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GENETIC STUDY AND AGRONOMIC EVALUATION OF *brachytic 2* MUTANT IN MAIZE

AGR07

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1 INTRODUCTION

1.1 Maize

Cereals represent the most important renewable source of food, forage and raw materials for industries. For this reason the research on cereals got a renewed attention from the biotechnological sector. Within the cereals, maize has a particular importance for its high yield and its enormous biomass production, both for food and for industrial products; indeed, it is considered the staple ingredient in feed rations for livestock.

The cultivation of maize in the EU occupies an area of about 5 millions hectares of which just less than 50% are used for green fodder or silage.

Italy is the second European Country after France for grain production and for cultivated surfaces: Italy produces about 10 millions tons of grain on a surface of about 1 million of hectares.

It is estimated that in industrialized countries the 85% of maize grain is utilized for livestock feed, 10% for human food and the remaining 5% for industrial uses.

Maize, for its particular physiology, is one of the most efficient plant in transforming solar energy and one of the most plastic for changing its productive features according to environmental conditions and human needs: indeed, at first it met the American populations food needs, then its cultivation has been moved to other continents where it has adapted to various climatic conditions, and today it is the third important crop after wheat and rice.

On a global level, it covers about 140 millions of hectares of soil surface, and it provides about 700 millions tons of grain. The average yield per hectare varies considerably: from 1.5 – 1.8 tons in developing countries, up to 7 – 8 tons in developed countries. The Italian yield per hectare is almost 10 tons, but the Lombard one is even greater.

As mentioned above, maize physiology can perform one of the best photosynthetic efficiency in the plant world. Indeed, it belongs to the group of C_4 plants, the plants that have as the first photosynthetic product a 4-carbon atoms compound (malic acid, oxaloacetic acid).

These plants don't have photorespiration, they are therefore less susceptible to energy losses, and can utilize CO_2 even at concentrations ten times lower than the C_3 plants. C_4 plants show at their superiority at best at high levels of temperature and light intensity.

Finally, maize is one of the model species most utilized to study the role of the genes in biosynthetic pathways and plants morphogenetic: thanks to its dimensions, the practices of dissection are easier,

and thanks to the abundance of tissues in the embryo, leaves and ear, great quantities of material are available for biochemical and molecular analyses.

Two features of maize plants helped largely genetic studies: the ear size and the development of separated male and female inflorescence.

A normal plant can produce about 4-700 seeds. The plants that sprout from them can easily be crossed or selfed producing a wide progeny particularly useful in genetic studies. This progeny can be obtained in a relative short period, between 100 and 150 days from the sowing.

Moreover the localization of male flowers in the tassel and female flowers in the ear allow to avoid pollutions with undesirable pollen, simply hooding the ear without emasculation.

1.1.1 The plant and its parts

Maize (*Zea mays* L.) belongs to *Poaceae* family; it is an annual, monoecious plant, with a spring-summer cycle with a single stem (Fig. 1). The size of cultivated varieties can reach a height of 4 meters.

The root system is sorted, composed of three orders of roots:

- seminal roots, derived from the seed; they don't grow more when the plantule has 5-6 leaves;
- adventitious roots, originated from the nodes of the stem, 2-3 cm below ground, constitute the real root system;
- aerial roots, formed from the first 2-3 nodes above ground, they have mainly function of anchoring.

The stem, called stalk, has a diameter of 2-4 cm, a height of 1-4 m, alternation of nodes and internodes with an increasing length from the base to the top. Internodes elongate separately thanks to a meristematic area just above each node. The presence of a growth point for each node determines a telescopic elongation and confers to the plant a rapid development that, in our environment, can easily reach and even surmount 7-8 cm per day. Leaves are alternate, one for each node, in a variable number: from 8 to 10 in short cycle varieties; up to 22-24 in late varieties. The leaf sheath surrounds almost completely the internode above the node of origin; the leaf blade, lanceolate in shape, has variable posture from horizontal to more or less built up. The average leaf area is about 500 cm^2 and reaches higher values in the median leaves; the ligule band closely the stalk. The male and female inflorescences are located in two distinct portions of the plant: the male one, commonly called tassel, is an ear brought at the top of the stalk, it is formed by spikes with

rows of spiklets grouped in pairs – one sessile and the other with a short peduncle; they have glumes and each of them brings 2 flowers with 3 stamens; each plant produces more than 20 millions of pollen granules. The female inflorescence, commonly called ear, is, more precisely, a spike with a great axis (spadix) insert at the axilla leaf, placed at the 6^{th} – 7^{th} node below the tassel. It is formed of the cob, a great central axis 5-20 cm long, on which are inserted 8-20 rows of sessile spiklets grouped in pairs; each spiklet has two flowers, one of each fertile. The ear is supported by a peduncle with 8-10 nodes from which leaves generate. These are modified in bracts which completely surround the ear, they are called husk. The female flower is composed of a sole big ovule and a filiform style that protrudes from the bracts at the top of the ear; all the styles are commonly called silk or beard. The first styles that are issued are those of the bottom of the cob, while the last are those from the top.

The fruit-seed (Fig. 2) is a sub-spherical caryopsis, more or less flattened, commonly yellow or orange, but, in some varieties, it can take white or dark colour; it is a dry indehiscent fruit, which consists of three parts:

- the pericarp, with maternal origin that is the external coating tissue;
- the endosperm, formed of a proteic external layer, called aleurone, and a internal layer rich of starch;
- the embryo, from which the new plant origins.

According to the way the proteic grid and the starch are disposed, the caryopsis may have a powdery, vitreous fracture or a semi-vitreous one.

The life cycle takes place in about 100-150 days, from the sowing to the harvest; the length of the cycle is normally indicated in days, or in conventional classes fixed by FAO, increasing with the duration of the cycle (classes from 100 to 800).

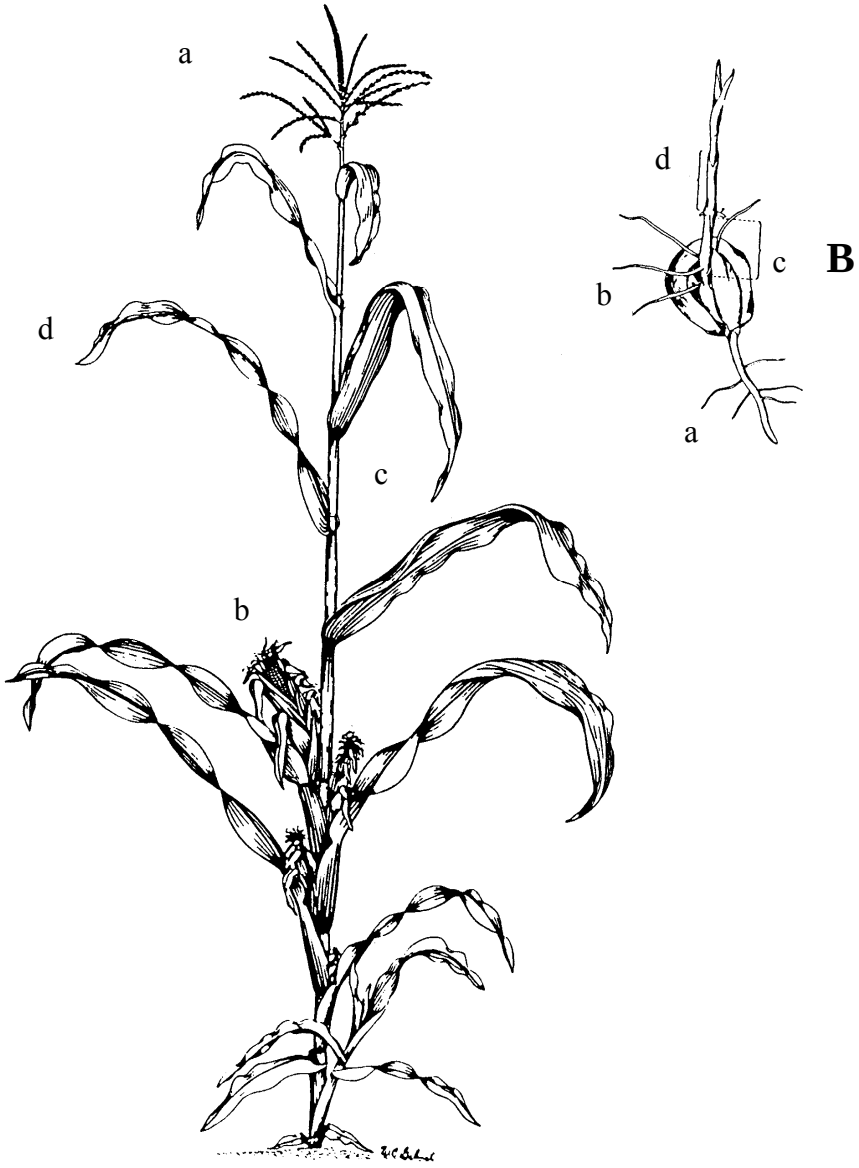


Fig. 1 Maize plant morphology.

A Adult plant: a) male inflorescence called tassel, b) female inflorescence called ear, c) stem or stalk, d) leaf.

B Seedling: a) primary root, b) seminal roots, c) mesocotile, d) coleoptile

(taken from Neuffer *et al.*, 1997).

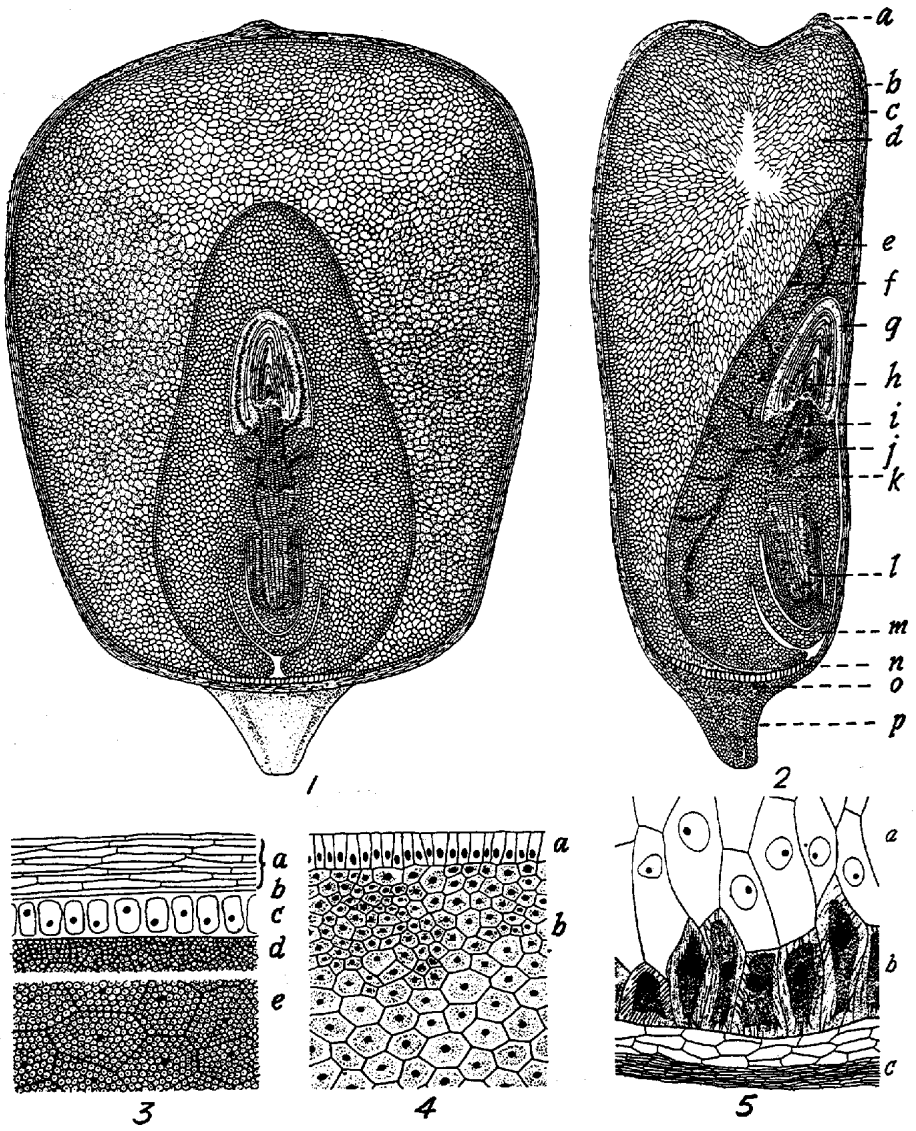


Fig. 2 Mature seed.

(**1**-**2**, 7X) Longitudinal (1) and transverse section (2) of the mature maize caryopsis: a) scar silk b) pericarp, c) aleurone, d) endosperm, e) scutellum, f) granular layer of the scutellum, g) coleoptile, h) leaves, i) first internode, j) seminal lateral root, k) scutellar node, l) primary root, m) coleorizza, n) endosperm conduction basal cells, o) dark abscission layer, p) pedicel of the flower .

(**3**, 70X) Magnification of the section on the pericarp and the endosperm: a) pericarp, b) nocellar membrane, c) aleurone, d) marginal endosperm cells e) internal endosperm cells.

(**4**, 70X) Magnification of the scutellum section: a) glandular layer, b) inner cells.

(**5**, 350X) Vertical section of the basal endosperm region: a) ordinary endosperm cells, b) run palisade endosperm cells, c) abscission layer.

(taken from Neuffer *et al.*, 1997)

1.1.2 Biological-Physiological Cycle of Maize

The biological cycle in higher plants is characterized by the alternation of two phases, the sporophytic phase (2n) and the gametophytic one (n). The sporophyte is represented by the plant. The male and female gametophytes are instead represented by the pollen granule and the embryo sac that arise in the male inflorescence (tassel) and in the female one (ear). According to the scheme in fig.3, a mature pollen granule contains three haploid nuclei: a vegetative one, and two generative (sperms); the embryo sac contains 8 nuclei, three at one pole (egg and two synergids) two at the centre (polar nuclei), and three at the opposite pole (antipodal).

Fecundation consist in the fusion of a generative nucleus with the egg to form a zygote from which the diploid embryo generates, and in the fusion of the second generative nucleus with the two polar nuclei to give origin to a triploid cell that, by continuous mitosis, produces the endosperm. This process, typical of the angiosperm, is called double fecundation.

In the soil with favourable conditions of temperature and humidity, the seed soaks water, mobilizes reserve substances and germinates emitting coleorhiza and coleoptile. After some days the seedling emerges from the soil and until the 4-6 leaves stage, it feeds on endosperm reserve substances. Subsequently the vegetative development starts. It lasts until the root system is completed and the foliar structure is complete. 35-40 days after sowing, the basal internodes start to elongate and the plants enter in a rapid growing phase that ends with the emission of the tassel. In the meanwhile also the root system ends its development emitting the adventitious and aereal roots. The whole duration of the vegetative phase, since the emergence up to the flowering, is directly related to the entire cycle duration.

At the same time or slightly after the issue of the tassel, the issue of the ear tip at the axilla of the 6^{th} – 7^{th} leaf below the tassel occurs. The release of pollen follows the issue of the tassel with consequent pollination of the silk and fecundation of the eggs. From now on the caryopsis thickens rapidly and since the third week it reaches the final size and it is filled with a milky and sugary liquid (milky ripeness). Then, the sugars condense into starch giving the grain at first a pasty consistency, wax type (waxy ripeness), and then floury. About 40 days after the flowering it reaches the physiological maturity with the formation of the so-called “black spot”; septum that interrupts nutrient relationships with the plant.

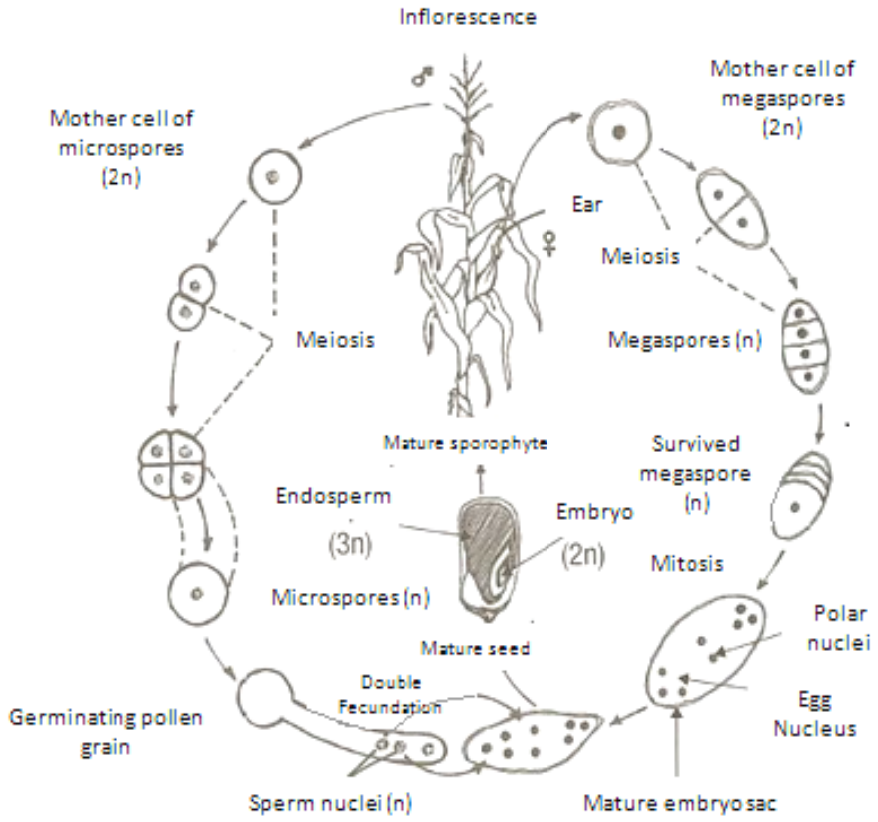


Fig. 3. Maize biological cycle

1.1.3 Development and growth of maize

The central problem in the study of plant development is to understand the role of the genes in the morphogenesis: which genes are involved, through which mechanism they direct and regulate this phenomenon, from fecundation up to the embryo maturation, and from germination up to gametes formation.

In higher plants, morphogenesis is the progressive development of a particular three-dimensional shape or structure that is distinctive of the species (Sheridan, 1988).

In the animals the majority of the morphogenetic events are accomplished during the embryo development: new individuals at birth have already all the organs and the structures of the adult. Instead, in the higher plants, it is not only accomplished during the embryo development, but also after germination, when the development of the meristems produces the juvenile and then the adult form of the plant. This is the reason why morphogenesis in higher plants can be studied both in embryo and in the meristems.

The isolation of mutants that present alterations in development or growth, is a direct approach to identify those genes that have an essential role in these processes; the subsequent genetic, morphologic and molecular analyses of these mutants will provide some information about the activities performed by these identified genes.

Morphogenesis depends principally on cellular activities that involve the asymmetrical distribution of cellular division and the cells elongation and growth; as these activities are due to genes performances, they can be object of mutations. The analysis of mutants affected in the morphogenetic process may also reveal which are the genes that control these cellular activities temporally.

Because of the complexity of the development processes, where many events occur simultaneously but in a precise sequence in relation to events that have occurred previously or later, it seems reasonable to expect that there are hierarchies of gene interaction in which groups of genes are regulated in a coordinated manner to accomplish a specific development program.

The research and the study of morphogenesis mutants leads to identify those genes that act at the different levels of the organism organization making the morphogenetic process.

A lot of mutations in many loci interfere with the morphogenesis and growth of maize plant. Many of these are pleiotropic, that is they affect different parts of the plant, while others affect just a single structure specifically.

The greatest part of the mutation that affects the morphogenesis of the maize stalk, implies a reduction in the size of the plant. Generally, these mutations fall into one of the following four groups: dwarf, brachytic-type, brevis-type and nana (Maize-GDB http://www.maizegdb.org).

Dwarf plants are characterized by shortened internodes and all their organs are smaller than normal ones (Galbiati M. *et al.*, 2002).
Brachytic-type plant has a reduced height, but its leaves, ears, tassel etc. have a normal size.
Brevis-type plants are characterized by a specific shortening limited to the internodes immediately adjacent to the ear.
In the nana plants the shortening affects all the internodes except the last one.
While some dwarf mutants can respond to the administration of gibberellins (Ross, 1994), the brachytic-type, the brevis-type and the nana are not sensitive to this treatment, showing, in this way, that these four group of mutants, even if showing a similar phenotype, identify genes with different mechanism of action and space-time regulation (Fujioka *et al.*, 1988 and Galbiati M. *et al.*, 2002).
In all these mutants are present some alterations in the synthesis and/or transduction of the signal of the hormones, essential molecules for plant development and morphogenesis.

1.2 Plant hormones

In plants the possibility to respond to environmental stimuli is limited by the physical immobility. In the course of evolution they had to develop a strategy that would allow them to be "plastic" to cope with environmental changes and thus ensure their survival. This strategy is based on the capability of the plant cells to use some signals as input for differentiation, for the maintenance of their differentiated state or for their reprogramming. Then the communication among the cells and the role of hormones is very important.
Generally speaking, hormones control the growth and the development of plants exerting effects on the elongation and cellular division. Certain hormones are also able to mediate short-time physiological responses of plants to environmental stimuli (tropism); some examples are the capability of growth towards light (phototropism) or following the gravitational direction (gravitropism). Each hormone has multiple effects, depending on the site of action, the stage of plant development and its concentration. Plant hormones are product in very low concentrations, however a very small quantity of a hormone can exercise deep effects on the growth and development of a plant organ. This implies that a hormone signal must somehow be amplified. A hormone can act altering the gene expression, exercising an effect on the enzymes already present, or changing some membranes property. Each of these actions can modify the metabolism and the development of a cell as response to the presence of a little number of hormone molecules.

