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Kiwifruit bud release from dormancy: effect of exogenous cytokinins

Presentata da: Dott.ssa Cristina Fabbroni

Coordinatore Dottorato

Prof. Luca Corelli Grappadelli

Relatore

Prof.Guglielmo Costa

Correlatore

Dr. Anna Maria Bregoli

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To Cricchio,

"Ciò che conta non è quello che fai, ma quanto amore metti in quello che fai"

(Beata Madre Teresa)

Contents

1.Introduction

KIWIFRUIT

Economical relevance

It's well know that Italy is the leading kiwifruit producer and marketer in the world (Della Casa, 2007). Since the beginning of the 1990 till now the world area cultivated by *Actinidia spp* has doubled and it is now established around the 140.000ha, half of which located in China. Without considering the production of this country, which is not relevant for commercial purpose in the foreign markets, Italy itself contribute to the 70% of the commercial production of the North Hemisphere that reaches the 600.000t per year. Estimation data in the near future shows additional investments which will establish in 2010 the national potentiality over the 500.000t per year. In parallel, the internal market for this crop has increased in the period 2000-2006 in contrast with the reduction of the 11% in the consumption registered in the fruit sector. As regard to the export, the commercial production, which is sold in the foreign markets is established around 70% mainly exported toward Europe (80%) (Miotto, 2007).

Plant architecture

Kiwifruit (*Actinidia spp*.) is a perennial climbing vine of horticultural importance and a subject of architectural interest (Ferguson, 1984; Warrington and Weston, 1990; Snowball, 1995; Snowball, 1997a; Seleznyova et al., 2002). At the whole plant levels, Actinidia conforms to the Champagnat architectural tree model (Hallè et al., 1978). This model is characterized by mixed axes, i.e. axes that have successive and distinct phases in their development. In Actinidia all branching is sympodial, with relay axes (dominant branches) developing in the region of curvature along the parent axes. Therefore, the vines are constructed by the superposition of such mixed axes, the proximal parts being included in the trunk and the distal parts becoming a branch.

In horticulture, kiwifruit vines are generally trained onto pergola or T-bar support structures (Sale, 1990). A single trunk is usually maintained and initially two relay axes, called 'leaders' or 'central leaders' are trained horizontally in opposite directions along the support structures. New relay axes, form along these, are left to bend under their own weight, these axes are eventually tied down in winter to the support structures as 'replacement canes'. The axillary shoots arising from these replacement canes in the following year produce the fruiting canopy. The fruiting canes are

generally replaced with new relay axes (canes) during winter pruning, thus bringing the vine back to a similar structure at the beginning of each season.

Annual growth cycle

A typical annual growth cycle of the kiwifruit cv. "Hayward" is shown in Figure 1. The first sign of growth after winter is bud swell which occurs in the first 10 days of March. Bud break subsequently takes place in mid March and at that time a burst of new root extension begins. After bud break, shoot arises from the buds start growing rapidly and flower bud development and enlargement occurs concurrently. The flower buds open in late May, some 2 months after bud release. After pollination and fruit set, the young fruit start expanding very rapidly in length and circumferences. The curve of volume growth from anthesis appears to be double-sigmoid. The vegetative growth declines in mid-summer when the competition with fruit becomes more marked. Fruit matures to a satisfactory harvesting stage by mid October, approximating 150 days after flowering. The dormant phase of the vine generally lasts from leaf fall through until March, when swelling of buds indicates the commence of the new season (Warrington and Weston, 1990).

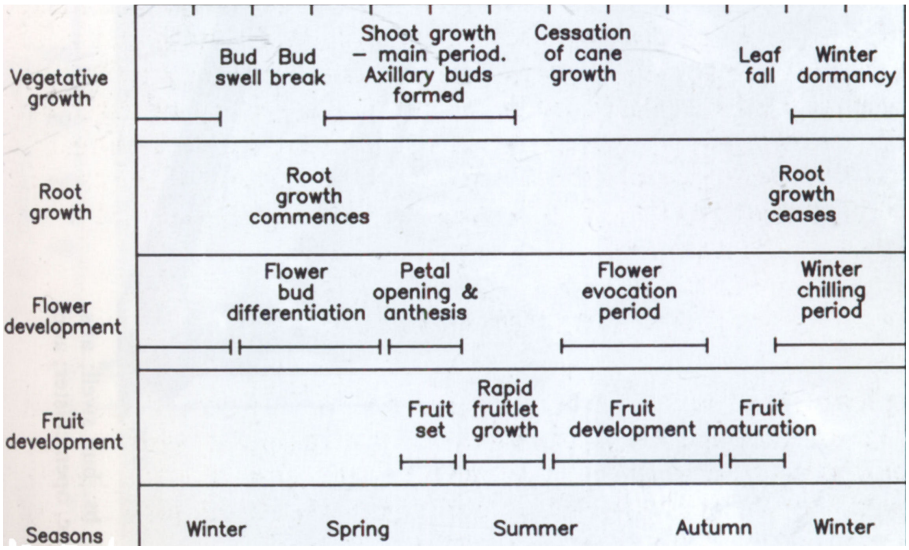


Figure 1. The annual growth cycle for kiwifruit cv. Hayward; (Warrington and Weston, 1990)

Chilling requirement

Kiwifruit as many deciduous woody perennial trees, enter a rest or dormant phase at leaf fall with the onset of cooler, shorter days in autumn. A period of cold temperature is required to overcome this dormancy and to enable the plant resume normal growth when external conditions become favorable. The amount of winter

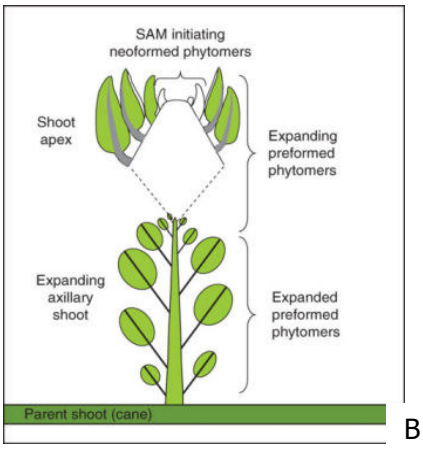
chilling required to break this dormancy varies with the species: kiwifruit plants that have just entered the dormancy phase require around 950-1000 hours of winter chilling (4°C) in order to resume vegetative growth in the minimum period of time (Brundell, 1976; Lionakis and Schwabe, 1984a). Data confirmed that cool winter temperatures dramatically increased flower numbers and increased the proportion of bud break (Mc Pherson et al., 1995) while warm temperatures during winter resulted in bud break being delayed and spread over a longer period (Mc Pherson et al., 1997).

Bud formation

In kiwifruit, growth and flowering occur in a 2-year cycle (Figure 2A), with the current season's shoots originating from axillary meristems (first-order axillary bud-FOABs) of the previous season's growth (Snowball, 1997b). Mature FOAB morphology has been described by Brundell (1975a). When observed while dormant in mid winter, the axils of the bud scales and leaf primordial of FOABs contain second order structures, namely, second order axillary buds (SOABs), and simple, dome shape meristem (SDSMm). By winter, at the end of the of the first growing season, the most basal SDSMm will have differentiated into second order buds (which do not usually develop further), while the others left remain as meristem. Early in the spring of the second growing season, those remaining meristems differentiate either into inflorescences or new axillary buds. Later in spring of the second growing season, these new buds will initiate third-order meristem, which are analogous to the SDSMm

Year	Season	Developmental stage
I	Spring	Budbreak (zero-order buds) and shoot extension
		Start of first-order bud development
		Initiation of second-order buds
		Initiation of second-order meristems
	Summer	Presumed start to floral commitment (evocation) in first-order buds
	Autumn	Leaf abscission
	Winter	Vine dormant
II	Spring	Budbreak and extension of first-order buds into shoots
		Differentiation of second-order meristems into inflorescences and flowers
		Flowering and fruit set
	Summer	Fruit development
	Autumn	Fruit harvest
		Leaf abscission
	Winter	Vine dormant

A



B

Figure 2. (A) Seasonal cycle of kiwifruit vine growth and first-order axillary shoot development (Walton et al., 2001); (B) Schematic illustrating shoot organization in Actinidia; (Forester et al., 2007)

initiated in the first growing season, thereby reiterating the developmental cycle (Walton et al., 2001). The rapid flush of bud growth which occur in spring it is correlated to the shoot apical meristem (SAM) ability described above, of initiating phytomers (repeating units of leaves, axillary meristem, node and internode) (preformation), which do not fully expand and mature until after a dormant period. (Puntieri et al., 2000; Sabatier et al., 1998). Furthermore, In *Actinidia*, as for other species, in some shoots additional phytomers are also initiated that expand without a dormant period (neoformation)(Halle et al., 1978; Barthelemy and Cariglio, 2007). In detail, in kiwifruit the active bud begins to swell and develops into an open cluster containing a few leaves (Brundell, 1975). In many shoots, growth cessation can occurs soon after this followed by the abortion of the shoot tip. Other shoots continue to grow and do not terminate until late in the season, resulting in a final number of phytomers that exceeds the number of preformed phytomers present during bud break (Figure 2B).

Bud break

Theoretically, in winter all dormant buds (Figure 3A) on kiwifruit vine have the potential to burst and produce flowers (Figures 3B-3C-3D). However by spring many buds lose or show reductions in this flowering potential (Grant and Ryugo, 1982). Bud break along the shoots does not result uniform, but displays distinct patterns, climatically dependent (Guédon et al., 2001). It typically rises from near zero at the cane base, to near 100% at the tip of pruned canes (McPherson et al., 1994). The frequency near the cane base is lower under warm winter conditions, and when growing shoots are trained upwards to favorite apical dominance (Snelgar and Manson, 1990). In addition, shoot orientation influenced bud break as well by inducing a bud burst rearrangement along the cane: ventral bud generally remained dormant, whereas high, but not uniform, bud-break levels were recorded for lateral and dorsal buds. This effect is probably linked to apical control and unrelated to phyllotaxis (Costa et al., 1991; Ferguson, 1990).

A B C D

Figure 3. Kiwifruit bud phenological stages dormant (A); bud swelling (B); bud burst (C); shoot growth (D).

Dormancy breaking agents

Bud break is high (>50%) when winters are cool, but can drop below 20% in warm-temperate regions, with flower numbers below economic level for crop production (McPherson et al., 1994). In Italy, bud break values normally reach 50%. This number can change according to the year and the area of production (Table 1). In the North, generally, the chilling requirements is easily satisfied while productivity is often limited in the South (Costa et al., 1995). Normally the number and type of buds left on the vine during winter pruning determine the desired potential productivity of the vines. In addition dormancy breaking agents (DBA) are applied to the vines to overcame the adverse effect of warm winter temperatures (Linsley-Noakes, 1989; Costa et al., 1995).

Dormancy breaking agents application induces different effects related to the area of production: in the South and Central areas DBA allow to increase the percentage of bud break and the flower fertility which are normally not sufficient to guarantee an high productivity (Inglese et al., 1996; Costa 2003). Moreover DBA application induce a bud break uniformity with positive effects on shoot development, and staminate/pistillate vine contemporary flowering. This two combine effects permit to increase the plant performances at harvest (Montefiori, 2003). In the North, where the chilling requirement is normally achieved, DBA applications do not increase the percentage of bud break but induce a simultaneous bud release and shoot growth. In these areas, another interesting effect, often induced by DBA, is the reduction of the later flowers of the kiwifruit inflorescence which permits to reduce time and money spent for this agronomical practice. The combination of an uniform bud break and the thinning effect caused an increase in yield and fruit size which can be related to the reduction of the competition among fruit for the resources during

their development (Montefiori et al., 2003). Among dormancy breaking agents Hydrogen Cyanamide (HC) has shown to be the most effective and it s normally applied in kiwifruit management (Schuck and Petri, 1995; Walton et al., 1998; Inglese et al., 1998). Despite of its efficacy this molecule can cause toxicity to the operator (Mazzini, 2008) and its use may not be sustainable in the long term (Walton et al., 2006). Recently, formulates containing Cytokinins are under investigation as dormancy breaking agents. In particularly, preliminary results showed that the application of the product Cytokins, containing natural Cytokinins, increased the bud break percentage and caused a reduction in the number of lateral flowers when applied in experimental trial carried out in the Emilia-Romagna region (Fabbroni et al., 2007).

Table 1. Values of bud break percentage registered in different part of Italy

Area	***Budbreak (%)***	***Font***
Emilia Romagna	56.9	Fabbroni et al., 2007
Friuli Venezia Giulia	52.7	Lamiani Mignani et al., 1983
Piemonte	55.7	Costa et al., 1998
Calabria	54.2	Inglese, 1992

DORMANCY

Plant dormancy in the perennial context

Plant dormancy has a major impact on the cultivation of plants, influencing such processes as seed germination, flowering and vegetative growth (Lang et al., 1987). Understanding the mechanisms controlling plant growth and dormancy is crucial to solving many problems in agriculture (Horwath et al., 2003). The diversity of plant tissue (buds, seeds, bulbs, etc.) that exhibit, or contribute to the manifestation of dormancy is great and there appear to be numerous mechanisms of dormancy induction or release (Lang et al., 1987). Perennial plants, such as trees, distinguish themselves from other plants in their ability to suspend and resume growth recurrently in response to environmental and often seasonal conditions (Rohde et al., 2007). Lang et al. (1987) distinguished three types of dormancy: eco dormancy, provoked by limitations in environmental factors; paradormancy, where the growth inhibition arises from other part of the plant; endo dormancy, where the inhibition resides in the dormant structure itself (Figure 4). This pragmatic classification is useful to describe the path to dormancy induction, maintenance and release but it is not exhaustive. In particular this definition is inadequate for unrevealing the molecular components that govern transitions into and out of dormancy, particularly at the cellular level. For this purpose a new definition of dormancy was established by Rohde et al., 2007 describing dormancy as the inability to initiate growth from meristems (and other organs and cells with the capacity to resume growth) under favorable conditions. A briefly description of all the stages identified by the two authors can help to a better understanding of the phenomenon.

Dormancy by Lang et al. (1987)

Lang define dormancy as "a temporary suspension of visible growth of any plant structure containing a meristem". As reported above he identified three type of dormancy:

1-Paradormancy: the dormant stage is originated in a structure other than the affected one. It is normally referred to apical dominance or correlative inhibition. Many studies have implicated basipetally transport of auxin as the primary signal regulating paradormancy. However, it should be realized that grafting studies have implicated other signals than auxin, produced in the roots and stem, as having a profound effect on shoot outgrowth (Beveridge et al., 2000).

2- Endodormancy : the dormant stage is the result of physiological internal changes to the bud that prevent untimely growth during seasonal transitions, when environmental conditions often fluctuate between those permissive or inhibitory to growth. Light and temperature both play a significant role in the induction and breaking of endo dormancy, with light playing the dominant role in most woody perennials. In deciduous trees, shortening day length induces a developmental change in terminal buds that results in a leaf primordium forming scales instead of leaf buds (Okuba, 2000). In trees such as birch (*Betula papyrifera*) and poplar (*Populus tremuloides*), additional changes take place that induce cold hardiness, cessation of cell division and induction of dormancy in the terminal meristems (Li, 2003). Extended chilling or freezing are often required to break the endo dormant state, but chemicals such as hydrogen cyanamide (HC) break endo dormancy (Henzell, 1991; Walton et al., 1998).

3-Ecodormancy: the dormant stage is imposed by external environmental factors such as cold or drought stress, which induce critical signals that prevent bud growth.

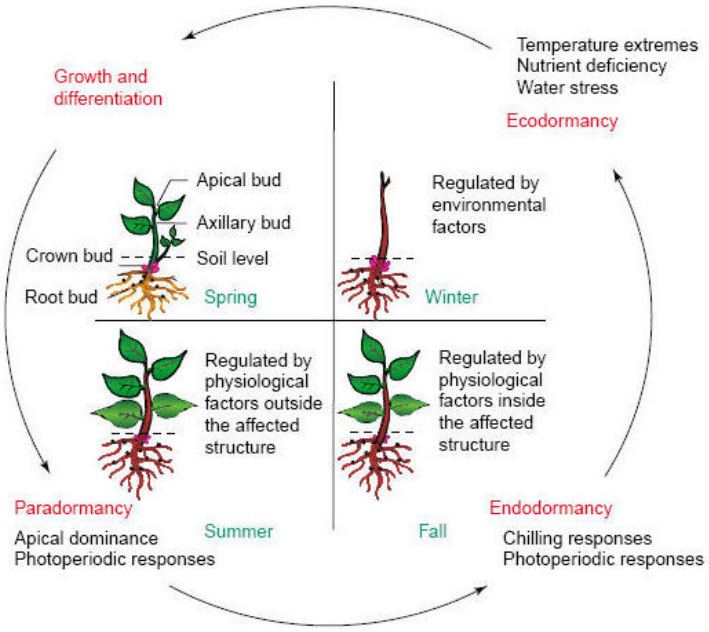


Figure 4. Diagram of signals and typical seasons corresponding to the different types of dormancy. Lang et al. categorized dormancy into paradormancy, endo dormancy and ecodormancy. This figure illustrates the cycle of three types of dormancy and the general signals and seasons that are associated with dormancy of perennial woody plants (shrubs and trees) and shoot buds of tubers (potato and yam); (Horwath et al., 2003).

These extended periods of environmentally unfavorable growing conditions generally

are required to signal the breaking of endo dormancy, while at the same time imposing eco dormancy.

Dormancy by Rhode et al. (2007)

Rodhe recently proposed a new definition of dormancy to better explain the perennial life style in plants. This definition refers to the ability to cease meristem activity and to establish a dormant state in which the meristem is rendered insensitive to growth-promoting signals for some time before it is released and can resume growth. *Populus sp*. is used as the model system. In Table 2 and Figure 5 are shown the stages identified by him.

Table 2. The growth–dormancy status and the corresponding meristem stages proposed by Rodhe et al. as model in the perennial tree

Stage	***Description***
I	cessation of cell division
II	establishment of dormancy
III	maintenance of dormancy
IV	release from dormancy state
V	resumption of cell division

The growth cessation is the first step towards establishing dormancy which is provoked by various factors such as photoperiod, cold or drought (*Populus sp)* (Sylven, 1940; Nitsch, 1957). It is followed by the dormancy establishment: once growth has ended, the dormant state becomes progressively established and results in the complete inability of the meristem cells to respond to growth-promoting signals. Currently, little is known about the changes that occur after growth cessation and before dormancy establishment, in part because this phase is masked by the concurrent bud formation. The bud is required for successful survival but not for dormancy. Release from endo dormancy requires exposure to chilling temperatures. Dormancy is released not by short-term but by long-term exposure to low temperatures, and chilling restores the ability to grow but does not promote growth. At last, an important issue in a perennial context is that once dormancy is released and growth resumes, resetting needs to occur. The state in which dormancy induction is possible together with the subsequent need for the chilling is re-established. If epigenetic mechanisms were involved in the induction of dormancy, epigenetic marks would have to be reset. In annual plants, resetting of an epigenetically fixed, vernalized state happens during meiosis and before seed

dormancy (Mylne, et al. 2006). Resetting in vegetative meristems has to occur through another, so far undescribed mechanism (Prince and Marks, 1982; Battey, 2000).

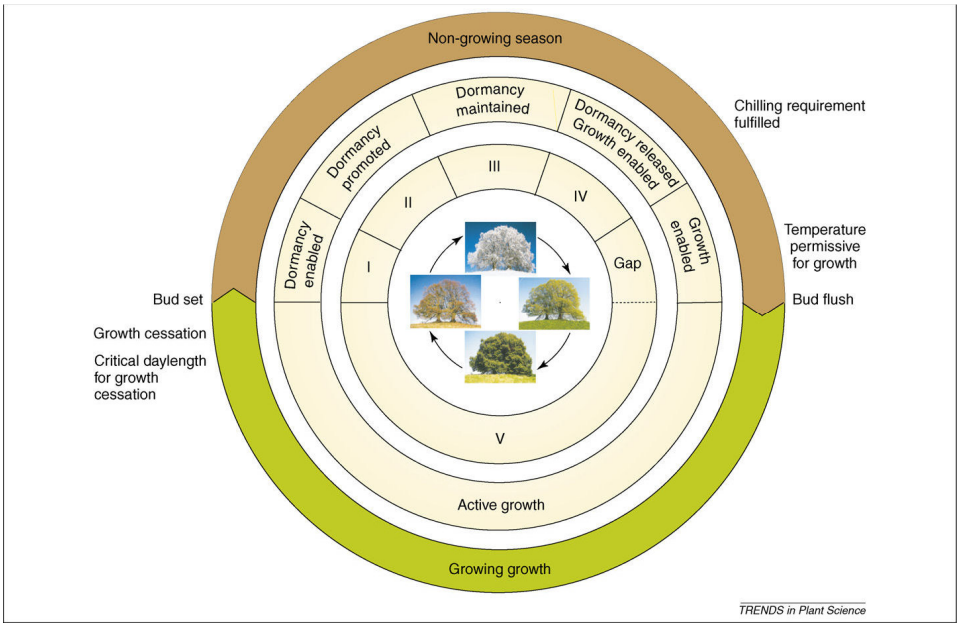


Figure 5. Transitions in seasonal growth–dormancy cycling in *Populus sp*. Poplars synchronize the onset of the dormant period mainly with changes in day length that are sensed by phytochromes. Bud flush and bud set delimit the growing season. Prolonged exposure to chilling temperatures will release plants from dormancy. Growth resumes once the temperature passes a critical threshold. Absence of growth before and after endo dormancy is caused by different environmental factors. The inner circles depict the growth–dormancy status and the corresponding meristem stages: I, cessation of cell division; II, establishment of dormancy; III, maintenance of dormancy; IV, release from dormancy; and V, resumption of cell division. 'Gap' between stage IV and V denotes the phase where growth does not occur because of purely environmental restraints (Rhode et al., 2007).

Bud release from dormancy

The ability to form buds and to undergo cycles of growth and dormancy has been central to the evolution of the perennial life strategy. After the evolution of branching, the subsequent acquisition of the bud structure enabled growth to be arrested in some, but not all, meristems. With this strategy, plants could adapt their branching to a greater variety of conditions, particularly seasonal environments. In this sense, bud dormancy evolved as a morphogenetic strategy and, secondarily, was adopted for adaptive purposes (Crabbe´, 1994). Nonetheless it is important to underline the adaptive purpose, plant growth and development typically occur in the context of a sessile existence. Consequently, plants have evolved elaborate mechanisms for surviving unfavorable growing conditions experienced in nature.

Production of vegetative buds provides plants with a safety net for re-growth or reproduction if environmental conditions results in the death of actively growing (Horwath et al., 2003). Often dormant bud regains the potential for further growth and development only after it has received adequate exposure to cold (Noode and Weber, 1978). This so-called `chilling requirement' is widespread, not only in the case of vegetative buds but also in floral buds (Salisbury and Ross, 1992). Several studies have shown that it is the bud itself, vegetative or floral, which must be exposed to low temperatures (Metzger, 1996) chilling of the plant body alone is insufficient. This is important as it shows that the bud cannot import the effect of chilling from the rest of the plant. It also implies that dormancy release is based on processes that are intrinsic to the bud itself (Metzger, 1996).

