DISSERTATION

Evaluation of Plant Characteristics and Disease Resistance in Cu-IPT Transformed Watermelon cv. Crimson Sweet

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY FAHRETTIN GOKTEPE ENTITLED EVALUATION OF PLANT CHARACTERISTICS AND DISEASE RESISTANCE IN Cu-*IPT* TRANSFORMED WATERMELON cv. CRIMSON SWEET AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

Evaluation of Plant Characteristics and Disease Resistance in Cu-*ipt* Transformed Watermelon cv. Crimson Sweet

Watermelon cv. Crimson Sweet was transformed with the copper inducible isopentenyl transferase gene (Cu-*ipt*) via *Agrobacterium* mediated gene transformation process. The *ipt* gene governs the rate-limiting step in the cytokinin biosynthesis pathway. The transformants were confirmed via polymerase chain reaction (PCR) in the plant with *ipt* specific primers. Cu-*ipt* transformed plants were treated with copper sulfate at a concentration of 5, 10, or 50 μM copper sulfate to determine if the gene could be activated by copper at three levels. Transformed plants treated with copper sulfate differed in evaluated horticultural characteristics from those non-transformed as well as transformed plants not sprayed with copper sulfate. Delayed leaf senescence, increased chlorophyll content, reduced apical dominancy and released axillary buds were significantly different in Cu-*ipt* transformants compared to non-transformant plants. Significant reduction of seed number in watermelon fruit was also observed in copper sulfate treated Cu-*ipt* plants as compared to the non-transformant plants. Other than some slight alterations, elevated endogenous cytokinin level didn't cause major interference with transformants

normal growth and development. The application of copper sulfate also induced resistance against Gummy Stem Blight disease in Cu-*ipt* transformants and their seedlings compared to the non-transformant plants.

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1.1 Introduction

Watermelon, *Citrullus lanathus (Thunb.) Mansf.* is a member of the *Cucurbitaceae* family, which includes muskmelon, pumpkin, squash and gourd. Watermelon is a tendril-climbing, annual, herbaceous plant (Compton and Gray, 1996; Mohr., 1986). Like other members of the *Cucurbitaceae* family, it is also sensitive to chilling injury and frost damage.

Most watermelon cultivars require a relatively long growing season and high temperature with abundant sunlight for plant growth and fruit development. Watermelons are relatively more productive in regions that have at least four months of frost-free growing season, with approximately 80 to 120 days of 21-27 °C days and 18-21 °C nights for adequate maturation of the fruit (Allred and Lucier, 1990). Since watermelon is normally not sensitive to extreme humidity, it can be cultivated in many regions of the world. However, atmospheric humidity is the factor that may affect the occurrence and severity of fruit and foliage diseases such as Gummy Stem Blight. Most watermelon cultivars require a well-drained, acidic (pH 5.0-6.5), sandy loam soil with a good water supply during the planting and fruit-development period. Watermelon tolerates dry conditions better than excess water, with good fruit quality based on warm, dry weather conditions.

Watermelon cultivars are generally monoecious, with staminate and pistillate flowers. Watermelon flowers, which are usually singly located in leaf axils, are relatively smaller and less attractive than the other species of cultivated *Cucurbitaceae* (Mohr,

1986). Although watermelon is considered to be a cross-pollinated crop, with honeybees providing the pollination, it possesses a high degree of self-pollination as well. Except for the seedless watermelon varieties, which are triploid with parthenocarpic fruit, cultivated watermelons are diploid (11 pairs of chromosomes) annuals and generally out-cross (Lee et al., 1996).

Since a number of wild types with bitter and also greater than normal fruit have been found together growing wild in the Kalahari Desert, watermelon is believed to have originated in Africa. From there it spread to Egypt, India and China, with the cultivation of watermelon dating back to pre-historic times, as demonstrated by pictures drawn in ancient Egypt (Mohr, 1986). Based on average growth as listed in United States Department of Agriculture's records, China had the greatest watermelon production over the past ten years (Figure1.1), with Turkey, Iran, Egypt and the United States ranking second, third, fourth and fifth, respectively. Production of China has steadily increased to around 1,180 million cwt in 2001.

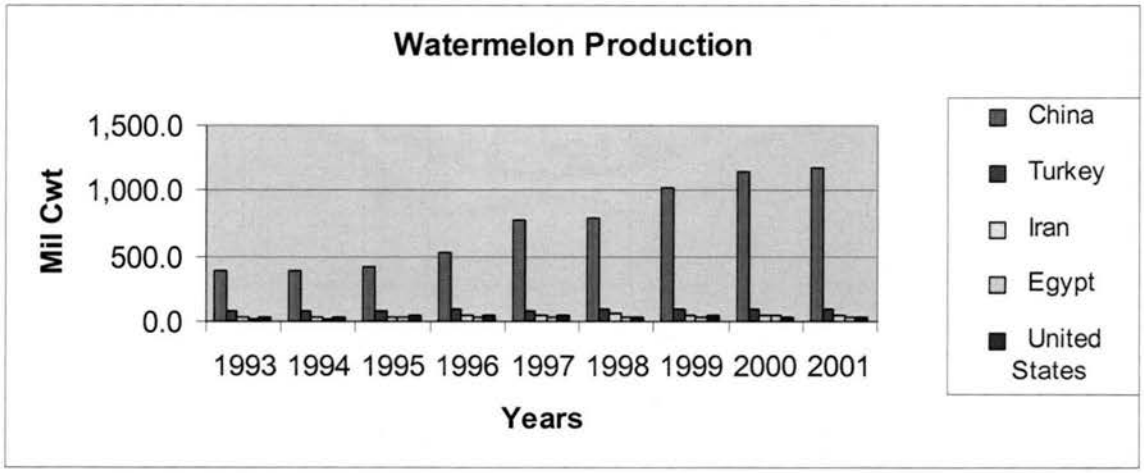

Figure 1.1. Countries with the greatest watermelon production from 1993 -2001 (http://usda.mannlib.cornell.edu/data-sets/specialty/89011/tab141b.xls)

As an important horticultural crop, especially for summertime consumption, watermelon has also become a major crop in various plant-breeding programs for the development of new cultivars with improved traits. With the help of successive breeding programs, it is now possible to see watermelons of differing size, shape, color, taste, etc., in today's market. The innovation of seedless watermelon cultivars has brought about a huge and positive increase in watermelon consumption, since a high percentage of consumers prefer seedless varieties. At the same time, the development of smaller varieties in 1986 may be another factor that has contributed to overcoming and even reversing an earlier decline in watermelon consumption (Cral et al., 1994).