The signal transduction pathways amplify the hormonal signal and collect it to the specific response of a cell. The response to the presence of a hormone, usually depends less on its absolute amount than on its concentration relative to that of the other hormones. It is the balance between different hormones, rather than isolated action of these, which controls the growth and development of the plant.

The major classes of plant hormones are auxin, cytokinin, gibberellins, abscisic acid, ethylene and brassinosteroid (Table 1).

Auxins are involved in the processes of distension and cell division, radical growth and in the phenomenon of apical dominance (Rayle D.L. and Cleland R.E., 1992).

Cytokinins act stimulating growth and differentiation, delaying senescence and promoting the formation of lateral buds; antagonize apical dominance (www.plant-hormones.info).

The gibberellins mobilize reserves in germinating seeds (Lovegrove and Hooley, 2000), are involved in the plant elongation process (Kende H. and Zeevaart A.D., 1997) and in the fruit set process.

Abscisic acid inhibits cell growth by blocking the cell distension, it is involved in stomatal closure, it causes the abscission of organs such as leaves, flowers, fruit, from the mother plant and is involved in dormancy of seeds and buds. It often antagonizes the action of growth hormones (Koornneef *et al.*, 1989; Koornneef, 1986; Gavazzi G. *et al.*, 2004).

Ethylene is produced in response to environmental stresses, in addition to being produced in response to the presence of high concentrations of auxin, it stimulates the ripening of fruits, the abscission of the leaves and it is involved in programmed cell death or apoptosis (Young and Gallie, 1999; Young and Gallie, 2000; Gavazzi G. *et al.*, 2004).

The brassinosteroids are steroids chemically similar to cholesterol and to animals sex hormones. They induce cell division and elongation, delay the abscission of leaves and promote the differentiation of xylem (Campbell N.A and Reece J.B., 2004).

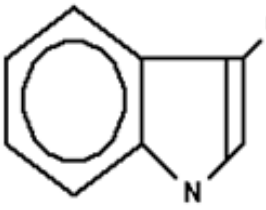
Table 1. Hormones in plants.

PLANT GROWTH SUBSTANCES

Any chemical substance causing growth of plant cells

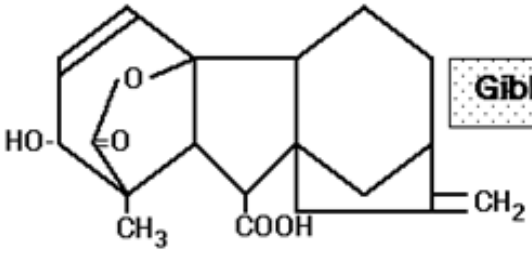
PLANT HORMONE

A chemical produced by one cell, transported to another cell, and causing a specific response. Of course a hormone may cause different responses in other cells.



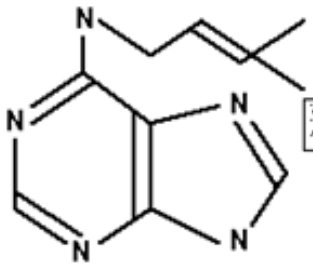
IAA (auxin)

Auxins are produced abundantly in stem and root apex. Transport is polar and basipetal. They stimulate cell growth, are responsible for apical dominance, stimulate adventitious root formation, and are active in tropic responses.



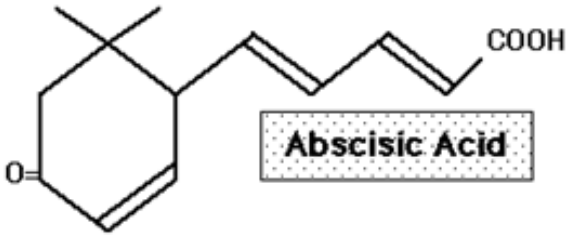
Gibberellin A_3

Gibberellins are produced primarily in young leaves. They cause sudden and rapid cell growth and also stimulate hydrolysis of starch in germinating seeds.



Zeatin (cytokinin)

Cytokinins are produced abundantly in roots and are transported toward the shoot apex. They stimulate cell division, release apical dominance (counteract auxin), and prevent senescence (counteract ethylene).



Abscisic Acid

Abscisic acid is produced in all plant parts especially when under stress. It causes and deepens dormancy (seeds and buds) and causes stomatal closure. It is probably not involved with leaf abscission.

Ethylene

Ethylene gas is produced in all plant parts especially when under stress. It stimulates senescence processes, including fruit ripening and leaf abscission.

1.2.1 Auxin

Auxin belongs to the class of signalling molecules; it is a plant hormone probably involved in every stage of plant growth and development. Auxin, indeed, controls many processes as the development of the embryonic axis (Friml *et al.*, 2003), the tropic response (Friml *et al.*, 2002), the light and gravity response (Davies P.J., 1995), the formation of lateral and adventitious roots (R.C. Reed *et al.*, 1998; Bhalerao *et al.*, 2002), roots, shoots and other part architecture (Davies P.J., 1995), phyllotaxis (Reinhardt *et al.*, 2003), and the vascular tissues development (Mattsson *et al.*, 1999).

Auxin is synthesized very early in the development phase of the plant and the presence of a pathway of transport is a prerequisite for moving forward from the stage of globular embryo with radial symmetry to subsequent stages with bilateral symmetry (Palme K. and Gälweiler L., 1999).

The biosynthesis of auxin is also important because it specifies the place where vascular differentiation occurs, it determines the direction of the formation of this tissue ensuring the continued development and growth of the plant (Palme K. and Gälweiler L., 1999).

Despite it was the first plant hormone to have been discovered and studied (Darwin C., 1880), much remains to be done to clarify the molecular basis of its mechanism of action.

The most common form of auxin is represented by the indole-3-acetic acid (IAA), that can be synthesized from an indole precursor of he tryptophan biosynthetic pathway or by a tryptophane-indipendent way (Woodward A.W., 2005).

However, none of these pathways has been fully characterized, and many points are still unclear, including, particularly, those of control. This happens because of functional redundancy of enzymes involved in the pathway, whereby it's difficult to find mutations with loss of function that are important for their identification (Woodward A.W., 2005). Auxin is synthesized at the shoot apex and in young leaves and it is then transported unidirectionally to the other tissues and organs of the plant. IAA transport is polar, basipetal and consumes energy. According to the chemiosmotic theory (Raven J.A., 1975), the proton gradient existing in the membrane is the driving force that allows the transport of auxin; indeed, ATP-dependent proton pumps present in the membrane mate metabolic energy to transport auxin (Fig. 9). Furthermore, auxin stimulates cell growth probably by binding to a receptor in cell membranes; the hypothesis of acid growth-dependent assign to proton pumps a key role in cell growth response to auxin. In the region of elongation of a stem, auxin stimulates proton pumps of the cytoplasmic membrane; this action causes in few minutes an increase of the voltage of the two sides of the membrane (membrane potential) and lowers the pH in the cell wall.

Wall acidification actives enzymes isolated for the first time in 1992, known by the name of expansin, which break the cross-linking of cellulose microfibrils with the consequent decrease in the rigidity of the wall texture (Rayle & Cleland, 1992). The increase of membrane potential stimulates

the recruitment of ions inside the cell that, in turn, causes the passive entry of water molecules through osmosis. The intake of water and the increased plasticity of the wall, allow the cell to elongate. Auxin also rapidly alters gene expression causing, in a short time, the production of new proteic molecules in the cells of the elongation region. Some of these proteins are transcription factors that activate or repress the expression of other genes. To maintain growth after this initial stimulation, cells must synthesize other cytoplasmic and wall material; not to mention that this hormone also stimulates the growth response in the longer term (Campbell N.A. and Reece J.B., 2004).

In addition to stimulating cell elongation in the primary growth, auxin stimulates also secondary growth by inducing cell division in the cambium and influencing the differentiation of secondary xylem (Campbell N.A. and Reece J.B., 2004).

The levels of free auxin in plants are not influenced only by the biosynthesis from scratch, but also from its conjugation and deconjugation: about 95% of total auxin, indeed, is linked to a great variety of compounds as amino acids, peptides and carbohydrates. Confirmation that auxin homeostasis is linked to the formation of conjugates was also provided through the study of *Arabidopsis* mutants, such as *fass* mutant. *fass* mutant, indeed, (Torres-Ruiz and Jurgens, 1994) shows a dramatic reduction in cell elongation along the apical-basal axis; this effect can also be provided by wild type embryo culture (hearth-stage) grown with high levels of auxin. The endogenous level of free auxin in *fass* mutants is variable but however at least 2-3 times greater than wild type, while conjugates are present in lower quantities. This observation suggests that *Fass* gene plays a role in auxin levels regulation, by its implication in conjugates formation. Conjugates may act as auxin sources that can be released by deconjugation. However, regulation of auxin levels in the plant is a very complex process, which involves many factors, many of which are still unknown. Some of these factors are involved in maintaining the polarity of transport. In particular, two types of transporters which are very important are the input and the output carriers of auxin from cells. Auxin can enter in the cells by diffusion, assisted and regulated, by a protonic co-transporter (Swarup *et al.*, 2001). However, it can leave the cells only through an efflux carrier, located in the basal region of the cell (Muday G. K. and DeLong A. 2001). Neither of the two classes of carriers was, however, definitively identified and studies continue through analysis of mutants with alterations in auxin transport, which generally show an aberrant morphology.

1.2.2 Gibberellins

Another class of hormones essential for the life and development of plants are the gibberellins (GA). Roots and young leaves are the main sites of production of gibberellins. The gibberellins (GA) stimulate the growth both in the leaves and in the stems, while have just little impact on root growth. In a growing stem, GA stimulates elongation and cell division together with the auxin. Moreover, the embryo of seeds is a rich source of GA; after the water has soaked the seed, the release of GA from the embryo signals to the seed to stop dormancy and start germination. The gibberellins, then, support the growth of cereal seedlings by stimulating the synthesis of digestive enzymes like α-amylase, phytase, protease, etc., that mobilize the reserves of accumulated substances (Campbell N.A. and Reece J.B., 2004).

The study of mutant and altered phenotypes played an important role in the identification of gibberellins and in the discovery of their role in the control of stem elongation and other developmental processes.

Studies on the extended phenotype exhibited by seedlings of rice affected by the fungus *Gibberella fujikuroi* led to the isolation of gibberellins in crystalline form (Yabuta and Sumiki, 1938) and then Brian and Hemming (1955) and Phinney (1956) began the study of mutant that had alterations in stem elongation.

The application of GA_3, a precursor in the biosynthesis of gibberellins, was shown to be effective in recovery of the wild type phenotype both in dwarf pea plants (*le*) and in corn (*d*), and so the gibberellins have been identified as regulators of growth in higher plants.

The analysis of mutants maintains a central role in the study of gibberellins and the main parameter for the screening of these mutants appears to be the elongation of the stem, despite the gibberellins are involved in many other processes such as flowering, fruit set , germination and mobilization of reserves. In particular, through the characterization of mutants blocked in different steps of the GA biosynthetic pathway, it has been possible to make considerable progress in knowledge of this pathway (Hedden P. and Proebsting M., 1999). Mutations that block the synthesis of gibberellins cause a dwarf phenotype that responds to the administration of GA. Among these, the mutation *ga1-3* in *Arabidopsis* led to the isolation of *GA1*, the first gene to be cloned in GA biosynthesis pathway (Sun et al., 1994). Functional analyses showed that the *GA1* gene encodes the enzyme CPS (Copalyl diphosphate Synthase) that catalyzes the first of two steps from GGDP (geranilgeranil diphosphate) to *ent*-kaurene. The mutant *ga1-3*, even if it doesn't produce the functional enzyme CPS, contains a certain amount of gibberellins, suggesting the presence in *Arabidopsis* of a second gene for the enzyme CPS.

In maize the synthesis of *ent*-kaurene is altered by mutations in the *an1* locus. *An1* gene has been cloned by gene tagging using transposon *Mu1* (Bensen *et al.*, 1995), and the sequence results homologue to GA1 and other cyclases; it seemed to be reasonable to assume that *An1* encodes for CPS enzyme. Some deletions of *An1* produce evenly *ent*-kaurene (up to 20% of the wild type level) showing that also in maize may be present a second gene encoding for CPS enzyme.

Another mutation in maize, *d3*, blocks the biosynthesis of GA before GA_{53} (Fujioka *et al.*, 1988), a precursor of GA_1. D3 gene has been cloned and the protein that shows sequence homologies with cytochrome P450 (Winkler and Helentjaris, 1995) could affect the GA pathway after *ent*-kaurene, although a functional assay of protein has not yet been reported and the exact passage blocked by a mutation *d3* has not been known yet.

Just as some enzymes are codified by multiple genes, some genes control more than one step in the biosynthetic pathway of GA, and an example is the *Dwarf-1* gene of maize. The mutation *dwarf-1* blocks the steps of the GA_1 precursors: from GA20 to GA5, from GA5 to GA3 and the conversion of GA20 to GA1 (3β-hydroxylation). Application of GA5, as GA1 and GA3, promotes the growth of *dwarf-1*, and it is more effective than GA20, showing that 3β-hydroxylation is not a prerequisite for biological activity (Spray C.R. *et al.*, 1996).

The response mutants can be divided into different classes based on stem length and type of response to the administration of GA. Therefore, there are mutants with elongated phenotype and constitutive response; mutants with elongated phenotype and an increased response to GA. Another class of mutants have reduced height and accumulation of GA, while another one shows reduced height and reduced response, but at high concentrations of GA it reaches height and response similar to those of the wild type. Finally, there are mutants with reduced height and weak response to GA.

1.3 The Green Revolution

The rise of agriculture led to the domestication of many species of plants and the exploration of natural resources. It took 10.000 years to the production of wheat to reach 1 billion tonnes in 1960 and only 40 years to reach 2 billion tons in 2000 (GS Khushi, 2001).

Since the '60ies, indeed, it appeared necessary to cope with the demand for food of a high growing population: due to the decrease in mortality resulting from advances of medical and health care, population increased and awareness emerged of an impending food crisis (Ehrlich P., 1968). Fortunately, the problems of global hunger and related economic and social consequences have been avoided thanks to an increase in cereal production started just from those years.

This result known as Green Revolution, was achieved mainly through the widespread adoption of techniques developed to generate genetically improved crop varieties with high yield and application of more efficient farming practices. Of crucial importance was the role played by Norman Borlaug, considered the father of the Green Revolution, who was the promoter of genetic improvement programs.

The Green Revolution had a huge impact on food production and on socio-economic and environmental sustainability. Between 1966 and 2000, the population of developing countries has almost doubled, but food production has increased by 125%.

The marked improvement in food production was caused by the application of advanced techniques for the development of varieties of cereals with high productivity. These varieties, particularly of rice, maize and wheat, were developed, at first, by IRRI (International Rice Research Institute) in the Philippines and by CIMMYT (Centro Internacional de Mejoramiento de Maiz y Trigo) in Mexico. Since then, many national breeding programs have been undertaken to produce many improved varieties of cereals. As rice, maize and wheat provide about 50% of the calories in human diet, the goal of these programs was to increase the production of these three species.

For example, since the marketing of the first high-yield varieties of rice in 1966, the area cultivated with rice has increased only marginally by 20% while the average production of rice has doubled. The total production of rice increased by 132% from 1966 to 1999. During the same period the production of wheat increased by 91% to 576 million tonnes. In many Asian countries, the growth in cereal production has increased so much compared to the population's needs, that it has promoted export. The increased availability of cereals and the reduction in production costs contributed to the decline in the price of rice but also of maize and wheat in the international and domestic markets. Production costs per unit fell by 20-30% for varieties with high yield compared with traditional

varieties and prices of rice, maize and wheat (adjusted for inflation) were lower by 40% in 2001 compared to those of the '60s (see Fig. 4).

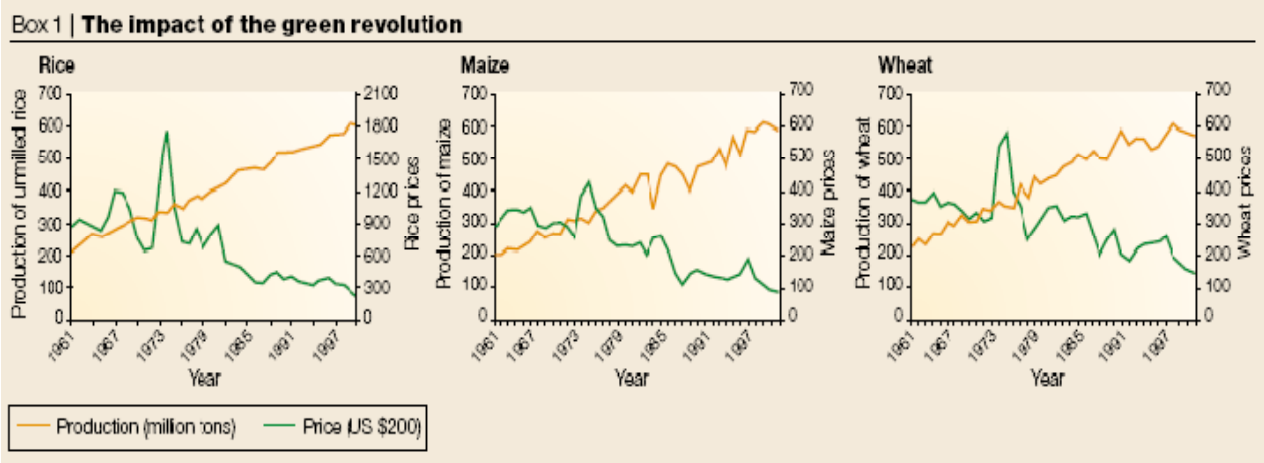


Fig.4: Impact of Green Revolution on production and price of rice, maize and wheat.
(Taken from: Gurdev S. Khush, 2001)

The spread of high yield varieties enabled many countries, especially Asia, to meet the growing food requirements and reduce the pressures on land, leaving intact the land used for forests, pastures, forests that would otherwise have been converted to cropland.

Many characters were genetically modified to increase production; through conventional techniques of crossing and selection (Fig. 5) it was possible to raise productivity, adapt the plants to different environments, shorten the growth cycle, tolerate biotic and abiotic stress and improve grain quality. A critical approach consisted in changing the architecture of the plant: plants with reduced height were selected, with a high number of kernels per spike, erect posture of the leaves and great resistance of the stem. The exploitation of the vigour of F1 hybrid plants allowed also to enhance the production especially for maize crop in which the size was only minimally altered.

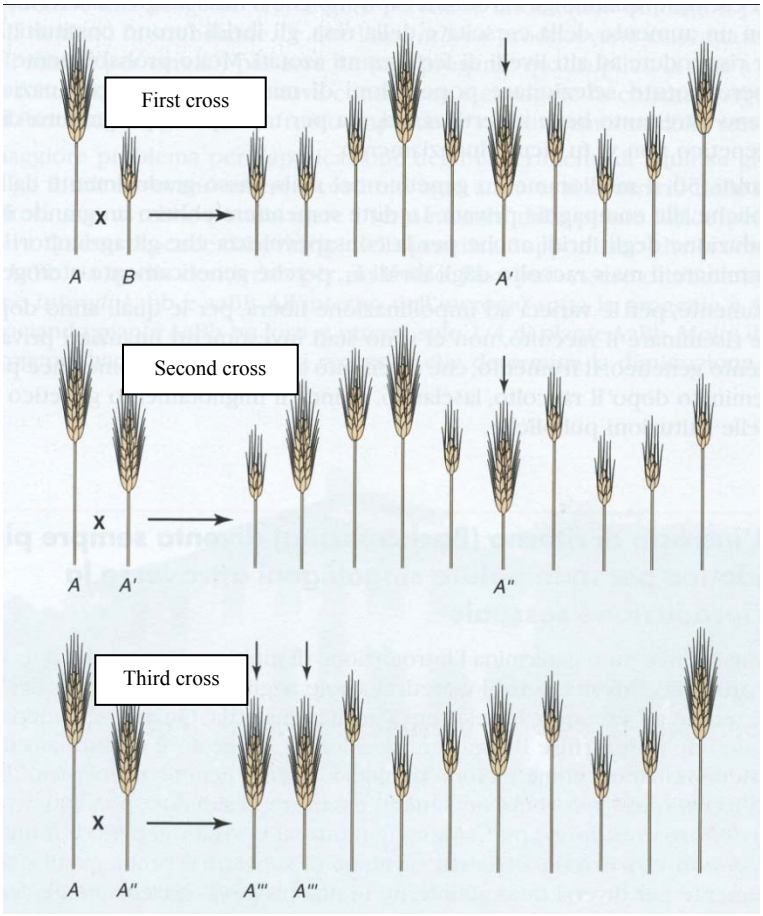


Fig. 5 Conventional breeding program of crossing and selection. Two parents (A and B) are crossed and the segregating generation is analyzed for the feature to be transferred. The aim is to introduce the dwarfing gene for reduced height from the genotype unproductive (B) to the high and productive wheat genotype (A). From the first cross (A x B), plants (A') are selected both for short stature and high production and back-crossed with the parent (A). Then plants (A'') are selected again both for short stature and high yield. These plants are back- crossed again with the genotype (A). After several cycles of crossing and selection, the dwarfing gene will become part of the genome of (A).
(Taken from Chrispeels M.J. and Sadava D.E., 2003)

The varieties of rice and wheat grown before the Green Revolution were high, with a low harvest index (harvest index = 0.3) and weak stems. In response to fertilization with high doses of nitrogen, the plants grew excessively in height, tillered too much, lodged and produced less grain than non fertilized plants. To increase the potential yield, it was therefore necessary to reduce the size of the plant with the introduction of a recessive gene, *sd1* (semidwarf) for short stature in rice from the

Chinese variety Dee-geo-woo-gen (Suh H.S. and Hue M.H., 1978), and one of the recessive genes *Rht1* or *Rht2* (Reduced height) to reduce the size of wheat (Rajanam S. and Van Ginkel M., 1996) from the Japanese variety Norin 10. Through classical breeding, the introgression of these "dwarfing genes" occurred; in this way, the majority of the resources of the plant was no longer used for vegetative growth but for grain production, improving the harvest index, the response to Nitrogen fertilization and increasing the resistance to lodging. This was achieved through the reduction of the height and thereby increasing the harvest index of more than 60%.

