
S186 x A 188	20	25%	5	2	0	3	0
A 188 x 182.1A	20	100%	20	8	2	12	5
A 188 x S 185.3	40	85%	39	23	2	16	6

B

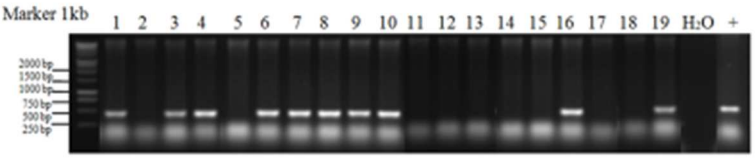

***Fig. 14**. Transformed seedling families analysed (A). Six independent T_0 plants were crossed, as pollen donor, with plants of the A188 inbred line and T_1 progeny families were analysed. (B) Single individuals were genotyped by PCR with Bar1-Bar2 primer. Wild-type: sample without the L1258-FDL-RNAi construct; T: sample carrying the L1258-FDL-RNAi construct.*

***Fig. 15**. Phenotype of RNAi transformed seedlings. RNAi transformed (A,B,C,D) phenotypes showing curly leaves and untransformed (E). The split on the first leaf (B) indicates a former adhesion with a different leaf when enrolled inside the coleoptile.*

Furthermore, SEM analysis of a transformed mutant second leaf revealed an uneven epicuticular wax distribution on its epidermal surface (Fig.16 D) resembling that of the fdl1-1 mutant epidermal surface (Fig.16 B). Similarly to the wild-type (Fig 16 A), this pattern was not detected on the second leaf of the transformed without fdl-like phenotype sibling plant (Fig.16 C).

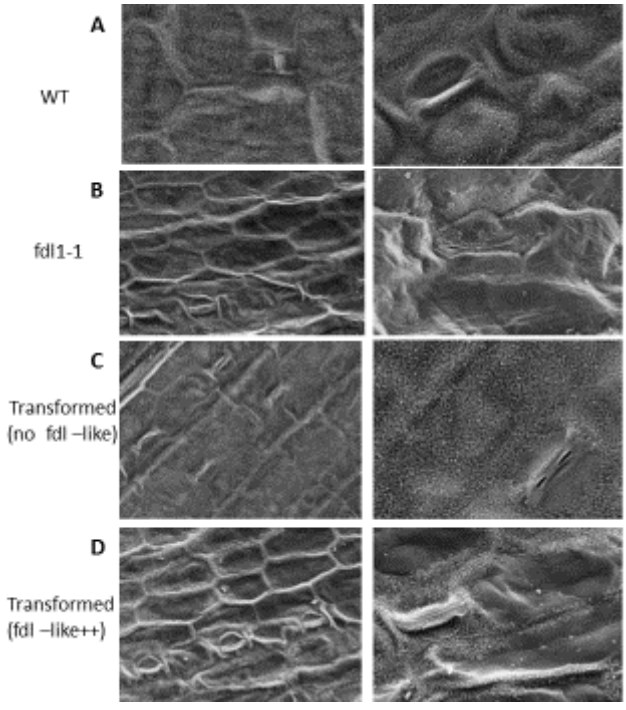

***Fig. 16.**. Second leaf of wild-type (A) and mutant (B) micrographs of epicuticular waxes at scanning electron microscopy (SEM). The epicuticular waxes are regularly distributed on the upper surface of the second leaf of wild-type (A) while they show a patchy deposition on the same leaf of fdl1-1 (B). An irregular distribution of epicuticular waxes can be seen on the second leaf of an RNAi transformed seedling (D) if compared with the second leaf of an untransformed seedling (C).*

In conclusion, we consider the data obtained from these experiments as the final proof of the gene identity. They show that the molecular lesion ascribing for the fdl1 phenotype

corresponds to an *En/Spm* insertion inside the third exon of a maize R2R3-MYB transcription factor whose subtending gene has been named *ZmMYB94* in the last version of the maize genome sequence (http://www.maizesequence.org).

The *fdl1-1* mutation affects embryonic shoot development and organ separation

Since the lack of *fdl1* action correlates with developmental defects, such as delayed germination and seedling growth histological analysis were performed on 18 DAP embryos to detect any aberration also in the seeds anatomy. Morphological analysis indicated that in mutant seeds the scutellum is less curved, leaving the embryo shoot uncovered. Moreover wide areas of coleoptile and first leaf fusion in the mutant embryo that were absent in wild-type embryos were observed (La Rocca et al. 2015).

To achieve a more detailed description of mutant embryo defects, in this work mutant and wild-type embryos were compared at later developmental stages. To this aim heterozygous +/fdl1 plants were selfed, and mutant and wild-type segregating kernels were collected at different days after pollination (25, 28 and 31 DAP).

Abnormalities in mutant embryo shoot organization were evident in every stages of development. Unlike the wild-type (Fig. 17 d-f), the mutant embryo was not entirely surrounded by the scutellum and the coleoptile (Fig.17 a-c). The scutellum was not detected in the germinal face of the embryo. A higher magnification of transversal sections disclosed the presence of wide areas of coleoptile and first leaf fusion in the mutant embryo (Fig. 18 a-c), as previously observed at earlier developmental stages. Areas of fusion were also detected between the first and the second leaf (Fig. 18 a-c), that were absent in wild-type embryos (Fig. 18 d-f). In the mutant at 32 DAP the fusion was evident also between the second and third leaf (Fig.18 c).

Taken together, these observations indicate that the effects of the fdl1-1 mutation are also evident at later developmental stages. In particular, they show that leaf fusions, which are evident in the early phases of seedling growth, are already present in the embryo prior to germination.