Recent focus on the nutritional value of watermelon has increased its consumption as well. Because it contains zero cholesterol and fat, mainly providing carbohydrates and vitamins such as A and C, as well as some minerals such as calcium and iron; watermelon is especially attractive to nutrition-conscious consumers. Modifications in the consumption patterns of consumers, the introduction of new varieties, year-long availability in today's market, and successful research and promotion programs are all important factors in the increase in watermelon consumption (Allred and Lucier, 1990).

Plant breeding is defined as the art and science of genetic improvement of plants for human benefit (Fehr, 1991). It is believed that genetic improvement has been an ongoing process since the earliest farming period, when farmers learned to search for superior plants to harvest for seeds. Selections for outstanding traits were increased with the development of the science of genetic (Fehr, 1991; Poehlman and Sleper, 1995). In fact, with few exceptions, today's cultivars have evolved from their wild ancestors with the

help of either natural or artificial selection, carried out by humans over a long period of time.

Selection was the earliest plant-breeding tool, in which breeders or even growers employed their skills to carefully select and maintain seeds from their best plants to grow for the next season. But while selection was an effective tool for increasing the productivity and quality of various plant species for a time, eventually selection alone was insufficient for meeting the increasing demands of a global food supply.

The recognition of genes as units of heredity and the exploitation of the scientific process for systematically manipulating these plant genes provided the foundation of plant breeding. Hybridization assisted in the eradication of major limitations in terms of genetic variations in a given gene pool in which breeders depended solely on their skills in finding chance variants as new cultivars (Poehlman and Sleper, 1995). The systematic cross-pollination of the same or closely-related species in order to seek and combine different genes for varying traits in their progeny provided superior individuals that exceeded the performance of the parents. Although hybridization was used as a key element in combining plant genes in various platforms for a long period of time, the rapid scientific development of molecular genetics introduced biotechnology as an alternative method of transferring gene(s) rather then relying on hybridization, which may be severely limited by compatibility factors. Instead, breeders are now able to generate artificial variations in an existing gene pool and then select the targeted trait(s) through hybridization.

Gene transformation techniques via biotechnology opened wholly different areas for plant breeders and molecular geneticists to either develop new cultivars or to manipu-

late and enhance the performance of existing ones. During the last two decades, highly successful studies of genetic engineering on plant species such as corn, soybeans, cotton, tobacco, etc., have been reported and some of these transgenic varieties have been cultivated on a large scale, especially in the United States.

The first genetically-modified plant was reported in the early 1980s by groups at the Max Planck Institute and at Monsanto (Gasser and Fraley, 1992). The geneticists involved in this pioneering work were able to transfer and express the NPT II, kanamycin resistant gene, into plants (Gasser and Fraley, 1992). Their studies were considered a major milestone for the genetic modification of plants by direct gene transformation. Since then, unique ideas and research projects have been proposed in this relatively new field.

One of the main factors that makes biotechnology more interesting and attractive for both public and private projects may be the availability of the application of technology to plant breeding, rather than simply depending on hybridization within a species. Biotechnology basically removes the species barriers for gene donation by practically allowing the transfer of genes from many living organisms into plants. This feature technically provides unlimited working material for plant breeders to combine their vision with their skills in order to generate novel cultivars that better serve consumer demands in the twenty-first century.

The other important characteristic of biotechnology is that it eliminates the chance of introducing unwanted genes together with a targeted gene. Carrying unwanted genes or traits, which might significantly interfere with desired traits, is an important issue for conventional plant breeding, and an extensive amount of work, time and expense are re-

quired to eliminate these extra genes (Borlaug, 2000). Biotechnology, on the other hand, eliminates this extra effort and expense by simply allowing plant breeders to pick a particular gene(s) and transfer it to improve a targeted trait. Although this technique requires more facilities as well as greater sophistication, the basic goal is identical to that of traditional plant breeding, which is to transfer new DNA into the plant genome (Gasser and Fraley, 1992). In fact, the impact of transferring specific genes and monitoring their activities may be more predictable and controllable than conventional plant breeding.

Although transgenic breeding might be considered an extension of plant breeding, this technique has opened new gene pools by allowing the transfer of gene(s) from unrelated organisms into plants, which was not possible by any earlier plant breeding technique (Klee and Romano, 1994; Zhong, 2001). Breeders, working with conventional plant breeding technologies, encounter strict limitations in present germplasm pools. The valid functions of cross-breeding permits breeders to work primarily within a species but rarely among species. However, in view of the rapid increase of human global population and the decrease in arable lands, this technique may not always be sufficient to meet consumers' demands and alleviate world hunger.

Another factor that makes transgenic plants more attractive is that today's agriculture requires the large-scale use of pesticides, herbicides and fertilizers that may not be economically feasible and can potentially affect human health or damage the ecosystem. To prevent these negative effects, specifically developed transgenic plants have been extensively tested and highly successful studies have been reported. By developing natural resistance with the help of transferred gene(s), these plants become more resistant against pests and pathogens. For example, pest control is mainly carried out by inserting

genes that encode for toxins. The genes encoding *Bacillus thuringiensis* endotoxins have been used on major crops plants in order to control their pests (Groot and Dicke, 2002). By transferring the genes that activate the plant defense system, it has been shown that plants become more resistance to various pathogens and the best-known example is the development of plants having genes that mainly trigger the activation of gene-encoded pathogenesis-related (PR) proteins.