As for rice, small size, stiff culms, erect leaves, number of panicles per unit area, high fertility of spikelets and contemporary maturation were positively related to higher productivity (Fig. 6). The utility of short and stiff culms is linked to resistance to lodging, while the erect leaves promotes better light penetration. Even today the formation of new varieties is mainly based on the technique of crossing followed by selection, and it seeks to obtain lines with the characteristics described above. Some historic and widespread varieties very much appreciated by consumers for their superior quality, but showing signs of adverse agronomic traits such as great size and susceptibility to lodging, were improved in their size; a classic example is the Carnaroli rice that was improved giving rise to Karnak, which should summarize in itself the Carnaroli qualities and better agronomic characteristics.

For wheat it was equally important to improve the stem as its height is inversely correlated with the resistance to wind and rain. In older populations the plants reached the height of 180-220 cm, while now, cultivated varieties do not exceed 70-80 cm. Even in Italy, at the beginning of the last century, Strampelli crossed fine Italian varieties with Japanese grains (eg Akagomughi) to decrease the size and the crop cycle, and with Dutch wheat to improve fertility. With this first improved materials, the following breeders made further steps towards the modern varieties. (Baldoni R. and Giardini L., 2000).

At the beginning of the last century, the first targets of genetic improvement in sorghum, achieved through selection, were the reduction of the size and length of growing season of sorghum populations imported from tropical regions. Later, artificial hybridization started with the consequent selection; but it was in the 50s that the discovery of cytoplasmic male sterility allowed the production of F1 commercial hybrids. The modern hybrids are characterized by size not exceeding 120-150 cm, culm erect and robust, close nodes, overlapping leaf sheaths; the internodes length is increasing from base to apex, with the last internode very long (combine character) to facilitate mechanical harvesting. (Baldoni R. and Giardini L., 2000).

Fig. 6 Different type of rice plant:

- Left: high, conventional plant.
- Center: improved high-production plant with high number of tillers.
- Right: new type of plant (Super rice) with low number of tillers but with more vigorous stems and greater production of grains per ear.

(Tratto da: Gurdev S. Khush, 2001)

1.3.1 The genes of Green Revolution

Only recently it was possible to identify the molecular mechanisms underlying the reduced stature of plants used to increase the yield during the green revolution. The genes conferring "semi-dwarfism" in rice and wheat have been identified, but much more must be done for a rational approach for genetic improvement, even for other species such as maize.

Hormones play a key role in development of higher plants, including stem elongation, and their alteration may lead to significant changes, sometimes lethal, in the development of the plant.

In wheat, the reduction in height was obtained thanks to the introduction of dwarfing semi-dominants alleles *Rht* (*Reduced height*), that cause a reduced response to gibberellins (GA). Two of these alleles are present in the Japanese variety Norin 10 and in the 70% of the global cultivars currently on the market that come from it.

The *Rht* gene has been cloned (Fig.7) and it's ortholog of *GAI* gene (Gibberellic Acid Insensitive) of *Arabidopsis thaliana* and *dwarf 8* maize gene that, if mutated, origins dwarf phenotypes insensitive to GA (Peng *et al.*, 1999).

RHT-1, D8 and GAI proteins forms a subset of the family of GRAS proteins that are regulators of transcription. Members of this subgroup contain two N-terminal conserved regions, including a pattern of 27 amino acids known with the name of DELLA domain. In the short stature wheat, some substitutions of bases in the Rht-B1b and Rht-D1b alleles have been identified; these substitutions introduce stop codons in the DELLA region. This fact suggests that RHT protein is translated from the methionine just after the stop codon and it is truncated.

Since the DELLA domain is not present anymore, it works as a constitutive repressor of growth, insensitive to GA. From these results it was possible to understand the function of the wild type proteins RHT, GAI, D8 that are negative regulators of GA. GA represses their inhibitory function, but the presence of N-terminal DELLA domain is necessary(Dill A. *et al.*, 2001).

In 2009 a new kind of *D8* allele was characterized and cloned by Pilu *et al*. The different alleles of *D8* so far isolated cause varying degrees of dwarfing to the phenotype, depending on the N-terminal mutations in DELLA domain, and only one (the D8-2023 allele) affecting the VHYNP domain (Peng et al. 1999). Pilu *et al.* discovered a mutation that is the first VHYNP single amino acid insertion isolated. This indicates the importance of this motif, so far not well characterized in maize. Indeed the *D8-2023* mutation previously isolated causes the loss of 12 amino acids in the region of the VHYNP motif but this mutation due the closeness of the DELLA domain and the width of the insertion could represent an indirect lesion of the DELLA motif due to a tridimensional change of the *D8* protein structure modifying the DELLA region.

Some progress has been made in understanding the role of protein RHT-like: these proteins are found in the nucleus and are rapidly degraded in the presence of GA where is required the domain DELLA. The transduction pathway of the upstream signal of RHT is not yet clear, but the degradation induced by GA is thought to be involved in a ubiquitin-mediated proteolysis (Olszewski N. *et al.*, 2002). Besides the signal domains for GA that consist of a DELLA domain and of a second conserved region (TVHYNP domain), other functional elements are involved in the regulation.

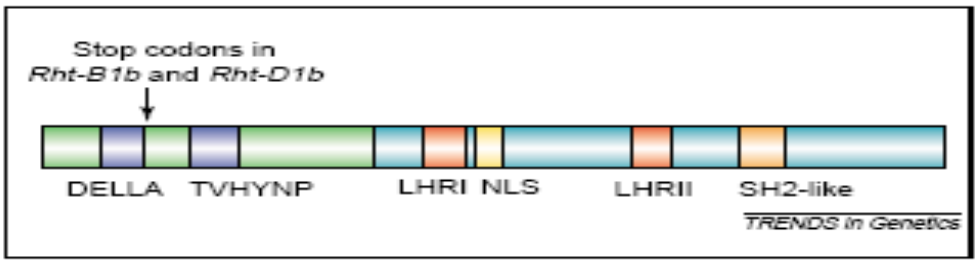


Fig. 7 Structure of Rht/GAI and related proteins. The C-terminus (light blue) is highly conserved in all GRAS proteins and contains the repressor activity. Functional domains identified in this region include two leucine heptad repeats (LHR), the first of which mediates dimerization, a nuclear localization signal (NLS) and a SH2-like domain, which could indicate the involvement of phosphotyrosine signalling. The N-terminus (green) contains the GA-signalling domain. It is more variable, but includes two highly conserved motifs (dark blue) that are required for GA-induced degradation. The arrow indicates position of stop codons in *Rht-B1b* and *Rht-D1b*. (Taken from: Hedden P., 2003)

In rice, instead, the reduction of size has been obtained selecting a recessive mutation of a different gene: *sd* (*semidwarf1*). The plant can recover a normal height as a response to GA application, showing defectiveness in the hormone production. The *sd1* gene codifies, indeed, for a GA20-oxidase (*GA20ox*), an enzyme of the GA biosynthetic pathway (Ashikari M. *et al.*, 2002). The semi-dwarf rice varieties coming from the cross with the Chinese variety Dee-geo-woo-gen have a *sd1* allele with a deletion of 383 bp in the GA20ox, that introduces a stop-codon in the reading frame, giving as result an inactive truncated enzyme. The Gibberellins 20-oxidases are 2-oxoglutarato-dependent dioxygenase catalyzing the loss of carbon-20 in the penultimate step of the biosynthesis of GA (Hedden P. and Phillips A.L., 2000) (Fig. 8). They are codified by a small gene family, whose members have a partial functional redundancy, probably due to the overlap of the expression

pattern or to the shift between the tissues of the intermediate products by these enzymes. For this reason, mutants that have lost the GA20ox functionality, usually have just a weak decrease of GA content and are semi-dwarf, in contrast to plants with lower levels of this hormone, which have a very small stature and are often sterile. With the selection of semi-dwarf rice varieties, were steadily produced more mutations in this gene (OsGA20ox) than in others, that would lead to serious consequences in the development or damage to production (Hedden P., 2003). It is no coincidence that mutations in the gene for GA20ox have been selected in screening programs for semi-dwarf rice plants.

For wheat is different because it is difficult to have mutations with loss of function in a hexaploid genome plant, unless they are present in all the three copies of the genome; in fact, the dwarf plants were identified by dominant mutations, with a gain of function. However, both cases have highlighted the importance of the role of GA in the regulation of developmental processes that are critical for agriculture (P. Hedden, 2003).

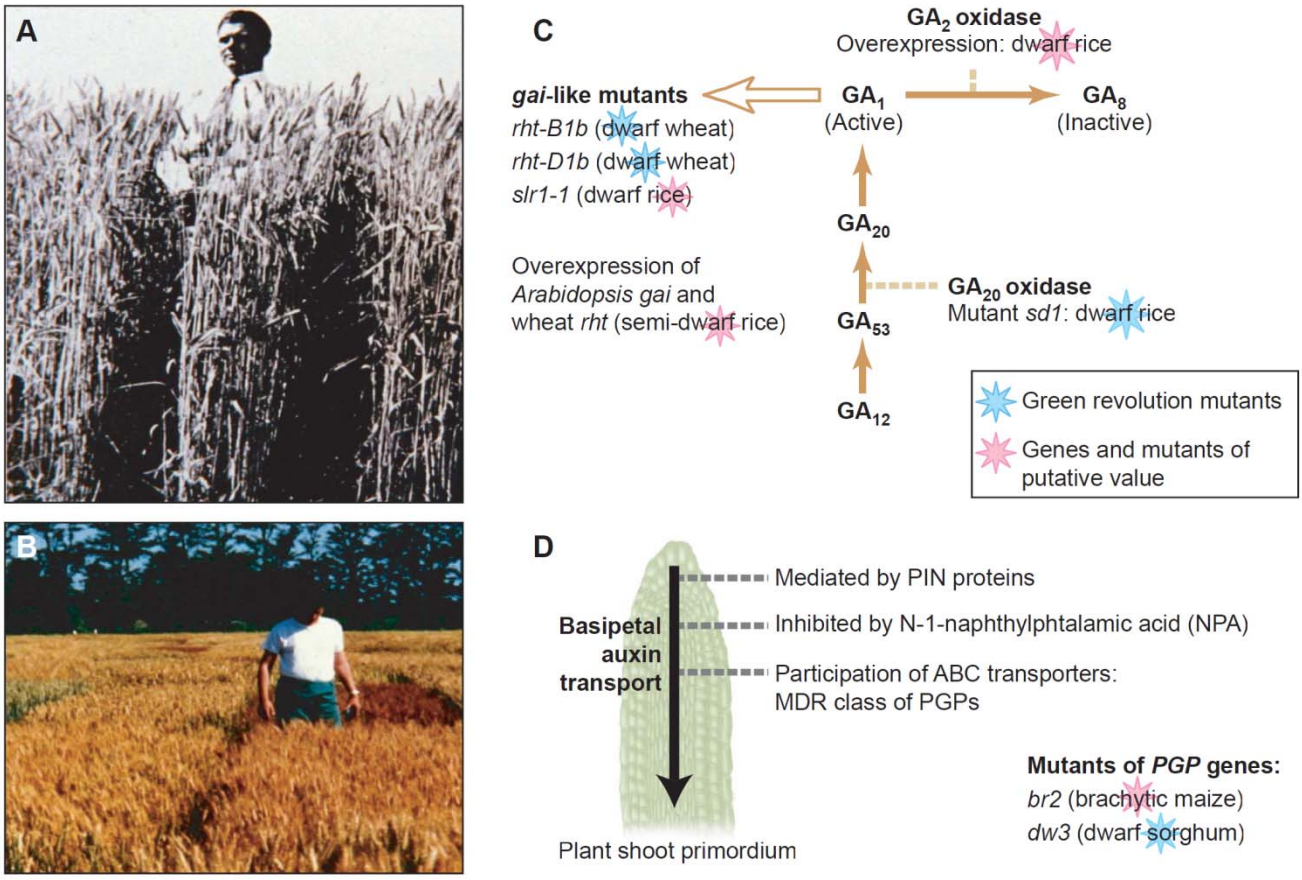


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Fig. 8 The benefits of being short. (**A**) Varieties of wheat from the early 1900s were almost as tall as a normal person. (**B**) In contrast, modern wheat varieties, particularly those that launched the green revolution, have stalks that are 40 to 100% shorter than the stalks of earlier cultivars. The short wheat varieties carry mutations in genes encoding proteins that regulate the synthesis and signaling of gibberellin (GA), a plant growth hormone. (**C**) The pathway of GA synthesis indicating mutants of agronomic interest in which GA synthesis or signaling is disrupted. GA directs degradation of the product of the GAI (gibberellic acid insensitive) gene, which is a transcriptional repressor. In gai mutants (rht, reduced height; slr, slender; sd, semidwarf), degradation of GAI is abrogated and GA-inducible genes are repressed (9). (**D**) Gene mutations affecting transport of auxin, some of which contribute to dwarfism. (PIN, pinoid phenotype; ABC, ATP-binding cassette; MDR, multidrug resistance; PGPs, P-glycoproteins; br, brachytic; dw, dwarf).

In sorghum four independent mutations, *dwarf1* (*dw1)*, *dwarf2* (*dw2)*, *dwarf3* (*dw3)* e *dwarf4* (*dw4)*, were and are utilized by the breeders to shorten the stature of plants. Typically three of these are combined for commercial inbred lines production; the *dw3*, thanks to its ability in improving harvest index, is often utilized in these combinations (Quinby J.R. and Karper R.E., 1954). Unfortunately, the sole available mutant allele of *dw3* is unstable and spontaneously reverts to the high phenotype with frequency from 0.1% to 0.5% depending on the genetic background (Karper R.E., 1932 and Quinby J.R., 1963). The mechanism underlying the instability of this phenotype has not been clear for long time. Unlike genes exploited to reduce the size in other species, *dw3* does not interfere with the synthesis, translocation or receiving GA, but with the polar auxin transport, and this phenotype does not revert administering any of the plant hormones involved in growth.

1.4 Polar Auxin Transport in plants

The plant hormone auxin (the predominant form of which is indole-3-acetic acid; IAA) is a major coordinating signal in the regulation of plant development. Many aspects of auxin action depend on its differential distribution within plant tissues, where it forms local maxima or gradients between cells. Besides local biosynthesis and the release of active forms from inactive precursors, the major determinant of differential auxin distribution is its directional transport between cells. This regulated

polar auxin transport (PAT) within plant tissues, appears to be unique to auxin, as it has not been detected for any other signaling molecule. Molecular biology and genetics approaches in the model system *Arabidopsis thaliana* have contributed fundamentally to our understanding of the mechanisms of auxin transport. Currently, a large body of evidence supports the concept that intercellular auxin movement depends on several auxin transporting mechanisms, which include both passive and active processes that transport auxin over long and short distances. Of these, the major mechanism for controlling auxin distribution during plant development appears to be the active directional cell-to-cell movement of auxin that is mediated by plasma membrane-based influx and efflux carriers.

In plants, auxin is generally transported by two distinct pathways. Throughout the plant, most IAA is probably transported away from the source tissues (young leaves and flowers) by an unregulated bulk flow in the mature phloem. In addition, a slower, regulated, carrier-mediated cell-to-cell directional transport moves auxin in the vascular cambium from the shoot towards the root apex (Goldsmith, 1977), and also mediates short-range auxin movement in different tissues. These two pathways seem to be connected at the level of phloem loading in leaves (Marchant *et al*., 2002) and phloem unloading in roots (Swarup *et al.,* 2001). A series of classical physiological experiments predicted the existence of carrier-type auxin influx and efflux components that mediate PAT. The asymmetric cellular localization of these transporters has been proposed to determine the direction of auxin flow. During the past two decades, candidates for auxin carrier proteins and for the relevant regulatory mechanisms have been identified (Fig. 9). Heterologous expression experiments in cultured plant cells, yeast, *Xenopus laevis* oocytes and mammalian cells have demonstrated the auxin-transporting capacity of these carrier proteins (Vieten *et al.,* 2007). Expression and localization studies of auxin carrier proteins, as well as specific defects in differential auxin distribution in plants that lack the function of these carriers, established that carrier-dependent PAT is absolutely required for the generation and maintenance of local auxin maxima and gradient.

1.4.1 Influx carriers

From the first studies conducted to understand the mechanisms involved in the transport of auxin into cells, it seemed that the auxin, in its lipophilic protonated form (the most abundant form in the apoplast) could easily cross the cell membrane and vesicles (Rubery P.H., 1974). It was however difficult to accept that the IAA could get inside the cells simply by diffusion, without a control in its entry. Only in more recent studies, in fact, it was found that the entry of auxin is a saturable process,

probably because of the presence of a carrier, although definitive evidence of an electrogenic proton symport for IAA were obtained subsequently by using specific inhibitors (K. Palme, 1999).

Further confirmation for this hypothesis was provided after the characterization of an agravitropic *auxin resistant 1* mutant (*aux1*) of *Arabidopsis* that shows resistance to an exogenous synthetic auxin, 2,4-D, led to the identification of the AUX1/LIKE AUX1 (AUX1/LAX) family of transmembrane proteins, which are similar to amino acid permeases, a group of proton-gradient-driven transporters (Swarup *et al.,* 2008) both in plants and in fungi. To date, four auxin influx carriers with specific functions have been described in *Arabidopsis*, and the functions of some homologs in other plants have also been studied. Recently, AUX1 and LAX3 has been shown to mediate IAA uptake when heterologously expressed in *Xenopus* oocytes (Swarup *et al.*, 2008), which provides biochemical evidence for their role as auxin influx carriers. Based on this similarity it can be supposed the involvement of AUX1 in the transport of signalling molecules similar to amino acids. Hormone IAA is structurally similar to tryptophan, thus appears to be a viable candidate (Estelle, 1998).

It is believed that the stimulus of gravity is perceived in the root in the region of the cap, which then transmits again the auxin signal to cells of the growth zone through epidermis. The specific expression of AUX1 associated with root epidermis, confirms its role in auxin transport specifically for the control of root gravitropism (Bennett *et al.,* 1996).

1.4.2 Efflux carriers

The investigation of several *Arabidopsis* mutants, namely of the allelic root mutants *agravitropic 1* (*agr1*), *wavy roots 6* (*wav6*) (Bell and Maher, 1990) and *ethylene insensitive root 1* (*eir1*) (Roman *et al.*, 1995), and the floral mutant *pin-formed1* (*pin1*) (Okada *et al.,* 1991), resulted in the identification of auxin efflux carrier candidates. The root agravitropic phenotypes, as well as the *pin1* phenotype with defects in organ initiation and phyllotaxy, can be phenocopied by the pharmacological inhibition of auxin efflux. Additionally, these mutants display decreased PAT in shoots and roots. The corresponding *PIN1* gene encodes a plantspecific protein with two transmembrane regions separated by a hydrophilic loop (Galweiler *et al.*, 1998). Concomitantly, the *agr1*, *wav6* and *eir1* mutants have been shown to be allelic with a mutant that carries a mutation in another PIN family member, *PIN2*. The *AGR1*, *WAV6*, *EIR1* and *PIN2* genes encode a homologous protein designated PIN2 (Muller *et al.*, 1998). Until now, eight members of the PIN protein family

have been isolated in *Arabidopsis* and are commonly referred to as PIN1 to PIN8 (Vieten *et al.*, 2007). A subgroup comprising PIN5, PIN6 and PIN8 has an educed middle hydrophilic loop and presumably regulates the auxin exchange between the endoplasmic reticulum and the cytosol (Mravec *et al.*, 2009). The PIN1, PIN2, PIN3, PIN4 and PIN7 proteins, by contrast, are localized at the plasma membrane, where they act as auxin efflux carriers (Mravec *et al.,* 2008; Petrásek *et al.*, 2006). PIN homologs in other plants have also been identified (Zazimalova *et al.*, 2007), and some of them have been functionally characterized.

A reverse approach in studying PIN protein in other species was that of Carraro *et al.*, 2006, who identified two novel putative orthologs of AtPIN1 in maize and analyzed their expression pattern during development. The expression studies were complemented by immunolocalization studies using an anti-AtPIN1 antibody. Interestingly, the maize proteins visualized by this antibody are almost exclusively localized in subepidermal meristematic layers. Both tassel and ear were characterized by a compact group of cells, just below the surface, carrying PIN. In contrast to or to complement what was shown in Arabidopsis, these results point to the importance of internally localized cells in the patterning process. The *barren inflorescence2* (*bif2*) maize mutant was chosen to study the role of auxin polar fluxes in inflorescence development. In severe alleles of bif2, the tassel and the ear present altered *ZmPIN1a* and *ZmPIN1b* protein expression and localization patterns. In particular, the compact groups of cells in the tassel and ear of the mutant were missing. The conclusion was that BIF2 is important for PIN organization and could play a role in the establishment of polar auxin fluxes in maize inflorescence, indirectly modulating the process of axillary meristem formation and development (Carraro *et al*, 2006)

Other proteins that play a role in auxin efflux are plant orthologs of the mammalian ATP-binding cassette subfamily B (ABCB)-type transporters of the multidrug resistance/phosphoglycoprotein (ABCB/MDR/PGP) protein family (Noh *et al.*, 2001). Some of these (ABCB1, ABCB4 and ABCB19) have been identified as proteins with binding affinity to the auxin transport inhibitor 1-naphthylphthalamic acid (NPA) (Noh *et al.*, 2001). The biochemical evidence for these ABCB proteins having a role in auxin transport has been provided by heterologously expressing them in tobacco cells, HeLa cells and yeast (Petrásek *et al.*, 2006. The importance of the ABCB proteins for auxin transport-related development has been also documented in other higher plants. Recently, a system for comparative analyses of transport activities and the structure of all three groups of auxin transporters (AUX1/LAX, PIN and ABCB) has been established in *Schizosaccharomyces pombe* (Yang and Murphy, 2009). It represents a valuable tool for testing the cooperation between these transporters, as well as with other regulatory proteins. Other auxin transporter candidates exist, for example the members of a group of aromatic and neutral amino acid transporters in *Arabidopsis*

(Chen *et al.*, 2001) or the transmembrane protein TM20 in maize (*Zea mays*) (Jahrmann *et al.*, 2005). However, their contribution to the intercellular transport of auxin is still unclear.