Factors affecting bud release from dormancy

It is clear nowadays that a wide variety of factors can break dormancy, in particularly environmental and hormonal influences have to be analyzed to understand the complex mechanism which start when the bud resume growth. All this data brings into question the existence of universal key components and pathways in dormancy cycling, as well as the validity of existing theories and concepts (Lang et al., 1987). As multiple effectors may lead to dormancy breaking, the process is unlikely to be reliant on a linear control pathway. Rather, it may depend on the collective behavior of interconnected metabolic pathways (Van der Schoot, 1996). A briefly description of the mains factor affecting bud release is reported below.

Environmental factors

Different factors affect bud release and dormancy in perennial species. Primarily studies discovered the importance of environmental factors including day length, temperature, water, and nutrient availability, which are known to play major roles in control of eco dormancy and establishment of endo dormancy in apical buds (Nooden and Weber 1978). Other experiments have found that sugars, nitrogen (Chao et al. 2000; Perry, 1971), water potential (Borchert, 1991), light quality and quantity (Nooden and Weber 1978), and developmental state (Nissen and Foley 1987) have direct effects on the levels and activity of hormones in apical, axillary, and adventitious buds. Recently, a detail study on vegetative peach-tree buds confirmed the importance of sugar: once environmentally condition allow growth, the

carbohydrate uptake capacity of the bud increases (Gevaudant et al., 2004) thus leading to the hypothesis that, at the time of dormancy release, bud meristem require a high import of sugars from the underlying tissue, which is essential to sustain bud growth.

Hormones

In the past excellent works led to the discovery of the importance of plant hormones such as auxin, cytokinin (CKs), Abscisic acid (ABA), and giberellic acid (GA) (Nooden and Weber 1978) in the regulation of bud release. In fact, all these molecules are implicated in the control of all three types of dormancy (Nooden and Weber 1978). In particular the role of the hormones auxin and cytokinin have been established in the control of dormancy of adventitious and axillary buds (Cline 1991; Nissen and Foley 1987; Nooden and Weber 1978). Auxin is almost certainly the signal produced by the expanding meristem that directly or (more likely) indirectly is responsible for preventing growth of more distal axillary and adventitious buds (Cline 1991) while elevated cytokinin levels have been implicated in breaking dormancy in adventitious and axillary buds (Stafstrom 1995). Both of these hormones have been shown to play an essential role in the control of growth and cell division in plants (Leyser et al. 1993; Soni et al. 1995). In addition, ethylene, another plant hormone, has been implicated in control of dormancy: addition of an ethylene response inhibitor increased the rate of precocious sprouting in potato microtubers (Suttle 1998).

Genetics of bud release

Although many significant mileposts have been reached, in the past 50 to 60 years, in our understanding on the induction and release of bud dormancy, only in the last 10 to 20 years researches included information about the molecular and genetic events occurring during bud release. A useful tools used by many author to better understanding the release from dormancy in many species is the construction of a cDNA microarray to discover differential genes expression profiling occurring during this process. In particular, comprehensive studies on raspberry, poplar, grape and other species were carried out (Mazzitelli, 2007; Rohde et al., 2007; Keilin et al., 1997; Horwath et al., 2008). The only work carried out on the molecular characterization of kiwifruit bud release from dormancy was reported by Walton et al. (2006) where microarray experiments were carried out to identify genes differentially

expressed between HC treated and untreated vines. The obtained results suggested the hypothesis, supported in other species of stress induction caused by the treatment which leads to a cascade of biochemical and molecular events inside the buds with consequent bud outgrowth (Mathianson et al., 2008). Comparison of these studies leaded to the identification of a wide range of genes whose expression profiling is activated or subjected to changes during the release from dormancy. In the table below (Table 3) the main classes of genes relevant for the bud outgrowth controlling mechanism are shown.

Table 3. Functional categories of genes expressed during bud release, identified from c-DNA microarray experiments in different plant species

	Functional Category
1	Stress/response/defense/detoxification
2	Sugar metabolism
3	Hormones-induced genes
4	Cell cycle and DNA processing
5	Energy generation
6	Transcriptional factor and signal transduction

In particular in all the researches carried out it appears evident the activation of stress-response/defense related genes as well as genes controlling sugar metabolism, cell cycle and affected by hormones. It is interesting to compare the gene list of differentially expressed genes because it is possible to determine several recurrent themes emerging as well as genes which are not described in other species (Mazzitelli et al., 2007). More studies are needed to a comprehensive understanding of the phenomenon.

CELL DIVISION AND CELL CYCLE

The concept of "meristem"

In contrast to animals, plant development is largely post-embryonic. New organs, such as roots, stems, leaves, and flowers, originate from life-long iterative cell divisions followed by cell growth and differentiation. Such cell divisions occur at specialized zones known as meristems (Inzè and Veylder, 2006). The Oxford English Dictionary defines the term "meristem" as: "Plant tissue consisting of cells which are actively dividing and giving rise to new cells; a region composed of such tissue, occurring especially at the tip of a root or shoot" (Online edition http://dictionary.oed.com). Especially, leaves and flowers are formed at the shoot and floral meristems, respectively, whereas the root meristems continuously add new cells to the growing root. The cells at the meristem are pluripotent so that their progeny can become committed to a spectrum of developmental fates. Initially, the shoot apical meristem (SAM) produces leaves, but under the right developmental or environmental conditions, the SAM will be converted into a floral meristem that produces flowers (Inzè and Veylder, 2006).

Structure

The SAM is a highly ordered structure (Evans and Barton, 1997) divided into different zones defined by genetic, cellular and functional parameters (Scofield and Murray, 2006). First, the angiosperm SAM has been divided into three different morphological and functional regions: the central zone (CZ) at the apex or centre of the meristem, the peripheral zone (PZ) surrounding the CZ, and the rib zone (RZ) beneath the CZ (Figure 6A). It is generally accepted that the CZ acts as a population of stem cells (Lenhard and Laux, 1999) generating the initials for the two other zones whilst maintaining itself. Cells proliferate at different rates in the different zones: a constant feature appears to be that cells divide more slowly in the CZ than in the PZ, on in the RZ (Lyndon, 1998). Superimposed on the partitioning into zones characterized by different proliferation rates of the individual cells, the SAM can be divided into different layers (Figure 6B). The typical angiosperm SAM is divided into two cell layers, both of which contribute to organ formation: one outer layer, called tunica, and one inner layer or corpus. In dicots, the tunica, in its turn, is composed of different sheets, usually two, called L1 and L2. The outermost L1 is only one cell

thick. The tunica cells remain clonally separated from the corpus because usually cells in the tunica divide anticlinally. In dicots, cells in the inner tunica layer, the L2 cells, divide in both anticlinal and periclinal orientations at the site of organ initiation. Cells from the corpus, or layer L3 (in dicots), divide in apparently random orientations. Laufs et al. (1998a) analysis on the cellular parameters of the SAM in *Arabidopsis* observed that the meristematic layers, i.e. dome shape meristem, could be further divided into subdomains that differ in terms of mitotic activity (inner zones of L1 and L2 show lower mitotic activity). This observation was confirmed by Grandjean et al. (2004) who found significant heterogeneity in cell cycle duration within living meristems where neighboring cells could have distinctly different cell cycle rates (i.e. one cell can divide twice within a given time whereas another does not divide at all). This asynchrony is reflected at the level of cell cycle gene transcription, as both S-phase and M-phase associated transcripts accumulate stochastically in scattered cells throughout the meristem (Fobert et al., 1994). Although many of the key regulatory meristem genes have been defined genetically, how they impact on the core cell cycle machinery to coordinate cell division remains unclear (Gegas and Doonan, 2006).

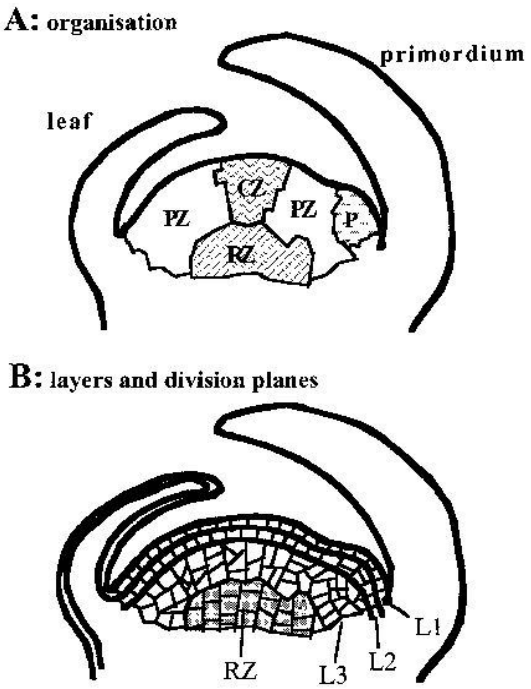


Figure 6. Zonation within the shoot apical meristem of a typical dicot plant. A. The SAM can be divided into three concentric zones: a central zone (CZ) at the apex of the meristem, the surrounding peripheral zone (PZ) where organ primordia (P) are initiated. The rib zone (RZ) gives rise to the central tissues of the shoot axis. B. The shoot meristem is also composed of clonally distinct horizontal layers. In dicots L1, L2, L3 layers can be distinguished. The L1 and L2 layers form the so-called tunica, the L3 layer the corpus. (Vernoux et al., 2000).

Cell cycle

The mitotic cell cycle encompasses four sequential ordered phases that temporally distinguish the replication of genetic material from the segregation of duplicated chromosomes into two daughter cells (Dewitte and Murray, 2003). It

comprises mitosis (M), cytokinesis (CK), post-mitotic-interphase (G1), DNA synthesis phase (S) and post-synthesis-interphase (G2) (Figure 7) (Inzè and Veylder, 2006). Lag or gap (G) phases separate the replication of the DNA (S phase) and the segregation of the chromosomes (M phase, mitosis). The G1 phase (the first gap) intercedes between the previous mitosis and the entry into the next S phase, whereas the G2 phase separates the S phase from the subsequent M phase. Cells in G2 are therefore discriminated from G1 cells by possessing a double DNA content. The gap phases allow the operation of controls that ensure that the previous phase has been accurately and fully completed, and not surprisingly the major regulatory points in the cell cycle operate at the G1/S and G2/M boundaries, which correspond to points of potential arrest as a consequence of evaluation of external conditions (Van't Holf, 1985). Passage of eukariotic cells through the cell division cycle is controlled by the cyclin-dependent serine-threonine protein kinase (CDKs). All eukaryotic organisms studied to date possess at least one CDK, in particularly, in plants four classes of CDKs have been classified: *CDKA* and *CDKB* which plays an important role during cell cycle and *CDKC* and *CDKE*, with still no clear role. Wide researches established that *CDKA*, plays a pivotal role at both the G1-to- S and G2-to-M transition points (Iwakawa et al. 2006), in particular it is seemingly the only CDK active at the G1 and S phases in plant cells, whereas the entry into mitosis is probably controlled by multiple CDKs. As regard to *CDKB*, this specific class of CDKs seemed to have been described only in plant (Hirayama et al., 1991; Joubes J et al., 2000; Boudolf et al.,2001). Among *CDKB* two subgroups were identified in *Arabidopsis CDKB1* and *CDKB2* (Vandepoele et al., 2002). The two *CDKB* subgroups are found in both monocotyledonous and dicotyledonous species, suggesting a conserved unique role for each of the *CDKB* subgroups in cell cycle regulation, but have a slightly different timing in cell cycle phase–dependent transcription. *CDKB1* transcripts accumulate during S, G2, and M phases, whereas *CDKB2* expression is specific to the G2 and M phases (Fobert et al., 1996; Segers et al., 1996; Meszaros er al., 2000). To be active, CDKs require regulatory proteins, the Cyclins. Cyclins are a diverse group of proteins with low overall homology that share a large, rather poorly conserved region responsible for their interaction with the CDK; this region is referred to as the cyclin core. The cyclin core stretches about 250 amino acid residues and is organized in two folds of five helices. The first fold is the cyclin box and comprises »100 amino acid residues (Joubes et al., 2003), representing the region of highest conservation, although it contains only five absolutely invariant

positions. The crystal structure reveals the cyclin box as the face of interaction with the cognate CDK (Fobert et al., 1996). Cyclins fall into many classes that share homology and, at least to some degree, conserved function between animals and plants. There are 13 classes of Cyclins in animals (A-L and T) (Pines, 1995) but only seven in plants i.e. A, B, C, D, H, P and T within which approximately 60 plants genes belong. The main classes involved in the cell cycle are A, B and D.

A-types generally appear at the beginning of S phase. These kind of cyclins are involved in S-phase progression, and are destroyed around the G2/M transition;

B-types appear during G2. They are known to control the G2/M and mitotic transitions, and are destroyed as cells enter anaphase;

D-types control progression through G1 and into S phase and differ from A and B types by generally not displaying a cyclical expression or abundance.

Cell cycle control

A universal model for the control of progression through the mitotic cycle has been proposed in *Arabidopsis* (Figure 7) and it involves the formation, activation and subsequent deactivation of the CKDs-cyclin complex (Nurse, 1990) formed by the proteins described above. In particular it has been proposed that CDKs bind sequentially to a series of cyclins that are responsible for differential activation of the kinase during cell cycle progression. CDKs activity is further modulated by activators (CAK or recently CDKD) and by inhibitors (CDKI). In particular the phosphorylation of Thr160 (or the equivalent residue) of CDKs is performed by CDK-activating kinases (CAKs) whose function is to induce a conformational change allowing proper recognition of substrates. *Arabidopsis* contains four CAK-encoding genes, divided into two functional classes (CDKD and CDKF) (Vandepoele et al., 2002; Umeda et l., 2005). The complex (CDK-cyclin) activity is also

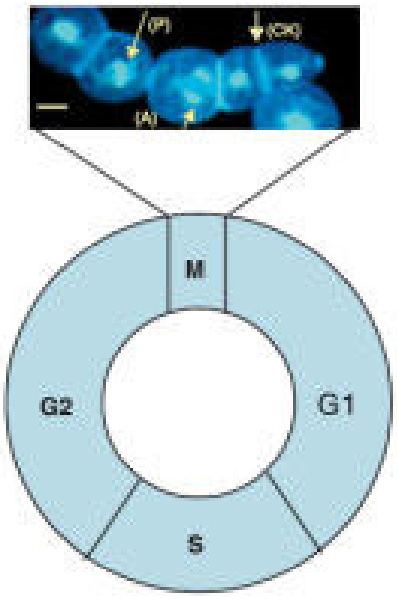


Figure 7. The plant cell cycle. Top panel:BY-2 cells transformed with fission yeast cdc25 and stained with Hoechst: prophase (P), anaphase (A), cytokinesis(CK).Bar, 100um. Bottom panel: mitosis (M), postmitotic interphase (G1), DNA synthetic phase (S) and premitotic interphase (G2); (Francis, 2006)

regulated by CDK inhibitors (CKIs) characterized by a conserved domain involved in binding CDKs and cyclins and is essential for the inhibitory activity of the proteins (Wang et al., 1997; Lui et al., 2000; De Veylder et al., 2001; Jasinski et al., 2002;Coelho et al., 2005). Despite remarkable differences between the plant and animal cell cycle, both types of organisms use the pRB/E2F (retinoblastoma protein/elongation factor 2F) pathway to switch from protein phosphorilation to DNA transcription activity (Hwang et al., 2002).

In addition to the endogenous mitogenic signals which drive the cell cycle there are others which modulate the mitotic activity in response to environmental situation, e. g., the availability of water and mineral nutrients. Environmental signals are mostly

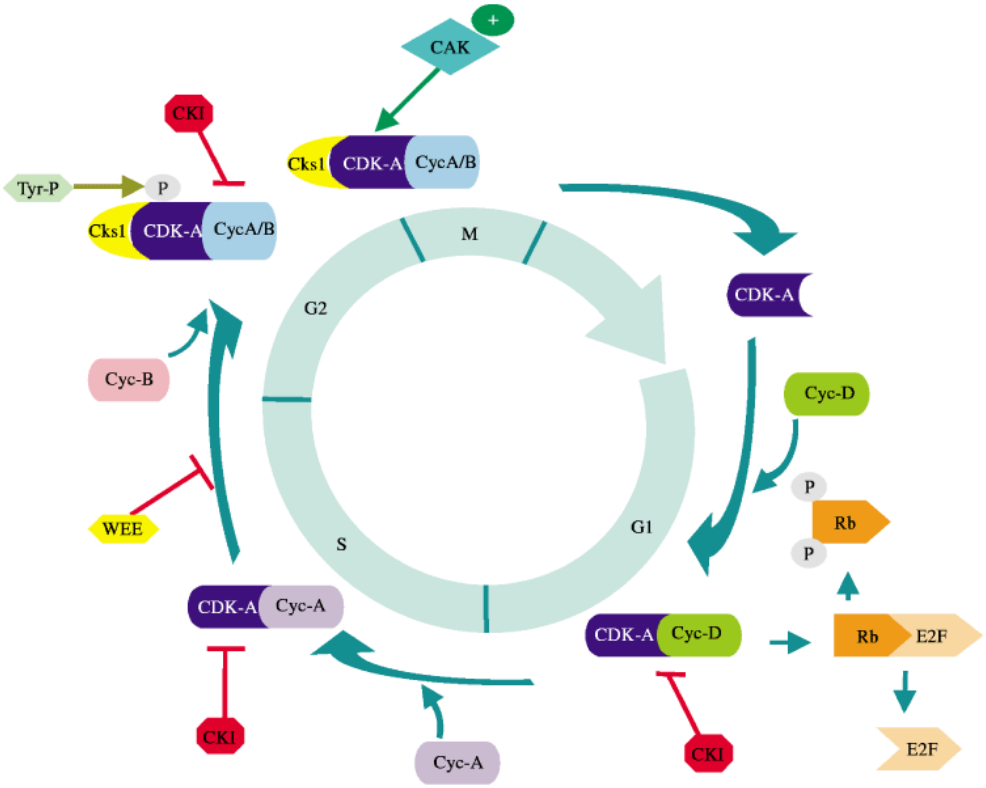


Figure 8. Schematic representation of cell cycle control in plants. Progression through the mitotic cycle involves the successive formation, activation and subsequent inactivation of cyclin-dependent kinases(CDKs). The kinases bind sequentially to a series of Cyclins, which are responsible for differential activation of the kinase during the cell cycle. The G1 to S transition is thought to be controlled by CDKs containing D-type cyclins that phosphorilate the retinoblastoma protein, releasing E2F transcription factors. E2F are involved in the transcription of genes needed for the G1 to S transition The G2 to M transition is carried by CDK complexes containing CycA and CycB cyclins. CDK complexes are kept inactivate state by phosphorylation by the WEE1 kinase, and by interaction with inhibiting proteins (CKIs). At the G2 to M boundary activation of the kinase is brought about by release of the CKI protein, by positive phosphorylation (by CAK kinase), and by *CDKD-dependent stimulatory phosphorylation;* (Hwang et al., 2002).

transferred by phytohormones such as ABA, cytokinins or jasmonate (Del Pozo et al., 2005). In particular ABA- auxin-, GA-, and ethylene responsive elements have been demonstrated in the promoters of Cyclins and CDKs (Richard et al., 2002) and direct interaction with Cytokinins with CDKs has been suggested (Redid et al., 1996). As regard to the molecular aspect, extensive researches established that the expression of D-type cyclin genes is modulated by plant growth factors, such as cytokinins, auxins, brassinosteroids, sucrose, and gibberellins (Soni et al., 1995; Riou-Khamlichi et al.,. 2000; Hu et al., 2000; Richard et al., 2002).

Role of Histones

Histones are well-conserved nuclear proteins that participate in the organization of chromatin. They can be classified into five subtypes: the four core histones H2A, H2B, H3, and H4, constituting nucleosomes with chromosomal DNA, and the linker histone H1, associated with DNA located on and near the nucleosomes resulting in a higher-order chromatin structure (Crane-Robinson, 1997).

The synthesis of these histone proteins is highly correlated to DNA replication. In fact in the S phase of the cell cycle, not only the chromosomal DNA but also chromatin structure is replicated, along with a two-fold increase in the total amount of histones. Each of the histone subtypes is encoded by a multigene family and, in general, the major species are expressed at high levels during the S phase. Analyses on suspension-cultured cell cycle (e.g., tobacco BY2cells) demonstrated that plant histone genes also expressed themselves in a DNA replication-dependent manner (Meshi et al., 2000). This makes their gene expression a useful marker for visualizing S-phase cells (Nemoto and Sugiyama, 2005).

CYTOKININS

Structure

Cytokinins (CKs) are N6-substituted adenine derivatives that play a role in almost all aspects of plant growth and development (Mok and Mok 1994, 2001). Conventionally Cytokinins are divided in isoprenoid CKs and aromatic CKs related to the nature of the group carried at the *N6* terminus (Strnad M. 1997; Mok and Mok, 2001), respectively isoprene-derived or an aromatic side (Figure 9). Both isoprenoid and aromatic CKs are naturally occurring, with the former more frequently found in plants and in greater abundance than the latter. Common natural isoprenoid CKs are *N*6-(_2-isopentenyl)- adenine (iP), tZ, *cis*-zeatin (cZ), and dihydrozeatin (DZ). Among aromatic CKs, BA, and topolins were identified in several plant species including poplar (Strnad M. 1997) and *Arabidopsis* (Tarkowska et al., 2003) but it is not yet clear whether they are ubiquitous in plants. Several synthetic derivatives possess CK activity (Skoog and Armstrong , 1970; Shudo, 1994; Iwamura, 1994) but have not been found in nature so far. Usually, all natural CK nucleobases have the corresponding nucleosides, nucleotides, and glycosides. The free bases and their ribosides are thought to be the biologically active compounds while glycosidic conjugates play a role in CK transport, protection from degradation, and reversible and irreversible inactivation (Letham 1994).