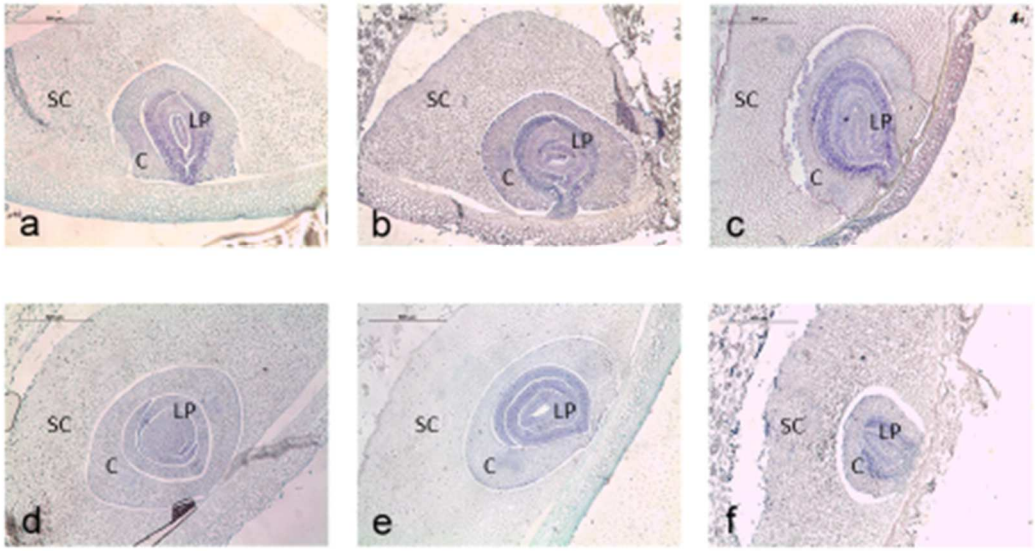

***Fig. 17**. Light microscope micrographs of transversal sections of mutant (a- c) and wild-type (d-f) seeds. Immature seeds at succeeding days after pollination (DAP) were analysed; (a, d) 25 DAP, (b, e) 28 DAP and (c, f) 32 DAP. The embryo is completely surrounded by the scutellum and the coleoptile in the wild-type (d-f) but not in the mutant samples (a-c). (C = coleoptile, SC = scutellum, LP =leaf primordia. bar = 500 mm)*

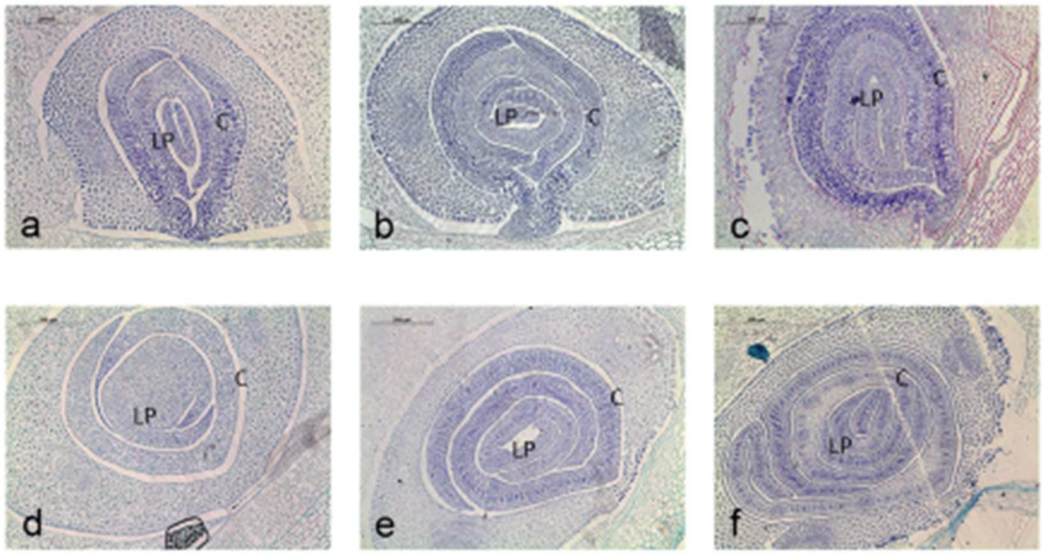

***Fig. 18**. Higher magnification of immature mutant (a-c) and wild-type (d-e) embryo transversal sections. In the mutant, the lack of tissue identity between coleoptile and first leaf and between the leaf primordia in the fused region is evident in every development stages analysed (a-c). In the wild-type, fused region are not present (d-e). (C = coleoptile, SC = scutellum, LP =leaf primordia. bar = 200 μm).*

Maize *fdl1-1* mutant shows altered cuticular wax composition

Previous analysis have shown that *fdl1-1* seedlings display organ fusion and a glossy phenotype and SEM analysis revealed an abnormal epicuticular wax deposition, which was less homogenous on the mutant than in the wild-type surfaces (La Rocca et al., 2015).

To asses any quantitative and qualitative changes in wax accumulation, we compared wild-type and mutant seedlings through chromatography-mass spectrometry (GC-MS) analysis

For both wax and cutin analysis, seedlings belonging to the same F_2 progeny were collected at four succeeding developmental stages, i.e. coleoptile, coleoptile and first leaf fused, first and second leaf fused and three-leaf stage, which correspond to the stages illustrated in Fig.12. In all stages the extraction with extensive chloroform/methanol treatment was performed from the whole seedlings. We could not analyse the leaves individually because of the fusion between the coleoptile and the first leaf and between the first and second leaves in the mutant samples.

Relative to the wild-type, the wax load of *fdl1-1* seedlings was dramatically reduced in the first three stages, while in the last one it was similar to the wild-type (Fig.19 A). In the first stage, the decrease measured in the mutant was largely the result of the reduced level of the main component of maize seedling waxes, the C32 1°-alcohol (Fig.19 A).

It is also evident in the first two-three stages, that the amount of primary alcohols with 30 or less carbons is higher in the mutant than in the wild-type, whereas the level of C31, C33, and C34 alcohols was reduced (Fig.19 B). If the relative level is considered, in wild-type seedlings the C32 alcohols are the vast majority (about 90%) in all stages (Fig.20 A), while the percentage of other compounds with less carbons, i.e. C28, C30, C31 is very low. Only C30 alcohols accumulate to a certain extent although they do not overcome the 7,5 % (Fig.20 B)

Main differences in mutants seedlings are visible at stage 1 where C26, C28 and C30 accumulate at higher level than wild-type, being 21, 21 and 28 % respectively (Fig.20 B), while C32 represent only the 30% of total alcohols (Fig.20 A). These discrepancies are much reduced in stage 2, while at later stages the mutant levels do not differ from those of wild-type. Similarly to the primary alcohols with less than 32 carbons, both alkanes and sterol levels were much increased in the first stage. Instead, the level of aldehydes with more than 30 carbons and

b-amyrin was lower (Fig.20 C). At stage 2 alkanes were still much higher in *fdl1-1* mutant, whereas at following stages mutant and wild-type profiles did not differ.

This result suggests that *fdl1-1* shows a partial block in the synthesis of long chain molecules. This block is evident at earlier developmental stages and affects the two major components of maize seedling wax produced by the alcohol-forming pathway, namely the C32 alcohol and C32 aldehyde. Moreover, the accumulation of alkanes with less than 32 carbons indicates that this block affects both the alcohol and the alkane forming pathway.