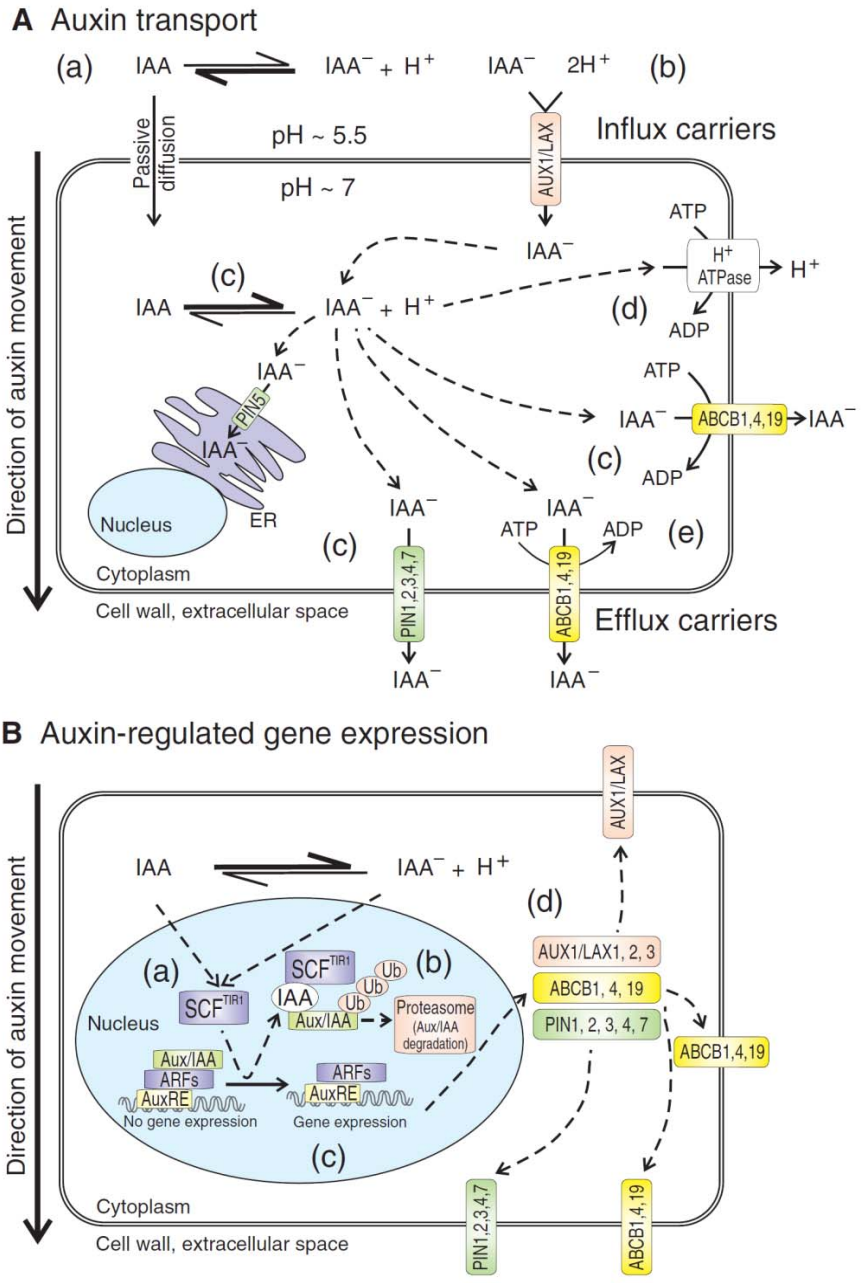


Fig. 9 Auxin transport across the plasma membrane and auxin-regulated gene expression. (A) Schematic depiction of auxin transport across the plasma membrane. Both passive diffusion and specific auxin influx and efflux carriers are involved in the transport of auxin (IAA) across the plasma membrane. Undissociated IAA molecules enter cells by passive diffusion (a), whereas the less lipophilic, and therefore less permeable, dissociated auxin anions (IAA–) are transported inside via auxin influx 2H+ cotransporters of the AUX1/LAX family (b). In the more basic intracellular environment (c), IAA dissociates and requires active transport through the PIN or ABCB efflux transporter proteins to exit the cell. Some cytosolic IAA is transported by PIN5 into the lumen of the endoplasmic reticulum (ER). This compartmentalization presumably serves to regulate auxin metabolism. Whereas PIN transporter activity is supposed to use a H+ gradient that is maintained by the action of the plasma membrane H+-ATPase (d), and possibly also the vacuolar H+ pyrophosphatase, ABCB transporters have ATPase activity (e). (B) Schematic depiction of auxin-regulated gene expression. Intracellular auxin binds to its nuclear receptor from the TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFB) family of F-box proteins, which are subunits of the SCF E3-ligase protein complex (a). This leads to the ubiquitylation and the proteasome-mediated specific degradation of auxin Aux/IAA transcriptional repressors (b). Subsequently, the auxin response factors (ARFs) are derepressed and activate auxin-inducible gene expression (c). Among other auxin-responsive genes, all known auxin transporters are regulated by this feedback mechanism (d). Ub, ubiquitin

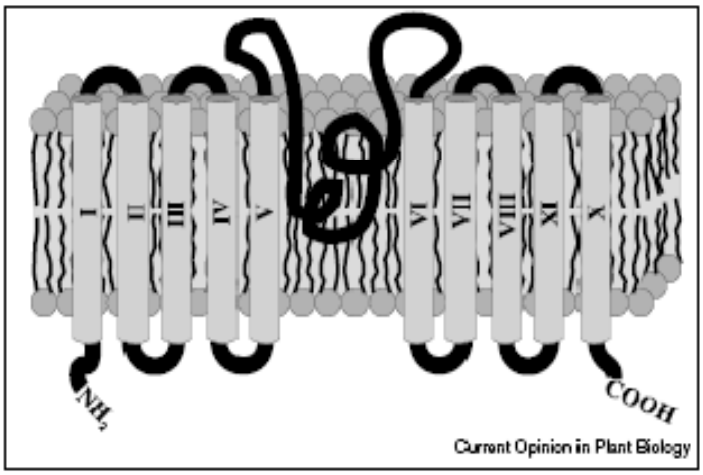
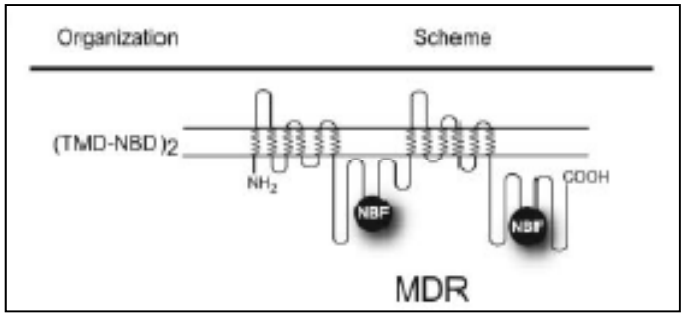


Fig. 10 The AtPIN1 protein is a plasma membrane protein with transmembrane segments and a large hydrophilic loop between transmembrane segments five and six. (Taken from K. Palme, 1999)

A



B

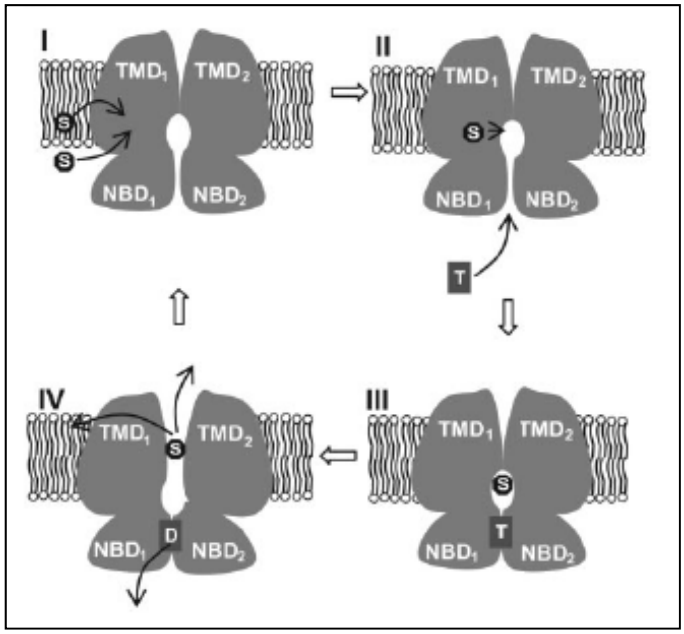


Fig. 11 Topology and putative catalytic mechanism of ABC transporters**. A**, Topology. The TMD, with the predicted membrane spans (zig-zag), and the NBD, containing the nucleotide binding fold, are shown for the three main types of full-size ABC transporters. **B**, Putative catalytic mechanism. The figures schematize an ABC transporter with its four domains, two of which (TMDs) are embedded in the membrane. A substrate (S) coming from the cytosol or already within the membrane binds to a substrate binding site in one of the TMDs (this site has not yet been localized precisely and might, as a matter of fact, be one among several binding sites) (I - II). This is followed by ATP (T) binding to the nucleotide-binding site localized at the interface between both NBDs (note that only one of the two nucleotide binding sites is represented) (II - III). ATP binding and hydrolysis is accompanied by a structural rearrangement that allows the substrate to access to, and to be translocated through, a membrane path (III - IV). Upon ADP (D) and phosphate dissociation, the protein returns to the initial state (IV -I). This still putative model is based on that proposed for the bacterial BtuCD (Locher et al., 2002) except for the substrate path, which is inverted. Alternatively, the central cavity might represent the substrate-binding site. Taken from Jasinski M., 2003.

1.4.3 Auxin transport routes during embryogenesis

Auxin and auxin transport is already important at the earliest stages of plant development. The analysis of *Arabidopsis* mutants, combined with the visualization of the auxin response by means of auxin-inducible promoters, demonstrated that differential auxin distribution mediates important steps during embryogenesis, such as apical-basal axis specification and embryonic leaf formation. The concerted action of PIN1, PIN4 and PIN7 efflux carriers (Friml *et al.,* 2002; Friml *et al.*, 2003) is required for the differential auxin distribution in embryogenesis (Fig. 12). Individual PIN proteins act redundantly, given that single pin mutants can still complete embryogenesis, hereas *pin1 pin3 pin4 pin7* quadruple mutants are strongly defective in the overall establishment of apical-basal polarity (Benková *et al*., 2003; Friml *et al.,* 2003). In contrast to pin mutants, mutants in other auxin transport components, such as the *abcb* and *aux1/lax* mutants, are not defective in embryogenesis, which suggests a major role for PIN-dependent auxin transport in patterning the embryo. Soon after the first anticlinal division of a fertilized zygote, increased auxin accumulation can be detected in the apical cell by the activity of the auxin-inducible element DR5 or by IAA immunolocalization. This differential distribution results from the activity of PIN7 that is localized apically in the adjacent suspensor cells. At this stage, PIN1 presumably mediates the uniform distribution of auxin between cells of the forming proembryo (Fig. 13). Both ABCB1 and ABCB19 contribute to auxin transport during the early stages of pro-embryo formation (Mravec *et al.*, 2008). ABCB1 is localized to all suspensor cells and pro-embryonal cells, and ABCB19 localization is restricted to the suspensor-forming cells. Both proteins are localized without obvious polarity. Later, during the early globular stage, PIN1 gradually relocalizes to the bottom plasma membranes of the embryo cells that face the uppermost suspensor cell, the hypophysis (Kleine-Vehn *et al.*, 2008). Simultaneously, the polarity of PIN7 shifts from apical to basal in the suspensor cells (Fig. 13). These coordinated PIN polarity rearrangements, which are later also supported by the action of PIN4, lead to an apical-to-basal flow of auxin and to auxin accumulation in the hypophysis. At this stage, the auxin distribution and response are crucial for the specification of the hypophysis as the precursor of the root meristem. Accordingly, mutants of the auxin-binding F-box proteins TIR1 and AFB, and of the downstream transcriptional regulators MONOPTEROS (MP, also known as ARF5) and BODENLOS (BDL, also known as IAA12) (Hamann *et al.*, 2002), show pronounced defects in embryonic root formation. Afterwards, during the development of the heart stage of the *Arabidopsis e*mbryo, additional auxin maxima are formed at the positions of the two initiating cotyledons, mainly through the action of PIN1 (Benková *et al.*, 2003). At this stage, the ABCB19 expression pattern is largely complementary to that of PIN1 and shows the highest expression in endodermal

and cortical tissues (Fig. 13). The *pin1 abcb1 abcb19* triple mutants, in contrast to the single *pin1* or double *abcb1 abcb19* mutants, are severely defective in establishing auxin maxima and show fused cotyledons, which hints at a synergistic genetic interaction between PIN1 and ABCB proteins (Mravec *et al.*, 2008). These results indicate a role for both the ABCB-mediated and PIN-dependent auxin transport pathways in the generation of differential auxin distribution at different stages of embryogenesis.

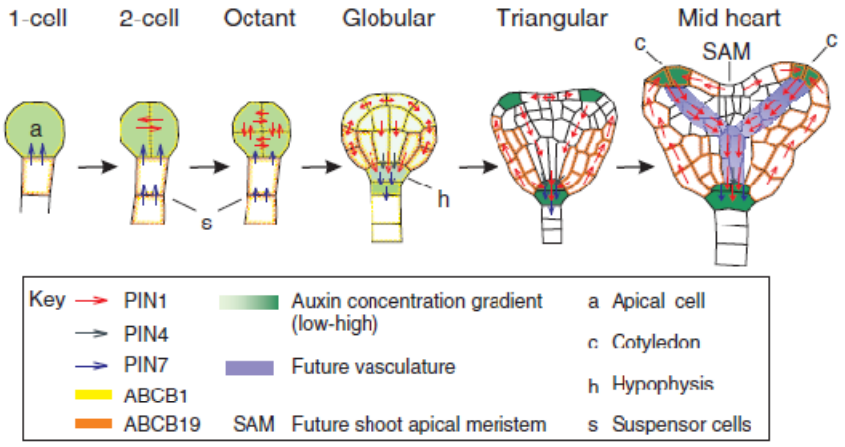


Fig. 12 Auxin gradients and auxin transporters during embryogenesis. Schematic depiction of the auxin distribution and the localization of auxin transporters during early plant embryonic development. Auxin distribution (depicted as a green gradient) has been inferred from DR5 activity and IAA immunolocalization. The localization of the efflux transporters PIN1, PIN4 and PIN7, as well as that of ABCB1 and ABCB19, is based on immunolocalization studies and on in vivo observations of green fluorescent protein (GFP)- tagged proteins. Arrows indicate auxin flow mediated by a particular transporter; dotted lines indicate the cell type-specific localization of particular auxin transporters with no obvious polarity. PIN7, localized at the apical sides of the suspensor cells (s), transports auxin towards the apical cell (a) that forms the pro-embryo; there, PIN1, which is localized at all inner cell sides, distributes auxin homogenously. ABCB1 and ABCB19 cooperate during this initial stage and are localized apolarly in all cells or only in the uppermost suspensor cell, respectively. The crucial moment in the setting of the basal end of the apical-basal embryonic axis occurs during the early globular stage, when PIN1 starts to be localized basally in the pro-embryonal cells, and PIN7 is simultaneously shifted from the apical to the basal plasma membrane of suspensor cells. These PIN polarity rearrangements reverse the auxin flow downwards and, with the aid of PIN4, lead to auxin accumulation in the forming hypophysis (h). At this stage, ABCB19 helps to maintain the auxin distribution in the outer layers of the embryo. In triangular- and heart-stage embryos, bilateral symmetry is established through auxin maxima at the incipient cotyledon (c) primordia. These auxin maxima are generated by PIN1 activity in the epidermis; in the inner cells of cotyledon primordia, however, PIN1 mediates basipetal auxin transport towards the root pole. SAM, future shoot apical meristem.

1.4.4 Auxin and postembryonic root and shoot development

Auxin plays an important role in the patterning of both shoot and root apices, as well as in the initiation and the subsequent development of root and shoot organs. Increased auxin levels at the incipient positions of the primary root and the cotyledons during embryogenesis are reflected in postembryonic development. Auxin maxima always mark the positions of organ initiation and, later, of the tips of developing organ primordia (Benková *et al.*, 2003). Correspondingly, the local application and production of auxin triggers the formation of leaves or flowers and of lateral roots (Dubrovsky *et al.*, 2008). Auxin fluxes and maxima in root- and shoot-derived organ primordia are similar and can be described in terms of fountain and reverse fountain models, respectively (Benková *et al.*, 2003). In general, all three auxin transport systems, using PIN, ABCB and AUX1/LAX proteins, contribute to postembryogenic auxin transport, although the exact contribution of each of these cooperating transport systems to total auxin transport remains unresolved.

1.4.4.1 Auxin transport routes during root development

In the primary root, auxin is transported acropetally towards the root tip by a PIN-dependent route through the vascular parenchyma and through the phloem, with subsequent AUX1-dependent unloading into protophloem cells (Friml *et al.*, 2002; Swarup *et al.*, 2001). Auxin flow towards the tip is maintained by the action of basally localized PIN1, PIN3 and PIN7 in the stele (Friml *et al.*, 2002). In the columella, the action of PIN3 and PIN7 redirects auxin flow laterally to the lateral root cap and the epidermis, where the apically localized PIN2 mediates the upward flow of auxin to the elongation zone (Friml *et al.*, 2003; Müller *et al.*, 1998). The PIN2-based epidermal auxin flow is further supported by the action of AUX1 and ABCB4, whereas PIN1, PIN3 and PIN7 recycle some auxin from the epidermis back to the vasculature (Blilou *et al.*, 2005). The concerted action of the PIN auxin efflux carriers is one of the major determinants of pattern formation in root tips. By concentrating auxin in the quiescent center, the columella initiates, whereas surrounding stem cells restrict, the expression domain of the auxin-inducible PLETHORA (PLT) transcription factors. PLTs are the master regulators of root fate and, in turn, are required for PIN transcription (Blilou *et al.*, 2005). The ABCB1 and ABCB19 auxin transporters seem to play a supportive role in controlling how much auxin is available for each PIN-based transport flow. *BCB1* is expressed in all root cells, except for the columella (Mravec *et al.*, 2008), whereas *ABCB19* expression is restricted to the endodermis and the pericycle, which might help to separate the acropetal and basipetal auxin

fluxes in the stele and the epidermis, respectively. Auxin transport is also crucial for lateral root initiation and development. In pericycle cells, auxin maxima specify the founder cells for lateral root initiation (Dubrovsky *et al.*, 2008). Subsequent rounds of coordinated divisions form the lateral root primordium, from which the lateral root emerges later. Indeed, the functionally redundant network of PIN efflux carriers facilitates the auxin transport that is needed for the correct development of lateral root primordia (Benková *et al.*, 2003). During the initiation phase, PIN1 is localized at the anticlinal membranes. The switch of the pericycle cell division plane from anticlinal to periclinal is accompanied by the redistribution of PIN1 to the outer lateral plasma membranes of inner cells. This guanine nucleotide exchange factor for ADP-ribosylation factors (ARF-GEF)-dependent, transcytosis-like PIN1 polarity switch (Kleine-Vehn *et al*., 2008) mediates the auxin flow towards the primordium tip, where an auxin maximum is formed. At later stages, the PIN2-mediated auxin transport away from the tip through the outer layers is established. AUX1 significantly contributes to lateral root formation, probably by controlling the overall auxin levels in the root tip (by unloading auxin from the phloem) and its availability in the region of lateral root initiation (by basipetal transport from the tip) (Marchant *et al.*, 2002). An interesting role is reserved for LAX3, which is induced in cells around the developing primordium, where it establishes the auxin maxima needed for the specific production of cell-wallremodeling enzymes, which is necessary for lateral root emergence (Swarup *et al.*, 2008). The ABCB1 and ABCB19 proteins are also expressed and required for lateral root formation, as indicated by the defects in the *abcb* and *pin abcb* mutants (Mravec *et al.*, 2008; Petràšek *et al.*, 2006).

1.4.4.2 Auxin transport routes during shoot development

In the shoot apical meristem (SAM), the main source of auxin is unclear, but auxin is probably partly supplied by the phloem (as in the case of roots) and by young developing organs in the vicinity. Auxin fluxes are largely reversed in shoots when compared with roots. Auxin arrives at the organ initiation sites through the epidermis layer L1 and is canalized through the interior of developing primordia into the basipetal stream of the main shoot. This stream is mostly maintained by the activities of PIN1, localized basally in xylem parenchyma cells, and of ABCB19 (Noh *et al.*, 2001), which, together with ABCB1, helps to concentrate auxin flux in the vascular parenchyma (Blakeslee *et al.*, 2007). Shoot lateral organs (leaves and flowers) are generated from the SAM in a highly periodic phyllotactic pattern. In *Arabidopsis* phyllotaxis, the 137°angle between developing primordia is marked by auxin maxima at the position of incipient primordia (Benková *et al.*, 2003).