Transgenic plants have also opened a new avenue for helping to explore the poorly understood role of genes on metabolic activities. Antisense RNA-mediated inhibition of genes is used to selectively suppress gene(s) in order to clarify their importance for the normal functioning of plants. Salicylic acid (SA) has been known to be involved in systemic acquired resistance (SAR) by acting as a signaling molecule. However, the significance of SA as a key element was demonstrated only through the help of recent technology, in which the inhibition of the gene for salicylate hydroxylase-production via an anti-sense RNA technique was used to block the accumulation of SA, eventually reducing resistance in these transgenic plants (Ryals et al., 1996). Thus, it will be seen that with the help of recent biotechnology, the specific roles of different plant growth regulators are being better understood than as recently as two decades ago.

Plant hormones are defined simply as organic substances that are sensitized by specific parts of plants, such as roots, shoots and apical nodes. They also have a great impact on plant growth and development at the micromolar level, both at the site where they are sensitized and the site to which they are transferred. One of the main characteristics which distinguish these naturally occurring organic substances from others is that they are able to influence physiological processes at very low concentrations (Davies, 1995).

Their activities depend mainly on the concentration of active hormone and tissue sensitivity. The interactions of plant growth regulators with each other are crucial for metabolic activities, and these interactions modulate plant growth and development (Carimi et al., 2003).

Cytokinins are responsible for inducing cell division, delaying senescence and stimulating leaf expansion. It can also stimulate lateral shoot development via the release of lateral buds inhibited by the apical meristem (Davies, 1995; Kunkel et al., 1999). Elevated cytokinin concentration overcomes the apical dominance controlled by auxins. Cytokinins respond antagonistically to auxin for root and shoot development in plant tissue culture (Carimi et al., 2003). Although the role of cytokinins has been specified by a tissue culture bioassay, cytokinin responses on whole plant levels are believed to be broader and sometimes unspecified (Mok and Mok, 2001). The continuous interaction with other phytohormones, especially with auxins, either synergistically or antagonistically, and the ability to mask one another's effects usually prevents the clear identification of the role of cytokinin *in vivo* (Mok and Mok, 2001). The role of cytokinins in inducing ethylene production also makes it difficult to relate the morphological changes directly to cytokinins (Bettini et al., 1998; Mok and Mok, 2001). The higher ratio of cytokinin as compared to auxin promotes shoot development, while the opposite ratio promotes root development.

In order to increase the level of endogenous cytokinin in plants, most work has been carried out by transferring the *ipt* gene from *Agrobacterium tumefaciens*, a soilborne plant pathogen that causes galling primarily in dicot species (Figure 1.2). A number of groups have utilized the *ipt* gene to elevate endogenous cytokinin levels for testing

certain traits, such as delaying senescence, increasing disease resistance, etc., on various plant species.

One of the most interesting applications of the *ipt* gene has been its use as an alternative marker gene in place of an antibiotic resistance gene in gene transformation protocol. It was reported that by using the *ipt* gene as a selective marker in the transformation of tobacco leaf discs, the transformation frequency was 2.7-fold higher than with kanamycin selection (Kunkel et al., 1999; Endo et al., 2001). Other than increasing the transformation rate, the use of *ipt* may reduce possible risks of gene transformation associated with an antibiotic-resistant gene. The presence and unknown effects of an antibiotic-resistant gene in genetically modified crops have raised a major debate in the scientific arena. Some people, especially those who reject the concept of genetically modified crops on principle, have used this unknown effect as a threat to ban these crops from the market.

The *ipt* gene encodes the enzyme isopentenyl transferase, which catalyzes the rate-limiting step of the cytokinin biosynthetic pathway in which dimethylallylpyrophosphate is condensed with 5'AMP to form isopentenyl AMP, a precursor of different cytokinins (Figure 1.3) (Ebinuma et al., 1997; Moris, 1995; Glick and Pasternak, 1998). By adding 5'AMP to an isoprenoid side chain, isopentenyl transferase forms the cytokinins isopentenyladenine and isopentenyladenosine. The subsequent hydroxylation of these two molecules by plant enzymes generates cytokinin transzeatin and transribosylzeatin (Glick and Pasternak, 1998; Martin et al., 1999). Since there is no evidence of isopentenyl adenosine monophosphate (IPA) accumulation in *ipt* transgenic plants, while an en-

hancement in zeatin-related cytokinins occurs, it is believed that the synthesis of isopentenyl transferase is the rate-limiting factor for cytokinin synthesis.

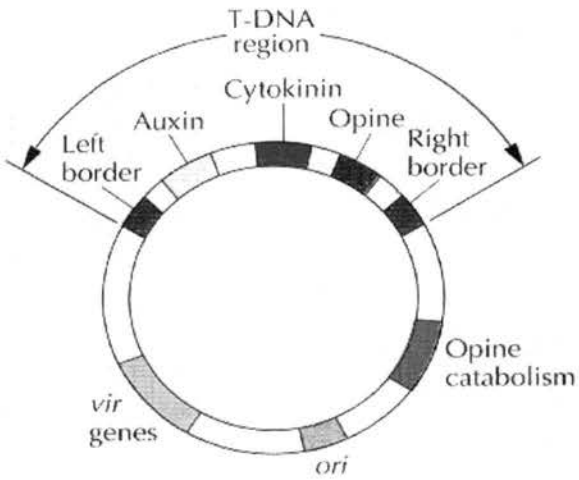

Figure1.2. Schematic representation of a Ti plasmid including the genes for the biosynthesis of auxin, cytokinin, and opine, which are transcribed and translated only in plant cells (Glick and Pasternak, 1998).