In the last stages analysed, the total wax load and the ratios of all wax components on mutant seedlings are very similar to that of the wild-type plants (Fig.19 A). This finding is consistent with the fdl1-1 phenotypic developmental profile, during which, starting form the third leaf stage, mutant plants become indistinguishable from wild-type siblings. .

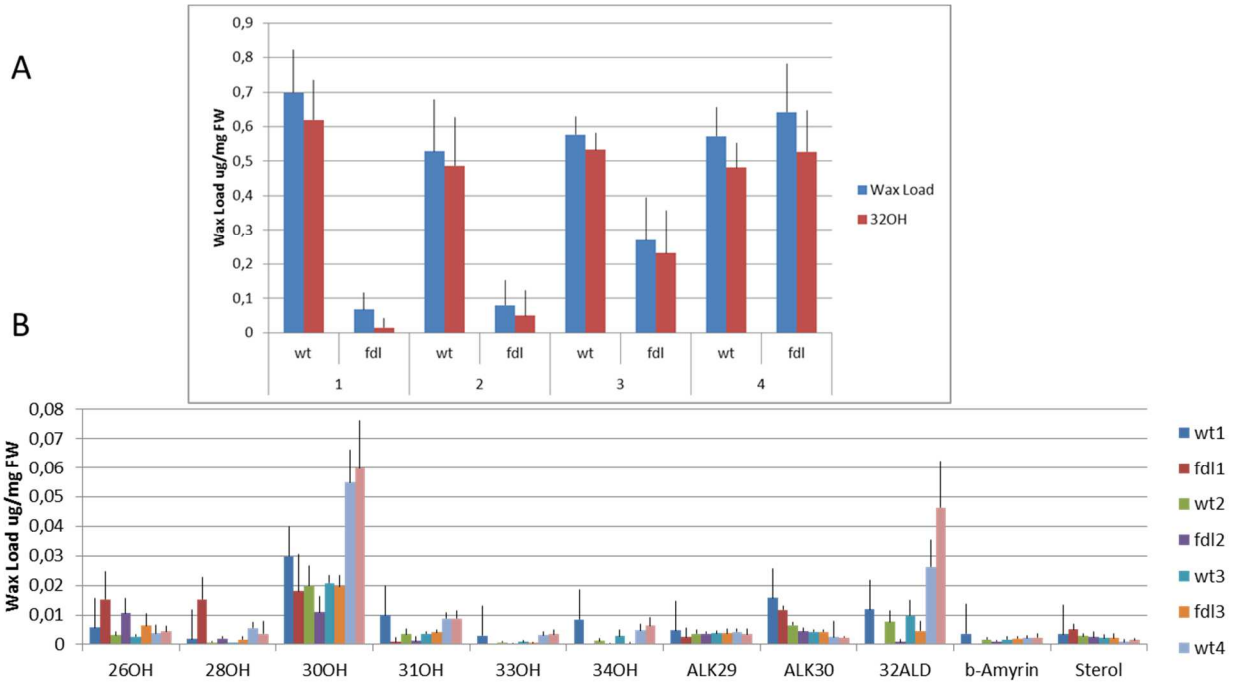

***Fig. 19**. Cuticular wax load and composition. Total wax and C32 1°alcohol load (A) and other wax compound load (B). Each wax constituent were analysed in four succeeding development stages in both wt and mutant samples. Wax coverage was expressed as μg/g of fresh weight (A,B). Each values represent the means and bars the ±SD of four replicates.*

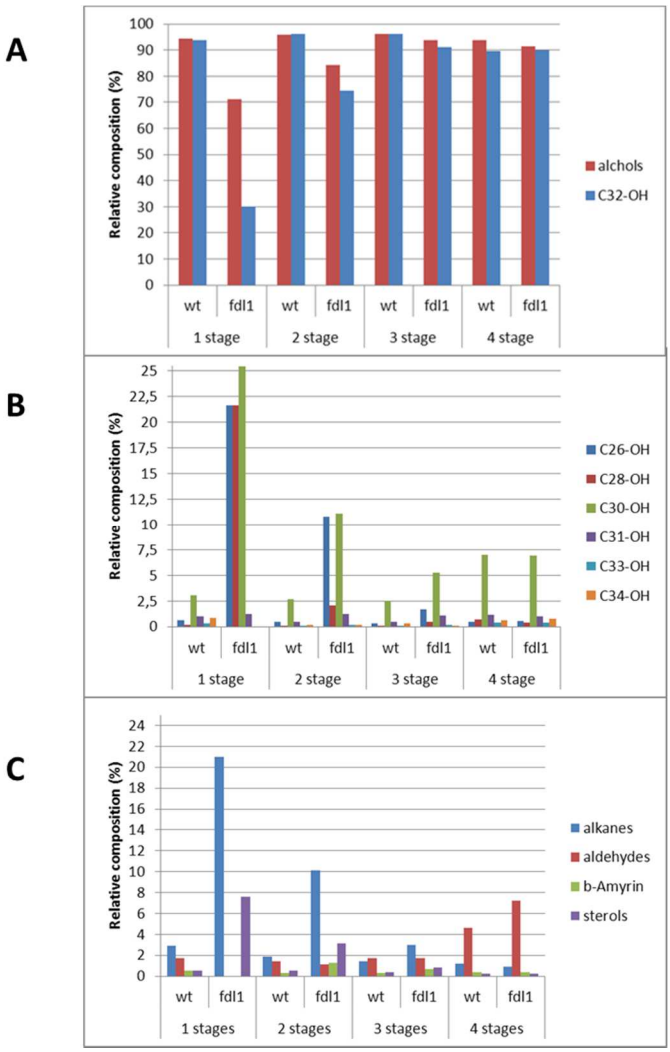

***Fig. 20**. Relative quantities of compound classes in wild-type and mutant fdl1-1 seedling waxes. (A)The percentage of primary alcohols and the main compounds of wax (C32 1°alcohol). The percentages of each chain length within the respective alcohol compound class (B) and the other compounds (C) in the total seedling at succeeding development stages.*

Waxes are part of the cuticle and derived, as cutin monomers, from fatty acid precursors; thus, the cutin polyester from mutant and wt seedlings was extracted and the cutin load and composition was analysed in detail. Gas chromatography-mass spectrometry (GC-MS) analysis of residual bound lipids after extensive chloroform/methanol extraction of whole seedling allowed the identification of 28 different compounds (Fig.21 B).

Contrary to the epicuticular waxes, the total cutin load and composition of mutants did not differ substantially from that of wild-type seedling (Fig. 21 A). The only difference detected is the relative amount of the lipids involved in the cutin formation (Table 16).