This highly organized auxin distribution is maintained by the cooperative action of AUX1, LAX1, LAX2 and LAX3 (Bainbridge *et al.*, 2008), as well as that of PIN1. PIN1 polarities in the L1 layer, which also undergo complex rearrangements relative to auxin maxima, appear to be responsible for generating the phyllotactic pattern of auxin distribution, whereas auxin influx activities largely restrict auxin to the L1 layer. Not only the positioning, but also the development of shoot lateral organs is regulated by auxin distribution, with the maximum concentration at the primordium tip, where it is maintained mainly by the activity of PIN1, which transports auxin through the epidermis towards the tip. From there, a new basipetal, PIN1-dependent, transport route is gradually established through the interior of the primordium. This marks future developing vascular tissues that will connect new organs with the pre-existing vascular network (Benková *et al.*, 2003). ABCB1 and ABCB19 also contribute to the establishment of this auxin sink (Noh *et al.*, 2001). Observations regarding the localization of the components of different auxin transport systems, combined with the defects in the corresponding mutants, show that all the transport systems that depend on ABCB, AUX1/LAX and PIN proteins are involved in shoot-derived organogenesis.

1.4.4.3 Auxin routes in tropisms

The role of auxin and auxin transport in the directional growth responses of plants to light (phototropism) and to gravity (gravitropism) played a major role in the discovery of auxin and in the formulation of the concept of plant hormones (Darwin, 1880). The negative gravitropism of stems, the positive gravitropism of roots and the positive phototropic curvature of stems are characterized by the uneven distribution of auxin at the different sides of stimulated organs. This differential auxin distribution activates asymmetric growth and subsequent organ bending (Went, 1974) in a context-specific manner: whereas higher intracellular auxin concentrations trigger elongation in shoots, they are inhibitory in roots. In roots, gravity is detected in the starch-containing root cap cells, in which PIN3 is relocalized from its originally uniform distribution to the bottom plasma membranes after gravistimulation (Friml *et al.*, 2002). Auxin flow is redirected towards the lower side of the root tip, from where it is transported through the lateral root cap and epidermal cells towards the elongation zone, where growth-inhibitory auxin responses are induced (Swarup *et al.*, 2005). This basipetal transport route requires both the epidermally localized PIN2 (Müller *et al.*, 1998) and AUX1 (Bennett et al., 1996; Swarup et al., 2001; Swarup et al., 2005). The flow along the lower side of the root is further enhanced by the vacuolar targeting of PIN2 and its degradation on the upper root side (Kleine-Vehn *et al.*, 2008). In addition, ABCB-dependent auxin transport might regulate the gravitropic response, considering that *abcb4* and *abcb1 abcb19* mutants show an enhanced

gravitropic response (Lewis *et al.*, 2007) and a genetic interaction with *pin2* (Mravec *et al.*, 2008). Moreover, flavonoids, the putative endogenous modulators of auxin transport, might contribute to root bending through their influence on PIN and ABCB4 expression and activity (Lewis *et al.*, 2007). In shoots, gravity is detected in endodermal cells (starch sheath cells), where PIN3 is localized at the inner plasma membrane. The corresponding *pin3* mutants are partially defective in hypocotyl gravitropism (Friml *et al.*, 2002). It is likely, but has not been conclusively demonstrated, that, similar to the root gravitropic response, the PIN3 relocation to the bottom side of endodermis cells triggers auxin accumulation in the lower side of the shoot, where the auxin response promotes growth and upward bending. The mechanisms that generate auxin asymmetry in response to light remain unclear, but studies with mutants or inhibitors show that phototropism also requires the activity of all auxin transport components, such as PIN3 (Friml *et al*., 2002), AUX1 (Stone *et al.*, 2008), ABCB1 and ABCB19 (Lin and Wang, 2005).

1.5 The *br2* mutant of maize and the *dw3* of sorghum

Agronomic interest in short plants derives largely from their ability to resist to lodging caused by wind, rain, or higher densities, which allows them to effectively convert increased fertilizer input to higher yields.

Elongation of plant parts is a complex phenomenon mediated by many plant hormones, including auxins, brassinosteroids and gibberellins. However, our understanding of how these hormones regulate cell elongation remains limited (Vogler H. and Kuhlmeier C., 2003). One way to resolve the mechanism that controls plant height is by characterizing mutants in which this growth component is compromised. This approach recently led to the elucidation of the mechanisms that cause dwarfing of the green revolution rice and wheat cultivars, which were found to have defects in the genes involved in the biosynthesis and signalling of GAs, respectively.

A maize dwarf mutant of agronomic potential contains the recessive *Brachytic 2* (*br2*) mutation, which results in shortening of lower stalk internodes. The other part of the plants like mesocotyl, coleoptile, leaves, ear and tassel, are more weakly affected by the mutation and their growth is not apparently strongly modified. The *br2* phenotypes could not be reverted by treatment with auxins, brassinosteroids, cytokinins or GAs, suggesting that *br2* does not contain a defect in the biosynthesisi of any of these growth regulators (Multani D. *et al*., 2003).

In contrast to the reduction in length, the girth of the affected stalk internodes is often enhanced. As expected, the length of *br2* stalk cells was found to be from 40 to 50% of the length of their normal counterparts. A similar reduction was observed in the diameter of *br2* stalk cells, and it was coupled with a substantial increase in cell numbers. This increase was especially evident in the hypodermal region where a parenchyma region up to 10 cell layers across developed immediately below the epidermis. Additional aberrations were observed in the vascular system, in which the structure, number, and distribution pattern were altered in *br2* plants. One consequence of these anatomical changes is that the strength of the *br2* stalk is substantially enhanced. This was demonstrated genetically by combining *br2* with *brittle stalk 2* (*bk2*), an easily breakable maize mutants impossible to propagate in the field. Double mutants *bk2-br2* were grown up to maturity in field without assistance (Multani D. *et al*., 2003).

Aberrations associated with stalk vascular and hypodermal proliferation suggested that some aspect of auxin homeostasis was perturbed in the *br2* stalk. This possibility was evaluated by comparing basipetal transport of [^{3}H] 3-indol-3-acetic acid (IAA) in *br2* and wild type plants. These assays showed a light dependent effect on auxin transport in middle to lower stem tissues of *br2* mutants. The transport of auxin in the first internodes of light grown plants was greatly reduced in *br2* mutants compared to that in the wild type. However, only slight differences were seen in *br2* dark-grown coleoptile or mesocotyl tissues. The noted differences were not a result of increased [^{3}H] auxin uptake at the site of application, given that br2 consistently showed increased loading of auxin into the upper coleoptile near the site of application.

These patterns of accumulation indicate that the polar transport of auxin is impaired in a light dependent fashion in the *br2* stem.

Multani et al. in 2003, cloned the *br2* gene by transposon tagging with *Mutator* (*Mu*). The gene is 7139bp long, has a codifying sequence of 4185 bp, and it consists of 5 exons (Accession Number: AY366085) (Fig. 13).

Tests have shown that the *br2* gene is expressed preferentially in the internodes elongating, confirming the hypothesis of its involvement in the determination of reduced stature phenotype. Furthermore, it was found that the element *Mu8* evolved by acquiring part of the *br2* gene.

Fig. 13 Scheme of *Br2* gene. Exons are represented as black rectangles, while introns as lines. In grey the part of the fifth exon that presents homology with the transposon *Mu8*.

The gene Br2 encodes a putative protein of 1394 amino acids (Accession Number: AAR00316) similar to ABC transportes (ATP- Binding Cassette) of the Multidrug resistant (MDR) class of P-glycoproteins (PGPs).

As with other PGPs the predicted BR2 protein consists of two similar halves, each containing six putative transmembrane domain and an intracellular ATP nucleotide-binding domain.

The closest homolog of BR2 is Atpgp1, with 67% identity. The classification of BR2 as PGP is consistent within recent findings from Arabidopsis that suggest that PGPs modulate auxin-dependent growth. Mutations in *AtPGP19* (*AtMDR1*), a close relative of AtPGP1, resulted in decreased auxin transport and pleiotropic auxin related phenotypes (Noh B., 2001).

A function in polar auxin transport was further suggested when AtPGP1, AtPGP2, and AtPGP19, were purified by affinity chromatography with the auxin transport inhibitor N-1-naphtylphthalamic acid and the putative auxin efflux carrier PIN1 was found to be mislocalized in *atpgp1* and *atpgp19* mutants, resulting in enhanced lateral auxin flux and hypertropic bending responses (Noh B., 2003). These studies strongly suggest that plant PGPs facilitate the polar movement of auxin.

Notably, the disruption of MDR genes has different consequences in maize than in Arbidopsis. Whereas mutations in maize Br2 affect primarily the growth of lower stalk internodes, the loss of the equivalent genes in Arabidopsis is associated with multiple morphologies (Noh B. Murphy A.S., 2001). Conversely the pattern of cellular proliferation, differentiation and vasculature remains largely unaffected in the Arabidopsis stem, whereas all of these features are substantially altered in the *br2* stalk. This disparity may simply reflect the spatial expression regimes of this subset of MDR genes, which occurs predominantly in elongating internodes of maize, but in overlapping expression patterns in meristems, shoot apices, flowers, stem nodes, and tissues associated with high auxin content in Arabidopsis. Additionally, maize and Arabidopsis have fundamentally diverse body forms, consisting of segmented and unsegmented stem, respectively. This difference may be critical, considering that each segment (internode) of maize stem has its own intercalary meristem. What role, if any, these meristems play in regulating auxin flow and homeostasis remains unknown, but this question can now be addressed using *br2* as a tool.

Agronomically, *br2* has not been exploited commercially in maize, partly because of the excessively severe nature of the original mutant allele, but similar mutations have been used extensively in sorghum production since the 1950s. Sorghum is closely related to maize both in genomic organization and in plant form. Four independent dwarfing mutations – *dw1*, *dw2*, *dw3*, and *dw4* – are available to sorghum breeders for reducing plants height, and typically three mutations are variously combined to develop commercial lines. Because of its ability to improve the harvest index of sorghum, the *dw3* dwarfing gene is often included in these combinations. However the only mutant allele of *dw3* available thus far is unstable and spontaneously reverts back to the tall type at a frequency of 0.1 to 0.5%, depending on the genetic background. The mechanism underlying *dw3* instability has long puzzled sorghum geneticists and attempts to rectify this problem have failed.
The dwarfing phenotype of *dw3* is remarkably similar to that of *br2* (Fig 14), and it is promoted to investigate whether the two genes were identical at the molecular level. They also share a common evolutionary origin, which was indicated by the finding that *dw3* is flanked by hm1 and PIO644, two markers that are tightly linked to *br2* in maize.

Fig.14 **A**. Comparison between wild type B73, left, and *br2* B73, right. **B**. Comparison between revertant phenotype of *Sorghum bicolor* (hybrid Puma), left, and the dwarf commercial variety, right.

After sequencing and alignment of the *br2* and *dw3* sequences of maize and sorghum respectively, these two alleles exhibited a high homology and a identity greater than 95% in the region corresponding to a part of exon 5 of *br2*. In addition, an 882 bp direct duplication was found in exon 5 of the *dw3* allele, which appeared responsible for the allele's loss of function. All the Dw3 revertants lacked this duplication, indicating that it was also responsible for the instability in *dw3*, because direct duplications are apt to undergo unequal crossing-over.

Interestingly, a dwarf plant with a restriction band diagnostic of wild type revertants was also found. The sequence analysis showed that unequal recombination had removed the duplicated part of the gene but introduced a number of simple nucleotides changes in the copy that was left behind. These changes disrupted the reading frame of DW3 and also truncated the protein by about 200 amino acids, thereby explaining the mutant nature of this new allele. As this allele lacks the duplication, it is expected to confer a stable mutant phenotype (Multani *et al.*, 2003).

These findings not only resolve a long-standing puzzle in sorghum genetics but also provide a simple strategy for effectively correcting *dw3* in the sorghum germplasm. Moreover new mutant alleles of sorghum *dw3* or of corresponding genes in other cereals may be generated by conventional mutagenesis approach. New perspectives have therefore been opened for maize breeding, by generating new and improved alleles of *br2*.

Some studies (Djisbar A. and Brewbaker J.L., 1987) tried to evaluate yield and agronomic attitudes of different lines and hybrids of tropical maize in which had been previously introgressed the *br2* allele. Tropical maize germplasm often shows an excessive height with consequent susceptibility to lodging in condition of high density and high nitrogen inputs.

The reduction of internodes number is quite easy to reach for the breeders, but it is linked to precocity and, often, to a lower yield. Actually it is possible to develop programs of genetic improvement aimed at reducing internodes length without affecting the production, but this is not an easy way and it is long term.

Among all the mutations that affect the length of the stalk, *br2* was considered the most interesting from a commercial perspective: the rate of maturation, the number of leaves and nodes are not affected by the action of the gene; indeed, the leaves seem to have a longer stay-green and to be more persistent on the plant. Beside some variability of expression among the various lines used and resulting hybrids, the aim of reducing the size of the plant and, especially, the height of ear insertion has been reached very well: average reduction of 44% for the plant and 55% for the ear. The interesting finding was that there was no statistical significant difference in production between the hybrids *br2* and normal during summer; instead in the case of winter crop, the *br2* showed a lower

yield. Maturation in all mutants is delayed by an average of 2.5 days (Djisbar A. and Brewbaker J.L., 1987).

Pilu *et al.* in 2007 isolated a brachytic maize mutant, originally named *br*-23*, in the progeny of a selfed B73 inbred line plant. This spontaneously arising recessive mutant has a short stature that resembles the brachytic phenotypes described to date (Scott and Campbell 1969). This decrease in the length of the plant's stature – approximately 50% compared to the wild type in the B73 isogenic line – is not caused by a decrease in the number of internodes but by the shortening of the basal internodes. The girth of the internodes is enhanced in the mutant plants, most predominantly in the second internode, which is approximately 30% larger than its counterpart in control plants. These structural changes enhance the strength of the brachytic stalk; as a result, the mutant shows a greater tolerance to wind lodging (data not shown). The size of the leaf epidermal cell is also influenced by the presence of the *br*-23* allele. In the allelism test, *br*-23* did not complement the *br2* mutant, suggesting an allelic relationship with the Br2 gene. Further evidence supporting this conclusion is provided by the map position of the *br*-23* mutation, which is the same as that described for the *br2* mutation previously isolated. Consequently, we renamed the new mutation *br2–23*. We found that the mutant allele in heterozygous *Br2/br2–23* plants with the B73 background exhibited consistent effects in terms of plant and ear height (which are 8 and 18% shorter in mutant plants than in *Br2/Br2* wild-type ones) and leaf angle. This observation may indicate that one single functional gene copy is not sufficient to restore the wild-type phenotype. These data indicate that plant height, leaf angle and, in particular, ear height are closely linked to the BR2 level. It is probable that differences in plant height and ear height that are characteristic of several inbred lines can be partially explained by different expression levels of Br2 alleles. One of the quantitative trait loci (QTLs) with the strongest effects on plant height has been mapped to the same region as *Br2*, indicating that this gene may play a crucial role in determining this trait (Beavis *et al.* 1991). This is in accordance with Robertson's hypothesis that major mutants studied by maize geneticists are null or near null alleles at a QTL (Robertson 1985). Modern corn (*Zea mays* L.) varieties have been selected for their ability to maintain productivity even at the dense plantings that are characteristic of industrialized agriculture. Factors such as plant shape (in particular, leaf angle), plant populations and row width will affect how photosynthetically active radiation (PAR) is intercepted, thereby influencing canopy photosynthesis and yield (Stewart *et al.* 2003). Although the *br2* phenotype may be an ideal plant type to increase crop productivity, to date it has not been used commercially because of the severe nature of this mutation. In fact, the effects of the *brachytic 2* mutation on maize yield and its components was partially explored by Djisbar and Brewbaker (1987), who demonstrated that grain yields were reduced markedly by the *br2* gene in all backgrounds tested.

Our finding that *Br2/br2* heterozygotes exhibit a useful intermediate phenotype in terms of plant height, ear height and leaf angle, suggests us that this effect is worth exploring when developing new hybrids. In order to investigate the molecular lesion in the *br2-23* allele, we designed specific primers on the basis of the sequence of the *br2* gene cloned by transposon tagging (Multani *et al.* 2003). Expression analysis performed using the RT-PCR technique revealed that *Br2* and *br2-23* have the same expression level, suggesting that this null mutation resulted from an alteration in the coding sequence. The alignment of the 3' *Br2* (B73 allele) and *br2–23* sequences, performed using the CLUSTAL W program, revealed that the mutant carries an eight nucleotide deletions in the coding region. The presence of this deletion in the coding region was also confirmed using allele-specific primers on segregating populations. This PCR-based molecular marker associated to the *br2–23* allele could be a useful tool for rapidly selecting *br2-23/br2-23* homozygous inbred lines with the purpose of developing new hybrids. Currently, when aiming to obtain the introgression of a recessive allele in an inbred line, it is necessary to test each generation that results from the cross with the recurrent parental line with a recessive homozygote in order to identify the genotype. Using the marker-assisted selection approach described here, it would be possible to select in each generation the desired plants for the next generation by rapid molecular analysis. In summary, the results presented here indicate that the *br2* mutation exerts effects not only on the stalk, as previously reported (Djisbar and Brewbaker 1987; Multani *et al*. 2003), but also on some morphological characters such as the dimensions of the epidermal cells and the leaf angle. These data are in agreement with the results published by Fellner *et al.* (2003) showing that auxin and polar auxin transport are involved in the elongation growth of corn seedlings and in the regulation of the leaf angle.

2 MATERIALS AND METHODS

2.1 Plant materials

The *brachytic 2* mutant studied in this work (originally named *br2-23*) was isolated in the progeny of a selfed B73 inbred line plant. *brachytic 1 (br1)* and *brachytic 3* (*br3*) mutants were provided by Stock Center Resources of MaizeGDB (http://www.maizegdb.org/stock.php).

br1 maps on the chromosome 1 (bin 1.07, 153 ± 2 cM) and it is associated with the phenotypic marker *f1* (bin 1.07, 158 ± 2 cM); *br2* maps on the chromosome 1 (bin 1.06, 142 ± 3 cM), *br3* on the chromosome 5 (bin 5.04, 105 ± 10 cM). The map coordinates were found by the Genetic 2005 (1) map for the cross (611A x T218 F2 1998 1) for *br1, br2, f1* and by Genetic 2005 (5) map for the cross (611A x T218 F2 1998 5) for *br3* (MaizeGDB htpp://www.maizegdb.org).

In the text the terms *br2* and *br2-23* are used as synonymous.

The double mutants were obtained through crossing. After four selfing cycles the double mutant near-isogenic lines were studied from several points of view.

The inbred lines Mo17 and W23 were originally supplied by Stock Center Resources of MaizeGDB and kept in purity in our field by self cross. All the commercial hybrids were bought at Consorzio Agrario di Milano e Lodi.

2.2 Methods

2.2.1 *br2-23* genotyping

Plant genotyping was performed using specific primers designed on the 3' deletion region of *br2-23* to obtain allele-specific amplified products. A sample of leaf was used for DNA extraction (Dellaporta *et al.,* 1983). PCRs were performed using the primers BracD2 and Brac16R (Pilu *et al.,* 2007). The two amplification fragments, one of 105 bp that was specific for the *Br2* allele and one of 97 bp that was specific for the *br2-23* allele, were fractionated by electrophoresis on 4% (w/v) agarose gels.

2.2.2 Semiquantitative RT-PCR

Total RNAs were extracted from wild type, *br2/br2*, *br3/br3* and *br2/br2 br3/br3* internodes at the tenth leaf stage and seedlings using the method described by van Tunen *et al.* (1988). Reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect the *br2*, *ZmPIN1a*, *ZmPIN1b* and *ZmPIN1c* genes transcripts. Total RNA was treated with DNAse I 1 U/µl (Deoxyribonuclease I, Amplification Grade, Invitrogen) for eliminating DNA during RNA purification procedures. First strand cDNA was synthesized with an oligo (dT) primer from total RNA using the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). The different samples of cDNA were then diluted to obtain a uniform concentration. First-strand cDNA was used as the template for PCR amplification. Amplification reactions were carried out on samples containing an aliquot of cDNA synthesized from 5 µg of total RNA, 5X Green Reaction Buffer, 2.5 mM $MgCl_2$, 200 µM each dATP, dCTP, dGTP, and dTTP, 0.1 µM each primer, and 1 unit of GoTaq (R) Flexy DNA Polymerase (Promega, Madison, WI) and were performed in a final volume of 50 µl. A set of primers specific for the *orange pericarp-1* (*orp-1*) gene, which encodes the β-subunit of tryptophan synthase (Wright *et al.,* 1992), was used to standardize the concentration of the different samples. *orp-1* specific sequences were amplified using the following primers: upstream primer, 5'-AAGGACGTGCACACCGC-3', and downstream primer, 5'–CAGATACAGAACAACAACTC-3'. The length of the amplified product was 207 bp. Several cycles of successive cDNA dilutions and *orp-1* amplifications were done in order to obtain similar amplification signals in the different samples and to ensure that amplification reactions were within linear ranges. For mRNA detection of the *br2* gene under analysis, BracD2 L (5'-GCCGCGTAGGACGGAATG-3'; Tm 62°, position + 6645 respect to the start codon), and Brac16 R (5'-TTGCGATCATGGAGTACCACCAT-3', Tm 62°, position +6749, 3' UnTranslated Region –UTR-) specific primers were used, both of which were designed on the basis of the sequence of the *Br2* gene deposited in GenBank (accession number: AY366085). For *ZmPin1a* gene (accession number: DQ836239) under analysis, PIN1A1F (5'-ATAAATCGCGTGCGGGAACAA-3', Tm 60°, position +1531 respect to the start codon) and PIN1A1R (5'-TCCTGCTCCACATCCCCATC-3', Tm 64°, position +1867, 3'UTR) specific primers were used. For *ZmPin1b* gene under analysis (accession number: DQ836240), PIN1B1F (5'-ATCATCGCGTGCGGGAACAA-3', Tm 62°, position +1513 respect to the start codon) and PIN1B1R (5'- ACCCCACGGGTCGGTCACAGG-3', Tm 68°, position +1754, 3'UTR) specific primers were used. For *ZmPin1C* gene under analysis (accession number: EU570251), PINCF (5'-TCATCCCCATGGAGTCGAGGATGCCACC-3', Tm 72°, position +2820, 3'UTR) and PINCR

(5'-GGATCCACCCAGACCCAATCCCCATACCTACTTCT-3', Tm 72°, position +3035, 3'UTR) (Gallavotti *et al*., 2008). The amplified product was 105 bp for BracD2 L /Brac16 R, 337bp for PIN1A1F/ PIN1A1R, 242bp for PIN1B1F/PIN1B1R and 216bp for PINCF/PINCR. PCR products were fractionated on 1.5% (w/v) agarose gels. The identity of the products was confirmed by sequencing (cDNAs were amplified by High-Fidelity PCR, Pfu polymerase; Stratagene).