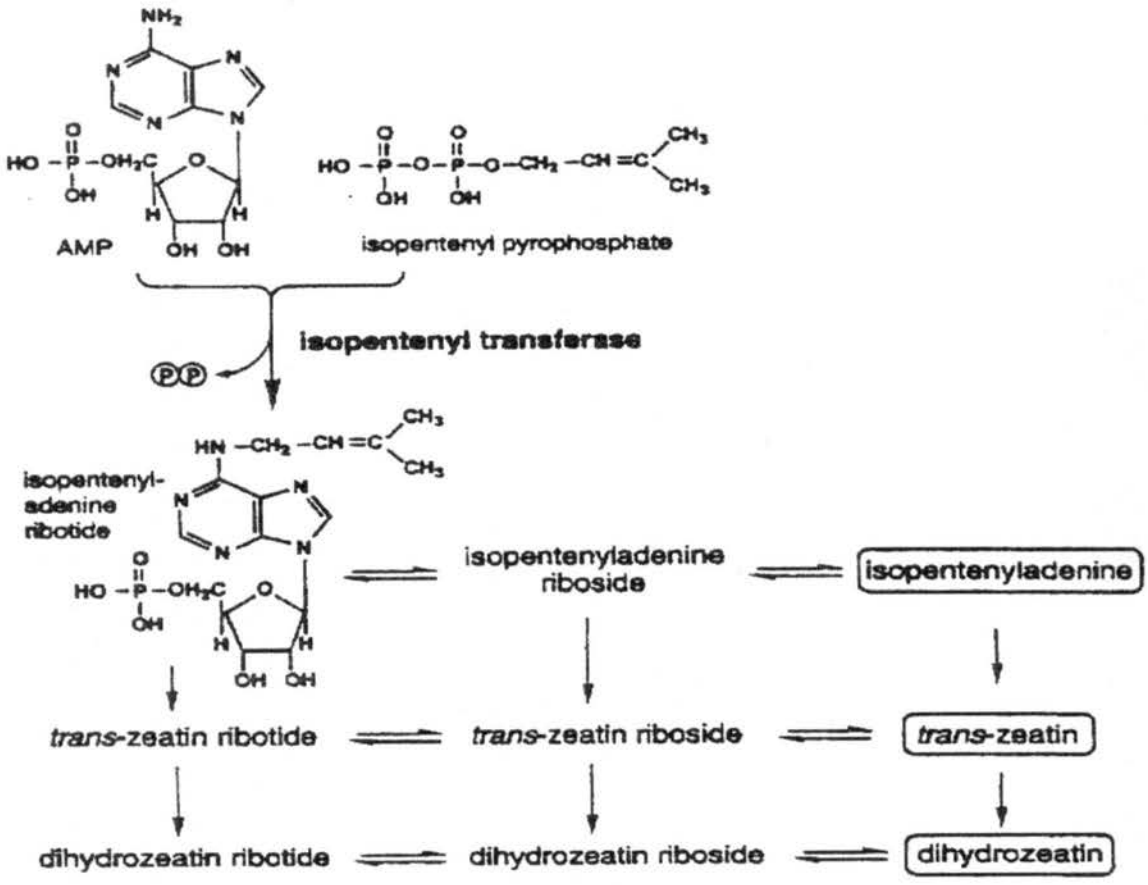

Figure 1.3. The schematic representation of the cytokinin biosynthesis pathway (Gan and Amasino, 1996).

In terms of native plant genes for cytokinin biosynthesis, nine putative genes for isopentenyl transferase have been identified to date by searching the *Arabidopsis* genome sequence (Kakimoto, 2001). Out of these nine native plant genes, the *PGA22* gene was activated and reported to encode the isopentenyl transferase enzyme and induce endogenous cytokinin biosynthesis in mutated *Arabidopsis* plants (Sun et al., 2003). The identification of these native plant genes involved in cytokinin biosynthesis has initiated fresh discussions of whether all plant species contain these genes, and if so, the total number of genes that are involved in cytokinin biosynthesis. These questions obviously require time and extensive effort for further exploration and understanding.

In addition to their previously identified main characteristics, cytokinins were also reported to have a great impact on plant defense systems by inducing disease and insect resistance (Dermastia and Ravnikar, 1996; Storti et al., 1994; Bettini et al., 1998; Smigocki et al., 1993). The primary signaling molecule for induction of resistance and production of pathogen related proteins (PR) upon pathogenic attack is known to be salicylic acid (SA) (Maleck and Dietrich, 1999; Delany et al., 1994; Gaffney et al., 1993; Gaffney et al., 1994). However, ethylene, xylanase, the polypeptide systemin, jasmonic acid, hydrogen peroxide, superoxide radicals and possibly some other regulators may also induce biosynthesis of the PR protein (Agrios, 1997; Vijayan et al., 1998). Apart from the identification of elevated PR protein levels in transgenic plants with cytokinins over produced, the involvement of cytokinin in the induction of PR protein still remains unclear. Cytokinin may either be directly involved in the production of PR protein or positively regulate other signaling molecules to induce PR protein. The only known fact to date is

that over- expressed cytokinin somehow induces the production of PR protein and activates the plant's defense system.

Since cytokinins were discovered in 1956, extensive research has been carried out to identify the role of cytokinins in plant growth and development. In the beginning, researchers tried to investigate these roles of cytokinin by exogenous application of this hormone; however, the uptake and inter- and intra-cellular mobilization of applied compounds was not clearly understood (Ditt et al., 2001; Gan and Amasino, 1996). The genetic engineering of plants has made it possible to explore these questions in greater detail and to more clearly understand the role of this phytohormone. Although native plant genes encoding cytokinin biosynthesis in *Arabidopsis* have recently been identified, researchers still prefer to use genes from other organisms, such as the *Agrobacterium ipt*, to investigate the effect of elevated cytokinin levels on various plant species. Plant biotechnology makes it possible to transfer genes among organisms that are not in any way related to each other. The applications of recent technology assists in the exploration of additional characteristic of cytokinins in plant growth and development. For example, it was reported that a high concentration of cytokinin reduces cell growth and proliferation, as well as induces DNA fragmentation in both carrot and *Arabidopsis* tissue (Carimi et al., 2003). In fact, cytokinin overproduction, especially in constitutively expressed *ipt* transgenic plants, has had a seriously undesirable impact on plant growth and development.

In order to delete the negative effects of elevated endogenous cytokinin level by *ipt* gene transformation, researchers have used either tissue-specific activation or inducible promoters. Some of the undesired morphological effects on the normal development

of plants are inhibition of root formation during the regeneration process, extensive lateral shoot formation, rosette structure, etc. The main features and expectations of an inducible promoter are low expression in the absence of the promoter as well as high expression in the presence of inducers, non-interference with the normal physiology of the plant, and finally, having no effect on the expression of the other genes (Mett et al., 1993).