In both wild-type and mutant samples, the major lipid classes found were the phenolic compounds, in particular coumarate and ferulate, as previously reported. Coumarate was more abundant in the wild-type and ferulate in the *fdl 1-1* seedlings (Fig. 23). However, the total accumulation of aromatic compounds in the mutant resulted lower (>17 %) than in the wild-type. Also the total level of the aliphatic compounds is lower (>7 %) in the mutant, in particular the ω-OH fatty acids and the 1-alcohols (Table 16). Interesting the ratio of 18:0 epoxy and 2OH fatty acids (22:0 and 24:0) were increased in the *fdl1-1* samples (Fig.24). The strongest difference observed about the cutin composition is the ratio of fatty acids (Fig.22); the amount of mutant fatty acids is 30% higher than in the wild-type, increase mainly due to an high level of 18:2 fatty acid (Table 16).

All the differences were detected only in the first stage of growth (Fig.22, 23, 24). Indeed, *fdl 1-1* seedlings resumed the normal ratio of the cutin component during the development.

The mutation on *ZmMYB94* seems to strongly affect the epicuticular waxes deposition, with a pronounced effect on the synthesis of long-chain compounds (C32), but only minor changes in the cutin load was observed.

sample	stage	load	% reduction	% monomers	% phenolics	%alliphatics
Wt	1	1.31	0.00	4.88	75.11	20.02
fdl1		1.16	10.98	30.10	59.26	10.64
wt	2	1.12	0.00	5.40	73.13	21.47
fdl1		0.86	22.79	8.59	69.28	22.13
wt	3	1.39	0.00	5.08	74.10	20.83
fdl1		1.07	22.86	6.25	67.94	25.81
wt	4	1.71	0.00	5.03	77.13	17.84
fdl1		1.23	28.03	5.21	71.20	23.59

***Table 16**. Relative cutin wax load and composition in wild-type and mutant seedling at succeeding development stages.*

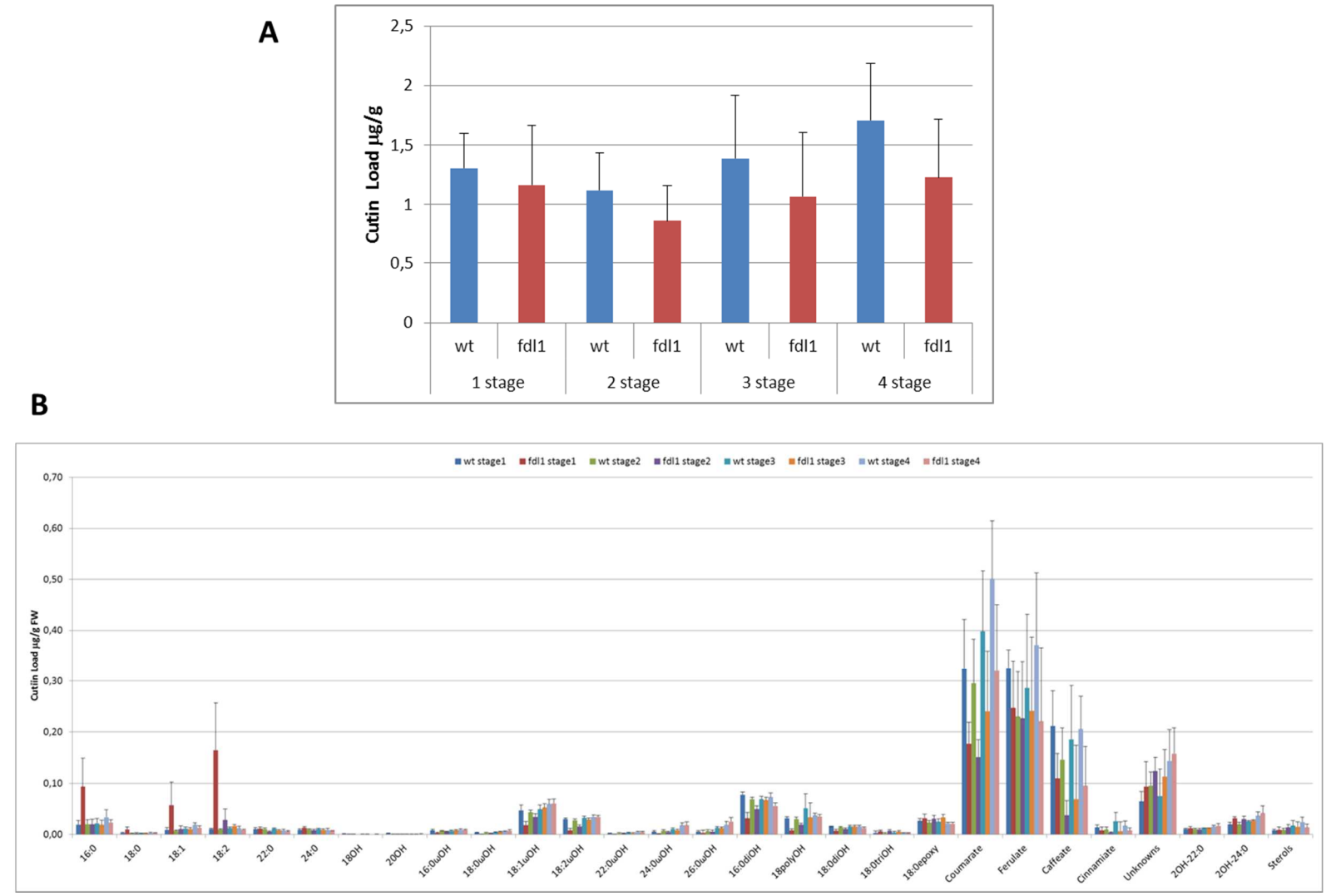

Fig. 21*. Cutin load (A) and composition (B) in wild-type and mutant seedling at succeeding development stages. Cutin coverage is expressed as µg/g of fresh weight (A,B).Each value represent the mean and bars the SD of four replicates.*