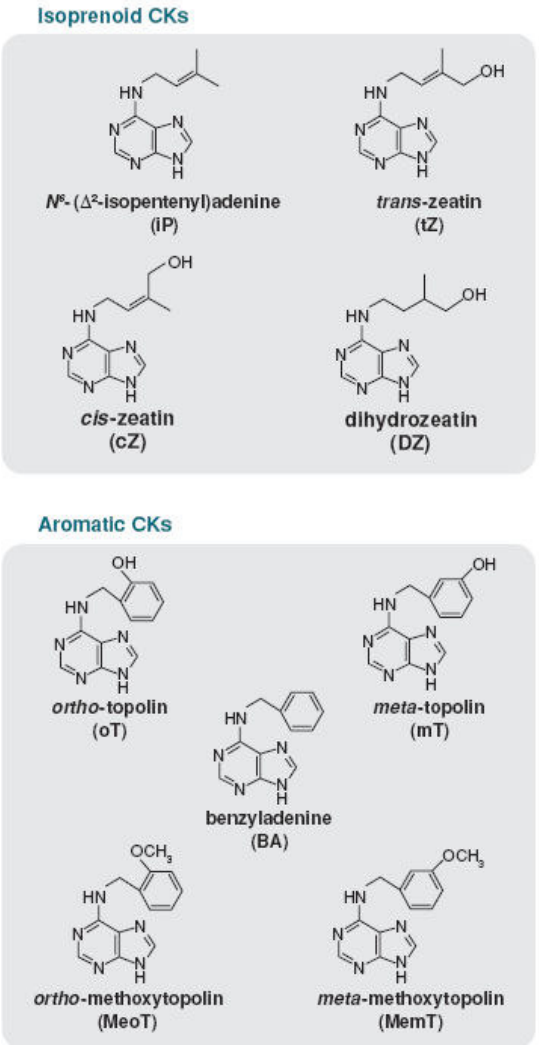


Figure 9. Structure of representative active Cytokinin (CK) species occurring naturally; (Sakakibara, 2006)

Biosynthesis and Metabolism

Despite the wealth of information concerning Cks chemistry and physiology, the transition from descriptive studies to molecular biology has been relatively slow compared with others hormones (Mok and Mok., 2001). Only recently, their biosynthesis and signal transduction has become clear. The progress was achieved by identifying a series of key enzymes and proteins controlling critical steps of these processes. In *Arabidopsis* the current model of isoprenoid cytokinin (CKs) biosynthesis pathways predominantly originate from the methylerythritol phosphate (MEP) pathway, whereas a large fraction of the *cis*-zeatin (cZ) side chain is derived from the mevalonate (MVA) pathway. The initial step of cytokinin biosynthesis is catalyzed by adenosine phosphate-isopentenyltransferase (IPT). In higher plants, the major initial product is an iP nucleotide, such as iP riboside 5-triphosphate (iPRTP) or iP riboside 5-diphosphate (iPRDP) because the IPT predominantly uses dimethylallyl diphosphate (DMAPP) and ATP or ADP (Kakimoto, 2001; Sakakibara et al., 2005). In Arabidopsis 9 different Isopentenyltransferase genes(AtIPT1-9) have been identified and their expression have been studied revealing a specific expression pattern of each gene (Miyawaki et al., 2004). Afterwards, iP nucleotides are converted into tZ nucleotides by cytochrome P450 mono-oxygenases CYP735A1 and CYP735A2 (Takei et al., 2004b). To become biologically active, iP- and tZ-nucleotides are converted to nucleobase forms by dephosphorylation and deribosylation, but genes encoding the nucleotidase (Chen and Kristopeit, 1981a) and nucleosidase (Chen and Kristopeit, 1981b) have not yet been identified. Recently, a novel pathway was identified that directly releases active cytokinin from the nucleotide, catalyzed by the cytokinin nucleoside 5-monophosphate phosphoribohydrolase called LOG (Kurakawa et al., 2007). Thus, it is likely that there are at least two cytokinin activation pathways in plants, although any functional differentiation between the pathways remains to be clarified. So far, no sufficient information are available to explain the biosynthesis and degradation pathways of the aromatic CKs (Sakakibara, 2006). However the mechanisms of glycosylation of this type their interaction with the cellular signaling system appear to be shared with isoprenoid CKs because the enzymes and receptors involved recognize members of both groups (Mok, 1994; Yamada et al, 2001; Mok et al., 2005).

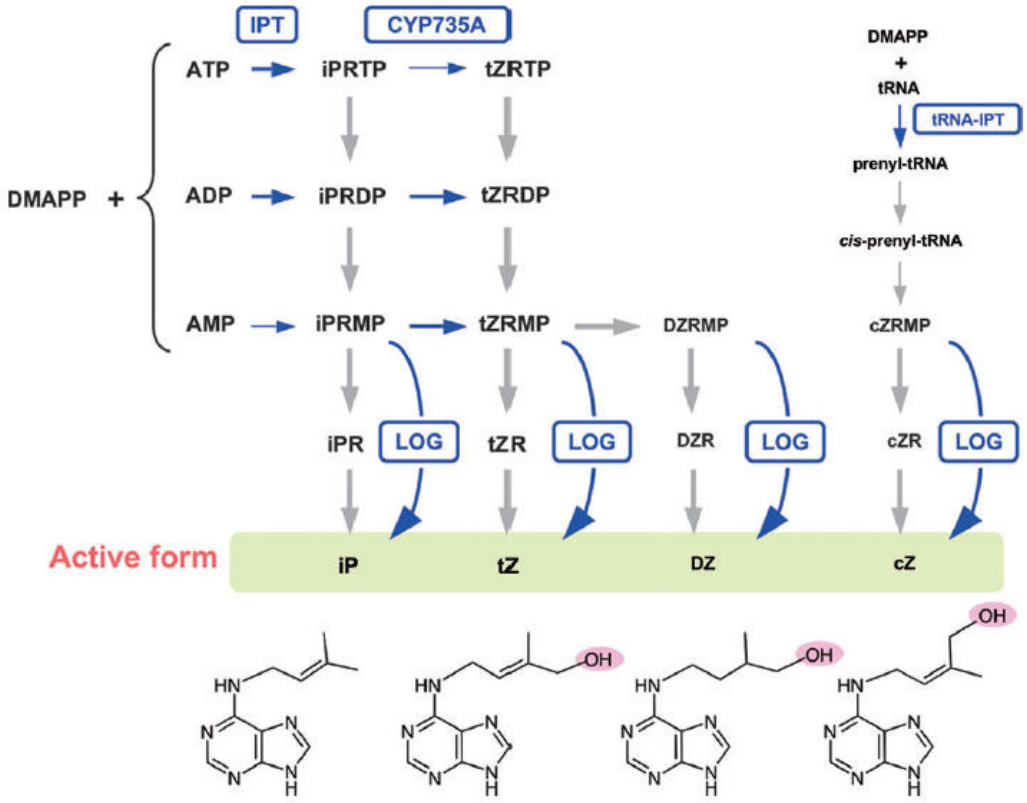


Figure 10. A current model of cytokinin biosynthesis and the two known activation pathways. iPRMP, iP riboside 5-monophosphate; tZRTP, tZnriboside 5-triphosphate; tZRDP, tZ riboside 5-diphosphate; tZRMP, tZ riboside 5-monophosphate; DZRMP, DZ riboside 5-monophosphate;ncZRMP, cZ riboside 5-monophosphate; DZR, DZ riboside; cZR, cZ riboside. Blue arrows indicate reactionswith known genes encoding the enzyme, and grey arrows indicate that the genes have not been identified. In this scheme, only biosynthesis and and activation steps are drawn (Hirose et al., 2007).

Signal Transduction

In Arabidopsis, the model recently proposed for the CKs perception and signalling is similar to bacterial two-component phosphorelays (Figure 9);(reviewed in: Kieber, 2001; Hutchison and Kieber, 2002; Heyl and Schmulling, 2003; Kakimoto, 2003). The cytokinin receptors are encoded by members of a gene family in Arabidopsis that is similar to bacterial two-component histidine kinase sensors (AHK2, AHK3 and CRE1) (Suzuki et al. 2001, Ueguchi et al. 2001, Yamada et al. 2001, Hwang et al. 2002). Activation of the cytokinin receptors by cytokinin binding is postulated to result in the phosphorylation of the AHP proteins, which move from the cytoplasm into the nucleus (Hwang and Sheen, 2001), where they transfer a

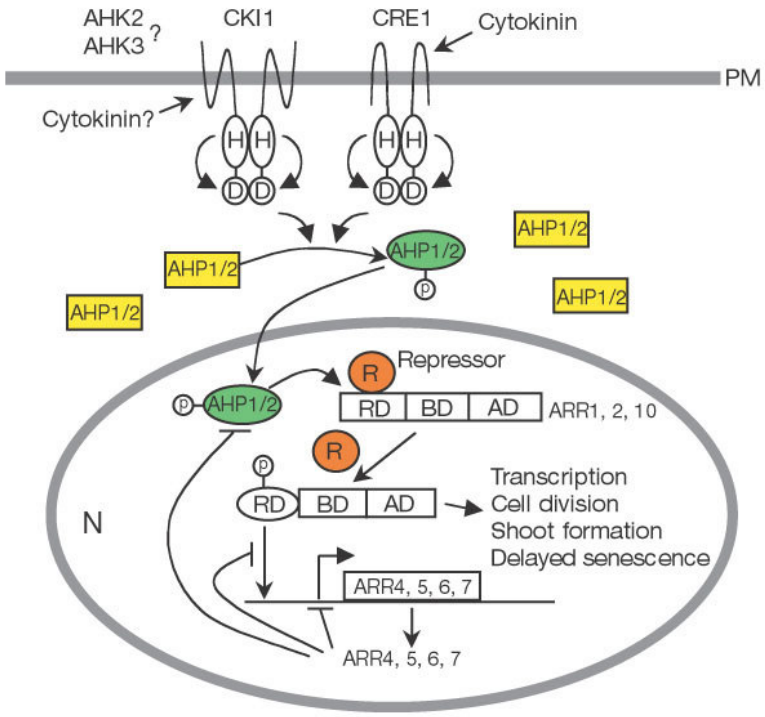


Figure 11. Model of the cytokinin signal transduction pathway in Arabidopsis. Cytokinin signal is perceived externally or internally by multiple histidine protein kinases at the plasma membrane. On perception of the cytokinin signal, histidine protein kinases initiate a signalling cascade through the phosphorelay that results in the nuclear translocation of AHP proteins from the cytosol. Activated AHP proteins interact with sequestered ARR proteins or ARR complexes, and release the activation type of ARR proteins from putative repressors in the nucleus. The liberated ARR proteins bind to multiple cis elements in the promoter of target genes. Activation of the repressor-type of ARR genes as cytokinin primary response genes provides a negative feedback mechanism. RD, response domain; BD, DNA-binding domain; AD, transcription activation domain;PM, plasma membrane; N, nucleus; R, putative repressor; H, histidine; D, aspartate (Hwang and Sheen, 2001).

phosphate moiety to Arabidopsis response regulators (ARRs). There are two classes of ARRs (type A and type B) that differ in sequence similarity, domain structure and their transcriptional response to cytokinin. The type-A ARRs are comprised solely of a receiver domain and their transcription is rapidly up-regulated by cytokinin (D'Agostino et al. 2000, Rashotte et al. 2003). In contrast, the type-B ARRs have, in addition to a receiver domain, a large C-terminal output domain that functions as a DNA binding and transcriptional activation domain and their transcription is not elevated in response to cytokinin (Sakai et al. 2000, 2001, Hwang et al. 2002).

Biological role

Cytokinins were originally described as substances able to promote cell division. Since the establishment of this definition it became evident that CKs play a critical role in plant growth and development. In fact they are active throughout the plant life cycle and in addition to the control of cell division, they take part to regulate other processes, i.e. leaf senescence (Gan and Amasino, 1995; Kim et al., 2006), apical dominance (Sachs and Thimann, 1967; Tanaka et al., 2006), root proliferation (Werner et al., 2001), phyllotaxis (Giulini et al., 2004), reproductive competence (Ashikari et al., 2005), and nutritional signalling (Takei et al., 2001b, 2002). .It is also known that CKs participate in the maintenance of meristem function (Werner et al., 2003; Leibfried et al., 2005; Kurakawa et al., 2007) as well as are indispensable for the progression of the plant cell cycle (Miller et al, 1955).

CROSSTALK AMONG BUD RELEASE, CELL DIVISION AND CYTOKININS

Bud release and cell division

Dormancy is an adaptive trait that has a profound effect on cell proliferation. For instance the meristems of many plant become seasonally dormant, promoting survival through adverse conditions such as drought, shade or cold. Tepfer et al., (1981) reported that cells in the dormant meristem of the Jerusalem artichoke tuber, are arrested in G1 phase and that this is the case for many other species. Following the breaking of dormancy, vegetative bud growth is often accompanied by increased cell division. Changes in cell-cycle-specific gene expression occur during release of axillary buds of pea (Pisum sativum) (Devitt and Stafstrom, 1995) potato (Campbell et al., 1996), adventitious buds of leafy spurge (Euphorbia esula) (Horvath et al.,2002) and Jerusalem artichoke (Freeman et al., 2003) from dormancy. Dormancy breaking results in an up-regulation of genes that act at G1-S phase transition such as Histones (Horvath et al., 2002). In addition, several post translational modifications to key enzymes involved in cell cycle regulation occur shortly after dormancy break in several plant systems (Campbell et al., 1996; Horvath and Anderson, 2000).

Cell division and Cytokinins

It is known that CKs regulate cell cycle progression and are involved in the regulation of both G1/S and G2/ M transitions. The main support for the involvement of CK in G1/S regulation is the observation that CKs increase the expression of CYCD3, one of the key regulators of the G1/S transition of the cell cycle (Soni et al., 1995).Furthermore, constitutive expression of AtCYCD3;1 produces CK-independent growth proliferation of Arabidopsis calli (Riou-Khamlichi et al., 1999). Consistent with its role in cell division control, CYCD3 is expressed in proliferating tissues, such as the shoot meristem, young leaf primordia, axillary buds, the procambium and vascular tissues of maturing leaves. The application of exogenous CKs increases CYCD3 transcript levels without changing the expression pattern of cycD3, indicating a tissue-specific response to CKs (Riou-Khamlichi et al. 1999). CKs are also important in the regulation of the G2/M transition. In tobacco synchronizable cultured BY2 cells, which are CKs-independent, endogenous Zeatin type CKs peak around the S and M phases (Redig et al. 1996). This suggests a likely implication not only in G1/S, but also in the G2/M transition. Indeed, the application of lovastatin, an inhibitor of

mevalonic acid synthesis that inhibits CKs biosynthesis, blocks mitosis. Z addition bypasses the block of mitosis by lovastatin, supporting the observation that sufficient Z levels are rate limiting or the G2/M transition, at least in tobacco BY2 cells (Laureys et al., 1998).

One of the main cell cycle points controlled by CK is probably the stimulation of tyrosine dephosphorylation and subsequent activation of CDK (Zhang et al., 1996). A strict requirement for CK in late G2 is also observed in Nicotiana plumbaginifolia suspension cultured cells which, in the absence of kinetin, arrest in G2 phase with an inactive CDKA kinase (Zhang et al., 1996). This inactive enzyme contains a large amount of inhibitory tyrosine phosphorylation. Recombinant purified yeast CDC25 phosphatase, specific for the removal of phosphate from tyrosine at the active site of the CDK enzyme, causes extensive in vitro activation of p13suc1- purified enzyme from pith and suspension cells cultured without CKs. CK stimulates the removal of phosphate, activation of the enzyme and rapid synchronous entry into mitosis.

Cytokininis and bud release

CKs are known to overcome acrotony and apical dominance by stimulating the growth of lateral and axillary buds, respectively (Helgeson 1968; Leopold and Kriedemann 1975). Flower bud formation in apple trees is related to a gibberellin:cytokinin balance (Luckwill 1970), cytokinins are also essential for flower bud development in grapevines (Lavee 1989) and, the CKs concentration in phloem is critical to the induction of flowering of the long-day-plant *Chenopodium murale* (Mahácková 1988). Cytokinins have also been implicated in bud regeneration (Leopold and Kriedemann 1975) and in root primordia initiation in cuttings (Sudeinaya, 1986). All these processes are associated with a specific and well-regulated cytokinin level. According to the model proposed by Kaminek and his team (Kaminek, 1988) cytokinins exert auto inductive effects on their own biosynthesis and accumulation (positive feedback) and degradation (substrate induction of cytokinin oxidase). Moreover, CKs play a distinctive role in apple bud burst (Faust et al. 1997). Studies evidenced that buds respond to dormancy breaking agents such as cytokinins (Faust et al. 1997) when endodormancy weakens. One of the first experiments with young apple trees (Plich et al. 1975) in which bud outgrowth was promoted by BA application, suggested that cytokinins were in short supply and that buds compete for them.

2.Aim of the research

Agronomical aspect

The research performed during these three years of Doctoral Thesis had the aim to investigate the kiwifruit bud break and the role of exogenous cytokinins on this phenomenon.

The most relevant aspect in this research was the agronomical one: the first goal was, in fact, to confirm the practical advantage to use cytokinins as a tool to avoid insufficient winter chilling and/or to obtain positive effects on bud release, that could increase uniformity on the vegetative and reproductive growth and, as a consequence, higher fruit quality and orchard productivity at harvest.

For this reason field trials were set up in two different geographical areas of the Emilia-Romagna region and a new formulate containing cytokinins, commercially available as Cytokin (Intrachem S.r.l., Bergamo , Italy), was applied at different application times before bud release on Hayward orchards. Assessments were performed throughout the kiwifruit growing season to record time and percentage of bud-break, flowering time, percentage of open flowers, and flower fertility. At harvest, the main productive parameters and quality traits were also measured. The obtained data were analyzed in relation to climate conditions and seasonal variations and to phenological stage of bud development at the time of cytokinin applications. Additional trial with pure cytokinins where performed to further investigation carried out on at physiological and molecular levels.

Physiological and molecular aspects

Besides the agronomical aspect, the physiological one was also approached in this study with the aim to better explain data obtained from the field trials. For this reason further investigations from a molecular and histological point of view were performed during the development of kiwifruit bud from dormancy up to bud break. Two main aspects were studied.

Firstly, the identification of genes in kiwifruit which are supposed to change their expression during bud release in other crop species and secondly, the identification of histological differences among buds at different developmental stages. These analyses were performed to obtain a new and more detailed picture about some of the most important process naturally occurring during kiwifruit bud development and to compare this picture with the one obtained from bud treated with cytokinins. Data obtained with this comparison were analyzed with the aim to identify the physiological level on which these hormonal substances exert their role during bud release.

3.Materials and Methods

Field trials treatments

Two kiwifruit cv. Hayward orchards, were chosen to perform a three year studies on the application of dormancy breaking agent on this crop. Both orchards are located in the Emilia Romagna region, the first one in a private farm in the Faenza district, (Sarna), and the second at the experimental farm of the Bologna University. Kiwifruit vines located in the Faenza area were planted in 1995 at a density of 740 plant/Ha and trained on T-bar system while in the Bologna area the orchard was planted in 2002 at 1100 plant/Ha with the same training system. Two commercial formulates were tested. Dormex (Alzchem, Nord) containing the Hydrogen Cyanamide as active molecule and Cytokin (Intrachem Production s.r.l., Bergamo, Italy) containing a font of three different cytokinins derived from natural sources. From 2006 to 2008, Cytokin and Dormex were applied at different concentrations and application times

using a randomize block scheme (4 blocks of 5 trees each) and a detailed scheme of the treatments is reported in Table .

Furthermore, a two years study with two kind of pure CKs, 6-benziladenine (6-BA) and kinetin (K) (Sigma Aldrich), has been carried out on the same orchard, located at the experimental farm of the Bologna University, with the aim to verify their effect on kiwifruit bud-break. The complete scheme of the treatments is reported in Table.

Assessment during the growing season

Assessments were performed throughout the kiwifruit growing season to record:

- ✓ Moment and percentage of bud-break (expressed as number of new growing shoot per total number of buds measured in selected vines);
- ✓ Time of flowering;
- ✓ Percentage of open flowers (expresses as number of open flowers per total number of flowers measured in selected vines);
- ✓ Flower fertility (expressed as number of inflorescence having terminal flower (single) or terminal and primary flowers (double) or terminal, primary and secondary flowers (triple);

Assessment at harvest and after cold storage

Assessments were performed at harvest to record the main productive parameters, i.e.:

- ✓ Yield (expressed as kilograms per plant);
- ✓ Average fruit number (expressed as number of fruit per plant);
- ✓ Average fruit weight;
- ✓ Fruit class distribution (expressed ad number of fruit placed iont the commercial size classes);

Additional assessments were performed at harvest and after four months of cold storage in normal atmosphere (-0°C; 2% O_2; 4% CO_2 C_2H_4 < 0.02ppm) to record the main quality traits, i.e. :

- ✓ Flesh firmness (expressed as kilograms per cm^2; measured with a penetrometer 8mm tip (Effegi, Italy);
- ✓ Soluble solid content (expressed as grade Brix; measured removing each end of the fruit and squeezing one drop of juice from each end onto an ATAGO digital refractometer;

✓ Dry matter (expressed as percentage; calculated as difference of weight in kiwifruit slides dried for 72 hours at 65°C in a oven)

Table 4. Scheme of the commercialized DBA applications performed from the 2006 to the 2008 kiwifruit growing seasons in two producing areas of the Emilia-Romagna region

Product	*Application time*	*Concentration (L/ ha)*	*Place*	*Year*
Control	-	-	Bologna, Faenza	2006-2007-2008
Cytokin	Ecodormancy (beginning of March)	0.5	Bologna	2006
Cytokin	Bud-swell (mid of March)	0.5	Bologna, Faenza	2006-2007-2008
Cytokin	Bud-swell (mid of March)	0.5 + 0.5 (after 1 week)	Faenza	2006
Cytokin	Endodormancy (end of January)	0.5	Faenza	2007
Cytokin	Endodormancy (end of January)	1	Faenza	2006-2007-2008
Dormex	Endodormancy (end of January)	18	Faenza	2006-2007-2008

Table 5. Scheme of the pure cytokinins applications performed at kiwifruit bud swelling in the experimental farm of the Bologna University.

Trial	*Product*	*Concentration (ppm)*	*Year*
Control	-	-	2007-2008
6-BA 100	6-Benzilaminopurine	100	2007
6-BA 200	6-Benzilaminopurine	200	2007-2008
K 100	Kinetin	100	2007
K 200	Kinetin	200	2007

Samples collection for molecular Analyses

During the 2007 and 2008 season kiwifruit buds were collected and immediately frozen in liquid nitrogen at three different phonological stages:

1. "Dormant", corresponding to the beginning of January (Endo dormancy);
2. "Breaking", corresponding to the mid of March (bud break), referring to the buds along the cane which are able to start growth;
3. "Non Breaking", corresponding to the mid of March (bud break), referring to the buds alone the cane which are not able to start growth;

Moreover kiwifruit untreated and treated buds were collected at three different fixed hours (24 hours, 3 days and 7 days) after 6-BA 200ppm application performed at bud swell.

Samples collection for histological Analyses

During the 2008 season untreated kiwifruit buds corresponding to the phenological stages ("Dormant", "Breaking" and "Non Breaking") and 6-BA treated buds after 24 hours from the application were fixed in FAA solution (50% (v/v) ethanol, 10% 5% glacial acetic) by applying vacuum for 15 minutes (2X) and then incubating in the fixative for 1 day at 4°C. The material was dehydrated in a 50%-60%-70% ethanol series, 1 hour each and stored in 70% ethanol at 4 C.