The replacement of *ipt*'s native promoter with fruit-specific promoter (Martineau et al., 1994), heat-inducible promoter (Medford et al., 1989), chalcone synthase promoter (Wang et al., 1997), senescence–specific SAG12 promoter (McCabe et al., 2001; Ori et al., 1999) and seed-specific promoter (Ma et al., 1998) has allowed some control of endogenous cytokinin levels and facilitated root formation. The negative aspects associated with these promoters are that they are either too weak to completely auto-control *ipt* gene expression or have too narrow an application due to their dependence on certain traits or plant parts. On the other hand, the copper inducible promoter (Mett et al., 1993; McKenzie et al., 1998) has been used to manipulate the cytokinin level by inhibiting *ipt* expression until inducers are present. With a copper inducible promoter, the activation of the targeted gene does not require any conditions other than the application of copper silver ions. Since the regeneration of transformed cell(s) is still largely dependent on the tissue culture process, a tight auto-regulation of the *ipt* gene increases the value of such a promoter.

Tissue culture is basically *in vitro* propagation of a plant's organs, tissues or cells from whole plants under aseptic conditions. *In vitro* propagation allows for the adjustment of nutrient concentration, including plant growth regulators and environmental con-

ditions, in order to reach optimal regeneration for that particular plant species. However, the nutrient concentration required for different plant species varies extensively even within the same species. Based on today's biotechnology, tissue culture is one of the most important and crucial steps for this transformation process, since it makes it possible to regenerate a whole plant from isolated transformed cells or tissue. Without an optimized regeneration protocol for a particular plant species, gene transformation is generally not feasible or even possible. While there have been some attempts to remove the plant tissue culture aspect of the transformation process (Hansen and Wright, 1999), these processes are still under development.

Although the first successful plant tissue culture study was reported by Gottlieb Haberlandt at the beginning of the twentieth century, numerous studies and lengthy procedures were required to reach the present level (Caponetti et al., 1996). The discovery of plant growth regulators and the development of laminar flow hoods were considered to be major milestones for plant tissue culture studies, making possible the extensive use of this technique on different plant species for various purposes, including genetic manipulation of plants via gene transformation.

1.2 Gene transformation

Generally, genetic transformation in plants has been achieved by particle bombardment of intact tissues, electroporation of protoplasts, and most commonly by using *Agrobacterium tumefaciens* as a vector. The *Agrobacterium* technique is most commonly used for diocots. However, with some modification of transformation protocols, such as the adding of a phenolic acetosyringone, this technique has been applied in the gene transformation of monocot plant species as well (Hiei et al., 1997; Peters et al., 1999).

The main factors that affect the efficiency of *Agrobacterium*-mediated gene transformations are bacterial infection, host recognition and the transformation competency of the targeted gene ((Delany et al., 1998; Trick and Finer, 1998).

Agrobacterium bacteria are gram negative, soil-borne pathogens that cause tumor growth (*Agrobacterium tumefaciens)* or hairy-root (*A. rhizogenes*) development on infected plants, especially diocots. *A. tumefaciens* was known as *Bacterium tumefaciens*, the causative agent of crown gall disease since the early twentieth century (De la Riva et al., 1998; Zupan et al., 2000), and since then different strains of this plant pathogen have been identified.

The presence of circular plasmids (Figure 2.2), completely separated from the bacterial nuclear genome and the ability to pass part of these plasmids into their host, has made this bacterium a unique tool for the genetic modification of plants. In nature, one of the main reasons for the transference of part of *Agrobacterium's* genome into its hosts is to change the genetic structure of the host thus supplying the bacteria with carbon and nitrogen. This modified environment assists in the growth and proliferation of the bacteria.

Thus far, it has been shown that the circular plasmid carries three distinct classes of genes. The oncogenic genes, involved in the synthesis of cytokinin and auxin, are located on the tumor-inducing (Ti) region of the plasmid; they aid in callus formation by their expression in the plant genome. This Ti region is separated from the rest of the plasmid by a repeated sequence of the 25 base pairs of the left and right borders. Bacteria attempt to transfer part of their plasmids (Ti region) to their hosts during inoculation with the help of virulent (*Vir*) genes, which are one of the other classes of genes located on the

plasmid. In practice, during the host-pathogen interaction, anything located between the left and right borders (25 bp. flanking direct repeat) of the Ti, region termed T-DNA (transferred DNA), will be transferred to its host. Consequently, modification of the Ti region, the removal of the naturally present genes and substitution with specific gene(s) of interest, makes it possible for *Agrobacterium* cells to be used in gene transformation.

Whenever the pathogen interacts with the wounded host, the released exudates from the host, mainly phenolic compounds, induce the expression of genes in the *Vir* region (Zupan et al., 2000). This induction is considered to be the key element for successful gene transformation. The presence of phenolic compounds such as asetosyringone in diocots makes these plant species suitable hosts for *Agrobacterium.* Although the mechanism of gene transformation and integration is not clearly understood, the consensus is that the protein produced by different *Vir* (*VirA,B,C,D,E,G*) genes plays varying roles in its occurrence. The phenolic compounds released from wounded plants first induce the expression of *Vir A* which activates the expression of *Vir G* which is the transcriptional activator for the remaining *vir* genes (Chilton, 2001). The proteins encoded by these *vir* genes have different responsibilities in removing, carrying and inserting the T-DNA located between the right and left borders into the plant genome.

1.3 Developing seedless watermelon

Seedless watermelon cultivars are mainly developed by crossing tetraploid varieties with diploids in order to produce triploid hybrid plants. The odd number in the chromosome distribution does not allow seed development, resulting in seedless watermelon fruits. Although this breeding technique is the key element for seedless water-

melon production, alternative methods, such as the application of various plant growth regulators, especially synthetic Cytokinin-1-(2-chloro- 4-pyridyl) -3-phenylurea (CPPU) (Hayata and Niimi, 1995) and the use of pollen irradiated with soft x-rays (Sugiyama and Morishita, 2002), have also been reported. Based on their developmental processes, each technique has its own advantages and disadvantages. For example, the costs of seed development for triploid seedless watermelon is relatively high, while the application of plant growth regulators may not be feasible for reasons of safety affecting both the environment and consumers.