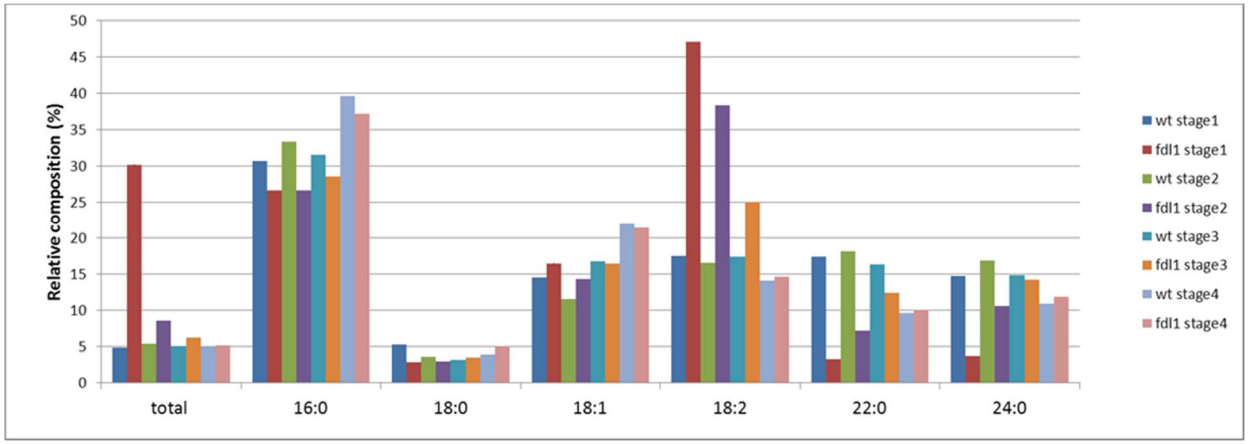

***Fig. 22**. Relative monomers composition of cutin in wild-type and mutant seedling at succeeding development stage*

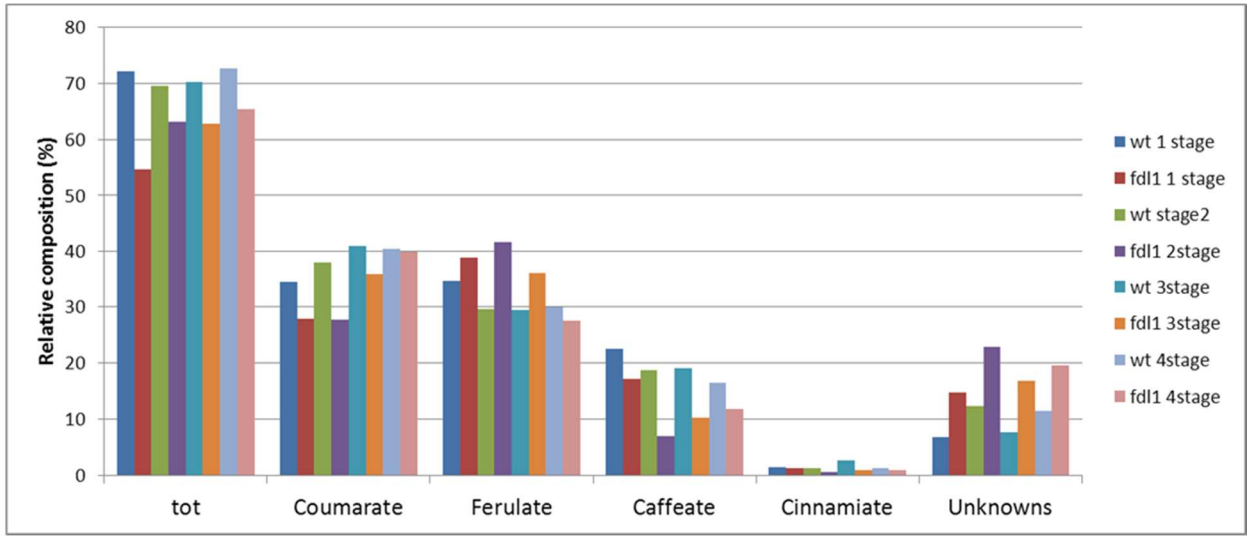

***Fig. 23**. Relative phenolic composition of cutin in wild-type and mutant seedling at succeeding development stages.*

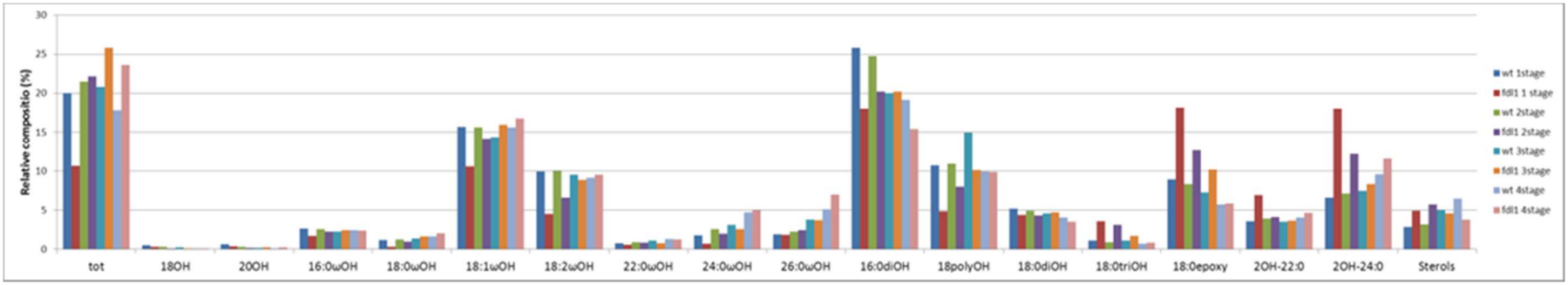

***Fig. 24**. Relative aliphatic composition of cutin in wild-type and mutant seedling at succeeding development stages.*

In mutant seedlings the rate of water loss is higher than in normal siblings

The rate of water loss was measured in seedlings and detached leaves at succeeding stages of development following air exposures.

For wild-type and *fdl1-1* sample, three different development stages (coleoptile/first leaf fused, detached second and third leaf) were analysed. This experiment was conducted under dark condition to exclude the stomata contribution. The values have been expressed as fresh weight decrease (%) in detached seedlings or leaves from both mutant and wild-type seedling and reported in Fig. 25

In all stages analysed the rate of water loss appeared significantly higher in the mutant versus wild-type siblings. Discrepancies were particularly evident in the first stage analysed. After two hours, wild-type samples lost about 10% of water at all stages analysed, whereas mutant ones at the first stage showed more than 20% of water loss (Fig 25 A). In the second and third detached mutant leaves the differences were still present although reduced. Mutants after 120 minutes lost less than 15% of water (Fig 25 B, C). Similar to what observed for other phenotypic trait visually detected, also this trait is more pronounced in the earliest stages of seedling development.