RNA extraction

Total RNA was extracted from kiwifruit buds by using the method of La Starza et al. (2003), modified following Lopez-Gomez and Gomez-Lim (1992) to obtain higher amount and purer RNA. Two days are necessary to complete this method. The first day, 0,2g. of frozen kiwifruit buds were ground with liquid nitrogen in a mortar adding 0.2g of polyvinylpolypyrrolidone (PVPP). The fine powder obtained was added to 20ml extraction buffer (1.5 % SDS, 1% Tween 80, 1% Triton X-100, 300mM LiCl, 5mM Tiourea, 10mM DTT, 10 m*M* ethylenediaminetetraacetate (EDTA),200m*M* Trizma base ph 8. After shaking, 5 ml of chloroform:isoamyl alcohol (24:1) and 5 ml of Phenol Solution ph 7, were added. The solution was incubated for 10 minutes in ice. Afterwards, the sample was centrifuged at 6000 *rpm* for 20 min at room temperature. The aqueous phase was extracted and cold precipitated for 1 hour at -80°C adding the same volume of isopropanol and 1/10 Vol. Of Sodium Acetate 3M, ph 6.1. A Second centrifugation in a cold rotor at *10000°rpm* for 20 min was applied to the sample after precipitation. The aqueous phase was removed and the pellet obtained was washed with ethanol 70% and added with 1.7 of millliq sterilized water. Sample was finally stored at 4°C after the addition of 0.45ml LiCl 8M and 0.40 ml TBE 4X. During the second day, the sample was centrifuged at 10.000 rpm for 30 min at 4°C. The supernatant was discarded and the pellet was washed with Etanol 70%. The pellet was resuspended in 0.7 ml of milliq sterilized water and cold precipitated with Isopropanol and Sodium acetate as described above. Pellet was washed another time with ethanol 70% and then resuspended in milliq sterilized water. An aliquot was quantified by using NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and run into a 1%

agarose gel to check RNA integrity. The RNA samples were stored in Eppendorf tubes at -80° C.

Dnasi Treatment

1 μg. of total RNA were treated with DNase to eliminate contamination by genomic DNA. The reaction was performed using 2 μl of DNase enzyme (Promega), 5 μl of Buffer 10X (Promega) in 10 μl of total volume and incubated at 37° C for 30 min. The reaction was stopped by adding 1μl of Stopping solution (Promega) incubating for 5°min at 65°C. An aliquot of RNA was quantified spectrophotometrically.

Reverse Transcription

RNA was (1 μg) retro transcribed following the High Capacity RNA-to-cDNA kit (Applied Biosystem) in a total volume of 20 μl. The reaction was carried out with 10 μl RT buffer and 1ul RTmix. The thermal condition applied were: 1 hour at 37°C- 10 minutes at 65°C). The cDNA samples were stored at −20°C.

Degenerative primers design

EMBL database was used to find the aminoacidic sequences of Histone H4, Cyclin D3 and isopentenyltransferase genes available for the main crop species. The program ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html) was utilized to align sequences available for other crop species to identify the most conserved region of each gene. Sequences analyzed and primers designed are reported in Table . RT-PCR was set up to amplify the conserved fragment from total RNA of kiwifruit buds. The amplified PCR products were run into an agarose gel and the amplified band was cut and extracted following the Quiagen gel extraction kit protocol.

Gene cloning and isolation

PCR product was cloned and isolated following the steps described below:

LIGATION: 20 ng of PCR product, extracted from the gel, was cloned by using the p-GEM T easy vector system. Ligation was set up by incubating oven night at 4°C by adding 5μl (2X) Ligation Buffer, 1μl of pGEM-T easy vector (50ng) and 1μl T4 DNA Ligase.

TRANSFORMATION: The product of the ligation was transformed in DH5 competent cell (Invitrogen) by using heat shock. In details, 5μl of ligation's product reaction was added to 50μl of competent cell. After 30 minutes on ice, heated shock was apply for 45s at 42°C water bath, followed by 2 minutes on ice. Finally 1ml of S.O.C. medium

was added to the sample. The sample was incubated for 1 hour at 37°C (shaking). Bacterial suspension was obtained after centrifugation for 1 minutes at 12.000rpm.
SCREENING BACTERIAL COLONIES: Bacterial colonies were grown by inoculating 100µl of Bacterial suspension on the surface of a 90-mm agar plate previously prepared by adding 100ug of antibiotic (ampicilline), 2% X-Galactosidase and 20% IPGT (isopropyl-β-D-thiogalactoside. After the inoculation the plate was incubate over night at 37°C. The following day the plate was removed from the incubator and put at 4°C for 3-4 hours. Colonies carrying the recombinant plasmid were finally identify (white colonies contained the recombinant plasmid; Blue colonies do not).
GROWHT OF BACTERIAL CULTURE: Each single transformed colony was picked up and grown in tubes containing 5ml LB medium with incubation for 12-16hours at 37°C with vigorous shaking. A total of 20 colonies were analyzed for sample. PCR, with 1 µl of bacterial suspension, was set up to verify the presence of the fragment. The thermal conditions applied were 94°C for 4°min;(94°C for 20°s; 60°C for 30°C; 72°C for 40 s) for 35 cycles;10 min at 72°C.
PLASMID DNA PURIFICATION: Bacterial cell were harvested by centrifugation at 10.000rpm in a conventional microcentrifuge for 3 minutes at room temperature. To perform the plasmid extraction the Quiagent Miniprep Kit (Quiagen) was followed.
SEQUENCING: The product obtained from after the plasmid extraction was quantified spectrophotometrically and sent to sequence to the Genome Laboratory (John Innes Centre). Primers required for the analyses were the Puc/M13 Forward and Reverse primers.

Semi quantitative RT-PCR

Conventional PCR was carried out on 0,4 µg of cDNA (obtained as previously described). In a total volume of 20 µl were mixed 1 µl of 10 µM suited primers (Table 4), 10 µl of Mister Mix (Qiuagen) and milliq sterilized water. The following PCR conditions were used: 4 min at 94°C, followed by a suitable number of cycles (depending on transcript abundance) of 94°C for 20 s, 60°C for 3°s, 72°C for 40 s and a final extension at 72°C for 10min. Each reaction was performed in presence of control (18S) and the result was visualized on 2% agarose gel.

***In situ* RNA Hybridization**

RNA was hybridised *in situ* according to Fobert et al. (1996). The following procedures were performed:

PROBE PREPARATION: The preparation of the probes was based on the digoxigenin labeling system described by Drea et al. (2005). Probe was generated by in vitro transcription using T7 polymerase from linearised plasmid (Pgem-T easy vector, Promega) containing 3' fragment of *hist4* c-DNA.

PRE-HYBRIDIZATION: Processing of the slides included the following steps: deparaffinization, hydration in EtOH series, digestion with Proteinase K, acetylation and final dehydration. Slides are placed on a sterilized glass box and deparaffinized in two changes of xylene for 10 minutes in a fume-hood. Hydration is then performed in the following ethanol solutions (all v/v in water) using a shaking platform: 100% ethanol 5 min twice and 95% ethanol, 90% ethanol, 80% ethanol+0.75% NaCl, 60% ethanol+0.75% NaCl, 30% ethanol+0.75% NaCl, 0.75% NaCl for 1 min. After washing the slides with 1XPBS, tissue was permeabilized by incubation in 10 ug/mL Proteinase K (in a buffer pH 7.5, 0.1 M Tris-HCl pH 7.5, 50 mM EDTA pH 8) for 30 minutes at 28 C degrees. The proteinase K activity was blocked by washing with 2 mg/mL glycine in 1XPBS for 5 min, followed by one wash in 1XPBS and postfixation in 4% paraformaldehyde for 10 min. Slides are again washed with 1XPBS and acetylated by incubating them with fresh 0.3 % acetic anyhydride in 0.1 M triethanolamine/H_2O for 10 min and constant shaking. Prehybridization steps were completed with two washes in 1XPBS, one wash in 0.75% NaCl, for 5 min each and dehydration in the same ethanol solutions prepared for the initial rehydration, but in reverse order. Slides are removed from the incubation box and were allowed to dry on the bench for 30 min.

HYBRIDIZATION: The following steps of the protocol, including hybridization and post-hybridization washes, were performed in the InsituPro VS slide processor. Each slide was covered by 250 ul hybridization buffer (50% Formarmide; 10 X salts, 5% dextrane sulfate, 1X Denhart's solution, 100 ug/mL yeast RNA), in which a single probe had been added in a given dilution and then denatured at 85 C for 3 min. A special counter slide with spacers was placed over each slide so that a small incubation chamber was created. Each slide pair was mounted into a sealing block and these blocks were placed in a heatable incubation tub. Hybridization was performed for 14 hours at 55 C.

POST-HYBRIDIZATION WASHING: After hybridization, the slides were washed for 20 min in 50% (v/v) formamide and 0.2 X SSC including a total of seven changes at 55 ^{o}C and three changes after the temperature control was switched off and the incubation tub was allowed reaching room temperature slowly. Post-hybridization washes were completed with 1XPBS for three times, 5 min each.

SIGNAL DETECTION: Detection of the signal started with incubation in blocking solution [1% Blocking Reagent (Roche) in maleic buffer] for 30 min twice and in 1.0% BSA in TBS-T for 60 min. Anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) is then diluted in a final concentration of 1:1250 and added in the slides. After incubation with the antibody for 1.5 hour, the specimens were washed 3 in 1.0% BSA in TBS-T, 30 min each and 4 in AP-buffer (100mM NACl; 50mM MgCl; 100mM Tris, pH 9.5), 5 min each. At this point the slides are taken out from the processor and submerged in a glass slide jar filled with AP-buffer. Using forceps, counter slides were removed carefully and individual slides were placed on paper horizontally. Rapidly, 100 ul of Western Blue substrate (Promega) were dispensed in each slide and a coverslip was placed on the top. The specimens were transferred in a sealed plastic box with its bottom covered with wet towels to avoid drying and incubated in the substrate in the dark overnight. During the next few days, the signal is monitored under a stereo microscope. When the staining reaction is complete, the slides were rinsed in TE twice for 5 min and mounted in Glycerol/TE (50% v/v).

Hystological Analyses

MATERIAL PREPARATION: Kiwifruit buds were sectioned under a stereo microscope (LEIKA) to minimize the size of the region containing the (SAM) shoot apical meristem.
PARAFFIN INFILTRATION: samples were transferred in plastic cages and then placed in Tissue-Tek VIP Vacuum Infiltration processor for dehydration, clearing and wax infiltration. The program reported in Table 6 was followed. The material, removed from the VIP processor, was placed in the hot wax tank of the Tissue Tek embedding station. A thin layer of wax was poured into a metallic mould, the specimen is orientated to the favorable direction, left shortly to cool down and then a plastic backing was applied. One more layer of wax was applied on the block and then it was allowed to set on adjacent cold plate. The wax blocks were stored at 4 C.
SECTIONING: Tissue sections, 8 µm thick, were prepared in a Leica microtome and placed on a polysine slide covered with a thin layer of water. After a minute, the water was removed and the slides were placed on a hot plate at 42 ^{o}C overnight. Slides were stored at 4 C for several months.
PARAFFIN REMOVAL: slides with paraffin sections were soaked in Xilene in a glass box for 2 X 10 minutes. Sections were then rehydrated with diminishing series of Ethanol (95%-90%-80%-60%-30%) for 1 minutes each step. Slides were incubated in NaCl 5M for 1 minute. A final wash in Posphate-Saline Buffer (PBS) was applied.

DAPI staining

For imaging nuclei, kiwifruit bud sections were stained 3 hours with 1ug/mL 4', 6-Diamino-2-phenylindole (DAPI) (Sigma-Aldrich) at room temperature.

Light Microscopy

Longitudinal 8 μm section of kiwifruit SAM were investigated microscopically with a Leica MZFLIII with a Plan 1.0x objective and recorded with a digital camera. Bright light came from a standard halogen light box with fibre arms, and it was applied to visualize *in situ* analyses while UV-filter (excitation 340-380nm and emission 420 LP) was applied to localize nuclei. Images were processed (brightness, color balance and size) with Adobe Photoshop 7.0 (Adobe System).

SAM organization and Dividing Cell Identification

In Figure 12 the shoot apical meristem organization, detailed described in the introduction, is reported. Analyses of three sample for each different phenological stage of bud development were carried out to identify:

- ✓ Mitotic Index (expressed as percentage of dividing cell per layers);
- ✓ Number of cell (expressed as total number per layers)

Statistic Analyses

All the data in the present study were analyzed following a completely random design, by one-way ANOVA. Means separation was derived directly from the ANOVA table by using the software STATISTICA Ver.7.1 (Statsoft Inc., Tulsa, OK,USA).. All levels of probability stated are at least P=0.05.

Table 6. Program followed for paraffin infiltration in the VIP-machine

	Solution	***Time***
1	Ethanol 70%	1:00
2	Ethanol 80%	1:30
3	Ethanol 100%	2:00
4	Ethanol 100% with Esol	1:00
5	Ethanol 100%	1:30
6	Xilene	2:00
7	Xilene	0:30
8	Xilene	1:00
9	Waxe	1:30
10	Waxe	1:00
11	Waxe	1:00
12	Waxe	2:00
13	Waxe	2:00

Table 7. EMBL accession numbers of the species aligned to design the degenerative oligonucleotide primers needed to isolate three partial c-DNA fragment of Histone H4(histH4), Cyclin D3 (CYCD3) and Isopentenyltransferase (IPT).

Sequence source	*Degenerative primers (From 5' to 3')*	*Kiwifruit Gene*
NP850660.1 BAD82897 BAF36442 CAN83554	F: ATG(A/T)(G/C)IGGI(C/A)GIGGIAA(G/A)GGIGGIAA R: (A/C)GI(A/C)GIAA(G/A)ACIGTIACIGCIATG	HistH4
CAN59802.1 NP190576 NP195142	F: GGI(C/T)TITA(C/T)TG(T/C)GA(A/G)GA(A/G)G R:CATIC(G/T)(C/T)TG(G/A/T)ATIGT(C/T)TTIGC(C/T)TC	CYCD3
CAN 73470.1 BAE 75936.1 BAB59029.1	F: GGIGTICCICA(T/C)CA(T/C)(T/C)TI(T/C)TIGG R: AA(C/T)TCGGIACICC(T/A/G)ATIGC(C/T)TTIC	IPT

Tabella 8. Primers used to perform RT-PCR expression analysis on kiwifruit treated and untreated buds

Genes	Primers *(From 5' to 3')*	Embl Ac. nr
Cycd3 F	CTCAGCAGCAATGGAACAAA	AM941204
Cycd3 R	TCCATGGCTTCTCTCTCTGG	AM941204
HistH4 F	GCCTCTTGAGAGCGTACACC	AM941203
HistH4	AATCAGCGGCCTGATCTATG	AM941203
IPT F	CTTTCTCCGTTGGAGTTTCT	AM941205
IPT R	CCCGAGTCAAGCATATCGTC	AM941205
Cdk-B F	AATGGAGAAATCAGCGATRGG	EF392719
Cdk-B R	AGTTTTGGTGGGGAGGTTCT	EF392719
SUS 1 F	ACCACTTTTCGTGCCAGTTC	AY339822
SUS 1 R	CAGGGTGGAACTTGGTCAGT	AY339822
SUS A F	TGTCCTCAAGTCTGCACAGG	AY339821
SUS A R	GAACGCCATTGCCAATAGATT	AY339821
18S F	ATGGCCGTTCTTAGTTGGTG	AB253775
18 R	TGTCGGCCAAGGCTATAAAC	AB253775

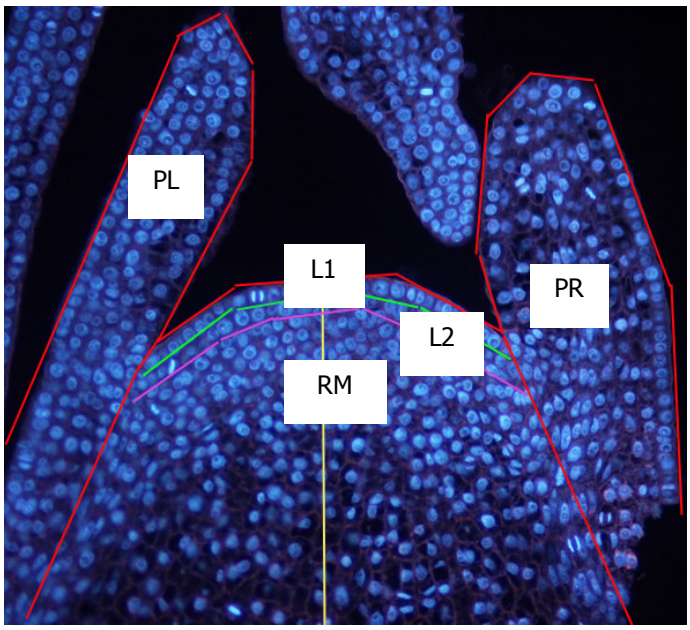


Figure 12. Shoot apical meristem organization; PL: primordial Left, PR: primordia Right; L1: first layer of cells; L2:second layers of cells; RM: Rib meristem.

4. Results

FIELD RESULTS

DBA applications at the Faenza farm

Cytokin application in the Faenza area showed positive effects on kiwifruit vines development through almost all the years of the research. First of all, bud break percentage was significantly increased with respect of untreated and Dormex-treated kiwifruit vines, during the season 2006 and 2008 (Table 9), while no significant effects were recorded in the 2007 season, probably due to an excessive mild winter as shown in Figure 13. Even if no higher bud break percentage was detected during this year, it is important to underline the induction of a more simultaneous bud release induced by this product when applied at bud-swell (Figure 14).

Cytokin applications also influenced flower fertility: treated plants showed, in fact, a general tendency to increase the number of inflorescence per shoot and to reduce the number of side flowers/inflorescence. All the results recorded during the three-year trials are reported in Table 10, 11 and 12. It is important to underline the effect induced with the application at the phenological stage of endo dormancy with 1 L/ha whose use seemed to cause a constant thinning of the kiwifruit lateral inflorescence.

Another positive effect induced from the product application was the synchronization of the flowering, in fact, as shown in Table 13, when almost all the DBA-treated plants have overcome the full bloom the control ones has not . It's important to underline that this event is related to the year and to the length of the flowering: in particular when the flowering period lasts for long, as in the 2007 season, Cytokin application at bud-swell (0.5 L/ha) as well as at endo dormancy (1L/ha) caused a significant concentration of flowering (Figure 15) allowing a better pollination of the female flowers.

Cytokin application also induced good results on the productive parameters at harvest. Yield is constantly increased as well as the fruit number carried by each plant without affecting the average fruit weight (Tables 14, 15 and 16). On the contrary, even if the weight measured at harvest did not significantly differ from treated and untreated plants, data on the fruit class distribution in the commercial size classes clearly showed a superior number of Cytokin-treated fruit in the higher classes compared to the control ones (Figures 16, 17 and 18). Among all the different treatments the application performed at bud-swell seemed to induce constantly a better fruit distribution in the higher commercial classes.

The quality traits analyses, performed each year of trial on a sample of fruits at harvest, showed that generally Cytokin applications did not affect them: differences were only measured during the 2006 season where treated fruit presented an higher flesh firmness and an higher soluble solid content (Table 17). In the following two years no differences were recorded on the same parameters (Tables 18 and 19). No clear effect was induced on the dry matter content during the 2007 season: Cytokin-treated fruit registered higher values compared to the control ones while the following year the opposite situation was detected. As regard to the effect on quality trait, measured after a period of cold storage during the 2007 season, it was possible to registered a light increase in the soluble solid content in treated fruit compared to the untreated ones (Table 18).

Table 9. Percentage of bud break measured on kiwifruit vines starting from the 2006 to the 2008 growing seasons. Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	*Bud break (%)*		
	2006	*2007*	*2008*
Control	48.5 b	49.4 b	49.9 b
Cytokin, bud-swell (1 trt.- 0.5 l/Ha)	63.8 a	46.5 b	58.4 a
Cytokin, bud-swell (2 trt.- 0.5+ 0.5 l/Ha)	59.0 a	-	-
Cytokin, endodormancy (1 trt.- 0.5 l/Ha)	50.4 b	46.2 b	-
Cytokin, endodormancy (1 trt.- 1 l/Ha)	-	45.6 b	60.1 a
Dormex, endodormancy (1trt.-18l/Ha)	55.3 b	56.2 a	48.4 b

Table 10. Percentage of inflorescences having a terminal flower (single) or terminal and primary flowers (double) or terminal, primary and secondary flowers (triple) and flower fertility recorded during the season 2006. Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	*Single (%)*	*Double (%)*	*Triple (%)*	*Flower fertility (N°/shoot)*
Control	86.6 b	5.5 a	7.9 a	34 a
Cytokin, bud-swell (1 trt.- 0.5 l/Ha)	90.9 ab	4.8 a	4.1ab	36,3 a
Cytokin, bud-swell (2 trt.- 0.5+ 0.5 l/Ha)	91.8 ab	6.0 a	2.2 ab	35 a
Cytokin, endodormancy (1 trt.- 0.5 l/Ha)	98.6 a	0.6 b	0.8 c	38.8 a
Dormex, endodormancy (1trt.-18l/Ha	92.6 ab	3.7 a	3.6 ab	37,8 a

Tabella 11. Percentage of inflorescences having a terminal flower (single) or terminal and primary flowers (double) or terminal, primary and secondary flowers (triple) and flower fertility recorded during the season 2007. Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	*Single (%)*	*Double (%)*	*Triple (%)*	*Flower fertility (N°/shoot)*
Control	82.1 c	9.5 b	8.3 b	18,5 a
Cytokin, endodormancy (1 trt.- 0.5 l/Ha)	84.0 bc	7.5 b	8.5 b	17 a
Cytokin, endodormancy (1 trt.- 1 l/Ha)	92.4 a	4.6 a	3 a	21.3 a
Cytokin, bud-swell (1 trt.- 0.5 l/Ha)	87.8 b	4.5 a	7.6 b	22 a
Dormex, endodormancy (1trt.-18l/Ha)	93,0 a	3.9 a	3,0 a	21 a

Table 12. Percentage of inflorescences having a terminal flower (single) or terminal and primary flowers (double) or terminal, primary and secondary flowers (triple) and flower fertility recorded during the season 2008. Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	Single (%)	Double (%)	Triple (%)	Flower fertility (N°/shoot)
Control	87.2 a	7.2 a	5.6 a	32.0 b
Cytokin, endodormancy (1 trt.- 1 l/Ha)	90.3 a	6.5 a	3.2 b	36,4 a
Cytokin, bud-swell (1 trt.- 0.5 l/Ha)	89.4 a	6.2 a	4.4 a	37.8 a
Dormex, endodormancy (1trt.-18l/Ha	88.7 a	6.2 a	5.1 a	35,2 a

Table 13. Number of open flowers measured on kiwifruit vines during the 2006, 2007 and 2008 seasons. Data collected at Full Bloom stage (50-60% open flowers). Different letters represent significant difference for P < 0.05 (Duncan's Test).