Although the application of tissue culture to clonal propagation was proposed as an alternative method of reducing the cost of seed production in triploid seedless watermelon, this technique still has not been widely applied, and it may require developing separate protocols for each genotype. X-ray treatment —in other words, the irradiation of pollen—may not be uniformly cost effective and requires greater experience and extra labor. Since these limitations cannot be ignored, alternative methods of producing seedless watermelon are sought by breeders. The utilization of plant biotechnology may offer still other processes for the development of seedless watermelon cultivars.

It was previously reported that the exogenous application of cytokinins could induce both parthenocarpic watermelon fruits from non-pollinated flowers and reduce the number of normal seeds from pollinated flowers (Hayata and Niimi, 1995). During this research, the elevation of endogenous cytokinin levels in plants was hypothesized, with the potential of producing parthenocarpic watermelon fruit. The basic idea is that when sprayed with a very low concentration of copper sulfate, the copper-inducible *ipt* gene would elevate endogenous cytokinin levels. This would activate the *ipt* gene for cyto-

kinin biosynthesis, which would eventually increase cytokinin concentration and lead to the development of seedless fruits.

1.4 Disease resistance

As hosts threatened by continuous and diverse pathogen attacks, plants possess a distinctly different defense mechanism from that of animals. Unlike vertebrates, plants do not have a circulatory system or antibodies against pathogenic infestation; their reactions and cures for diseases are also different from those of animal systems. By means of a combination of constitutive and induced defenses, each plant cell, unlike those of animals, is capable of defending itself (Staskawicz et al., 1995). In the plant system, symptoms are the primary feature for diagnosing the type and severity of a disease. Diseases usually first appear locally and then may or may not spread over the entire plant. Since diseased plants usually end up dying or malfunctioning, most cultivation practices are focused on preventing diseases before it appears rather than curing infected plants. Disease resistance is one of the most effective and efficient means of managing diseases.

In terms of resistance, plants demonstrate two types of responses against pathogenic attack; these are race specific (vertical resistance) and non specific (horizontal resistance). Vertical resistance is based on the specific interaction between a dominant resistant gene in the host and the product of a recessive virulent gene in the pathogen. The gene-for-gene interaction was first proposed to demonstrate the interaction between flax rust fungus and rust reaction in the host (Flor, 1955). As a result of this gene-for-gene interaction, a complex series of biochemical reactions occurs that leads to plant resistance. Biochemical reactions, triggered by host pathogen interactions, take place in cells and produce substances that are either toxic to the pathogen or create conditions that in-

hibit the proliferation of the pathogen in the host. The number of R genes present in the host and Avr genes present in the pathogen may vary, and a single host and pathogen may carry many such genes respectively (Bent, 1996). With the help of recent technology, mainly the isolation and sequencing of genes, plants have been shown to carry relatively large numbers of genes in their genome which interact with pathogens by identifying and destroying them (Crute and Pink, 1996).

The greater the numbers of R genes carried by the host, the greater the chance of creating resistance and eradicating disease development. Consequently, in their disease-resistance breeding programs, plant breeders' main objectives are to introduce as many R genes as possible into the targeted genotype. Combining different R genes is therefore one of the main factors that could provide long-lasting resistance against different races of a particular pathogen.

Unlike vertical resistance, horizontal resistance is based on a series of aspecific defense responses that leads to resistance against various races of pathogens. As a result of several successive studies, it has been reported that phytohormones are also involved in the defense response of plants against pathogen infection. Exogenous application of growth regulators can play a role in either inducing or inhibiting the hypersensitive response, possibly due to their antagonistic and synergistic interactions. Thus far, one of the best-studied examples of horizontal resistance is considered to be Systemic Acquired Resistance (SAR). When a host is infected with a weakly virulent pathogen, it develops a long-lasting, broad-spectrum systemic resistance to subsequent infections (Bettini et al., 1998). One of the most important features of the acquired resistance is its independence

of any specific pathogen. Once it is induced, it may protect plants against consecutive pathogenic threats of various organisms.

1.5 Gummy Stem Blight

Gummy stem blight or black rot generated by the pathogen *Didymella bryoniae* is one of the most destructive stem and foliage diseases of cucumber, cantaloupe, pumpkin, and watermelon (Keinath, 1996; Zhang et al., 1999). *D. bryoniae* may also cause fruit rot of watermelon and other cucurbits. Gummy stem blight disease or black rot disease is a major problem for watermelon production, especially where the relative humidity is fairly high, for example, in the southern United States. High relative humidity plays as a key factor in the release of ascospores and successful infection (Thomas, 1996). The symptoms of gummy stem blight on cantaloupe and cucumber leaves are expressed by irregularly circular tan spots. On watermelon leaves, circular dark brown lesions appear, generally at the leaf margin at first and later rapidly expanding through out the leaf blade. If the disease develops on watermelon fruit, it is called black rot. The pathogen can survive over winter for nearly two years on infected host debris; hence, crop rotation at least every two years is an important strategy in managing soil-borne inoculums of this pathogen in previously-infected fields (Thomas, 1996; Keinath, 2002). Factors which accelerate the decomposition of infected debris and negatively affect the survival of the pathogen may help to reduce the required rotation period. In addition, burying disease-infested vines for approximately thirty weeks significantly reduces the survival of the pathogen (Keinath, 2002). The disease mainly reduces yield and marketability of watermelon fruit, and fungicide use increases costs for watermelon growers. However, since two

years crop rotation may not be economically feasible for all growers, breeding of resistance cultivars is a more economical method of managing gummy stem blight disease. Although some cultivars and plant introductions (PI) have been shown to carry some degree of disease resistance (Norton, 1979; Wehner and Shetty, 2000), complete resistance against this disease has yet to be achieved.