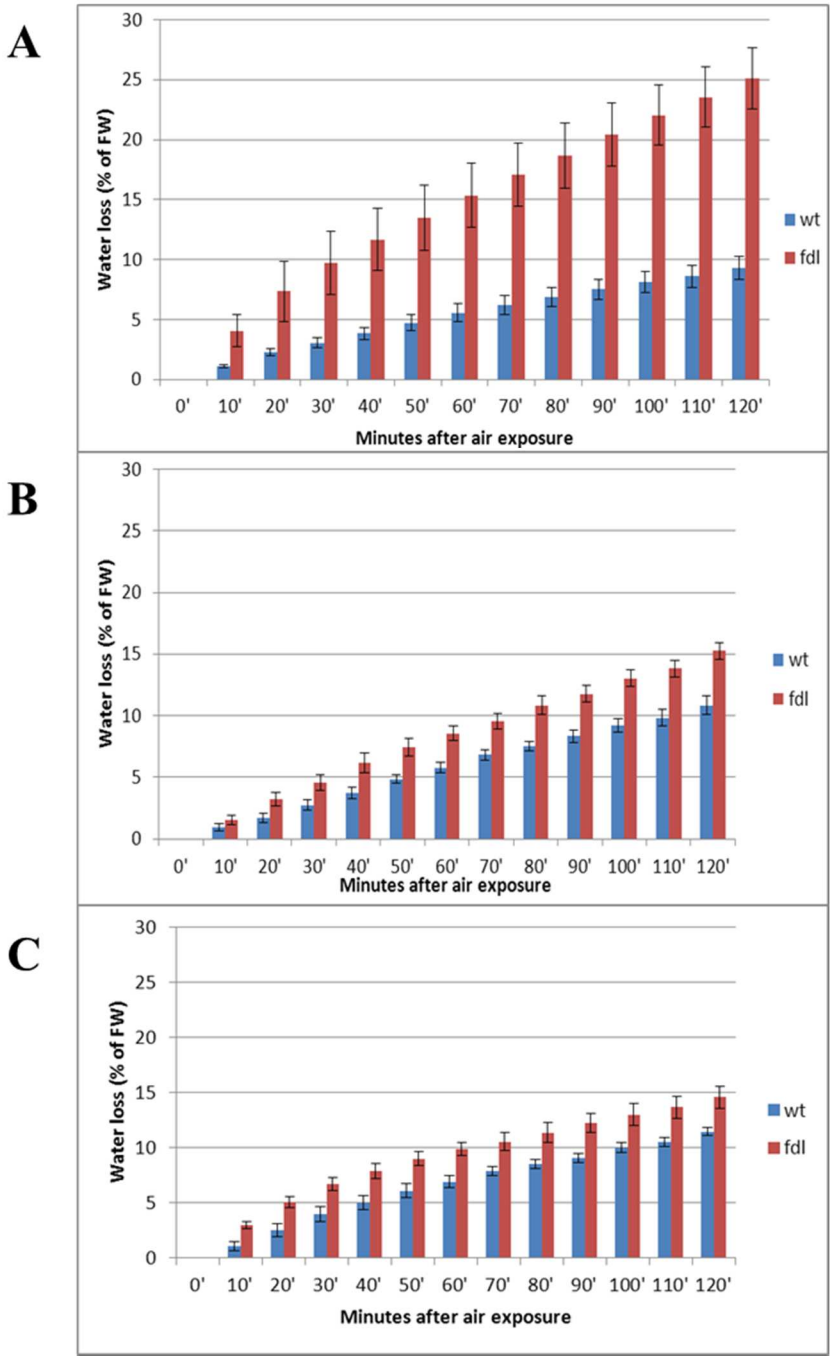

***Fig. 25**. Water loss assay. Coleoptile and first leaf (A), second (B) and third (C) leaf of dark-acclimated seedling were excised and weighed at the indicate time points. Five samples in two biological replicate were averaged at each time point. Bars indicate SE of the mean.*

ZmMYB94 is induced by drought stress

To assess the role of maize *MYB94* in abiotic stress, seedlings of the B73 genotype were subjected to water stress and quantitative RT-PCR were performed on RNA obtained from third leaves using the AW-F and AW-R *ZmMYB94* specific primers.

To this aim, seedlings were well watered for 14 days and then subjected to drought stress by complete termination of irrigation. Leaf samples were collected from seedlings at the three-leaf stage after 24, 48 and 72 hours of water deficit. The expression profile in response to the water stress was thus analysed.

After 24 hours of treatment an increment in the level of gene transcript was observed. A similar level was detected also in the samples subject to drought stress for 48 and 72 hours without any significant increment (Fig.26).

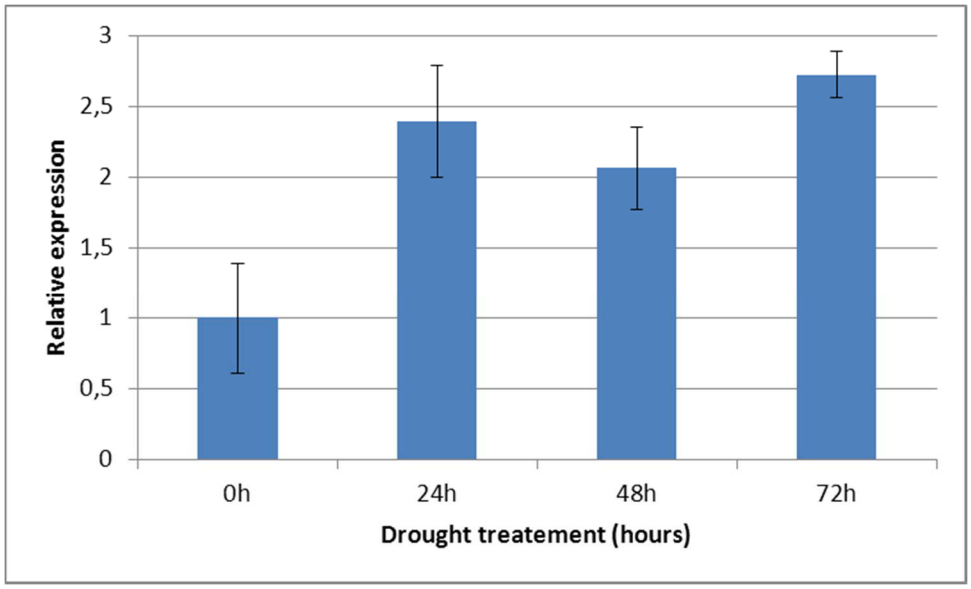

***Fig. 26**. Effect of drought stress on maize fdl1 transcript levels. Maize B73 seedlings at the three-leaf stage were grown in a controlled chamber, deprived of water for 72 hours. Total RNA was extracted from leaves at 0, 24, 48 and 72 hours after water was withheld. Transcript levels of ZmMYB94 were normalized to levels of orange pericarp transcripts measured in the same sample, and are shown relative to transcript levels. Values represent the mean of three RT-PCR replicates ± SE from three pooled plants collected in two biological replicates.*

Discussion

Previous work, based on Southern-based co-segregation analysis, provided evidence that the *fdl1-1* mutation identifies a novel gene encoding ZmMYB94, a transcription factor of the R2R3-MYB subfamily (La Rocca et al., 2015). In this work, proof of gene identity was confirmed using an RNAi approach that ascertains the lesion in the third exon of the sequence encoding ZmMYB94.The molecular lesion in the *fdl1-1* mutant was also confirmed by the analysis of the mutant cDNA sequence that showed that the *fdl1-1* mutant transcript retains the *En/Spm* element. The mutant transcript is predicted to encode a non-functional protein.