	Data apx 50% Open flowers		
Trial	***20-mag-06***	***10-mag-07***	***20-mag-06***
Control	42.6 b	35.2 c	45.2 b
Cytokin, bud-swell (1trt.- 0.5 l/Ha)	40.1 b	41.5 b	51.4 a
Cytokin, bud-swell (2 trt.- 0.5 + 0.5 l/Ha)	50.5 a	-	-
Cytokin, endodormancy (1 trt.- 0.5 l/Ha)	32.9 b	64.8 a	-
Cytokin, endodormancy (1 trt.- 1 l/Ha)	-	23.9 d	48.1 b
Dormex, endodormancy (1trt.-18l/Ha)	35.5 b	72.1 a	49.8 ab

Table 14. Productive parameters measured during the 2006 harvest (30.10). Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	***Yield (kg/ tree)***	***Fruit number (N°/ tree)***	***Fresh weight (g)***
Control	41.3 b	374.6 b	110.3 a
Cytokin, bud-swell (1 trt.- 0.5 l/Ha)	54.6 a	507.2 a	107.7 a
Cytokin, bud-swell (2 trt.- 0.5+ 0.5 l/Ha)	50.6 a	476.1 a	106.3 a
Cytokin, endodormancy (1 trt.- 0.5 l/Ha)	53.6 a	462.1 a	116.0 a
Dormex, endodormancy (1trt.-18l/Ha	47.6 a	462.2 a	103.0 a

Table 15. Productive parameters measured during the 2007 harvest (20.10). Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	***Yield (kg/ tree)***	***Fruit number (N°/ tree)***	***Fresh weight (g)***
Control	26.7 a	250.3 a	106.7 a
Cytokin, endodormancy (1 trt.- 0.5 l/Ha)	18.0 b	177.0 b	101. 7a
Cytokin, endodormancy (1 trt.- 1 l/Ha)	31.7 a	328.1 a	96.6 a
Cytokin, bud-swell (1 trt.- 0.5 l/Ha)	28.3 a	283.0 a	100.0 a
Dormex, endodormancy (1trt.-18l/Ha)	24.2 a	226.9 b	106.7 a

Table 16. Productive parameters measured during the 2008 harvest (23.10). Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	***Yield (kg/ tree)***	***Fruit number (N°/ tree)***	***Fresh weight (g)***
Control	37.9 b	394.2 a	96.2 a
Cytokin, endodormancy (1 trt.- 0.5 l/Ha)	39.9 b	426.7 a	93.6 a
Cytokin, bud-swelling (1 trt.- 0.5 l/Ha)	46.8 a	457.6 a	102.4 a
Dormex, endodormancy (1trt.-18l/Ha	39.7 b	394.3 a	100.7 a

Table 17. Quality traits measured during the 2006 harvest (30.10). Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	***Flesh firmness (kg/cm^2)***	***Soluble solid content (°Brix)***	***Dry matter (%)***
Control	6.0 bc	5.8 c	16.7 a
Cytokin, bud-swell	6.0 bc	5.9 bc	15.9 a
Cytokin, bud-swell	6.3 ab	6.1 ab	16.9 a
Cytokin, endodormancy	5.8 c	6.2 a	16.7 a
Dormex, endodormancy	6.6 a	5.9 bc	17.1 a

Table 18. Quality traits measured during the 2007 harvest (20.10) and after 120 days of cold storage. Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	***Flesh firmness (kg/cm²)***		***Soluble solid content (°Brix)***		***Dry matter (%)***
	Harvest	120d. storage	Harvest	120d. Storage	Harvest
Control	7.2 a	1,8 a	5.6 a	13,6 b	16.2 b
Cytokin, endodormancy (1 trt.- 0.5 l/Ha)	7.6 a	1,6 a	5.3 a	14,0 b	17.8 a
Cytokin, endodormancy (1 trt.- 1 l/Ha)	7.4 a	1,2 a	5.8 a	14,0 b	17.6 a
Cytokin, bud-swell (1 trt.- 0.5 l/Ha)	7.2 a	1,6 a	5.6 a	15,3 a	17.4 a
Dormex, endodormancy (1trt.-18l/Ha)	7.6 a	1,5 a	5.6 a	14,4 ab	17.7 a

Table 19. Quality traits measured during the 2008 Harvest (23.10). Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	***Flesh firmness (kg/cm²)***	***Soluble solid content (°Brix)***	***Dry matter (%)***
Control	6.2 a	7.2 a	18 a
Cytokin, endodormancy (1 trt.- 0.5 l/Ha)	6.4 a	7.1 a	16.8 b
Cytokin, bud-swell (1 trt.- 0.5 l/Ha)	6.4 a	6.7 b	16.9 b
Dormex, endodormancy (1trt.-18l/Ha	6.3 a	6.7 b	17.8 a

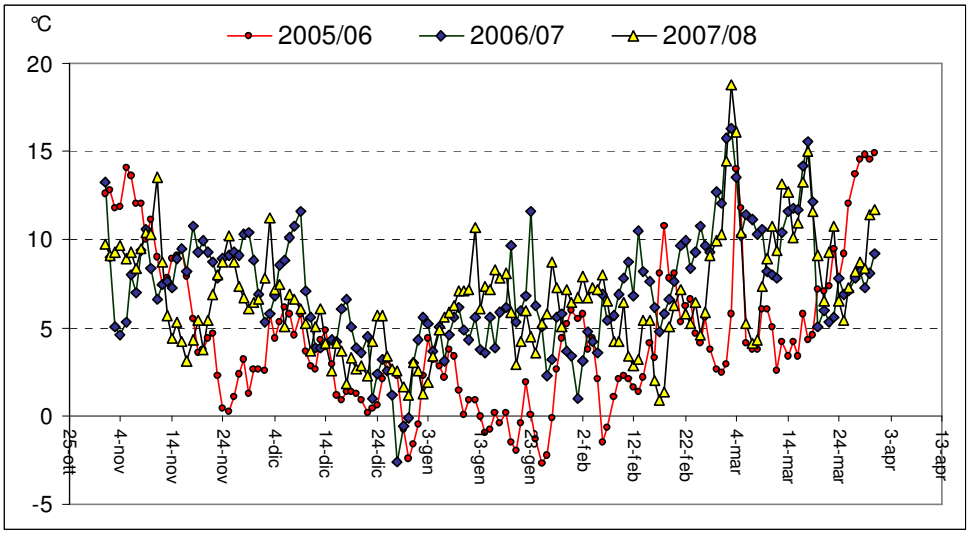


Figure 13. Daily temperatures measured in the 2006, 2007, 2008 years during the winter and spring periods of the Faenza kiwifruit producing area**.**

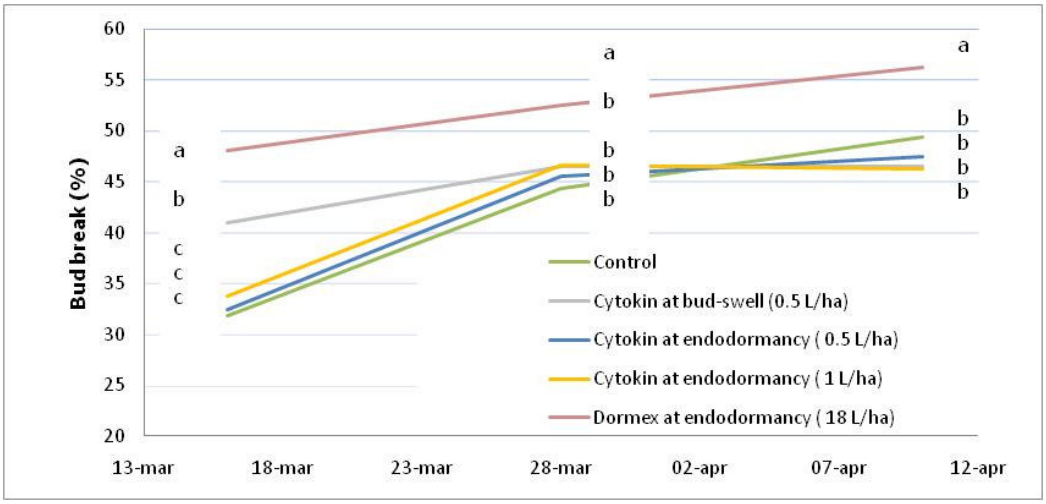


Figure 14. Variation of the bud break percentage recorded on kiwifruit vines during the growing season 2007. Different letters represent significant difference for $P < 0.05$ (Duncan's Test).

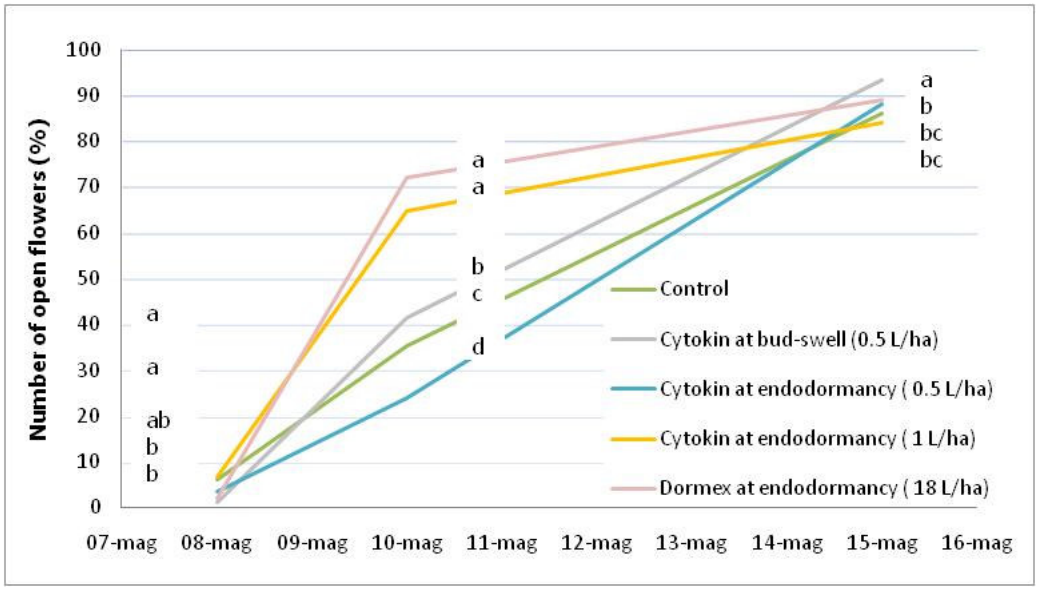


Figure 15. Time and number of open flowers recorded on kiwifruit vines during the growing season 2007. Different letters represent significant difference for $P < 0.05$ (Duncan's Test).

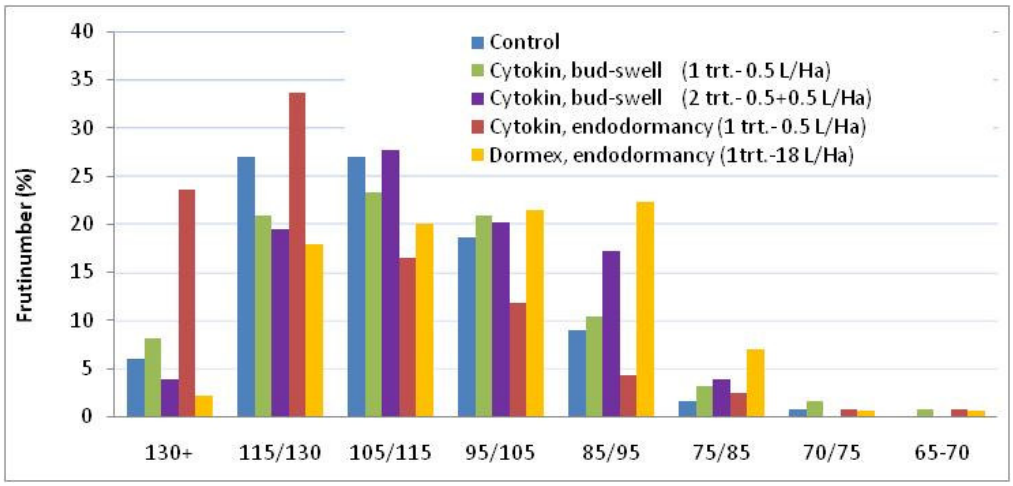


Figure 16. Fruit size class distribution measured at harvest (30.10) during the season 2006

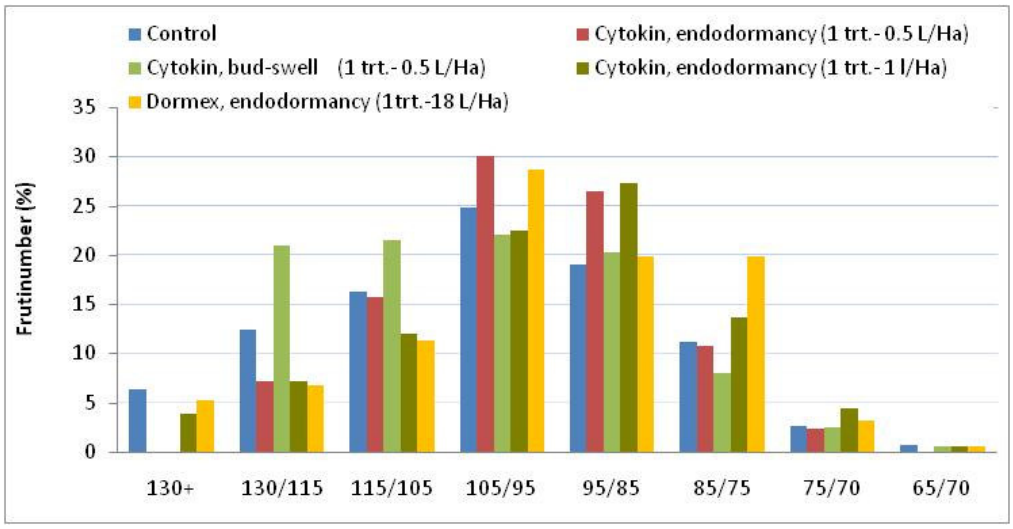


Figure 17. Fruit size class distribution measured at Harvest (20.10) during the season 2007

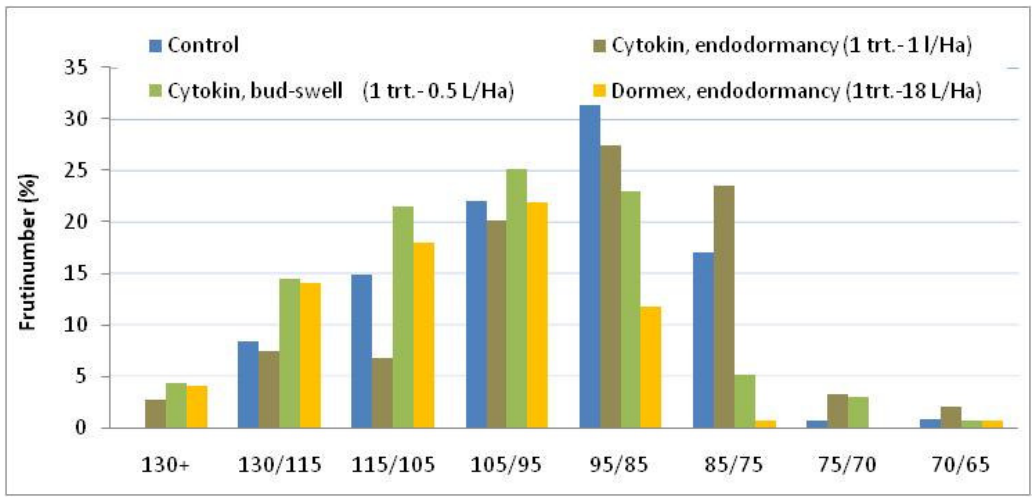


Figure 18. Fruit size class distribution measured at Harvest (23.10) during the season 2008

DBA applications at the Bologna farm

Trials carried out with Cytokin at the experimental farm of the Bologna University confirmed the results obtained in the Faenza area. In particular, as regard to the bud break percentage, positive results were obtained during the 2006 and 2007 seasons (Table 20) while in the 2008 season, untreated plants reached a percentage of bud break higher than normal and it was not possible to record any significant additional effect induced by the treatments. Despite the increase in the number of buds which start breaking, a constant effect on the simultaneity of young shoots growth in treated plant were recorded (Figure 19).

The thinning effect of side flowers was particularly induced: more in detail the number of single flowers differed in treated and untreated vines only in the 2007 season, when Cytokin applied at bud-swell registered higher values compared to the control one while the reduction in the number of double and triple inflorescences were recorded during all the three-year trials (Table 21).

The synchronization of the flowering was also detected, in particular Cytokin application 15 days before bud-swell seemed to be the most effective during the 2006 season while the following year the best results were obtained with the application at bud release. No effect was recorded during the 2008 probably due to the good weather condition occurred during the flowering period (Table 22). The percentage of open flowers measured during the 2007 is shown in Figure 20. From this figure it clearly emerged that treated plant registered significantly higher values compared to the control ones.

Cytokin applications did not affect the productive parameter measured at harvest. No differences on yield and fruit number per tree were measured during the three-year of trials with the exception for the 2008 season where treated plant had a significant increase in yield (Table 23). Analyses on the average fruit weight and the fruit size class distribution confirmed better weight reached by treated fruit, in fact each year of trial an higher percentage of Cytokin treated fruit are located in the best fruit class size (>95) compared to the control ones (Figure 21).

Analyses on the quality traits measured at harvest showed no clear results: during the 2006 season Cytokin treatments induced a slight delay of ripening: treated fruit contained a lower soluble solid and dry matter content. In contrast, during the 2007 season a lower flesh firmness and an higher soluble solid content were detected. No differences were recorded during the 2008 season (Table 24).

Table 20. Percentage of bud break measured on kiwifruit vines starting from the 2006 to the 2008 growing seasons. Different letters represent significant difference for P <0,05

Trial	Bud break (%)		
	2006	2007	2008
Control	57.8 b	51.5 b	68.9 a
Cytokin, 15d before bud-swell (1trt 0.5 l/ha)	70.6 a	-	-
Cytokin, bud-swell (1trt 0.5 l/ha)	60.7 ab	60.3 a	69.6 a

Table 21. Percentage of inflorescences having a terminal flower (single) or terminal and primary flowers (double) or terminal, primary and secondary flowers (triple) recorded during the seasons 2006, 2007 and 2008. Different letters represent significant difference for P < 0.05 (Duncan's Test).

Year	Type of inflorescence (%)	Control	Cytokin, bud-swell (1trt 0.5 l/ha)	Cytokin, 15d before bud-swell (1trt 0.5 l/ha)
2006	Single	95.7 a	96.7 a	95 a
2007		85.7 b	90.3 a	-
2008		87.4 a	84.7 a	-
2006	Double	1.2 a	1.7 b	3.6 a
2007		6.7 a	4.7b	
2008		18.3 a	13.9 b	-
2006	Triple	3.2 a	1.6 b	1.4 b
2007		7.5 a	4.9 b	-
2008		4.5 a	5.6 a	-

Table 22. Number of open flowers measured during the 2006, 2007 and 2008 seasons. Data collected at Full Bloom stage (50-60% open flowers). Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	Data apx 50% Open flowers		
	20-mag-06	11-mag-07	21-mag-06
Control	41.7 b	47.4 b	50.3 a
Cytokin, bud-swelling (1trt.- 0.5 l/Ha)	38.2 b	59.6 a	50.0 a
Cytokin, 15d. before bud-swelling (1 trt-0.5 l/Ha)	52 a	-	-

Table 23. Productive parameters measured at harvest in the 2006 (20.10), 2007 (17.10) and 2008 (22.10) seasons .Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	*Yield (kg/ tree)*	*Fruit number (N°/ tree)*	*Fresh weight (g)*
2006			
Control	18. 2 a	158.3 a	114.9 a
Cytokin, bud-swell (1 trt.- 0.5 l/Ha)	21.0 a	186.1 a	113.0 a
Cytokin, 15d. before bud-swell (1 trt-0.5 l/Ha)	18.4 a	158.7 a	115.9 a
2007			
Control	12.0 a	137.7 a	87.1 a
Cytokin, bud-swell (1 trt.- 0.5 l/Ha)	12.5 a	131.8 a	94.8 a
2008			
Control	19.7 b	167.9 a	117.3 a
Cytokin, bud-swell (1 trt.- 0.5 l/Ha)	21.8 a	199.8 a	109.0 a

Figure 19. Shoot growth uniformity induced by Cytokin treatments at bud-swell.

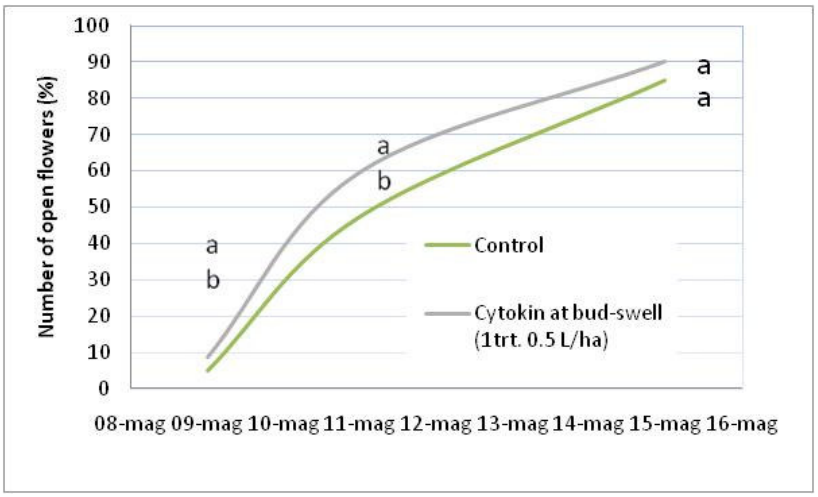


Figura 20. Percentage of open flowers recorded during the growing season 2007. Different letters represent significant difference for P < 0.05 (Duncan's Test)

Table 24. Fruit quality parameters measured at harvest during the 2006, (20.10), 2007 (17.10) and 2008 (22.10) seasons. Different letters represent significant difference for P < 0.05 (Duncan's Test).