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Chapter 2

Watermelon transformation with Copper inducible constructs of Isopentenyl Transferase

2.1 Abstract

The construct of isopentenyl transferase (*ipt*) gene with copper inducible promoter (Cu-*ipt*), in *E. coli* (pMJM100) was obtained from Dr. Marian J McKenzie at University of Otago. It was then transferred into *Agrobacterium tumefaciens* (LBA 4404) for use in watermelon transformation studies. Watermelon seeds were surface sterilized with 1.0% sodium hypochlorite and soaked in sterile water for 2 days prior to germination on Murashige and Skoog's basal medium (MS) in dark at 25 °C for16 hrs and 18 °C for 8 hrs. Etiolated cotyledons of 'Crimson Sweet' were cut around 2-3 mm above the apical node and cultured on solid MS media containing 5 μM BA for 5 days under 16/8 light/dark at 25/18 +/- 2 °C. The *A. tumefaciens* cells was grown in liquid LB+ Kan^{50} at 30 °C and 200 rpm shaker overnight or until the absorbance at A_{600} was between 0.1 and 0.2. The precultured explants were then inoculated with Agrobacterium cells for 1 hr then co-culture with *A. tumefaciens* cells on filter paper covered solid MS medium for 2 days. They were then transferred to a selective media containing 5 μM BA, 250 mg/l carbanacilin, and 100 mg/l of kanamycin with subsequent subcultures at 2 week intervals. The shoots that formed were transferred to MS without BA for root formation. Rooted plants were then transferred to soil media covered with plastic bags for 7-10 days for adaptation to the growth chamber. Young leaves from these transgenic plants were used for genomic DNA extraction and tested with specifically designed PCR primers to confirm the presence of the *ipt* gene. The lines that were PCR positive as well as clearly indicated the activation of the Cu-*ipt* construct with application of copper sulfate, were clonally propagated for further experiments. The phenotypic modifications as related to the elevated cytokinin levels such as release of axillary buds and leaf expansion were used as indications of the activation of the Cu-*ipt* construct.

2.2 Introduction

As a warm season vegetable, watermelon (*Citrullus lanatus*) is frost-sensitive and requires a long growing season with relatively high temperatures (Mohr, 1986). With 95% water content, watermelon is a unique vegetable for summer consumption as refreshment. Realization of its nutritional value has resulted in consumption. Since watermelon contains zero cholesterol and fat, mainly providing carbohydrates and vitamins such as A and C as well as some minerals such as calcium and iron, it is especially attractive to nutrition-conscious consumers. Modifications in the consumption patterns of consumers, the introduction of new varieties, year-long availability in today's market, and successful research and promotion programs have all been important factors in the increase in watermelon consumption (Allred and Lucier, 1990; Lucier and Lin, 2001).

Hybridization and selection have been used as the major breeding techniques for developing new watermelon cultivars and F1 hybrids. Higher yield and disease resistance are among the traits that have been prioritized in most breeding programs. As a member of the *Cucurbitaceae* family, watermelon is host to a wide variety of plant pathogens and insects. Its vigorous growth and large foliar parts usually make it vulnerable to plant diseases. In addition to yield and disease resistance (Martyn and Netzer, 1991; Carl et al., 1994), fruit qualities such as sweetness and edible flesh proportions are equally important traits in breeding programs (More and Seshadri, 1998). Except for seedless varieties, which are triploid with parthenocarpic fruit, cultivated watermelons are diploid annuals, generally out-crossing with 11 pairs of chromosomes (Lee et al., 1996). Consequently, with its relatively smaller genomic size, this crop becomes very attractive for molecular genetic studies. Although watermelon is considered a cash crop and one of the most important horticultural products, especially for summertime consumption, intensive gene transformation and molecular genetic studies have not yet been conducted on this vegetable. However, the introduc-

tion of relatively inexpensive *Agrobacterium*-mediated gene transformation techniques has brought new opportunities to re-evaluate various horticultural crops in terms of gene transformation and molecular genetic studies. As a diocot plant species, watermelon is a natural host for *Agrobacterium.* Although not extensive, there are some published researches relative to *in vitro* regeneration of watermelon cultivars. Therefore, like other horticultural crops such as tomatoes, watermelon is equally available for transformation in order to improve various traits.

In recent years, -propagation and regeneration of watermelon utilizing cotyledonary tissue for organogenesis and immature zygotic embryos for somatic embryogenesis has been undertaken (Choi et al., 1994). In this research, cotyledons were generally used as explant materials for *in vitro* watermelon propagation. Compton and Gray, in 1996, reported that it normally takes 6-10 weeks to obtain shoots with complete, rooted plants from culture initiation. Choi et al. reported the first Agrobacterium-mediated gene transformation of watermelon in 1994, with the GUS reporter gene transferred into two watermelon cultivars, 'Sweet Gem' and 'Gold Medal'. Since then, *in vitro* propagation and transformation of watermelon via *Agrobacterium* protocols is considered an efficient technique and available for further research.

Cytokinins alone have promoted shoot formation in regeneration of watermelon from cotyledons and hypocotyls (Srivastava et al., 1989). By transferring the shoots, which were greater than 2 cm in length, into 0.001 mM IBA-containing MS media, roots of at least 1 cm in length were formed within a 2-week growing period (Compton and Gray, 1996). Cytokinin studies in plants have been carried out by transferring the *isopentenyl transferase* (*ipt*) gene from *Agrobacterium tumefaciens* into plants. The introduction of the *ipt* gene into the plant genome results in elevated levels of cytokinin in the transformed tissue (Bettini et al., 1998; Faiss et al., 1997; Medford et al., 1989; Martineau et al., 1994; McKenzie et al., 1994; McKenzie et al., 1998; Jordi et al.,

2000). Isopentenyl transferase, encoded by the *ipt* gene is considered to regulate the rate-limiting step in the cytokinin biosynthetic pathway (Akiyoshi et al., 1984). By condensing dimethylallylpyrophosphate with 5'-adenosine mono phosphate, isopentenyl transferase forms cytokinins isopentenylanenine and isopentenyladenosine, which are then hydrolyzed by other plant enzymes to produce the cytokinins transzeatin and transribosylzeatin, respectively (Moris, 1995; Glick and Pasternak, 1998).