The *fdl1-1* seedling phenotype is characterized by curly leaves with regions of adhesion between the first leaf and the coleoptile or between the first and the second leaves (Fig.12) and the same regions of adhesion were found in the immature embryo (Fig.17, 18). However, after the third/leaf stage the homozygous individuals resumed a wild-type phenotype (Fig.12). This observation, together with previous experiment of mRNA expression profile, suggests that *fdl1* gene action is exerted during a strict developmental window, from embryo development to third/leaf stage.

A previous study showed that fusions could be attributable to the alterations in cuticle deposition and highlighted, through SEM analysis, showed that wax distribution on the mutant leaf surfaces was irregular (La Rocca et al., 2015). The same defect was also detected in this study in the analysis of the leaf surface of RNAi mutant phenotype (Fig.16).

Altogether these observations lead to the hypothesis that the *ZmMYB94* action is involved in the control the cuticle-related pathways. This is also supported by the finding that its closest Arabidopsis related genes, i.e. MYB30, MYB94 and MYB96 have all been implicated in the regulation of cuticular wax biosynthesis in Arabidopsis (Shepherd et al. 2006; Raffaele et al. 2008; Seo et al., 2011; Lee et al., 2014).

To test this hypothesis a deeper characterization of cuticle components were therefore undertaken in this study by comparing, through gas chromatography-mass spectrometry (GC-MS) analysis, mutant and wild-type tissues. The first observation that arose from this analysis is the significant reduction of the amount of waxes in the mutant samples (Fig.19 A). In particular, it is evident that the production of C32 primary alcohols, which was the major compound of cuticular waxes in maize, was drastically reduced and replaced by shorter chain alcohol (C26, C28 and C30) and shorter chain alkane (C29) (Fig.20 B, C). On the contrary, the total cutin load and composition of mutants did not differ substantially from that of wild-type seedling (Fig.21

A). On this basis, we speculate that *ZmMYB94* specifically affects elongation of chain molecules at the C30-C32 step.

In maize, at least 30 loci (the GLOSSY or GL loci) have been found that affect the quantity and/or the composition of cuticular waxes on the surface of seedling leaves. Mutants in these loci are impaired in the juvenile wax pathway. They are named "glossy" because of the appearance of the young leaves of such mutant seedlings that in addition retain applied water droplets on their surfaces (Neuffer et al., 1997; Schnable et al., 1994). Some of these genes could be under the control of ZmMYB94.

In particular, glossy1-2-4 are expressed in young leaves of the maize seedlings and in other tissues of the adult plants. However, the difference between mutant and wild-type phenotypes is evident only until six-leaf stage (Lorenzoni and Salamini, 1975). Similarly, the highest expression of *ZmMYB94* was observed in adult tissues, such as ear silks, but at the third/fourth leaf stage, homozygous *fdl1-1* plants resume a normal appearance, indistinguishable from that of wild-type siblings (La Rocca et al., 2015) Both glossy2 and 4 mutants show the same block as in *fdl1-1*, i.e. a block in the elongation of very long chain fatty acids from C30 to C32 (Bianchi et al., 1975). Both glossy2 and glossy4 mutants displayed a reduced amount of surface waxes to one-fifth of wild-type level in the first six seedling leaves and the C30 chains (C29 in the alkane fraction) prevail while the normal plant synthesizes almost entirely the C32 homologues (C31 for the alkanes) (Bianchi et al., 1975; Bianchi et al., 1985).

Glossy1 juvenile leaves showed a wax load reduction of 73% compared to wild-type due to a decrease of both aldehydes and long chain primary alcohols (C32) (Bianchi et al., 1977). Differently from gl2-4, gl1 mutant did not affect the alkane-forming pathway (Hansen et al., 1997), but only the alcohol-forming pathway, indicating that the block is successive.

A recent work revealed that glossy3 gene encodes an R2R3 type myb transcription factor (Stracke et al., 2001). The GL3 protein is most similar to the Arabidopsis protein MYB30 and MYB60. Consistent with the phenotype of the gl3 mutant, the Arabidopsis MYB30 gene regulates the biosynthesis of very-long-chain fatty acids (Liu et al., 2012).

Interestingly, the ancestor of GL1 is the same of that of WAXES2 (78% homology) and ACERIFERUM1 (35% homology) genes of Arabidopsis. Mutations in both genes caused dramatic alteration in composition of cuticular waxes (Lorenzoni and Salamini, 1975; Bianchi et al., 1985; Jenks at al., 1995; Chen at al., 2003). *Cer1* mutant is deficient only in the alkane (which are the major compounds in Arabidopsis waxes) and had increased aldehydes, whereas

wax2 is deficient in both aldehydes and alkane, suggesting that the *wax2* blockage is precedent to cer1 in the waxes biosynthetic pathway. The overall change in wax composition on *gl1* seedlings is similar to that observed on wax2 mutant leaves. In conclusion, these biochemical data on composition of mutant waxes also support the conclusion that GL1 is more closely related to WAX2 than to CER1. However, because of the differences in wax composition between maize and Arabidopsis, it is not conclusive in defining the homology in gene function (Sturaro et al., 2005).

In rice, Glossy1 (GL1)-homologous gene OsGL1-3 is ubiquitously expressed at different level in rice plants except root and transgenic experiments showed an increased cuticular wax amount in plants with OsGL1-3 overexpressed and a decrease in the knock out. As well as in gl1, these alterations were mainly due to the prominent changes of C30-C32 aldehydes and C30 primary alcohols (Zhou et al., 2015).

CER2 from Arabidopsis and its ortholog GL2 showed the same localization in the ER in epidermal cells at the inflorescence stem apex, which actively synthesize cuticular lipids (Haslam et al., 2012; Velasco et al., 2002). Hence, even though the chemical compositions of the cuticular waxes of maize seedling leaves and Arabidopsis stems and siliques are quite different, the mutations at the gl2 and CER2 loci of both maize and Arabidopsis affect the terminal elongation reactions in VLCFA biosynthesis. Thus, the structurally similar CER2 and GL2 proteins share a similar (but not identical) function. This is particularly interesting because even though the cuticular waxes on Arabidopsis leaves are derived from 32- (like those of maize) and 34-carbon fatty acids (Hannoufa et al., 1993; Jenks et al., 1995), the cer2 mutation does not affect the constituents of leaf waxes (Xia et al., 1996).