Year	***Quality trait***	***Trial***	
		Control	**Cytokin, bud-swell (1 trt.- 0.5 L/Ha)**
2006	Flesh firmness (kg/ cm^2)	7.7 a	7.5 a
	Soluble solid content (°Brix)	7.0 a	5.8 b
	Dry weight (%)	19.2 a	17.9 b
2007	Flesh firmness (kg/ cm^2)	8.3 a	7.9 b
	Soluble solid content (°Brix)	7.0 b	7.8 a
	Dry weight (%)	20.5 a	20.2 a
2008	Flesh firmness (kg/ cm^2)	7.7a	7.6 a
	Soluble solid content (°Brix)	6.9 a	6.4 a
	Dry weight (%)	18.9 a	18.6 a

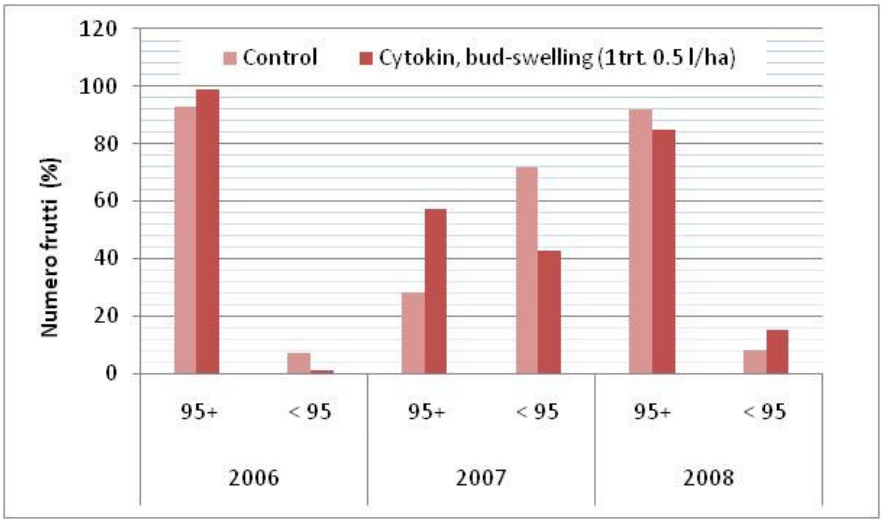


Figure 21. Fruit size class distribution, dividing two classes of commercial weight (>95;<95), measured at Harvest during the season 2006, 2007 and 2008

Phytotoxicity

Treatments with an increasing level of Cytokin performed at bud-swell did not cause phytotoxicity on the plant and on the fruit (data not shown). As regard to the influence on the vegetative parameters, the effect on the bud break percentage, the thinning of side flowers and the synchronization of flowering was confirmed (Table 25 and 26). In particular applications with 0.5 L/ha 4 L/ha increased significantly the number of buds which start growth. As regard to the number of lateral inflorescences, the thinning effect was particularly induced by the applications with 2 and 4 L/ha, while data collected at the moment of flowering showed a significant effect of 0,5 L/ha and 1 L/ha applications.

Table 25. Percentage of bud break measured during the 2007 growing seasons at the Bologna experimental farm. Different letters represent significant difference for P < 0.05 (Duncan's Test).

		Bud break (%)		
Trial	***L/ha***	***22-mar-07***	***28-mar-07***	***11-apr -07***
Control	-	45 b	47.2 b	51.5 b
Cytokin, 1trt. bud-swell	0.5	47.2 b	48.5 b	60.3 a
	1	43.1 b	49.1 b	51.5 b
	2	45.9 b	45.9 b	48.2 b
	4	54.3 a	62.9 a	65.9 a
	8	51.9°	51.9 b	53.4 b

Tabella 26. Percentage of inflorescence having a terminal flower (single) or terminal and primary flowers (double) or terminal, primary and secondary flowers (triple) and number of open flowers, recorded during the seasons 2007. Different letters represent significant difference for P < 0.05 (Duncan's Test

		Type of inflorescence (%)			***Number of open flower (%) aprx. 50%***
Trial	***L/ha***	***Single***	***Double***	***Triple***	***11-mag-07***
Control	-	85.7 a	6.7 a	7.5 a	47.4 b
Cytokin, 1trt. bud-swell	0.5	90.3 a	4.7 a	4.9 a	59.6 a
	1	87.1 a	6.1 a	6.9 a	60.9 a
	2	90.1 a	7.6 a	2.2 b	37.8 b
	4	92.3 a	6.4 a	1.3 b	43.6 b
	8	85.8 a	7.1 a	7.1 a	43.4 b

Pure Cytokinins application at the Bologna farm

Pure CK applications seemed to induce some of the effects obtained with the commercial DBA application. In particular, the 6-BA and the Kinetin applied at 200ppm significantly increased the final bud break percentage during the 2007 season (Figure 22). The same effect was not observed during the 2008 season when 6-BA 200ppm was applied on whole plant at bud-swell (Data not shown). As regards to the thinning effect on the side flowers, it was particularly visible only during the season 2008 where 6-BA treated plant presented a significant reduction in the number of side flowers compared to the control ones (Table 27). Data on the 2007 flowering apparently did not differ from treated and untreated plants. Nevertheless, it has to be underline that the flowering percentage of BA-treated plants was significantly lower than control ones two days before the full flowering (9th of May) thus leading to a shorter flowering period. This effect was also induced by the same application in the 2008 season (Table 28). Assessment performed at harvest during the season 2008 showed no influence on the productive and quality parameters caused by 6-BA application (Table 29).

Table 27. Percentage of inflorescences having a terminal flower (single) or terminal and primary flowers (double) or terminal, primary and secondary flowers (triple) recorded during the seasons 2007 and 2008. Different letters represent significant difference for $P < 0.05$ (Duncan's Test).

Trial 2007		Type of inflorescence (%)		
	Year	Single	Double	Triple
Control	2007	83.0 a	11.2 a	5.8 a
	2008	87.4 a	9.9 a	4.5 a
6-BA 100ppm	2007	86.2 a	6.3 a	7.5 a
6-BA 200ppm	2007	82.1 a	11.3 a	6.5 a
	2008	93.9 a	4.3 b	2.4 b
kinetin 100ppm	2007	94.6 a	3.3 a	2.1 a
Kinetin 200ppm	2007	84.9 a	7.4 a	7.7 a

Table 28. Percentage of open flowers recorded during the vegetative growing season 2007. Different letters represent significant difference for P < 0.05 (Duncan's Test).

Trial	***Number of open flowers (%)***				
	9-Mag-07	***11-Mag-07***	***19-Mag-08***	***21-Mag-08***	***23-Mag-08***
Control	7.8 a	54.5 a	33.9 a	50.3 a	75.2 a
6-BA 100ppm	14.2 a	50.7 a	-	-	-
6-BA 200ppm	2.7 b	56.3 a	24.5 b	52.8 a	87.3 a
kinetin 100ppm	15.8 a	61.1 a	-	-	-
Kinetin 200ppm	15.1 a	63.1 a	-	-	-

Table 29. Productive and quality parameters measured during the 2008 harvest (22/10). Different letters represent significant difference for P < 0.05 (Duncan's Test).

Parameter	***Trial***	
	Control	*6-BA 200ppm*
Yield (kg/ tree)	19.7 a	20.5 a
Fruit number (N°/ tree)	117.3 a	117.4 a
Fresh weight (g)	167.9 a	174.7 a
Flesh firmness (kg/ cm^2)	7.7 a	7.5 a
Soluble solid content (°Brix)	6.9 a	6.2 a
Dry weight (%)	18.9 a	18.2 a

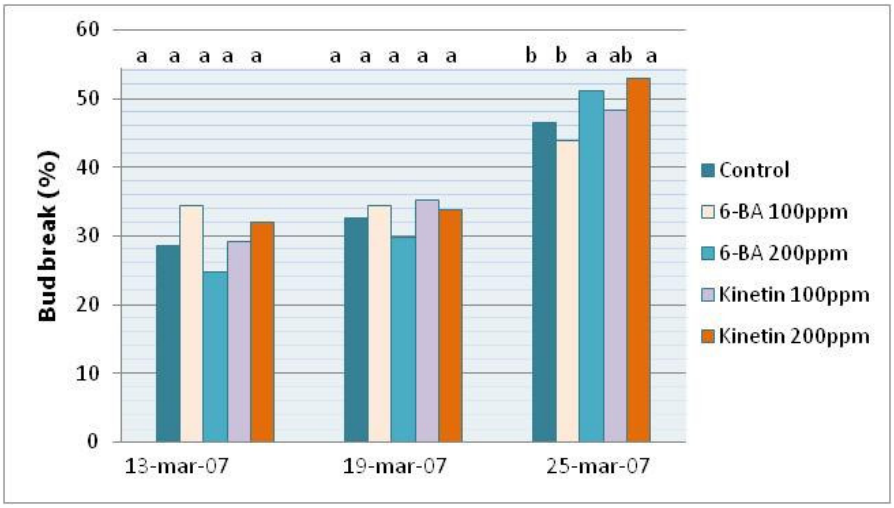


Figure 22. Variation of the bud break percentage recorded on treated and untreated kiwifruit vines during the growing season 2007 in the Bologna area. Different letters represent significant difference for P < 0.05 (Duncan's Test).

MOLECULAR AND HYSTOLOGICAL ANALYSES

The nucleotide sequence of Histone H4 mRNA

In order to determine the sequence of the coding region of Histone H4 cDNA, PCR was performed using cDNA synthesized from total RNA of kiwifruit buds and a set of degenerative primers HistH4-F: ATG (A/T)(G/C)I GGI (C/A)GI GGI AA(G/A) GGI GGI AA; HistH4-R: (A/C)GI (A/C)GI AA(G/A) ACI GTI ACI GCI designed base on the alignment of *Arabidopsis thaliana* (AT3G46320), *Fragaria x Ananassa* (AM197150), *Nicotiana tabacum* (AM280787) and *Vitis vinifera* (AM69706) aminoacids sequences (http://www.ebi.ac.uk/Tools/clustalw2/index.html). A 300 bp long cDNA was obtained from this RT-PCR, subcloned and sequenced, and the results revealed a 299 bp coding sequence for the histone H4 for Kiwifruit. The sequence was submitted in the EMBL database (http://www.embl.org/). The accession number is AM941203 as shown in Table 30.The similarity-based searching was carried out by using BLASTn on NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) which showed high similarity with histone H4 mRNA sequenced form *Eucalyptus globulus* (85%), *Zea Mays* (85%) and wheat (84%) (Table 31).

The nucleotide sequence of Cyclin D3 cDNA

The same procedure was followed to determine the sequence of the coding region of Cyclin D3 cDNA. PCR was performed using cDNA synthesized from total RNA of kiwifruit buds and a set of degenerative primers CycD3F: GGI (C/T)TI TA(C/T) TG(T/C)GA(A/G)GA(A/G)G;CycD3-R: ATI C(G/T)(C/T) TG(G/A/T)ATI GT(C/T) TTI GC(C/T) TC, designed base on the alignment of *Vitis vinifera* (AM454724), *Populus trichocarpa* (AM746118), *Arabidopsis thaliana* (AT4G34160) aminoacids sequences. A 450 bp long cDNA was obtained from this RT-PCR, subcloned and sequenced, and the results revealed a 416 bp coding sequence for Kiwifruit. The sequence was submitted in the EMBL database and the accession number is AM941204 as shown in Table 30.

Results from the nucleotide homology carried out by using BLASTn on NCBI of CYCD3 identified that the sequence with the highest similarity are an NtcycD3-1 mRNA from *Nicotiana tabacum* (84%), a sequence from a fruit crop, a 19-2 cyclin D3 from apple (76%) and with a partial D3-type cyclin sequenced in *Populus trichocarpa* (72%), (Table 32).

The nucleotide sequence of Isopentenyltransferase cDNA

PCR was performed using cDNA synthesized from total RNA of kiwifruit buds and a set of degenerative primers IPT-F: GGI GTI CCI CA(T/C) CA(T/C) (T/C) TI (T/C)TI GG; IPT-R: AA (C/T)TC GGI ACI CC(T/A/G) ATI GC(C/T) TTI C, designed base of the alignment of *Vitis Vinifera* (AM428301), *Pisum sativum* (AB194604), and *Arabidopsis thaliana* (AB061400) aminoacids sequences to identify the sequence of the coding region of isopentenyltransferase cDNA. A 450 bp long cDNA was obtained from this RT-PCR, subcloned and sequenced, and the results revealed a 431 bp coding sequence for the Isopentenyltransferase gene in Kiwifruit. The sequence was submitted in the EMBL database and the accession number is AM941205 as shown in Table 30.The similarity-based searching carried out with BLASTn on NCBI showed that The IPT sequence has the highest similarity with an IPT1 mRNA from Brassica rapa (67%) and from *Medicago truncatula* (65%), (Table 33).

Table 30. Submission of three partial c-DNA sequences isolated from Hayward kiwifruit buds to the European Molecular Biology Laboratory (EMBL)

Description	***Accession number (EMBL)***	***Fragment length (bp)***
Actinidia deliciosa var. deliciosa, partial mRNA for histone H4	AM941203	299
Actinidia deliciosa var. deliciosa, partial cycd3 gene for cyclin D3	AM941204	416
Actinidia deliciosa var. Deliciosa, partial IPT gene for isopentenyltransferase	AM941205	431

Table 31. Similarity results of the sequence AM9412043 isolated from Hayward kiwifruit buds obtained by BLAST analyses on NCBI (http://www.ncbi.nlm.nih.gov/BLAST/)

Accession	Description	Query coverage	E value	Max ident
EU977003.1	Zea mays clone 995955 histone H4 mRNA, complete cds	99%	7e-91	85%
NM_001051264.1	Oryza sativa (japonica cultivar-group) Os01g0835900 mRNA, complete cds	99%	9e-90	85%
AY263810.1	Eucalyptus globulus histone H4 mRNA, complete cds	99%	9e-90	85%
AY389648.1	Hyacinthus orientalis histone H4 mRNA, complete cds	97%	5e-86	84%
AC233794.1	Oryza minuta clone OM__Ba0207E07, complete sequence	99%	2e-85	84%
EU966759.1	Zea mays clone 297048 histone H4 mRNA, complete cds	98%	2e-85	84%
M12277.1	Wheat histone H4 TH091 gene, complete cds	98%	8e-84	84%

Table 32. Similarity results of the sequence AM941204 isolated from Hayward kiwifruit buds obtained by BLAST analyses on NCBI (http://www.ncbi.nlm.nih.gov/BLAST/

Accession	*Description*	*Query coverage*	*E value*	*Max ident*
AB015222.1	Nicotiana tabacum NtcycD3-1 mRNA, complete cds	76%	2e-54	75%
AM445921.2	Vitis vinifera contig VV78X016110.8, whole genome shotgun sequence	78%	5e-50	74%
AP006667.1	Lotus japonicus genomic DNA, chromosome 5, clone: LjT31L14, TM0366, complete sequence	78%	6e-43	73%
AJ002589.1	Lycopersicon esculentum mRNA for D-type cyclin	76%	2e-37	71%
EU325651.1	Solanum tuberosum D-type cyclin family 3 subgroup 2 (CycD3.2) mRNA, complete cds	76%	2e-36	71%
AJ132929.1	Medicago sativa cycD3 gene, type I promoter and exons 1-4	78%	8e-35	71%
AM746118.1	Populus trichocarpa partial mRNA for D3-type cyclin (CYCD3;3 gene)	69%	4e-32	72%
AY347854	Malus x domestica clone 19-2 cyclinD3 mRNA)	54 %	2e-14	76%

Table 33. Similarity results of the sequence AM9412045 isolated from Hayward kiwifruit buds obtained by BLAST analyses on NCBI (http://www.ncbi.nlm.nih.gov/BLAST/

Accession	Description	Query coverage	E value	Max ident
AB186132.1	Brassica rapa subsp. pekinensis BrIPT1 mRNA for isopentenyltransferase, complete cds	57%	7e-11	67%
AC154089.20	Medicago truncatula clone mth2-91j8, complete sequence	78%	1e-07	65%
AM428301.1	Vitis vinifera contig VV78X048063.16, whole genome shotgun sequence	45%	1e-06	67%
NM_105517.1	Arabidopsis thaliana ATIPT1 (ISOPENTENYLTRANSFERASE 1); adenylate dimethylallyltransferase (ATIPT1) mRNA, complete cds	64%	1e-06	65%
DQ108748.1	Arabidopsis thaliana clone 157146 mRNA sequence	64%	1e-06	65%
AB062607.1	Arabidopsis thaliana AtIPT1 mRNA for Adenylate isopentenyltransferase, complete cds	64%	1e-06	65%
AB061400.1	Arabidopsis thaliana AtIPT1 mRNA for cytokinin synthase, complete cds	64%	1e-06	65%
AB239805.1	Oryza sativa Japonica Group OsIPT8 gene for adenylate isopentenyltransferase, complete cds	47%	5e-06	66%

Gene expression analyses

Semi quantitative RT-PCR

The CYCD3, CDKB, and H4 transcript levels were analyzed during the development of kiwifruit bud from dormancy up to bud break performing RT-PCR experiments and normalization with respect to 18S rRNA.

CYCD3, CDKB, and H4 transcripts were expressed in the final phases of bud development from swelling up to breaking in both years of trials (Figure 23 A-B). As indicated by the RT-PCR cycle number, H4 mRNA showed the highest levels of accumulation followed by the CDKB and CYCD3 mRNA (Figure 23 B). As regard to their pattern of accumulation during bud release, H4 an CYCD3 did not significantly changed while CDKB showed a pick in advance bud swell just prior to bud break.

Based on the above reported results, the mRNA levels of these genes in advanced bud swell were compared to those of dormant, non breaking and treated breaking buds as shown in Figure 24. In both years of trials H4 and CDKB transcripts showed a clear increase starting from dormancy up to breaking. In particular, the H4 changed gradually while the CDKB markedly increased at bud break. CYCD3 mRNA also

showed a similar trend in the 2008 trial, while its low expression in the 2007 did not allow to observe the same. As regard to the CKs application, analyses on the 6-BA treated buds showed an up regulation of all genes, and in particular for the CYCD3 despite its low expression level.

The IPT gene expression was also analyzed by RT-PCR from bud swell up to bud break in treated and untreated vines. As shown in Figure 25, a similar trend of transcript accumulation was detected in both samples with a final increase when the bud break stage is completed. Moreover, 6-BA application constantly enhanced the transcript accumulation (Figures 25, 26 A-B). On the contrary, dormant and "non breaking buds" showed none or very low IPT gene expression in both years of trials (Figure 26 A-B).

The transcript level of two sucrose synthase genes (SUS1 and SUSA) were also investigated as reported in Figure 27. In general dormant buds showed lower values in mRNA accumulation in both years when compared with buds collected at bud swell, both breaking and non breaking. Comparison of the latter showed an higher transcript accumulation in breaking buds. As regard to the CKs application, it was not possible to observe a constant effect of up regulation induced by the treatment. Relative band intensity of SUS1 transcript, in fact, was increased only during the 2008 season while the one of SUSA seemed to be affected in the 2007.

In situ hybridization

In situ localization of HistH4 transcript in the shoot apical meristem (SAM) of kiwifruit buds collected at three different phenological stages of development (dormant, "breaking", "non breaking") during the 2008 season clearly showed that H4 is expressed only in the SAM of bud which have started growth (Figure 28 A-B). In particular, photos of SAM median longitudinal sections, which were taken under the bright-field, emphasized the strong dark signal detected in "breaking buds" with respect to the "non breaking" and dormant ones (Figure 28 A-C-D). Moreover, it clearly emerged from the magnification showed in Figure 28 B, that the HistH4 – expressing cell occurred most frequently at the periphery of the shoot apex and at the leaf primordial levels. In Figure 29 A-C-E, obtained combining bright and UV filters, the concomitant presence of dividing cell in the SAM of "breaking buds" is showed (E). No activity was detected in the ones collected in the endo dormant stage and at bud swell which had not started their growth (A-C).

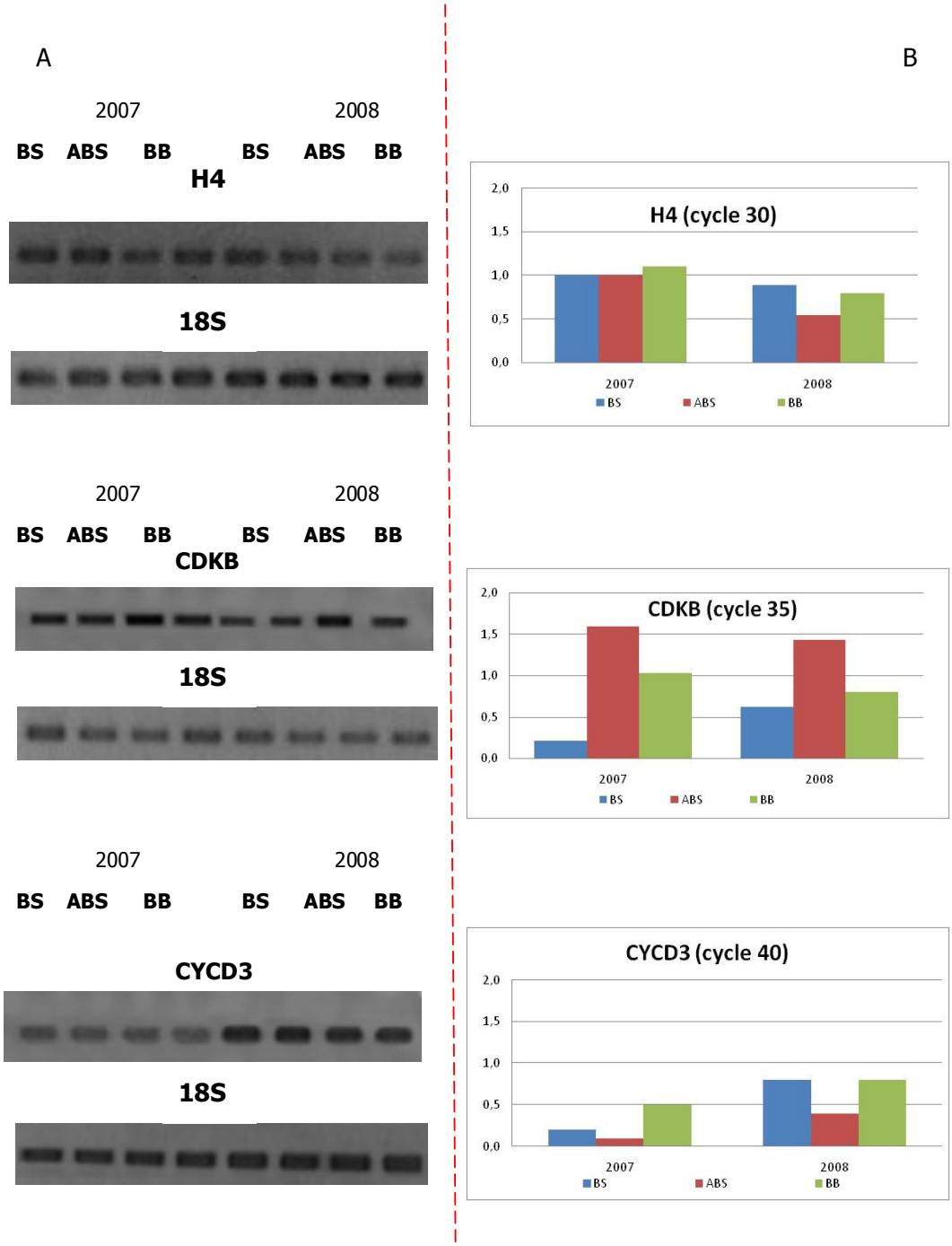


Figure 23. A-B Semi-quantitative RT-PCR of first-strand cDNA synthesized from total RNA of kiwifruit bud collected during bud release for Histone H4, CDKB and CYCD3. 18S RNA was amplified as internal control. In A the Et-Br stained gel is shown. In B the computer-assisted quantification of each gene is presented as the ratio of the gene signal divided by the 18S signal. Bs: bud-swell; ABS: advance bud swell; BB: bud-break.