2.2.3 Histological analyses

To determine epidermal cell sizes, *br2/br2* , *br3/br3* and *br2/br2 br3/br3* and wild type control seedlings were grown in normal conditions and at the stage of 2 leaves were collected and treated with a clearing solution (160 g chloral hydrate, 20 ml glycerol in 60 ml water). Cleared leaves were mounted on slides, and interference contrast images were taken using a Zeiss IMAGE R.D1 microscope equipped with a AxioCam MRc1 digital camera.

2.2.4 Embryo Rescue

br2 br3 embryos and wild type control embryos were removed aseptically and transferred to Murashige and Skoog salt mixture (pH 5.6) (Sigma, product no. M5519) containing 2% sucrose, solidified with 0.8% agar (Plant agar, Duchefa, Haarlem, The Netherlands). Cultures were incubated in a growth chamber at 25°C with a 18/6 light/dark photoperiod. Seedling observations were taken after 10 days. The light source consisted of four cool white (F36T12/CW/HO) fluorescent lamps from GTE SYLVANIA (Lighting Products Group, Danvers, MA).

2.2.5 Root gravitropism test

The test was set to evaluate the root growth in response to changes in gravitropism. Seeds of B73 wild type, *br2* and *br3* were germinated in boxes with an agarose media. When primary root reached approximately the length of 1 cm, the boxes were turned of an angle of 90°. Then the changing of direction of root growth was observed at different intervals.

2.2.6 Machineries and protocol for field tests

The sowing operations were performed with a Gaspardo plot pneumatic seed drill. The machine was set to have a final density of 7 plants per square meter. The appropriate experimental design was the randomized block with two replications. The elementary plot was composed by 2 rows (70 cm of distance) 6 m long. Each rank of plot was divided from the others by a path 80 cm wide. Threshing was carried out by a Laverda 3300 combine harvester adapted for plot harvesting with Husky weighing system. The assessments were: final number of plants, plant height (measured from soil to flag leaf insertion), ear height (from soil to ear insertion), fresh grain weight, humidity content.

3 RESULTS

3.1 Agronomic trials on three-way hybrids

We found that the mutant allele in heterozygous *Br2/br2–23* plants with the B73 background exhibited consistent effects in terms of plant and ear height (which are 8 and 18% shorter in *Br2/br2–23* than in *Br2/Br2* wild-type ones) and leaf angle. This observation may indicate that one single functional gene copy is not sufficient to restore the wild-type phenotype.

After having tested that the heterozygous effect was not hidden by the hybrid vigour in two simple hybrid combinations – B73xMo17 and B73xW23 - (Pilu *et al.*, 2007), we observed also that the ears of the heterozygous plants (Fig. 1) were longer than the wild type ones (data not shown). This phenomenon let us suppose a higher potential yield of these heterozygous plants. Even if the measurements on the grain production did not confirm this hypothesis, the possibility of enhance and exploit this feature was still open. Therefore, we decide to start a preliminary screening work to evaluate the attitude of different elite genetic background in which the allele was previously introgressed.

So different combinations of 3-way hybrid were produced crossing B73 *br2/br2* and different commercial hybrids. These materials heterozygous for *br2* have been tested with their corresponding wild type control in different location in plots for two consequent years.

The data obtained were analyzed by ANOVA with two factors and confidential intervals were applied to check any statistical difference (Table 1). These data suggest that the effect of heterozygous is confirmed also in the hybrids at different levels of expression (according to the specific genetic background) but however generally without penalizing the productions (a little penalization can occur depending on the genetic background). In some cases the heterozygous seems even to improve the yield.

3.2 Double mutants

In order to study the interaction between the mutations *br1*, *br2-23* e *br3*, double mutants were made: *br1/br1 br3/br3*, *br2-23/br2-23 br1/br1*, *br2-23/br2-23 br3/br3*.

These three loci map on the following positions: *br1* associated to the phenotypic marker *f1* (*fine stripe*) on chromosome 1(bin 1.07), *br2* on chromosome 1 (bin 1.06), *br3* on chromosome 5 (bin 5.04).

3.2.1 Constitution and analysis of the double mutant *br1/br1 br3/br3*

We produced a *br1 br3* double mutant by crossing *br1/br1* with *br3/br3* plants (Fig. 2). The *Br1/br1 Br3/br3* F1 obtained were selfed and the progeny were planted and screened for brachytic phenotypes. About 9 wild type: 7 brachytic segregation ratio was observed as expected (9 *Br3/- Br2/-*; 3 *br3/br3 Br2/-*; 3 *Br3/- br2/br2* and 1 *br2/br2 br3/br3*). Among the brachytic plants we noticed few very short plants. These plants showed shorter internodes compared to the monogenic brachytic mutation (Fig.3). We conjectured that these plants were *br1/br1 br3/br3* double mutants (expected in a 1 out of 16 ratio) and this strong additive effect was caused by the presence of two brachytic mutations. To test our hypothesis we crossed gnome plants with *br1* and *br3* homozygous mutants to perform a test cross assay (Fig. 8). The seeds obtained from these two crosses were sown in the following season and all the plants grown were brachytic (data not shown). The absence of complementation in the F1 generation confirmed that gnome plants were homozygous for *br1* and *br3* mutations.

3.2.2 Constitution and analysis of the double mutant *br1/br1 br2-23/br2-23*

Since the association existing between the loci *Br1* and *Br2*, distant 10 cM only (bin 1.07 and bin 1.06 respectively), the realization of the double mutant was a bit more laborious. Therefore the subsequent procedure was followed (Fig. 4).

The two single mutants (*br2/br2 Br1/Br1 F1/F1* x *Br2/Br2 br1/br1 f1/f1*) were crossed obtaining a F1 generation triple heterozygous (*Br2/br2 Br1/br1 F1/f1*). The phenotypic marker *f1* was used to follow the *br1* allele since it was just 5 cM distant. These plants were back-crossed with *br2* mutants obtaining a recombinant R1 population. The plants with a *brachytic* phenotype were selected and then selfed and crossed with homozygous *Br2 br1 f1* with the aim of isolating

recombinants gametes carrying the two mutations in cis. The following spring in field the brachytic striped plants were selected because of their nature of recombinants *br2-23 br1 f1*.
Also these plants showed a very small size with shorter internodes (Fig. 5).

3.2.3 Constitution and analysis of the double mutant *br2-23/br2-23 br3/br3*

With the aim of elucidating the relationship between *brachytic 2* and *3* mutations, we produced a *br2 br3* double mutant by crossing *br2-23/br2-23* with *br3/br3* plants (Fig. 2). The *Br2/br2-23 Br3/br3* F1 obtained were selfed and the progeny were planted and screened for brachytic phenotypes. About 9 wild type : 7 brachytic segregation ratio was observed as expected (9 *Br3/- Br2/-*; 3 *br3/br3 Br2/-*; 3 *Br3/- br2-23/br2-23* and 1 *br2-23/br2-23 br3/br3*). However, among the brachytic plants we noticed a few very short plants roughly resembling the extremely dwarf phenotype caused in maize by mutations in the Gibberellins pathway (Fig. 6). These plants, named "gnome", showed shorter internodes with curled and wrinkled leaves compared to those of the monogenic brachytic mutation. We conjectured that these plants were *br2-23/br2-23 br3/br3* double mutants (expected in a 1 out of 16 ratio) and this strong additive effect was caused by the presence of two brachytic mutations. The measurement of these plants (n at least 15) showed that gnome plants were shorter by about 85% percent compared to the wild type plant (Fig. 7). To test our hypothesis we crossed gnome plants with *br2* and *br3* homozygous mutants to perform a test cross assay (Fig. 8). The seeds obtained from these two crosses were sown in the following season and all the plants grown were brachytic (data not shown). The absence of complementation in the F1 generation confirmed that gnome plants were homozygous for *br2* and *br3* mutations. Furthermore we checked the presence of the *br2-23* allele in the double mutant using a codominant Sequence-Specific Amplified Polymorphism (S-SAP) marker based on the deletion present in the *br2-23* 3' sequence (Fig. 9). Taken together, these data confirm that gnome phenotype is caused by the simultaneous presence of the two brachytic recessive mutations. Starting from these gnome plants we performed four cycles of selfing to produce near-isogenic lines to use for the following studies. We noticed the high sterility of these plants with the presence of only a few seeds in the ears harvested.

3.3 Aberrant phenotype observed in the *br2/br2 br3/br3*

With the aim of understanding whether the *br2/br2 br3/br3* seedlings were in some way affected by interaction between these two brachytic mutations, we germinated on paper the offspring of selfed gnome (*br2* and *br3* seedlings do not show any obvious difference vs. wt seedlings, data not shown). We observed a strong effect regarding seedling morphology, in fact, as shown in Fig. 10, we identified defective seedlings roughly classified in four phenotypic classes: stunted plants like a dwarf mutant (Fig. 10B), seedlings with the first leaf that remains closed, named tube (Fig. 10C), seedlings with distorted growth (Fig. 10D) and seedlings without a shoot, named shootless (Fig.10E). We also noticed a high level of non-germinated seeds, as reported in Table 2 where we summarized the quantitative data regarding this phenotypic distribution (taking together all the segregation data obtained from the progeny of gnome plants). To strengthen the assumption that the *br2 br3* double mutant causes the abnormalities observed in embryo development, we also scored on imbibed paper the progeny of *Br2/br2 br3/br3* and *Br2/br2 Br3/br3* selfed plants: in the first case the segregation ratio expected would be 3 wild type: 1 (seedling aberrant phenotype plus non-germinated seeds); in the second case it would be 16:1. As reported in Table 3 the $\chi 2$ value did not exclude these segregation ratios, confirming in this way that the double mutant alters the normal embryo development. We also observed a different percentage of distribution of seedling abnormalities among the different selfed *br2br2 br3br3* near the isogenic lines developed, ranging from only non-germinated seeds to almost only stunted seedlings (data not shown). Of course, the only *br2/br2 br3/br3* phenotypic class able to grow was the stunted seedlings that at maturity became gnome plants.

By analogy we investigated also the offspring of the other two double mutants, to verify any possible interaction between the mutations. Analyses on the F2 populations (*Br1/br1 Br3/br3* selfed) seemed to show that there was no interaction between *br1* and *br3* in the embryo and first stages development. Indeed, the segregation ratio 15:1 expected for the double mutant was not found among the seedling. Only the R1083(203)⊗ family of seedlings showed two tube phenotypes. Also the seedling obtained by selfing *br2-23 br1/br2-23 Br1*, didn't seem to show any aberrant phenotype in seedling except for some not germinated seed (data not shown).

3.4 Embryo rescue analysis

With the aim of investigating the cause of the germination failure of a high proportion of *br2/br2 br3/br3* double mutant seeds, we performed embryo rescue analysis of the mature seeds. We used the double mutant sub-lines with the higher percentage of non germination. Excised embryos were cultured on MS media for ten days and the results obtained showed that the inability to germinate was caused by a deep upset of the bipolar embryonic axis introducing an asymmetry into the body plan (Fig.11).

3.5 Light microscopy analysis

Histological analyses showed that epidermal leaf cells of *br2/br2* and *br2/br2 br3/br3* mutants were characterized by alterations in size and in shape compared to the wild type (Fig. 12). Measurement of the major and minor axis of epidermal cells and the corresponding statistical analysis confirmed that *br2/br2* and *br2/br2 br3/br3* leaf epidermal cells were shorter and wider compared to the wild type (Table 4) and in *br2br3* double mutants the stomata cell length was also slightly but significantly reduced.
This found seems to show an alteration in the elongation of epidermal cells in the presence of the *br2* and *br3* mutations.

3.6 Expression analysis of *br2, ZmPIN1a, ZmPIN1b* e *ZmPIN1c* genes

In *Arabidopsis*, the auxin efflux carrier *pin1* is one of the main actors in controlling polar auxin transport during many aspects of plant development. In maize there are at least three pin1-like genes, *zmpin1a, zmpin1b* and *zmpin1c* (Carraro *et al*., 2006; Gallavotti *et al*., 2008), and the expression profiles of these maize *pin1* orthologs were analyzed by RT-PCR to assess their role in the phenomenon observed. As shown in Fig. 14 there was no difference in the *zmpin1b* and *zmpin1c* genes expression among *br2/br2*, *br3/br3* and *br2/br2 b3/br3* double mutants whilst *zmpin1a* was up-regulated in the presence of the *br3* mutation either in seedling or internodes sampled from

plants at the ten leaf stage. Furthermore, the presence of the *br2* mutation seems to down-regulate the expression of *zmpin1a,* in particular in the *br2/br2 b3/br3* seedling tissue. In the *br2* mutant the expression of *br2* gene is lower compared to the wild type and we observed a slight up-regulation in the presence of the *br3* mutation (Fig. 13).

3.7 Root gravitropic response

The role of auxin and auxin transport in the directional growth responses of plants to light (phototropism) and to gravity (gravitropism) played a major role in the discovery of auxin and in the formulation of the concept of plant hormones (Darwin, 1880). The negative gravitropism of stems, the positive gravitropism of roots, and the positive phototropic curvature of stems are characterized by the uneven distribution of auxin at the different sides of stimulated organs. This differential auxin distribution activates asymmetric growth and subsequent organ bending (Went, 1974).

For these reasons, we decide to set up a simple test, just to verify and give a further evidence of the involvement of *brachytic 2* and *3* in auxin transport (Fig. 15).

6 hours after the germination boxes were turned, it was already visible a change in the direction of growth in the wild type roots.

24 hours later, the primary root of the wild type seedlings had clearly changed the direction of growth, tending to vertical direction; on the contrary, the root of the *brachytic* seedlings seemed to have almost no response in the *br2/br2* and very weak response in *br3/br3*.

Tab. 1 Results of the field tests carried out in three locations per two years. In the HYBRID column the commercial hybrids used as parents are indicated. Of course all of these were crossed with B73 wild type and B73 *br2/br2* and compared. All the raw data were analyzed with ANOVA with two factors and confidential intervals were used to check any statistical difference. The hypothesis of the test was:

- Plant height of *Br2/br2* < WT
- Ear height of *Br2/br2* < WT

And our hope was to find some hybrids in which the yield of *Br2/br2* was greater than the wild type yield.

		2008			2009		
LOCATION	**HYBRID**	**PLANT**	**EAR**	**YIELD**	**PLANT**	**EAR**	**YIELD**
CALEPPIO (MI)	B73 x Mo17	++	++	++	+	+	-
	DK 440	++	++	-	-	+	-
	DKC 6530	++	++	+	+	+	+
	DKC 6040				++	+	-
	ALISEO				-	-	-
	ARMA				++	+	+
	PR33A46	++	++	+	+	-	++
LUIGNANO (CR)	B73 x Mo17				+	++	-
	DK 440						
	DKC 6530						
	DKC 6040						
	ALISEO				+	+	+
	ARMA				++	+	+
	PR33A46				+	+	++
LANDRIANO (PV)	B73 x Mo17	++	++	-	-	+	++
	DK 440	+	-	-	+	++	++
	DKC 6530	+	-	-			
	DKC 6040				++	+	++
	ALISEO				-	+	-
	ARMA						
	PR33A46	++	++	+	++	++	-

Legend:

++ = significant effect

+ = there is effect but not significant

- = the effect is opposite of that expected

-- = the effect is the opposite and significant

Table 2. Segregation of mutant seedling phenotypes obtained by selfing the *br2/br2 br3/br3* gnome plants. The seeds were germinated on imbibed paper.

cross	segregation		
br2/br2 br3/br3 selfed	gnome	abnormal seedlings	not germinated
	116 (38.66%)	49 (16.33%)	135 (45%)

Table 3. Segregation of mutant seedling phenotypes observed in the F_2 progeny obtained by selfing the *Br2/br2 Br3/br3* and *Br2/br2 br3/br3* plants. The expected segregation values for the mutant phenotypes (gnome + abnormal seedling + not geminated) was 15:1 in the case of selfed *Br2/br2 Br3/br3* and 3:1 in the case of selfed *Br2/br2 br3/br3*. The seeds were germinated on imbibed paper.

cross	Segregation				$\chi 2$ value	p
	wild type	gnome	Abnormal seedlings	not germinated		
Br2/br2 Br3/br3 selfed	860	25	23	22	2.59	0.2-0.1
Br2/br2 br3/br3 selfed	242	20	19	29	1.55	0.3-0.2

Table 4. Measurements (adaxial side of 2nd leaves) at seedling stage of leaf epidermal cells size and stomata cells length in wild type, *br2/br2*, *br3/br3* and *br2/br2 br3/br3* homozygous plants.

	Wild type	*br2/br2*	*br3/br3*	*br2/br2 br3/br3*
Epidermal cell length (µm)	135.74 ± 10.34	87.95 ± 5.19[a]	117.14 ± 10.83	85.65 ± 5.90 [a]
Epidermal cell width (µm)	30.08 ± 1.51	41.97 ± 1.66[a]	28.70 ± 2.18	40.63 ± 1.61[a]
Stomata cell length[a] (µm)	41.91 ± 1.26	38.70 ± 1.51	36.95 ± 1.28	37.70 ± 1.59

[a] confidence interval significantly different from wild-type plants at $P < 0.05$. Mean calculated from > 40 measurements

Fig.1 Comparison between wt ears on the left and heterozygous ears on the right. The ears in the picture are a representative sample of the two genotypes.

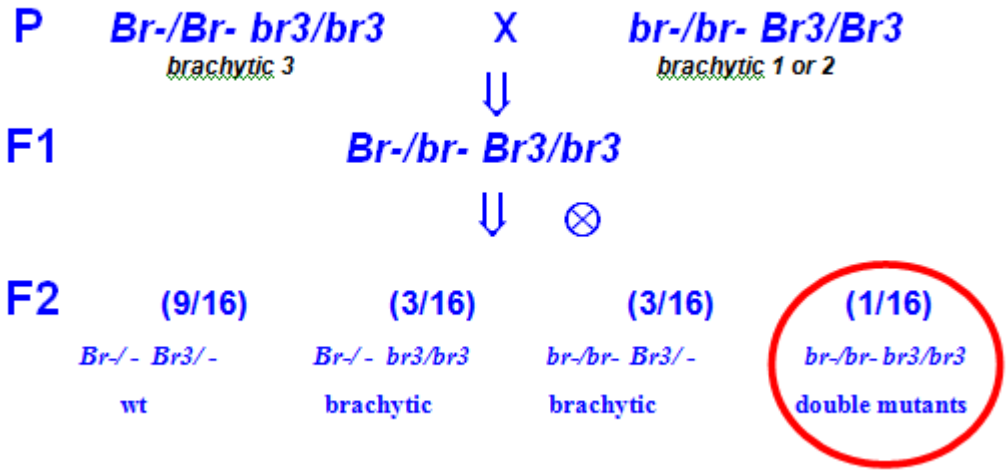


Fig. 2 Crossing scheme to obtain double mutants between *br3* and both *br1* and *br2*. The symbol "*br-*" indicates both *br2* and *br1*.