Contrary to *fdl1-1*, none of glossy mutant of maize characterized showed post-genital organ fusion. This difference could be due to a greater decrease (more than 90%) of epicuticular waxes in our mutant. Differently to the glossy mutant characterized, which are involved in specific step of the biosynthetic pathway, ZmMYB94 could affect directly or indirectly the expression of a set of genes involved in the biosynthesis of very-long-fatty acids. It is conceivable that the failure of multiple genes with the consequent greater decrease of waxes accumulation has caused a worsening of the phenotype. Alternatively, ZmMYB94 could regulate also some genes of cutin, but only minor changes in the cutin load were observed in the mutant. However, a correlation between cuticle membrane defects and post genital organ fusions were not observed in maize and other monocots. Maize seedlings treated with an inhibitor of cutin synthesis have no visual phenotypic alterations (Lequeu et al., 2003). On the contrary, some

Arabidopsis mutants displayed also the cutin morphology altered, as wax2, which shows fused leaves and a cuticle membrane thicker. Collectively, these results suggest a different role of maize and Arabidopsis cuticles in the prevention of post genital organ fusion.

Recent studies strongly support the idea that cuticular wax accumulation is also associated with drought resistance response (Aharony et al., 2004; Zhang et al., 2005, 2007; Kosma et al., 2009). The cuticle acts as a barrier against nonstomatal water loss (Riederer and Shreiber, 2001).

The most closely related dicot of ZmMYB94 of Arabidopsis AtMYB30, AtMYB94 and AtMYB96 are positive regulators of wax biosynthesis during stress. AtMYB94 and AtMYB96 are able to up-regulate the transcription of cuticular wax biosynthetic genes, although they activate distinct target genes, with the exception of *KCS2/DAISY* gene (*KCS1, KCS2, KCS6, KCR1,* and *CER3* are direct targets of AtMYB96, while *WSD1, KCS2/DAISY, CER2, FAR3,* and *ECR* genes are targets of AtMYB94). In AtMYB94- and AtMYB96-overexpressing plants the total wax loads increase in leaves as compared to those of wild-type plants, and at a lower level also in stems, even if this effect is not so evident, probably because total wax load in this structure is already high (Lee et al. 2015). The rate of cuticular transpiration in leaves of plants overexpressing one of the two genes was reduced under drought stress (Seo et al. 2009 and Lee et al. 2015). Moreover, a knockout mutant for AtMYB96 was characterized and it showed the opposite phenotype to the overexpressing lines, particularly the down-regulation of the wax biosynthetic genes and the decrease by 34% of total wax load (Lee et al. 2015). Hence, AtMYB96 and AtMYB94 TFs may act as master transcriptional activators of wax biosynthesis and accumulation in response to drought. Indeed, in plants overexpressing MYB30 inoculated with the bacterial pathogen Xcc (*Xanthomonas campestris pv campestris*), *CER2* and *CER3*, two genes encoding the VLCFA elongase subunits, expression were found up-regulated, indicating that wax synthesis might be under MYB30 regulation in biotic stress (Raffaele et al. 2008).

In our study, an increment of water loss in the mutant seedlings has been demonstrated (Fig.25). Moreover, a correlation between the severity of the phenotype and the rate of water loss was revealed, confirming the important role of waxes accumulation in the preventing.

The expression of *MYB94* was also analysed following administration of water stress to young seedlings. The increased level of gene transcript observed suggests that *ZmMYB94* transcription is stimulated by drought. It is conceivable that the gene product itself stimulates the activity of genes involved in cuticular waxes biosynthesis thus contributing to increase drought tolerance in the early phases of maize seedling growth.

Detailed knowledge of waxes biosynthesis and deposition should greatly improve our ability to develop the understanding of the molecular mechanism involved in protecting leaves from biotic and abiotic stress.

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Conclusion

Water is a major limiting factor affecting plant growth, development and yield mainly in arid and semiarid regions where plants are often exposed to periods of water deficit stress also known as drought stress. Drought is one of the major causes for crop loss worldwide, reducing average yields with 50% and over (Wang et al., 2003). One of the earliest responses to drought is stomatal closure. Stomatal closure allows plants to limit transpiration, but it also limits CO_2 absorption, which leads to a decreased photosynthetic activity (Nayyar and Gupta, 2006 and Yang et al., 2006).

Maize is the third most important crop worldwide, and is cultivated in both temperate zones, such as the U.S. Corn Belt and the Mediterranean area, and in the tropics, including Mexico, Central and Latin America. The scarce and highly variable precipitations in this region make efficient planning of water use for irrigation necessary for most summer crops. Maize crops are impacted by drought throughout the life cycle, with the greatest losses being observed when stress occurs in the development phase just before and after flowering (Passioura, 1996).

Breeding drought tolerant maize cultivars could limit the losses to yield in water limiting conditions (Campos et al., 2006). Alternatively, the identification and transfer or manipulation of genes that confer resistance/tolerance to drought stress through transgenic technology is often projected as one solution for protecting crops against a water stress environment and increasing crop yields worldwide, particularly in less developed areas that are threatened by food scarcity and low crop productivity (Nelson et al. 2007). Thus, understanding how plants respond to drought can play a major role in stabilizing crop performance under drought condition.

During the response and adaptation to diverse abiotic stresses, many stress-related genes are induced and numerous genes have been reported to be up-regulated under stress conditions in vegetative tissues (Seki et al., 2002; Zhu, 2002).

To gain insight into the regulatory mechanisms for drought stress tolerance, characterization of drought-resistant and drought-susceptible mutants is important.

In this study, we performed the characterization of two mutants involved in seedling development. The functional analysis of the subtending genes revealed their putative involvement in the drought stress response. In particular, the *lil1-1* mutant, deficient in active BRs, seems more efficient in preventing water loss, resulting in a higher tolerance to water

stress. On the contrary, *fdl1-1* resulted more sensitive to drought due to the aberrant cuticular waxes biosynthesis in its young leaves.

The characterization of *lilliputian1-1* and *fused leaves1-1* mutants may result of importance for the understanding of molecular mechanisms underlying drought stress response and ultimately genetic variants of these genes could be useful in future application aimed at enhancing drought tolerance in agriculturally and ecologically important plants.

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