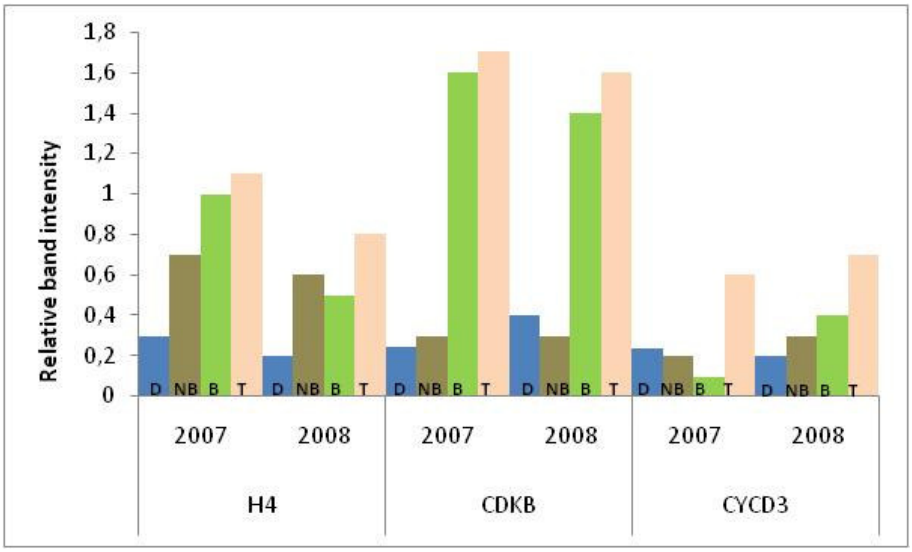


Figure 25. Computer-assisted quantification of semi-quantitative RT-PCR of cDNA synthesized from total RNA of kiwifruit bud collected at different phenological stages, using specific primers for Histone H4, CDKB and CYCD3. 18S RNA was amplified as internal control. Transcript quantities are presented as ratio of the gene signal divided by the 18S signal. D: dormant; NB: "non breaking"; B: Breaking; T: 6-BA (200 ppm) treated

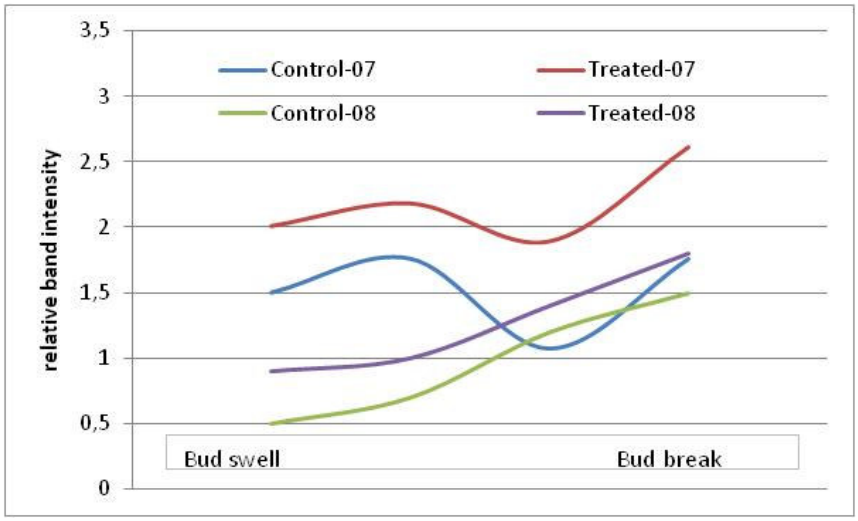


Figure 24. Computer-assisted quantification of semi quantitative RT-PCR of cDNA synthesized from total RNA of treated and untreated kiwifruit buds starting from bud swell till bud break, using specific primers for isopentenyltransferase gene (IPT) during the 2007 and 2008. 18S RNA was amplified as internal control. Transcript quantities are presented as ratio of the IPT signal divided by the 18S signal

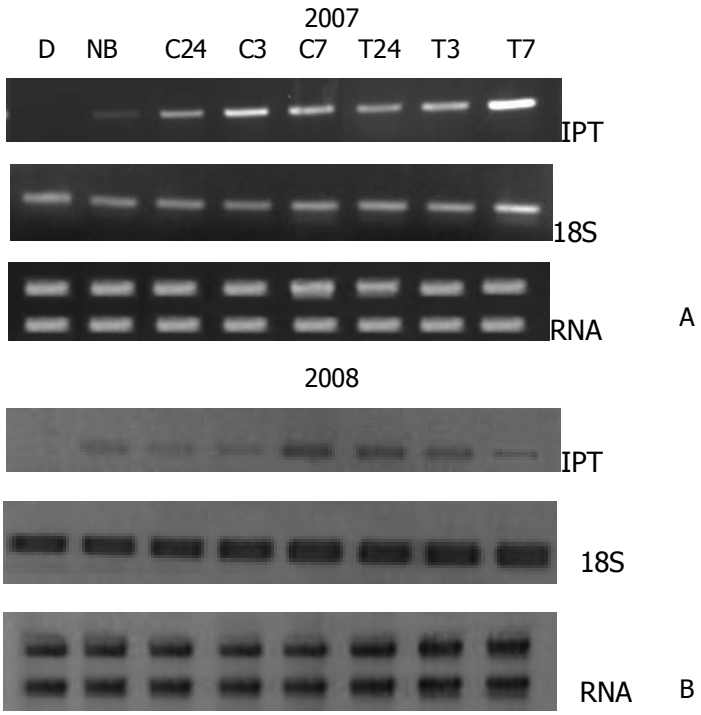


Figure 26. A-B Semi-quantitative RT-PCR of first-strand cDNA synthesized from total RNA of kiwifruit bud collected at different phenological stages of development during the 2007 (A) and 2008(B) seasons, using specific primers for isopentenyltransferase gene (IPT) and stained in Et-Br. 18S RNA was amplified as internal control. D: dormant; NB: "non breaking"; C: control breaking. T: treated with 6-BA 200ppm at bud swell; 24: 24h, 3:3 days, 7:7 days after the 6-BA application

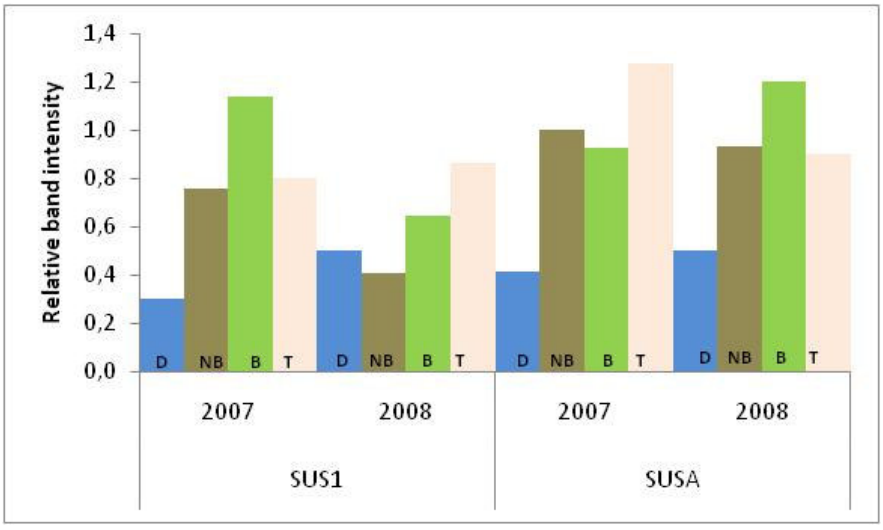


Figure 27. Semi-quantitative RT-PCR of first-strand cDNA synthesized from total RNA of kiwifruit bud collected at different phenological stages during the 2007 and 2008 seasons, using specific primers for SUS1 and SUSA. 18S RNA was amplified as internal control. Transcript quantities are presented as ratio of the gene signal divided by the 18S signal. D: dormant; NB: "non breaking"; B: Breaking; T: 6-BA 200 ppm treated.

Histological analyses on the SAM

DAPI staining on median longitudinal shoot apical meristem of kiwifruit buds, collected at different phenological stages of development, showed differences in the number of dividing cell between buds collected at the beginning of January (dormant) and the ones collected at bud swell (mid of March) both breaking and "non breaking" ones (Figure 29 B-D-F). In particular in the SAM of dormant buds, it was not possible to detect any dividing cells. A similar situation was observed in "non breaking" buds characterized by a low cell division rate. The proliferation in the SAM increased in untreated and treated breaking buds as shown in Figure 28 (A-F). In these tissues in fact, it was possible to count a consistent number of dividing cells.

The correspondent analyses of the mitotic index confirmed that there is no activity in dormant buds while an activation of cell division at bud swell, which is stronger in the one which are able to resume growth, occurred (Table 34). It's important to underline that a very low rate of activity was detected also in buds which did not break. In addition, from the statistic analyses it clearly emerged that the number of cell division is different in treated and untreated breaking buds particularly in the peripheral zone and in the rib meristem. Moreover, treatment with 6-BA (200 ppm) seemed to further enhance the number of cell divisions in breaking buds, especially at the primordial levels . In contrast, the analyses on the total cell number measured in the SAM layers of different samples evidenced no differences in the SAM of all the analyzed samples (Table 35).

Table 34. Cell division rate, expressed as mitotic index percentage, of the tissue layers in the SAM of kiwifruit buds at different phenological stages: L1 is the external layer of the apical meristem, L2 is the underlying layer, RM is the inner one, PL and PR are the leaf primordia on the left and on the right respectively;. Different letters represent significant difference for $P < 0.05$ (Duncan's Test)

	Mitotic Index (%)					
	L1	***L2***	***RM***	***PL***	***PR***	***Average***
Dormant	0,0 a	0,0 a	0,0 b	0,0 c	0,0 c	0,0
Non Breaking	0,0 a	0,0 a	0,1 b	0,0 c	0,4 c	0,1
Breaking (control)	3,9 a	1,4 a	2,4 a	1,6 b	2,4 b	2,3
Breaking (treated)	1,9 a	1,7 a	1,7 a	3,2 a	3,9 a	2,5

Table 35. Number of total cells (dividing and non dividing ones) of the tissue layers measured in the SAM of kiwifruit buds at different phenological stages: L1 is the external layer of the apical meristem, L2 is the underlying layer, RM is the inner one, PL and PR are the leaf primordia on the left and on the right respectively; Different letters represent significant difference for P < 0.05 (Duncan's Test)

	Total Cell number				
	L1	***L2***	***RM***	***PL***	***PR***
Dormant	22,7 a	19,3 a	290,0 a	141,7 a	163,3 a
Non Breaking	22 a	20,7 a	280,0 a	113,3 a	100,0 a
Breaking (control)	24,3 a	23 a	323,3 a	143,3 a	150,3 a
Breaking (treated)	21,7 a	21,7 a	266,7 a	103,3 a	101,7 a

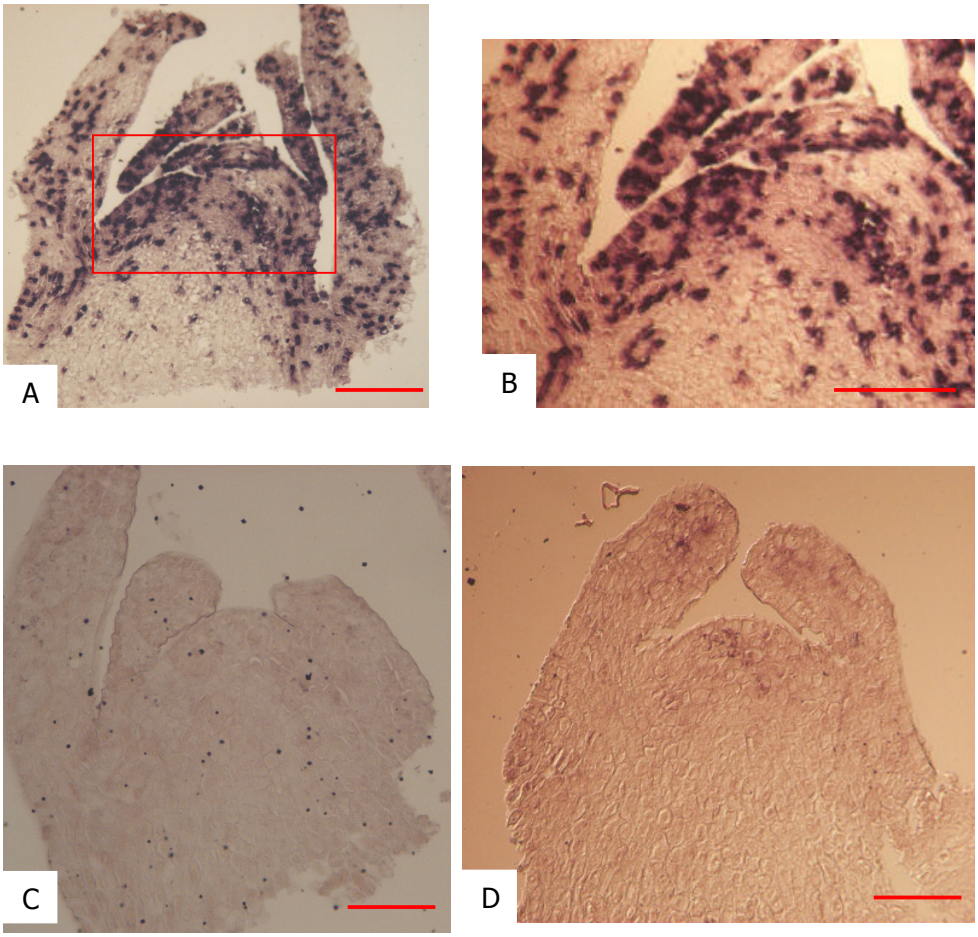


Figure 28. *In situ*-hybridization analyses with DIG-labeled histone H4 probe on median longitudinal section of kiwifruit's SAM collected from breaking dormant (A-B), non breaking (C) and dormant buds (D). Black color indicate the presence of the transcript. Detail of the area box in B (20X magnification with bright field. Bar= 100μm

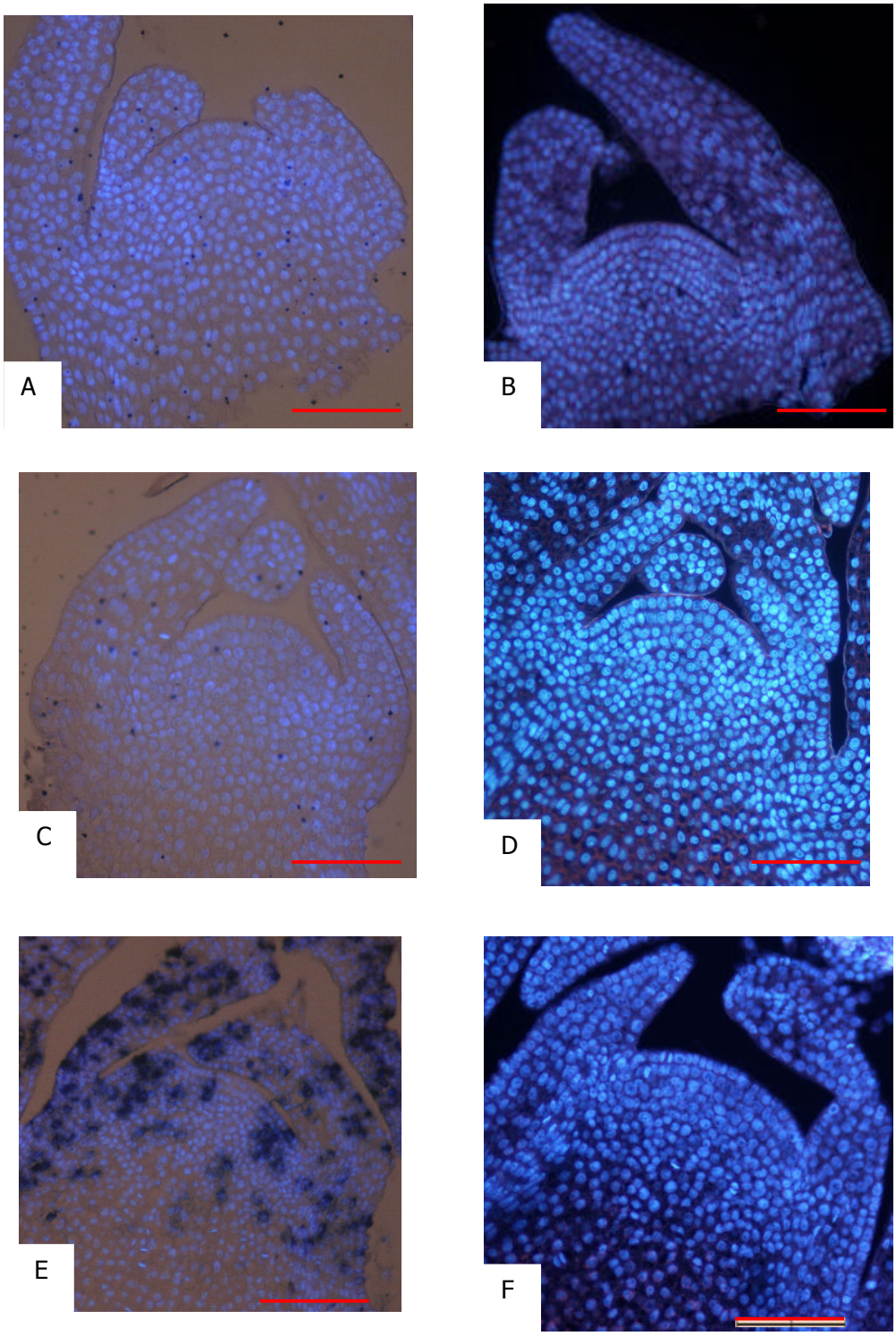


Figure 29. *In situ*-hybridization analyses with DIG-labeled histone H4 probe and DAPI staining on median longitudinal section of kiwifruit's SAM collected from dormant (A-B), non breaking (C-D) and breaking buds (E-F). Black color indicate the presence of the transcript. In A-C-D a combination of bright and UV filter was applied. In B-D-F only UV-filter was applied. Bar= 100μm

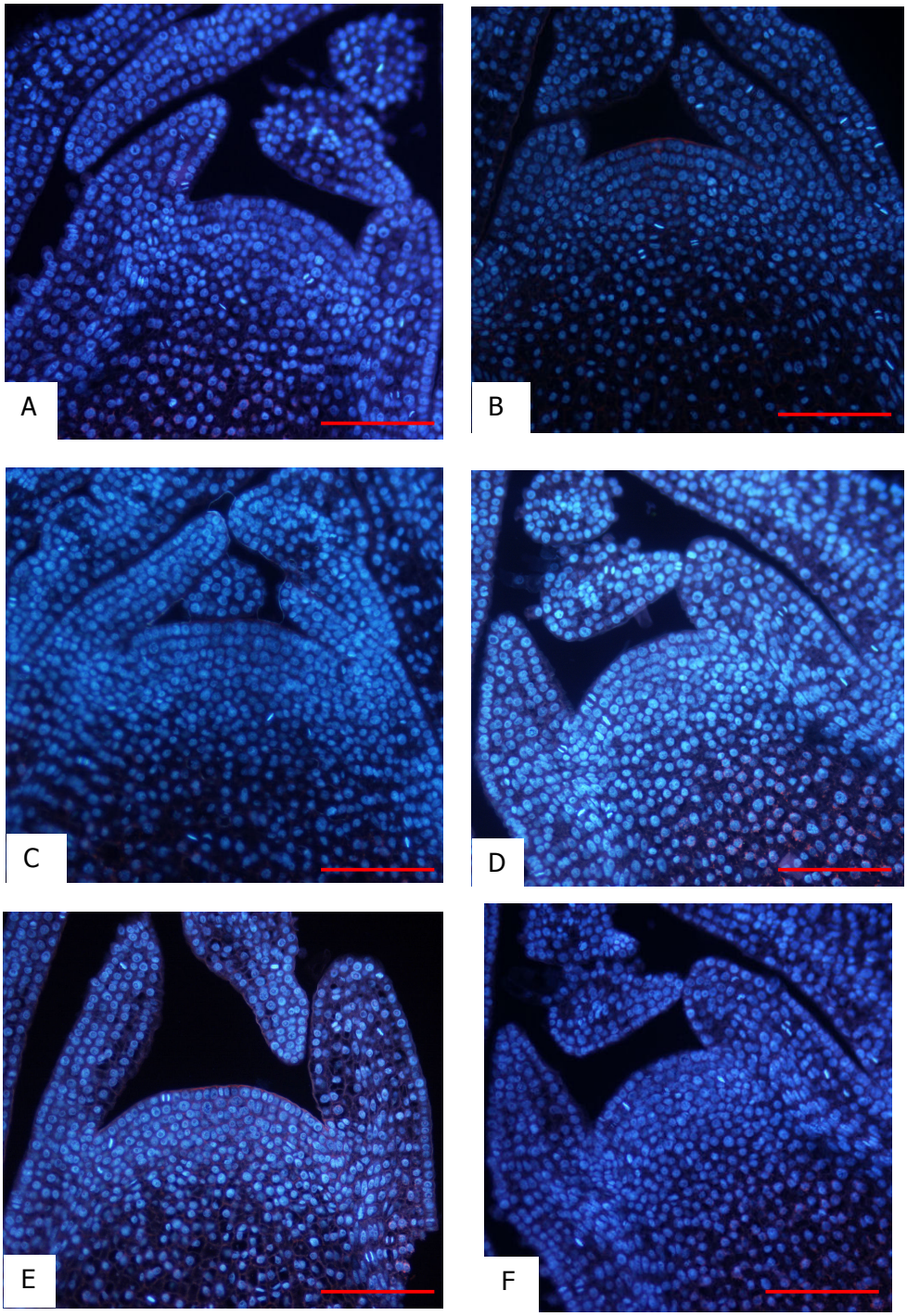


Figure 30. DAPI staining on median longitudinal section of kiwifruit's SAM collected from untreated (A-C-F) and treated (B-D-F) breaking buds

5.Discussion

Cytokinins as dormancy breaking agent

The current research, planned to test the natural molecules CKs as DBA, confirm that these hormones may have a possible agronomical use to this purpose In fact, both the commercial product Cytokin, applied as a source of natural CKS, and the pure CKs, kinetin and much more 6-BA, showed a clear action on kiwifruit bud release in the producing areas selected for the trial, where winter chilling is normally satisfied. Implication of CKs to promote budburst are reported in other crop species. In particular, some of them were successfully applied to stimulate the growth of dormant apple buds (Steffend and Stutte, 1989) as well as to supply for the chilling requirement in dormant peach buds (Weinberg, 1969). Except for these studies, no additional and more recent data are available on CK applications in open field conditions, especially for kiwifruit. During the three-years trial of this research, different CK positive effects were recorded on bud break, flowering and fruiting development, fruit size and quality. The obtained results will be discussed in different pharagraphs mainly in comparison with the only data available in literature, that are data from the use of HC, even if it is to underline that this compound completely differs from CKs both in the chemical nature and in the mode of action.