Fig. 3 Phenotypes from left to right: Wild type, *br3/br3*, *br1/br1*, double mutant *br1/br1 br3/br3*

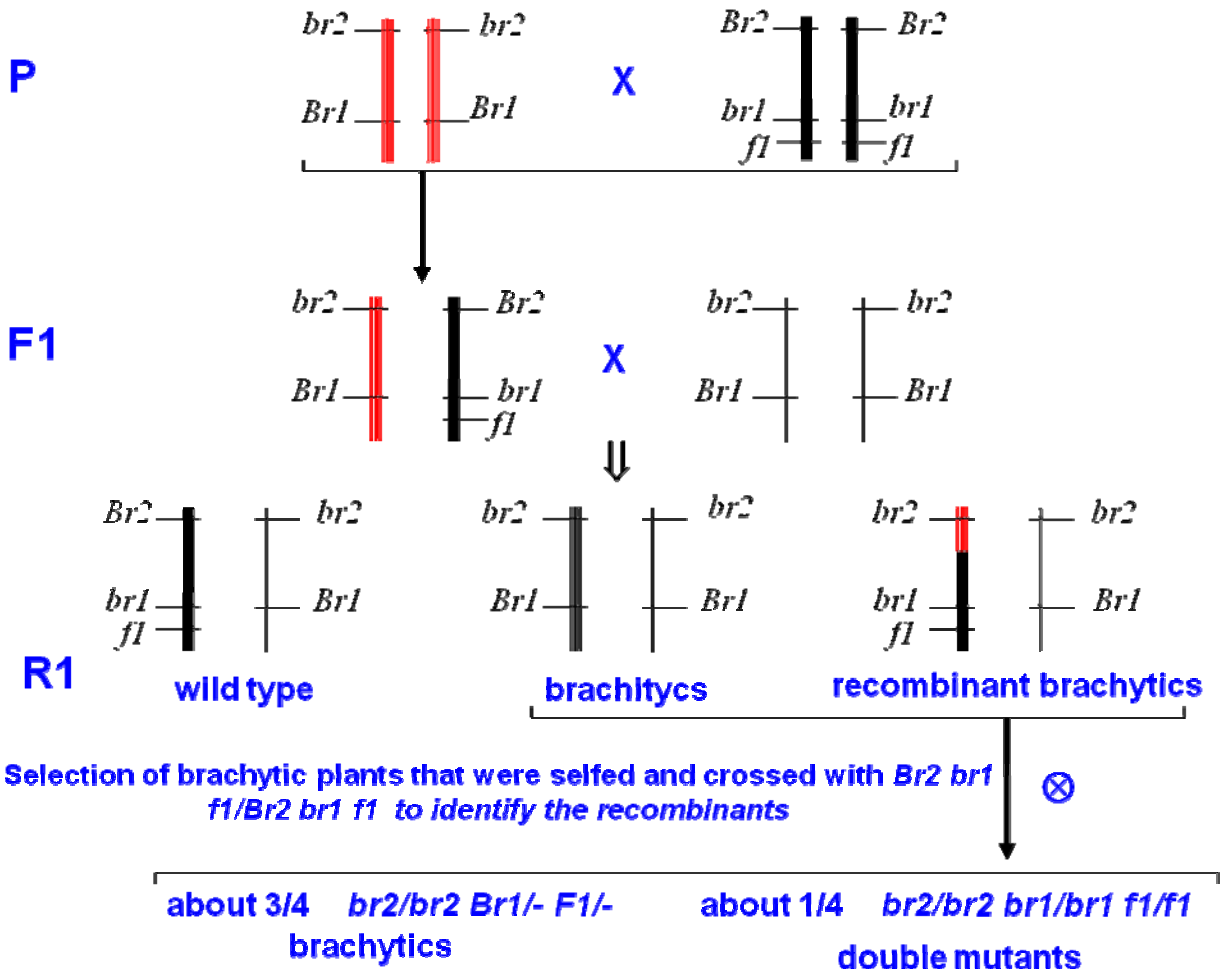


Fig. 4 Crossing scheme for the constitution of *br1/br1 br2/br2* double mutant. In the crossing scheme *F1* has been omitted and the wild type recombinant *Br2/br2 Br1/Br1* is not reported in R1 generation.

Fig. 5 Phenotypes from left to right: Wild type, *br1/br1*, *br2/br2*, double mutant *br1/br1 br2/br2*

Fig. 6 Phenotypes from left to right: Wild type, *br2/br2*, *br3/br3*, double mutant *br2/br2 br3/br3*

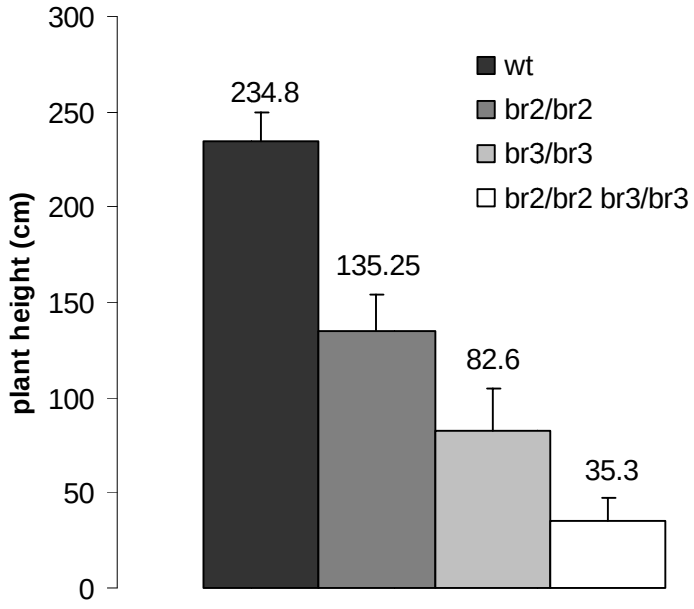


Fig. 7 Measurements on plant height

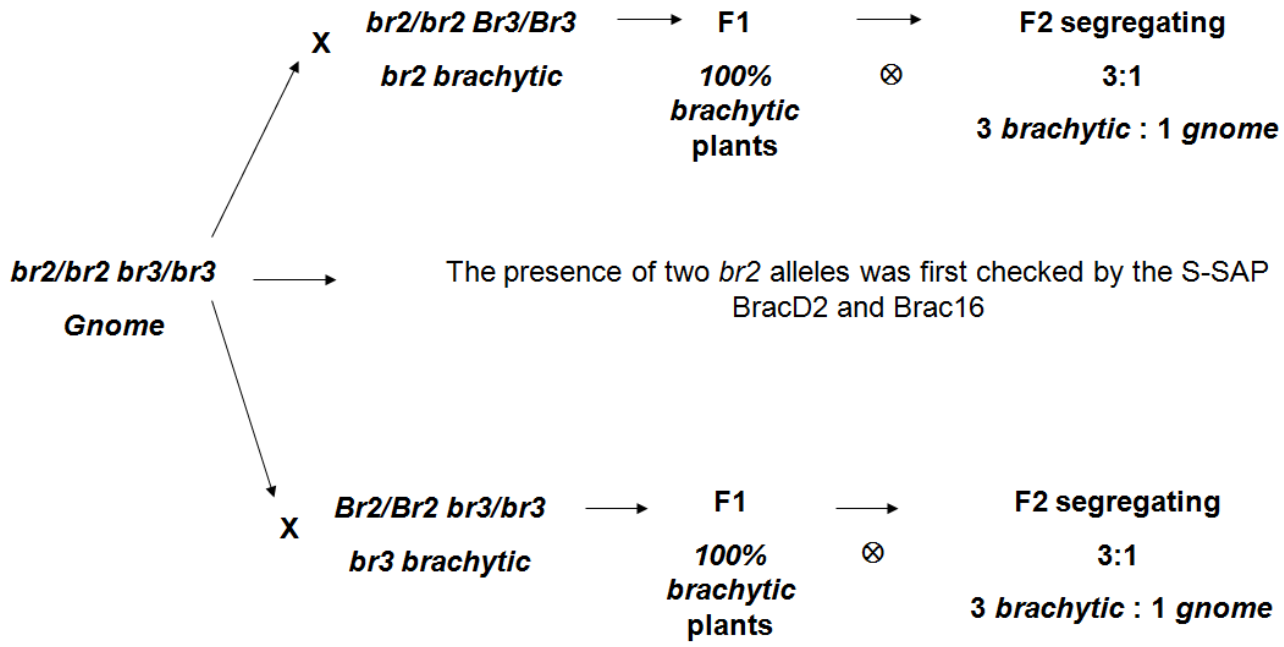


Fig. 8 Scheme of complementation test to verify the contemporaneous presence of the two brachytic mutations in gnome plants. Here *br2* and *br3* are indicated but this test can be done for any combination. On the contrary, we don't have any S-SAP for *br1* and *br3* but only for *br2*.

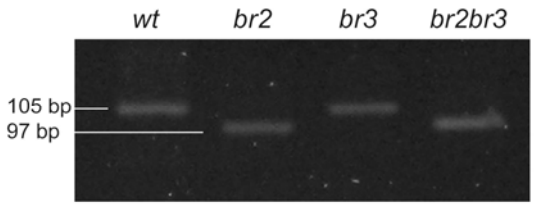


Fig. 9 Genotyping carried out using S-SAP molecular marker (Brac16/BracD2 specific primers). A specific 97 bp amplified product was obtained in the presence of *br2-23* allele while in the presence of wild type *Br2* allele a 105 bp amplified product was present. The genotypes shown are wt (*Br2/Br2 Br3Br3*), br2 (*br2-23/br2-23 Br3/Br3*), br3 (*Br2/Br2 br3/br3*) and br2br3 (*br2-23/br2-23 br3/br3*).

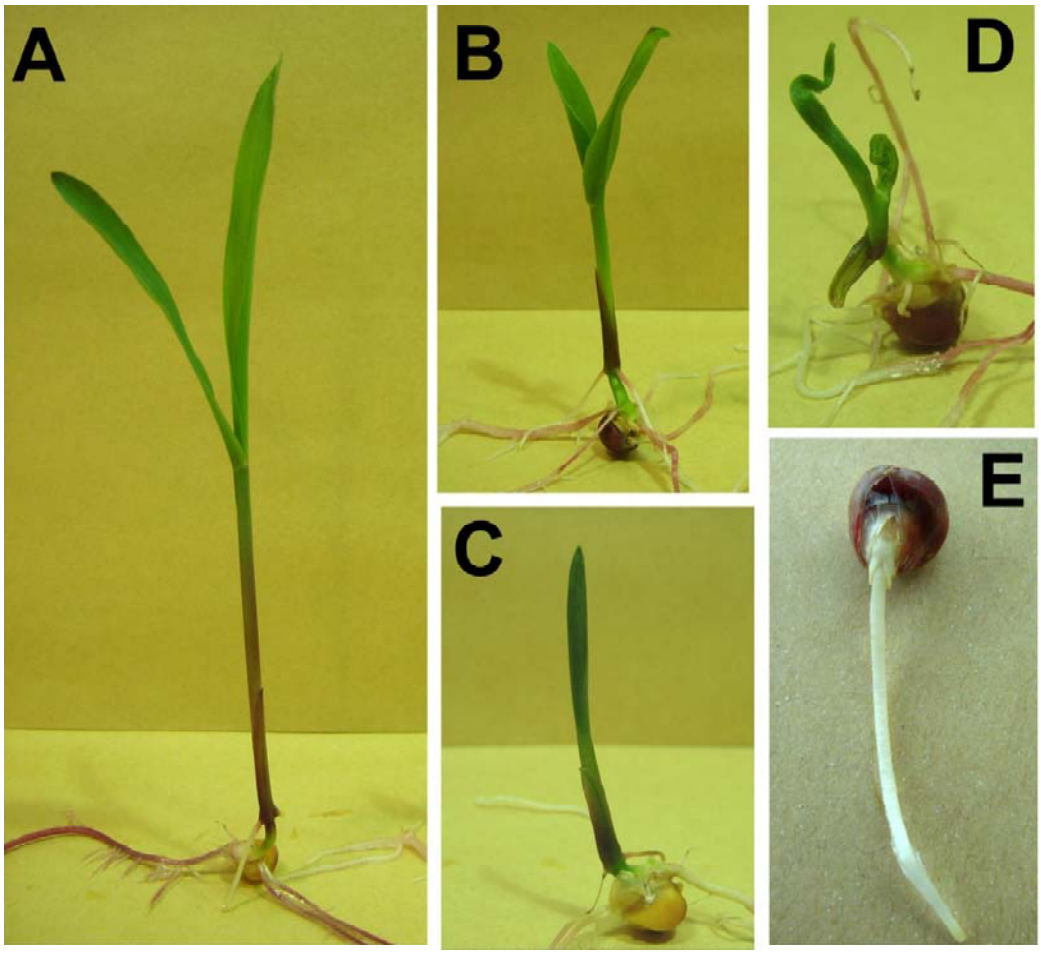


Fig. 10 Range of abnormalities showed by *br2br2 br3br3* seedlings. (A) wt seedling, (B) dwarfing-like seedling that will became gnome at maturity, (C) "tube" seedling: the first leaf remains closed, (D) seedling with distorted growth, (E) shootless seedling.

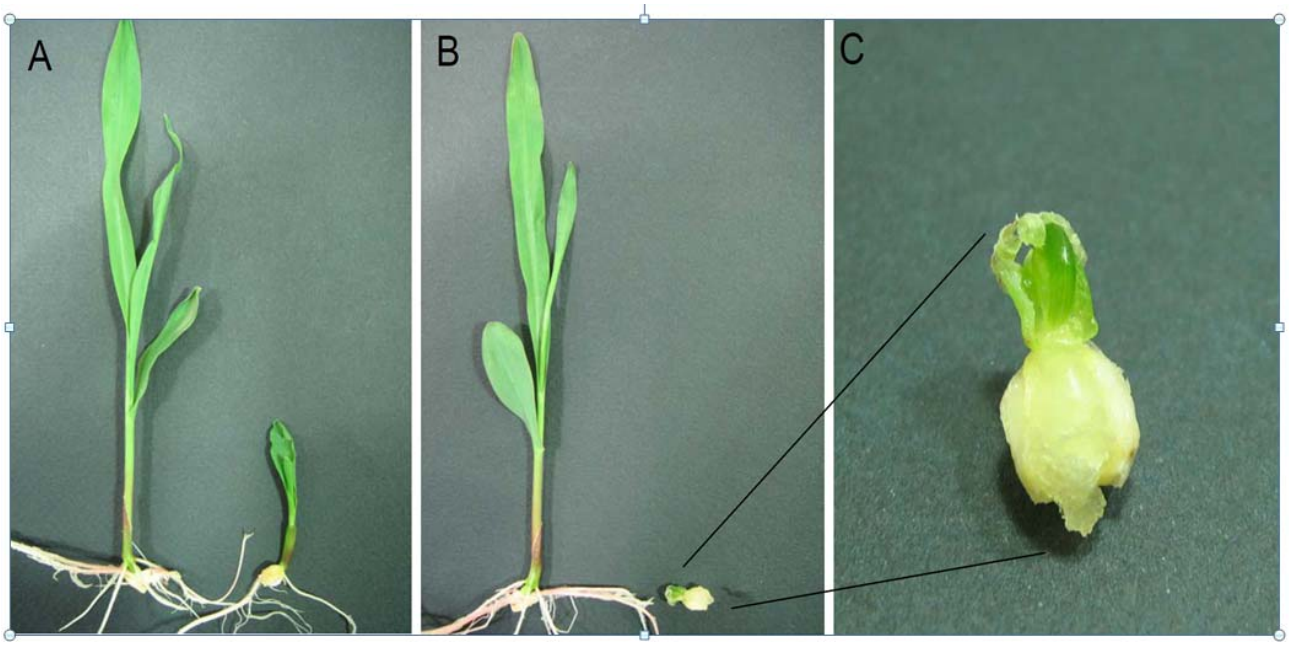


Fig. 11 *In vitro* culture of *br2br2 br3br3* embryos. Seedlings were obtained from embryos removed aseptically from the mature seeds and transferred to Murashige and Skoog tissue culture medium. (A) on the left the wild type and on the right the double mutant, (B) on the the left wild type, on the right the non germinated double mutant (C) magnification of the double mutant present in the previous image.

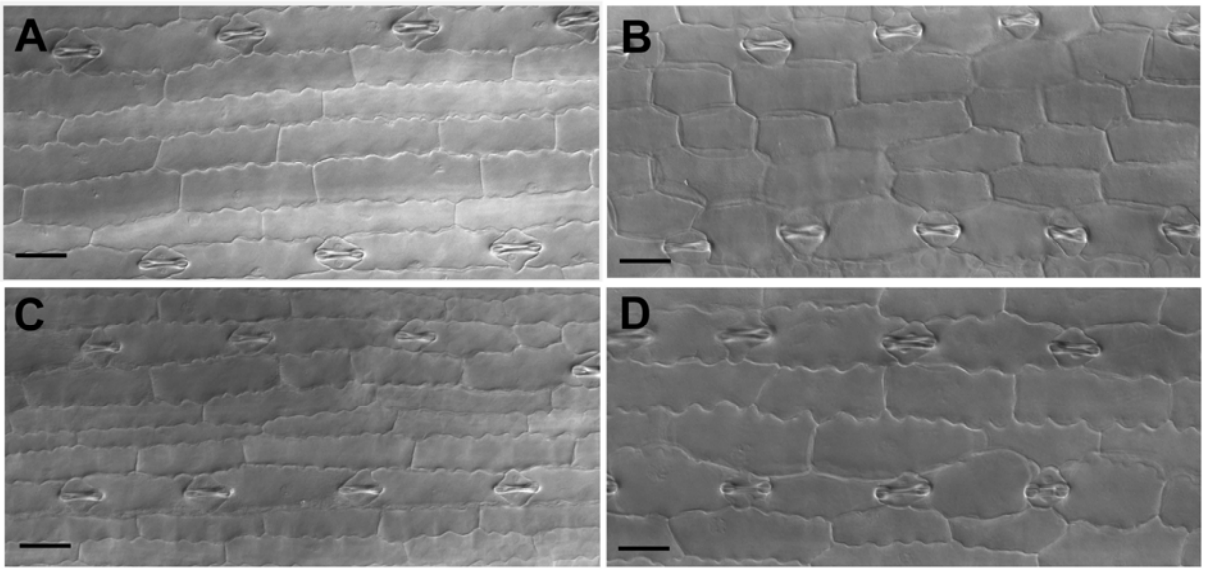


Fig. 12. Histological analysis of leaf epidermal cell (adaxial side of 2rd leaves). (A) Wild-type, (B) *br2/br2*, (C) *br3/br3*, (D) *br2/br2 br3/br3*. bars = 40 μm

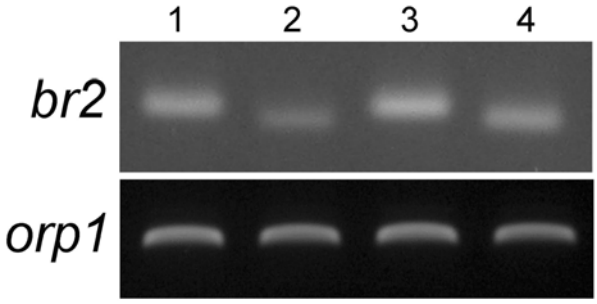


Fig. 13 Expression analysis of *Br2* gene. Semi-quantitative RT-PCR analysis of *Br2* was carried out using total RNA prepared from seedlings. The tissue analyzed was seedling of wt (lane 1, *Br2Br2 Br3Br3*,), *brachytic 2* (lane 2, *br2/br2*), *brachytic 3* (lane 3, *br3br3*) and double mutant (lane 4, *br2br2 br3br3*). The primers used were Brac16/BracD2 able to evidence the S-SAP polymorphism also at the cDNA level. *Orp-1* was used as control.

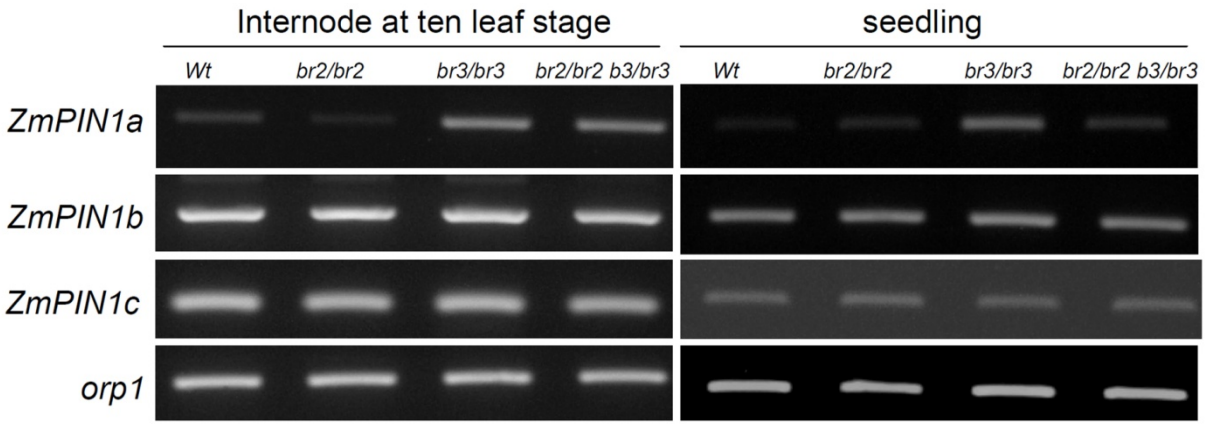


Fig. 14. Semi-quantitative RT-PCR expression analysis of *ZmPIN1a*, *ZmPIN1b* and *ZmPIN1c* genes. The tissues analyzed were internodes at ten leaf stage and seedling tissues of wt (*Br2Br2 Br3Br3*), *br2/br2*, *br3br3* and *br2br2 br3br3* double mutants. *Orp-1* was used as control.