Bud break

As regard to the main effect desired from DBA application, i.e. to increase and uniform bud break, Cytokin was able to induce higher bud break percentage as well as higher uniformity of bud outgrowth. The best results were obtained with the application at bud swell, just before resumption of growth; while a lower efficacy was

recorded with an earlier application (45 days before bud break). In both cases, and even at much more higher concentrations, Cytokin application did not cause any side effect neither on the vegetation nor on the fruits. This is in contrast with the effect induced by HC, whose application close to the time of kiwifruit bud break caused severe phytotoxicity with a reduction of bud which start breaking (Costa et al., 1995). Thus, differently from HC, it is possible to suggest the application of Cytokin later during the growing season when the incidence of bud outgrowth along the canes becomes more easily predictable and it appears the real need of an agronomical action.

In addition, an earlier bud break was never observed after Cytokin applications while it was often reported after HC treatments (Walton et al., 1998; Inglese, 1998; Powell et al., 2000). This data has especially to be considered in area where frost can occur in late spring with a possible damage to the young shoots.

Considering the well known relationship between exogenous application of growth substances and climatic conditions, the obtained results showed that Cytokin efficacy seemed to be more evident when the winter chilling is satisfied. During the 2007 season, in fact, when warm winter temperatures characterized the quiescent period, Cytokin decreased its efficacy compared to HC treatments in the Faenza area. Despite this lower efficacy, positive results were obtained on flower fertility, reduction of side flowers and fruit production at harvest. It has also to take into account that warm temperatures could positively influence the incidence of bud outgrowth, as observed during the 2008 year in the Bologna area. In this area and in that year, in fact, the lower efficacy recorded for Cytokin is more likely to be related to the warm conditions occurred just before bud break which enhanced bud release of both treated and untreated plant more than to a satisfaction of the winter chilling.

Flowering and fruiting

Flower differentiation in mature overwintering buds started just before bud break (Brundell, 1975; Politi and Grant, 1984). For this reason a direct effect of DBA application on flower fertility is predictable. Results showed that Cytokin constantly and positively influenced the complete development from undifferentiated inflorescence into female flower: treated plant, in fact, always showed a number of flower per shoot higher than the untreated ones. This constant effect is not always present after other DBA applications. In particular, contrasting results were reported in literature on HC effects. Some authors reported an increase of kiwifruit flower fertility after HC

treatments (Walton and Fowke, 1993; Powell et al., 2000; Montefiori, 2003); on the contrary, a study carried out in two different areas of Italy reported a decrease of kiwifruit flower fertility as well as of the number of fertile shoots in the Northern orchard (Vizzotto et al., 1996).

The Cytokin positive effect on flower development was exerted also on the reduction of side flowers. In kiwifruit both staminate and pistillate flowers are potentially a compound dichasium comprising a terminal flower and primary and secondary lateral flowers. In Hayward, the primary and secondary lateral flowers often aborted, but in some years it can occur that they are able to complete their development and 2-3 flowered inflorescences are produced thus preventing the correct development of the central flower (Warrington and Weston, 1990). For this reason the thinning effect induced by Cytokin application could be particularly appreciated when an increasing number of side flowers develops during the growing season. This effect is consistent with the well known action of CKs as thinning agent. 6-BA, in fact, is successfully applied as chemical agents to reduce crop load in different apple cultivars (Costa et al., 2001; Bregoli et al., 2006; 2007). As emerged from these researches and other in literature, the thinning efficacy of natural substances is closely related both to weather conditions and phenological stage of development at the application time. In this trial, the thinning effect on side flowers was induced in both producing areas with a different intensity probably linked more to the earlier application time than to an effect related to the geographical area. The highest effect, in fact, was constantly achieved with the earliest application (45 days before bud break) when buds are supposed to be in the endo dormant period. These results are in accordance with those reported for HC in Northern areas, where early applications (at mid of January) caused a clear reduction of the 3-flowered inflorescences (Montefiori et al., 2003).

As regard to flower development, Cytokin treatments can induce an anticipation of the pistillate flowering thus increasing the simultaneity with staminate open flowers and consequently improving fruit pollination. In this specie, characterized by dioeicious nature of the vine, an adequate pollination is necessary to permit adequate fruit set. For this reason, it becomes of relevant importance the synchronization of pistillate and staminate flowering. Among the various factors that could affect the flowering period, once more time, the weather conditions play the most important role: kiwifruit flowering, in fact, is strongly influenced by climate and weather (McPhearson et al., 1994). The relative mild temperatures, recorded during the trial immediately before and at flowering time in both the Faenza and Bologna areas, could explain the positive

effects on fruit pollination that were always recorded. Moreover, the obtained results were in accordance with the enhancing flowering and consequently higher crop levels reported for HC (Henzell and Briscoe, 1986; Walton and Focke, 1993).

Fruit size and fruit quality

In this study positive effects were constantly achieved at harvest in both areas of trials. In particular, Cytokin applications always increased fruit fresh weight without any reduction in the crop load. The reported increase in fruit size was also confirmed by the analyses on the fruit size distribution at harvest: Cytokin treated fruit were always distributed towards the highest size classes. This positive effect was in some cases reflected on yield: in the Faenza area, in fact, Cytokin constantly enhanced the production. No data are available on the effects of early CK applications on the productive parameters at harvest and, as above stated, it is also possible a comparison with data obtained after HC treatments. From this comparison it emerged once again that the effect induced by CKs treatment are more constant and uniform with respect to those obtained by HC applications on the same specie. Many authors reported a better class distribution for HC-treated fruits (Velosa and Oliveira, 2002; Montefiori et al., 2003). Moreover, Vizzotto et al. (1996) observed that crop productivity was increased after HC treatments performed in a orchard located in the South of Italy. Also Costa and coauthors (1995) observed a fruit weight increase after HC treatment, but, differently from the present and above reported data, they recorded a reduction in the crop load. The absence of any effect on the quality parameters measured at harvest as well as after cold storage agrees with similar analyses obtained on HC treated fruit (Powell et al., 2000).

Bud release mechanisms

The positive results obtained from the field trial performed in this research suggested that CKs can exert an important role in the control of bud release from dormancy as previously demonstrated for HC (Keilin T et al., 2007; Mathiason et al., 2008). In particular, molecular studies revealed that temporary oxidative stress and respiratory stress induced by HC treatment seemed to stimulate a cascade signalling that leads to bud break. Also in kiwifruit a similar mechanisms was evidenced by Walton et al., 2006. In contrast, no studies are available in this specie, as well as in more studied ones, on the exogenous CKs mode of action at physiological level. For this reason, in the present research pure CKs were applied in open field conditions to

obtain a controlled system and to perform molecular and histological analyses on untreated buds and buds treated with 6-BA, the CK that showed the highest efficacy.

A stress effect hypothesis can be excluded as explanation of the CKs mode of action for two main reasons that could be easily deduced from the literature cited in the "Introduction" of this thesis. Firstly, they are completely different from the other DBA normally utilized in field and in particular from HC: they are, in fact, molecules of natural origin having an hormonal role that did not interfere with basal survival mechanisms of the cells, as for example respiration. Secondly, they were applied in field at concentrations lower than those normally used in more sensitive systems, as for example poplar or apple shoot explants growing in greenhouse (Cline and Dong-Il, 2002; Tworkoski and Miller, 2006.

A more suitable hypothesis takes into account the CKs involvement in cell division and suggests a direct effect on kiwifruit bud break and shoot growth with implication in cell cycle entry. No data are available on cell cycle and on cell division activation caused by exogenous CKs during the early phases of kiwifruit bud break in open field condition. Only Moncalean et al. (2001) reported in *in vitro* cultured kiwifruit buds an immunocytochemical localisation of endogenous CKs in response to exogenous 6-BA and gibberellins.

Natural release

Histological analyses in the SAM of kiwifruit buds collected at different phenological stages (dormant, non breaking and breaking) were carried out to verify the predictable reactivation of cell division which occurs during bud release. This goal was achieved by performing DAPI staining of nuclei and by calculating the mitotic index and the cell number of the different SAM tissues. These analyses confirmed that, in kiwifruit, the cell division activity of the meristem is arrested during the endo dormant phase while a reactivation occurred during the release from dormancy. This not surprising result is consistent with data from the literature that usually report the absence of cell division activity in dormant plant tissues and are extensively reviewed by Horwath et al., 2003, Rhode et al., 2007) . This lack of cell divisions disappeared when buds overcome dormancy and regain their ability to growth. As reported for other species, a consistent proliferation occurred at kiwifruit bud break, with differences in the mitotic index between the peripheral and the central zone of SAM. More in detail, the former showed higher mitotic index than the latter and this data is consistent with the observations reported in literature. Lyndon and Robertson (1976) ,

in fact, found that cells in the central zone are considered to be undifferentiated and they can divide indefinitely; cell proliferation in the central zone caused the displacement of cells into the pheripheral zone where they divided more quickly and contribute to the formation of the stem and lateral organs. It is also of particular interest to note that in the present research a low rate of cell division was also found in the SAM of buds that will not break during the current season. This is in accordance with the hypothesis of Foster (2007) who stated that in this specie all the overwintering buds has the potential to restart growth.

The obtained results induced further molecular investigations on genes involved in the reactivation of cell division and cell cycle, as for example histones, cyclins, and cyclin-dependent kinases (Horvath et al., 2003). A coding sequence for histone H4 was for the first time identified in kiwifruit bud and cloned to perform *in situ* hybridization in buds collected at different phenological stages. *In situ* detection of histones genes, in fact, has proven to be a powerful tool for identifying cells that are in a specific phase of a cell cycle (Lee et al., 2005). In particular, it is well known that Histone H4 expression is primarily DNA replication dependent in plant and thus, under normal growing condition, high H4 expression levels are considered to be indicative of the S-phase (Gaudin et al., 2000). *In situ* analyses on the SAM of kiwifruit bud samples (dormant, "non breaking", "breaking") confirmed the cytological changes in the SAM of breaking buds compared to the dormant and "non breaking" one. In kiwifruit, the histone-expressing cells distribution, which resulted to be higher in the pheripheral zone, is similar to the one detected in lettuce vegetative shoot apices where the i*n situ* analysis allowed to identify the moment of floral initiation, phenomenon characterized by an increment of the meristematic activity in the central zone of the apex induced to flower (Lee et al., 2005).

After the identification of histological and histone H4 expression differences among the selected samples, a comprehensive molecular study was performed by using semi quantitative RT-PCR analyses on dormant, "non breaking" and breaking buds. The expression pattern of the above reported genes involved in the cell cycle (Histone H4, CYCD3 and CDKB) were considered as well as of others which are known to exert a role in the reactivation of cell division (IPT, SUS1 and SUSA). In detail, analyses of the transcript accumulation of Histone H4 confirmed the highest levels measured in "breaking buds" compared to the ones collected in the endo dormant phase. This result is consistent with similar results obtained by Mazzitelli and coauthors (2007) in raspberry where a cDNA microarray was constructed and used to determine

differential gene expression during dormancy release. In this study, a gradual increase in mRNA histone H2 and H3 levels was recorded towards dormancy release. Moreover, in kiwifruit, it was also possible to identify an H4 transcript accumulation in non breaking buds, which is in accordance with the presence of a low rate of dividing cell in the correspondent SAMs. CYCD3 are well know studied protein which are involved in the regulation at the G1 level during the mitotic cell cycle (Riou-khamlichi et al., 1999); for this reason, CYCD3 gene was for the first time identified in kiwifruit bud, cloned and its transcript profile was analyzed by semi quantitative RT-PCR. In general, it was difficult to understand the pattern of expression of this gene in kiwifruit bud samples due to its low level. Despite this, results seemed to show a slight increase in the mRNA abundance of this gene from the endo dormant phase towards bud swell. This low accumulation in the dormant period is in accordance with the down regulation of CYCD3 which was found in inactive meristem of dormant poplar buds occurring after four weeks of short day length (Ruttink et al., 2007). The poplar inactive meristem, in fact, is characterized by nuclei with less developed nucleoli, indicating reduced synthesis of ribosomal structure and more than four-fold down regulation of genes, including the core cell cycle regulators such as Cyclin A1 and A3, Cyclin B2, Cyclin D3, Cyclin Dependent Kinase B1 and B2. As regard to the expression measured during kiwifruit bud release, the relative increase detected is in accordance with the localization of Cyclin D3 gene measured in the snapdragon apical meristem and floral buds (Murray, 1997). In these tissues, cyclin D3 gene expression remained relatively constant during the cell division cycle suggesting the idea that D-cyclins operate in a regulatory pathway upstream of the cell cycle and that locally increased levels of D-cyclin may enhance local potential for proliferation.

Cyclin dependent kinase B1 was also analyzed by RT-PCR to investigate its role during bud release (Ruttink et al., 2007). During the kiwifruit dormant period, a down regulation similar to that reported for the other genes involved in the cell cycle was observed. The low expression levels detected in the dormant phase is in accordance with the down regulation detected in poplar buds inactive meristems (Ruttink et al., 2007). In contrast with the other two genes, at kiwifruit bud release CDKB transcript profile changed dramatically, with a sharp increase was detected in buds which have started to growth. This high expression is related to the CDKs B stimulus role in the initiation of the G2-M transition during the cell cycle (Mironov et al., 1999). CDKs B complex, in fact, are known to initiate the phosphorylation and the expression of genes

required for cytokinesis. No further evidences are reported in other plant species on CDKs B pattern of expression during bud release.

In accordance to works where overexpression of CK biosynthetic genes increased CK levels and reduced correlative inhibition in axillary buds (Faiss et al., 1997) as well as to the fact that increased CK level can induce Knat1, a gene involved in meristem growth and development (Frugis et al., 1999), the first key enzyme of the CK biosynthetic pathway was firstly identified in kiwifruit bud and its expression was investigated by semi quantitative RT-PCR. IPT transcripts were found to increase during bud break in both year of trials. This IPT accumulation is in accordance with the changes in mRNA ATIPT1 and ATIPT5 transcripts found in Arabidopsis floral and axillary buds (Miyawaki et al., 2004). An absent and a relative low expression were detected in kiwifruit dormant and "non breaking" buds respectively, in accordance to the fact that IPT genes are expressed ubiquitously, with highest expression in proliferating tissue.

Semi-quantitative RT-PCR analyses of the transcript level of two sucrose synthase genes, SUS1 and SUSA, whose sequences are available for kiwifruit, were also analyzed. Data showed the presence of transcripts during the dormant period with an increment reported at bud release. This data are partially in contrast with the work of Richardson et al. (2007) who measured the sucrose content in kiwifruit meristems from dormancy to bud break phases. He found that sucrose concentration increased in autumn till the beginning of winter, afterwards it remains stable suggesting the low metabolic activity in dormant buds. Sucrose concentration started to decrease rapidly during early spring before visible bud growth with increment in hexose concentration, which indicates that meristem had been released from dormancy. In this study, the pattern reported in the expression of both genes during the dormant period confirmed the low rate of metabolic activity during the 2007, while during the 2008 the higher transcript level founded suggested that even if no visible growth was detectable, bud has released from dormancy and it is probably working as driving force for the sugar supply. Sugars in fact represent the most important organic resources and it is indispensable for cell division (Wobus and Weber, 1999). No clear differences were detected between breaking and "non breaking" buds thus suggesting that buds which do not resume growth represent an active sink who is competing for the reserves. Their inhibition of growth is likely to be stimulate by other factors. This increment in the expression of sucrose synthase genes is in accordance with other works where

genes involved in carbohydrate metabolism are stimulated during bud release (Ruttink et al, 2007; Mazzitelli et al., 2007).

Cytokinins role

Molecular and histological analyses performed on kiwifruit treated bud samples evidenced the role exerted by exogenous CKs when applied at bud swell. In particular an up regulation of all the genes involved in the cell cycle was recorded. CYCD3, despite its low expression seemed to be particularly up regulated. This is in accordance with previous works where D- cyclins are proposed to transduce extracellular growth signals by altering the potential for cell proliferation (Sherr, 1995). In particular Gaudin (2000) found a 2.5-fold induction for CyclinD3a and CyclinD1 after exogenous CKs application to snapdragon seedlings. Similar results were observed in *Arabidopsis* where exogenous zeatin caused an increase in CycD3 mRNA levels (Riou-Khamlichi et al., 1999; Oakenfull et al., 2002). A lower effect was measured on Histone H4 and CDKB. This slight increment of both genes expression could be however be sufficient to enhance cell proliferation. No evidences were found in other species on the effects of CKs on the transcript profiles of CDKs B. The overall data on the transcript levels found in treated buds are in accordance with the higher mitotic activity detected. Hystological analyses, in fact, showed that exogenous CKs application increased the rate of cell division particularly in the pheripheral zone of the SAM of breaking buds. This zone is known to be the one where lateral organs formation starts biblio cri, thus it is possible to speculate that CKs application can enhance organ formation mainly acting on this pheripheral zone. A constant up regulation was also detected in the IPT gene expression and consequently in the CK biosynthesis. Higher values of transcript were found in treated buds compared to the untreated ones during bud release. This data was expected as CKs application can increase the endogenous CKs level. Kuiper et al. (1988) demonstrated that the endogenous CK budget of *Plantago major* ssp. *Pleiosperma* respond quickly to the supply of an artificial CK. However, this correlation is not always so stict, in fact, in Arabidopsis exogenous CK application induced a down regulation of their own biosynthesis (Miyawaki et al., 2004). As regard to the effects of exogenous CK application on sugar accumulation, no clear relationship was found, and from the obtained results it could stated that any direct effect does not exist. These not surprising findings are in accordance with other studies in other crop species, which stated that sucrose synthase were not CK responsive (Roitsch and Ehneβ, 2000).

6. Conclusions and Future Perspectives

The application of Cytokinins in different producing areas of the Emilia-Romagna region positively affected kiwifruit bud release from dormancy thereby improving productive and quality traits at harvest. These positive effects were recorded almost throughout all the years of the research even if applications were performed at different stages of bud development. Kiwifruit buds, in fact, seemed to be always receptive to the exogenous Cytokinins and the different sensitivity of the phenological stage generated differences in the final responses (reduction in the number of side flower rather than synchronization of bud release and young shoots growth), but did not cause any negative effect. This aspect joint to the absence of any phytotoxicity on fruit and tree (even at concentration 16 times more than the labeled dose), as well as to the Cytokinins natural origin, makes these hormonal substances particularly suitable in a modular schedule planning consecutive treatments and/or the use of different substances.

In addition to the above reported positive effects induced on plant development, the observed Cytokinins tendency to thin lateral flowers could allow a reduction of the time and money required for the manual practices that are usually necessary to obtain satisfactory productive performances from kiwifruit vines at harvest.

In conclusion, data obtained from this three-years trial showed that it is possible to obtain useful information on the role of exogenous Cytokinins during kiwifruit bud release and to induce effects on this phenomenon that could differ in relation to the real grower expectations.

Future trials that could be planned on these hormones have also to consider the different chilling exposure of the kiwifruit producing areas in Italy. In fact, even though this trial was carried out in two different producing district, the limited geographical area, extended only in the Emilia-Romagna region, did not allow to verify the Cytokinins efficacy when warm winters are usual conditions.

Considering that from this research it clearly emerged that Cytokinins could be successfully utilized to manipulate bud break timing an understanding of the biological mechanism involved in bud-dormancy release is crucial. Although extensive studies have been performed on various physiological aspects of dormancy, a characterization of the complex network of biochemical and cellular processes responsible for the regulation and execution of bud dormancy release has not yet been achieved in any

crop species. In kiwifruit, especially, few data are available, and this is one of the first research investigating the reactivation of the cell cycle and the role of exogenous Cytokinins both at cellular and subcellular level in bud tissues. Thus, this work did not pretend to completely elucidate the complex mechanism which control bud release from dormancy. The purpose was simply to identify a starting point, suggested by the field observations, to understand if Cytokinins can exert a role in this process as reported for other natural or artificial agents.

For the first time, some of the genes most involved in the regulation and reactivation of cell cycle were identified in kiwifruit buds. The isolated sequences of Histone H4, Cyclin D3, and isopentenyltransferase were submitted on the EMBL public database and they revealed high homology with other plant H4, CycD3 and IPT sequences.

Additional and new information on the reactivation of cell division in kiwifruit bud were obtained by performing histological and molecular analyses. In particular, histological investigations on the mitotic index and "*in situ* hybridization" analyses of histone H4 expression in the apical meristems of kiwifruit buds collected at different phenological stages showed that, as also reported for other more deeply studied species, dormant tissue and "breaking buds" are at the opposite side while "non breaking" buds were in an intermediate situation. Thus, as also hypothesized by other in the same specie, we can conclude that the onset of organogenesis is the default state in kiwifruit bud and that all the buds along the cane have initially equivalent developmental potential and they could be receptive to additional external stimuli, as for example application of hormones.

RT-PCR analyses confirmed the above described situation, the transcript expression profile of H4, CycD3, CDKB1, SUS1, and SUSA genes being gradually up-regulated starting from buds collected in the endo dormant period through the "non breaking" ones up to those collected at bud swell. Moreover, the latter showed also the highest expression of IPT. Considering that the analyzed sequence correspond to the conserved region of a gene codifying for a rate-limiting enzyme in the Cytokinins biosynthesis, an endogenous accumulation of these hormones could be suggested in kiwifruit buds which restarts their growth, as also well established in other plant systems.

Starting from this natural conditions, analyses performed on the effect of exogenous Cytokinins had shown that the 6-benzyladenine was particularly active, causing an up regulation of the above reported genes, except for SUS1 and SUSA

involved in the carbohydrate metabolism, in breaking treated buds. The comparison with the natural situation allows to draw a possible hypothesis on the mode action of exogenous Cytokinins on kiwifruit bud release. All the buds along the cane have the competence to resume growth, but some of them are blocked at the "non-breaking" status characterized by the low expression levels of the main genes involved in the process; the supply of exogenous Cytokinins seems to overcome this block either by directly stimulating the expression of the histone H4, the Cyclin D3, and the Cyclin dependent Kinase B1, or, more likely, in an indirect manner by increasing the levels of IPT transcripts and thus of the endogeonous CKs.

7. References

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10. Abbreviations

6-BA: 6-Benziladenine

ABA: Abscisic acid

CDKB: cyclin-dependent-kinase B

CDK-CDKs: cyclin-dependent serine-threonine protein kinase

CK: cytokinesis

CKs: cytokinins

CYCD3: cyclin D3

CZ: central zone

DBA: dormancy breaking agents

FOABs: first-order axillary bud

G1:post-mitotic-interphase

G2: and post-synthesis-interphase

GA: giberellic acid

HC: Hydrogen Cyanamide

IPT: adenosine phosphate-isopentenyltransferase

M: mitosis

PZ: peripheral zone

RZ: rib zone

S: DNA synthesis phase

SAM: shoot apical meristem

SDSMm: dome shape meristem

SOABs: first-order axillary bud

SUS1: sucrose synthase 1

SUSA: sucrose synthase A

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