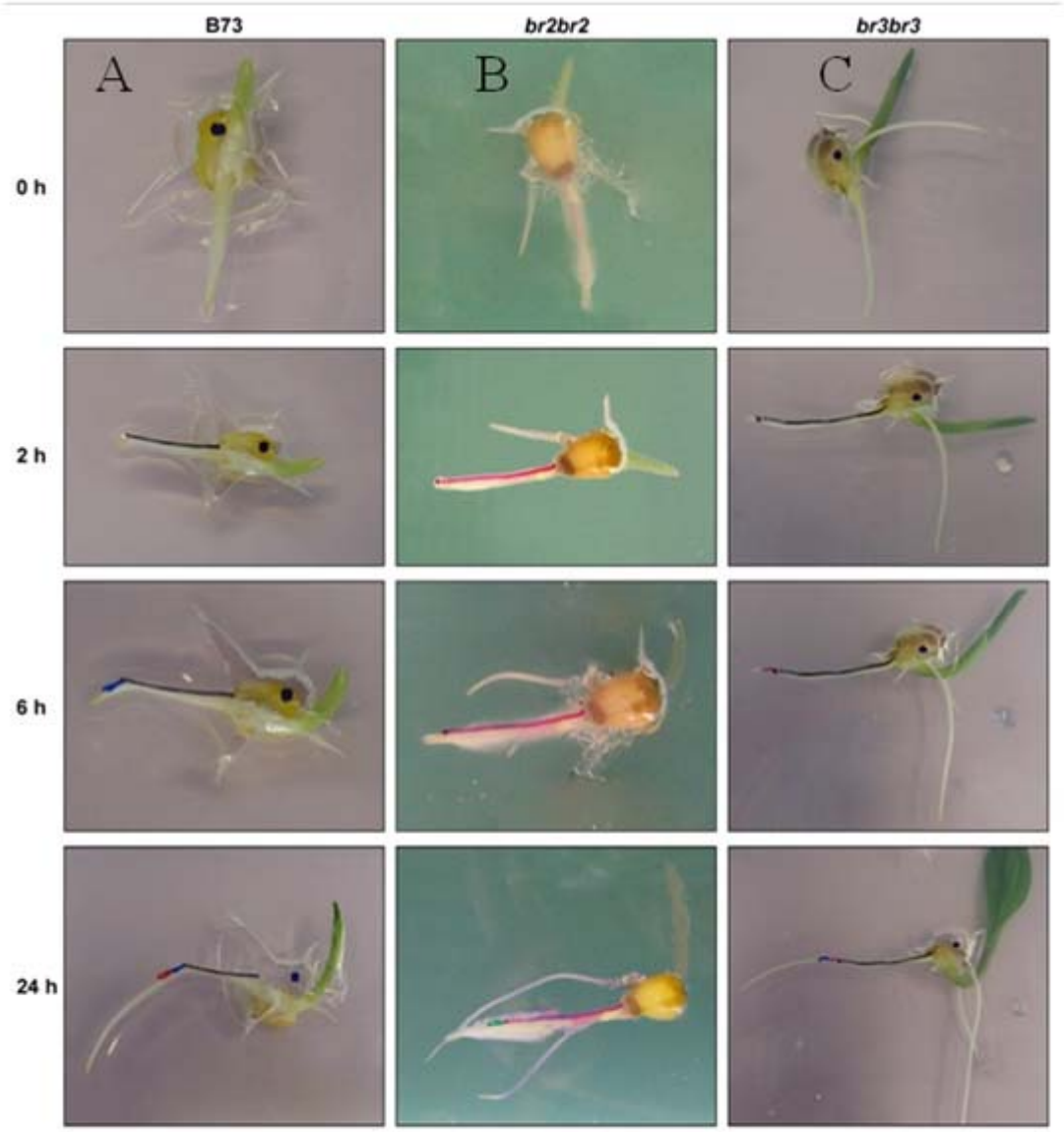


Fig. 15 Root gravitropic responses of: B73 seedling as control (A column), *br2/br2* mutant (B column) and *br3/br3* mutant (C column)

4 DISCUSSION

The rise of agriculture led to the domestication of many species of plants and to the exploration of natural resources. It took 10.000 years to the production of wheat to reach 1 billion tonnes in 1960 and only 40 years to reach 2 billion tons in 2000 (GS Khushi, 2001).

Since the ‘60ies, indeed, it appeared necessary to cope with the demand for food of a high growing population: due to the decrease in mortality resulting from advances of medical and health care, population increased and awareness of an impending food crisis emerged(Ehrlich P., 1968). Fortunately, the problems of global hunger and the related economic and social consequences have been avoided thanks to an increase in cereal production started just from those years.

This result, known as Green Revolution, was achieved mainly through the widespread adoption of techniques developed to generate genetically improved crop varieties with high yield and application of more efficient farming practices. Of crucial importance was the role played by Norman Borlaug, who was considered the father of Green Revolution and the promoter of genetic improvement programs.

The Green Revolution had a huge impact on food production and on the socio-economic and environmental sustainability. Between 1966 and 2000, the population of developing countries has almost doubled, but food production has increased by 125%.

The marked improvement in food production was caused by the application of advanced technology for the development of varieties of cereals with high productivity. These varieties, particularly of rice, maize and wheat have been developed, at first, by IRRI (International Rice Research Institute) in the Philippines, and by CIMMYT (Centro Internacional de Mejoramiento de Maiz y Trigo) in Mexico. Since then, many national breeding programs have been undertaken to produce many improved varieties of cereals. As rice, maize and wheat provide about 50% of the calories in human diet, the goal of these programs was to increase the production of these three species.

For example, since the marketing of the first high-yield varieties of rice in 1966, the area cultivated with rice has increased only marginally by 20% while the average production of rice has doubled. The total production of rice increased by 132%, from 1966 to 1999. During the same period the production of wheat increased by 91% to 576 million tonnes. In many Asian countries, the growth in cereal production has increased so much compared to the population's needs, that it has promoted export. The increased availability of cereals and the reduction in production costs contributed to the decline in the price of rice but also of maize and wheat in international and domestic markets.

Production costs per unit fell by 20-30% for varieties with high yield compared to traditional varieties and prices of rice, maize and wheat were lower by 40% in 2001 compared to those of the '60s.

The genetic characters modified to improve yield were a lot; in fact, the development of productivity, the adaptation of plants to different environments, the shortening of vegetative cycle, the resistance biotic and a-biotic stress, the improvement of kernel quality, were achieved thanks to conventional techniques of cross and selection.

A very important approach was the modification of plant architecture: plants with reduced height, higher number of kernels per ear, erect leaves shape and greater stalk resistance were selected.

Before the Green Revolution, wheat and rice varieties were high, had a low harvest index and had weak stems. In response to high nitrogen fertilization, they grew too much, spread too much tillers, lodged, and produced less grains than not-fertilized plants. To improve the productive potential of rice, it was necessary to reduce plant size introducing a recessive trait for short stature *sd1* (*semidwarf*), coming from the Chinese variety Dee-geo-woo-gen (Suh H.S. and Hue M.H., 1978). Instead, the recessive traits *Rht1* and *Rht2* (*Reduced height*) coming from the Japanese variety Norin 10 were exploited for shortening the stature of wheat (Rajanam S. and Van Ginkel M., 1996). These "dwarfing" genes were introgressed through conventional breeding and in this way the greatest part of plants resources were no longer utilized for vegetative growth but for grains production, so as to improve the harvest index, the response to nitrogen fertilization and resistance to lodging. The improvement of wheat stem was particularly important because in this crop, the plant height was inversely proportioned to wind and rain resistance. Old varieties reached the height of 180 – 220 cm whilst today the new ones do not exceed 70 – 80 cm.

On the contrary, in maize the selection did not focus on the reduction of height but breeders improved other characters like the resistance to lodging, eradication, corn borer and other biotic adversity, the increase of stay green, reduction of sterility, the adaptation of the plants to high density of population.

The Green Revolution, therefore, has seen a strengthening of maize yield due to the exploitation of hybrid vigor (Khush G.S., 2001). Still now, modern breeding programs pursue the same objectives.

The varieties with a short stature have not been commercially exploited because it seems that the excessive reduction of height caused by original mutant alleles confers an extreme phenotype penalizing the production. Moreover, the decrease of the size through the selection of quantitative traits, is a very long procedure and often leads to the reduction of the number of internodes with a consequent shortening of the crop cycle.

However, mutations working on plant stature have been widely exploited in sorghum since the ‘50ies. At the beginning of the last century, the first goals of breeding were the reduction of height and the reduction of the vegetative cycle of the imported tropical sorghum varieties. Later, breeders started with artificial hybridization and selection, but only the discovery of cytoplasmatic male-sterility allowed the commercial production of F1 hybrids. Modern grain hybrids are characterized by a size not exceeding 120 – 150 cm, erect stem and leaves, close nodes with overlapping leaf sheathes; the length of internodes is increasing from base to apex and the last internode is very elongated (combine character) to facilitate mechanic harvest (Baldoni R. e Giardini L., 2000).

The mutant of maize *br2-23* isolated in our laboratory recalls in a very strong way the phenotype of grain sorghum varieties, according to the homology of the sequence between *Br2* and *Dw3* in sorghum. As *dw3* is widely used to shorten the size of grain sorghums (the mechanism of this mutation has been clarify by Multani in 2003), we supposed that also *br2* could be an interesting gene for maize breeding.

In addition, a preliminary study of Micu V.E. and Solonenko T.A. in 1984, that studied 11 agronomic features of homozygous *br2/br2*, *Br2/Br2* and heterozygous *Br2/br2*, showed that in the heterozygous there was a little reduction in the height but an increase in the resistance to lodging and an improvement in grain production in comparison to the wild type. In our case, the effect on stature seems to be even stronger, with a greater reduction of height, probably because of the peculiarity of the isolated allele.

Thus, we decided to start a preliminary breeding program to test this mutation – both as homozygous and heterozygous – in different genetic backgrounds. Indeed, as the expressivity of the allele partially depends on the NIL in which it is introgressed, maybe even the influence on yield depends on the genetic background.

A finding of our group was that the mutant allele in heterozygous *Br2/br2–23* plants with the B73 background, exhibited consistent effects in terms of plant and ear height (which are 8 and 18% shorter in mutant plants than in *Br2/Br2* wild-type ones) and leaf angle (Pilu *et al.*, 2007). This observation may indicate that one single functional gene copy is not sufficient to restore the wild-type phenotype. This feature could be interesting for the selection of inbred lines for the production of commercial hybrids heterozygous for the mutation. In fact, if this mutation as homozygous seems to be too severe for temperate corn materials, as heterozygous it seems to give favourable agronomic features to the plants like more erect leaves and lower insertion of the ear.

After having tested that the heterozygous effect was not hidden by the hybrid vigour in two simple hybrid combinations – B73xMo17 and B73xW23 - (Pilu *et al.*, 2007), we observed also that the ears of the heterozygous plants (Fig. 1) were longer than the wild type ones (data not shown). Then we

decided to test the phenomenon in a modern elite genetic background. In this way, different combinations of 3-way hybrid were produced crossing B73 *br2/br2* and different commercial hybrids. These materials heterozygous for *br2* have been tested with their corresponding wild type control in different locations in plots. The data obtained suggest that the effect of heterozygous is confirmed also in the hybrids at different levels of expression (according to the specific genetic background, see Table 1) but however generally without penalizing the productions (a little penalization can occur depending on the genetic background); in some cases the heterozygous seems even to improve the yield (Villa *et al.* 2010).

This preliminary study could support the hypothesis of using this mutation in corn breeding and give indication of the plant materials from which the program could start. Of course, it is a long and hard way to reach selected and elite inbred lines which are adapt to produce performing maize hybrids for the market and it will take a lot of time, but the premises are encouraging.

However, the breeding work is facilitated thanks to a tool in our possession; in fact, the alignment of the 3' *Br2* (B73 allele) and *br2–23* sequences, revealed that the mutant carries an eight nucleotide deletions in the coding region (Pilu *et al*., 2007). The presence of this deletion in the coding region was confirmed using allele-specific primers on segregating populations. This PCR-based molecular marker associated to the *br2–23* allele could be a useful tool to rapidly discriminate the wild type, *Br2/Br2* heterozygous *Br2/br2* and *br2/br2* homozygous plants. Currently, when aiming at obtaining the introgression of a recessive gene in an inbred line, it is necessary to test each generation that results from the cross with the recurrent parental line with a recessive homozygote in order to identify the genotype. Using the marker-assisted selection approach described here, it would be possible to select in each generation the desired plants for the next generation by a rapid molecular analysis (Fig. 9).

A possible further and future application could be the selection of inbred lines characterized by high production of biomass and high quality of the fiber with the aim of producing hybrids for biomass for renewable energy and silage for fodder. We have just started a preliminary work (data not shown) of introgression of *br2* in an inbred line affected by the mutation *brown midrib* (*bm3*). This mutant is characterized by low lignin content and a consequent high digestibility of the fiber. Thanks to this peculiarity it could be an interesting fodder and biomass for anaerobic digester (Barriere Y. *et al.*, 1998). Unfortunately, *bm3* mutants are susceptible to lodging (Vignols F. *et al.*, 1995) because of their typical characteristics; these plants are also susceptible to pests and disease and at maturity they have a fast senescence and then collapse. The mutation *br2* could be exploited to strengthen the stalk of *bm3* producing inbred lines double mutants *br2/br2 bm3/bm3* in accordance to the work of Multani who crossed *br2* and *bk2* (*brittle stalk 2* a mutation that

undermine the stability of maize stalk causing lodging and collapsing) obtaining double mutants which are able to survive in field. In this way the *brachytic* mutation could compensate, with its positive agronomic features, the negative characteristics of *bm3*. As mentioned before, the *br2/br2* mutant has a greater stalk at base that would confer a greater resistance to lodging, a more acute leaf angle and a greater stay green with an elongation of the vegetative cycle.

Besides, we are introgressing the *br2* allele in different tropical varieties. At our latitude these plants have difficulties in turning from vegetative phase to reproductive one. Indeed, they have a very long cycle with a very late flowering and often they are unable to issue the ear. These plants reach and exceed easily 4.5 m of height and of course are very susceptible to lodging. Our intention is to cross these varieties with lines brachytic for *br2* with the aim of producing new elite lines characterized by very tall size even if they are homozygous for *br2*. Our final goal is to select elite inbred lines gifted of the positive features of *br2*, *bm3*, and tropical variety: high size and great production of biomass, high quality of the fiber with low lignin content, high stand ability and resistance to lodging.

New perspectives, therefore, could be opened in this field for the breeding of corn through the creation of new alleles *br2*. The reduction in stature and its synergistic effects on the quality of the stem would be a character of great interest for production increase. The brachytic-type mutants can also open new perspectives for genetic improvement of maize for cultivation in marginal areas of developing countries where often the environmental conditions are not optimal for the crop.

In order to study the interaction between the mutations *br1, br2-23* e *br3*, double mutants were made: *br1/br1 br3/br3*, *br2-23/br2-23 br1/br1*, *br2-23/br2-23 br3/br3*. Indeed, only *br2* has been cloned by Multani in 2003, while nothing is known about the other two mutations. Our aim was to try to understand if some interaction could have happened among the three mutations and in this way try to discover something about their action.

All the three double mutants have an extreme shortened phenotype resembling the extremely dwarf phenotype caused in maize by mutations in the Gibberellins pathway (Fig.3, 5, 6). They had a size reduced of about 80% of the wild type on average, with very short internodes. This strong additive effect was caused by the simultaneous presence of the two brachytic mutations.

In addition, among the double mutants *br2-23/br2-23 br3/br3* (we named this phenotype *gnome*), we discovered a considerable portion of seeds which were affected in morphology, and a great amount that didn't germinate (Fig. 10). We identified defective seedlings roughly classified in four phenotypic classes: stunted plants like a dwarf mutant (Fig. 10 B), seedlings with the first leaf that remains closed, named tube (Fig. 4 C), seedlings with distorted growth (Fig. 4 D) and seedlings

without a shoot, named shootless (Fig. 4 E). These abnormalities in seedling development, in particular in the shoot, (mirroring an altered embryogenesis) indicate an involvement of these genes in embryo development. In particular, the presence of a shootless phenotype is diagnostic of a severe failure of the shoot apical meristem (SAM) as reported at the histological level in the case of the *sml* (shootmeristemless) mutant (Pilu *et al*., 2002).

We also noticed a strong variability among the *br2/br2 br3/br3* sublines regarding the relative percentage of abnormalities observed (ranging from non-germinated seeds to gnome) and suggesting that different factors may modulate the phenotype observed (data not shown). Furthermore, epidermal cell size showed a significant difference between wt and the seedlings carrying *br2* and *br2 br3* mutations (Fig 12. Table 4) as reported for tobacco cells cultivated *in vitro* without auxin (Mravec *et al*., 2008).

These data strongly suggest for the first time that the *Br2* gene may play an important role not only in plant growth and architecture but also in embryo development.

The gene *Br2* encodes a putative protein of 1394 amino acids (Accession Number: AAR00316) similar to ABC transportes (ATP- Binding Cassette) of the Multidrug resistant (MDR) class of P-glycoproteins (PGPs).

As with other PGPs, the predicted BR2 protein consists of two similar halves, each one containing six putative transmembrane domain and an intracellular ATP nucleotide-binding domain.

The closest homolog of BR2 is Atpgp1, with 67% identity. The classification of BR2 as PGP is consistent within findings from Arabidopsis that suggest that PGPs modulate auxin-dependent growth. Mutations in *AtPGP19* (*AtMDR1*), a close relative of AtPGP1, resulted in decreased auxin transport and pleiotropic auxin related phenotypes (Noh B., 2001).

Auxin distribution within tissues contributes to developmental cues in embryogenesis and organogenesis and mediates the response to environmental stimuli such as gravitropism and photomorphogenesis. PAT (Polar Auxin Transport) allows the auxin gradient created by several influx and efflux systems to transport auxin across the plasmalemma. This important process is explained by a chemiosmotic polar diffusion model: undissociated native auxin (IAA) can diffuse passively in the cytoplasm from an acid extracellular environment (induced by a membrane H^+-ATPase activity) where the more alkaline environment causes the dissociation of IAA to IAA^- that remains trapped in the cytoplasm because it is not able to cross the plasma membrane again. The IAA^- molecule is transported across the plasma membrane from the cytoplasm to the extracellular environment by the active auxin efflux transporters, representing the limiting step of this transport (Goldsmith, 1977). In *Arabidopsis* the general picture of PAT has been quite well characterized using several experimental approaches in particular forward genetics, even though further works are

needed to elucidate in detail this fundamental process (reviewed by Petrášek and Friml, 2009). Now we know that the auxin influx is helped by the proton cotransporters belonging to the AUX/LAX family (auxin resistant and like auxin resistant mutations) of proton-gradient-driven transporters (Bennett *et al*., 1996). As already mentioned, the limiting step of the PAT is represented by the capacity of the cells to transport the IAA^- across the plasma membrane in the extracellular space, in fact, several efflux transporters have been isolated in different species belonging to two transmembrane protein families: the PIN and ABCB/MDR/PGP protein family (reviewed by Petrášek and Friml, 2009). In maize, the three brachytic mutations (*br1*, *br2* and *br3*) conferring short stature caused by shortening of the internodes, seem to be involved in auxin transport: in fact no hormone treatment is able to revert these phenotypes (Anderson and Chow 1960; Scott and Campbell, 1969; Pilu *et al*., 2007). To support this hypothesis, in 2003 Multani and colleagues cloned the *Br2* (*ZmPGP1*) gene that was the first auxin efflux ortholog of arabidopsis PGP1 (also named ABCB1) isolated in maize. To our knowledge no other auxin efflux carriers has been isolated in maize, while a reverse genetics approach was able to discover three novel putative orthologs of the *AtPIN1* gene: *ZmPIN1a*, *ZmPIN1b* and *ZmPIN1c* (Carraro *et al*., 2006; Gallavotti *et al*., 2008).

As *Br2* is a gene involved in auxin transport and its mutation causes approximately the same phenotype observed for *br3* mutants, we suppose that even *Br3* could be involved in this process as well.

We can conjecture that BR2 and BR3 proteins work in same way in functional redundancy: in fact single mutants do not display these alterations.

We can consider three hypotheses concerning the origin of the embryo abnormalities observed in the offspring of the double mutant:

i) BR3 is another auxin efflux protein paralogous to ZmPGP1 (BR2). For a correct embryo development the activity of only one gene is sufficient, nevertheless both of them are able to cause the plant brachytic phenotype. This hypothesis is supported by the fact that *Atpgp1 Atpgp19* (ABCB1 and ABCB19) double mutant in *Arabidopsis* confers enhanced pleiotropic auxin-related phenotypes (Noh *et al*., 2001).

ii) BR3 belongs to the PIN family and a single mutation in *Br2* (*ZmPGP1*) plus a mutation in a PIN gene (*Br3*) cause a synergistic effect on embryo development. In fact in *Arabidopsis* it is known that ABCB19 and PIN1 might physically interact for a correct work in a way which, so far, has not been understood (Blakeslee *et al*., 2007). It is also known that *Atpgp1 Atpgp19 AtPin1* triple mutants enhance the effect of the *pin1* single mutation during embryogenesis (Mravec *et al*., 2008).

Furthermore, as shown in Figure 7, *ZmPin1a* gene was up-regulated in the presence of the *br3* mutation and in a similar way mutations in a PIN protein in *Arabidopsis* cause an up-regulation of other PINs in the same expression domain (Blilou *et al*., 2005). As mentioned in the introduction, in maize three *ZmPIN1* genes (a, b and c) have been isolated so far and *ZmPin1b* sequence (DQ836240) maps on the bin 5.06 in the same position where *br3* mutation maps. This finding may indicate that the *Br3* gene was *ZmPin1b* even though the expression of this gene is not altered in the presence of the Br3 mutation (Fig.14).

iii) BR3 might be another auxin transporter or protein involved in PAT not so far characterized: in fact in 2005 a new auxin transporter candidate in maize ZmTM20 was identified by Jahrmann and colleagues; however its role in PAT is not clear.

The expression analysis carried out using RT-PCR technique (Fig. 13) showed a lower level of *Br2* transcript in a *br2* plant than in the wild type (RNA extracted from seedling). This finding was in contrast with the results published in 2007 by our group (Pilu *et al*., 2007) where we did not observe any difference between wild type and mutant (RNA extracted from second internode at the ten leaf stage). This result could be explained by the difference in the tissue taken for this expression analysis: we can conjecture that a different *Br2* expression may depend on the developmental stages and on the tissues studied. Furthermore, in the presence of the *br3* mutation we observed a slight up-regulation of the *Br2* gene and these data also suggest a relationship in the PAT of these two genes.

In conclusion, the results here reported suggest, for the first time, an important new role of *Br2* and *Br3* genes in maize embryogenesis which could contribute to answering several open questions regarding PAT and embryo development in maize and other cereals. Further work will be necessary to better characterize this new finding, in particular regarding the biochemical and cellular localization of BR2 protein and the isolation of the *Br3* gene. Of course, also the other two brachytic mutants have to be better studied and characterized, and maybe a cloning of *br1* and *br3* will be necessary in order to clarify the nature of these gene and their relative proteins. A further approach could be also the realization and the consequent study of the triple mutant *br1/br1 br2/br2 br3/br3* to check the phenotype resulted by the additive effect of the three mutations.

Another interesting approach would be analyze modern elite inbred lines compared to old and not improved lines or varieties and check if *Br2* gene has been target of selection in the breeding programs. Association methods such as Tajima's D test of selection (Jeffry M. Thornsberry, *et al.,* 2001) could be a useful statistic test to analyze the selection pressure on this gene.

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