One of the main problems involved in elevated cytokinin levels is associated with certain physiological response, primarily the inhibition of root formation during regeneration. In order to reduce these undesirable traits in cultivated varieties, inducible promoters have been used in various plant species. Fruit-specific promoter (Martineau et al., 1994), heat- inducible promoter (Medford et al., 1989), chalcone synthase promoter (Wang et al., 1997), senescence –specific SAG12 promoter (McCabe et al., 2001; Ori et al. 1999), seed-specific promoter (Ma et al. 1998) and copper inducible promoter (Mett et al., 1993; McKenzie et al., 1998) are some of the main promoters utilized to regulate *ipt* gene expression. Among these, the copper inducible construct is considered to provide a tight auto regulation over the targeted gene expression. This construct was originated from the yeast copper-metallothionein (MT) system and it contains activating copper- MT expression 1 (*ace1*) and four copies of the metal response element (MRE) (Fig. 2.1). The constitutive expression of the *ace1* gene generates a metal responsive transcription factor which acts as a regulatory protein (ACE I protein) by changing its conformation with the help of copper ions (Mett et al., 1993). When this conformational change occurs, ACE I protein becomes compatible to the specific upstream site of the yeast copper-MT promoter (Mett et al., 1993). Once the ACE I protein binds to the promoter site it activates the whole system by inducing the gene expression located downstream (Mett et al., 1996). The presence of copper or silver ions is crucial for the acti-

vation of the inducible promoter. As a result, the transcription of the targeted gene, linked downstream of the promoter, can be controlled by the application of these two ions. The main objective of this study was to develop watermelon lines with the copper inducible *ipt* gene.

2.3 Material and Methods

2.3.1 The Cu-*ipt* construct

Once the vector was obtained in *E. coli,* it was cultured in selective media. The vector pMJM100 was grown on spec^{100} plates at 37 °C until single colonies started to appear. A single colony from the spec^{100} plate was removed with a sterile toothpick and placed in a sterile test tube field with 3 ml liquid LB and spec^{100}. The test tube was sealed and left in a shaker at 250 rpm at 37 °C overnight. These cells were then used for DNA extraction for both restriction enzyme experiments and transferring these targeted constructs to the *Agrobacterium tumefaciens* (LBA4404) cells.

In order to verify the presence of these gene constructs, restriction digestion experiments were conducted on both vectors. The Not 1 restriction enzyme was used during the restriction digestion experiments (Figure 2.1). The plasmid DNA of *E. coli* cells was digested with these enzymes and run on a 0.7% agarose gel for 1 and 1/2 hour.

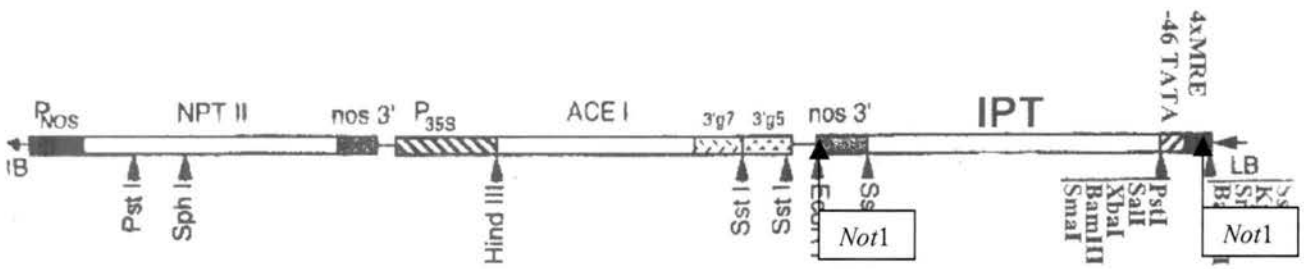

Figure 2.1 The schematic representation of gene construct (Cu-*ipt*) and the restriction digestion sites for different restriction enzymes.

DNA extraction from *E coli* and *Agrobacterium tumefaciens* cells:

The Boiling-Lysis mini-prep protocol was used to extract the DNA from both the *E. coli* and *Agrobacterium* cells. A 3 ml aliquot of cells was spun down twice in a 1.5 ml eppendorf tube at 12000 rpm for 2 minutes. The final pellet was re-suspended in 350 µl of STET buffer (0.1 M NaCl, 10 mM Tris-Cl pH 8, 1m MEDTA pH 8, 5% Triton X-100). A 25 µl aliquot of freshly-prepared lysozyme (10 mg/ml in 10 mM Tris_HCL pH 8) was then added to each tube and vortexed for 3 seconds. These tubes were placed in boiling water for exactly 40 seconds. At the end of this time the bacterial lysate was spun down at 12,000 rpm for 10 minutes at room temperature. The bacterial pellet was removed with a sterile toothpick and then 40 µl of 2.5 M NaAcetate pH 5.2 and 420 µl of propanol was added and mixed by vortexing. The tubes were left at room temperature for 5 minutes. They were spun for 5 minutes at 12,000 rpm at 4 ^{o}C and the supernatant was carefully removed. The pellet was washed with 70% ethanol and spun 2 minutes longer. The supernatant was then drained and the pellet left to dry. The pellet was resuspended in 50 µl of TE (10 mM Tris pH 7.5, 1mM EDTA pH 8) or in 50 µl of H_2O.

2.3.2 Stock cell in glycerol

A single colony of *Agrobacterium* for *ipt* construct was removed by sterile tooth picks from an LB and Kan^{50} plate and then placed in sterile tubes that contained 3 ml of liquid LB and Kan medium. The tubes were left overnight in a 30° C shaker. Once the medium appeared cloudy, 500 µl of culture and 500 µl of glycerol were pipetted into a marked 1 ml tube. These tubes were immediately vortexed and placed in liquid nitrogen for 10-15 minutes. Finally, the tubes were stored at minus 80 °C for future use.

2.3.3 Seed sterilization and germination

The watermelon cultivar Crimson Sweet was used for regeneration and transformation experiments. The seed coats were removed manually to increase germination rate and to decrease time to germination. The embryos were surface disinfected in 20% commercial bleach (sodium hypochlorite) and one drop of Triton x-100 for 15 minutes. They were then rinsed 3 times with sterile water, followed by soaking in sterile water for 2 days in a dark growth chamber. During the sterilization period, the seeds were constantly shaken in order to obtain even penetration and obliterate all pathogens present. Seeds were placed on a sterile filter paper for 30 minutes in a laminar flow hood in order to remove excess water. The final step was to place 2-3 seeds in 10-15 ml of basal MS media in 8 oz baby jars (120 ml). These seeds were germinated in a dark growth chamber at 25 °C (18 hours) and 18 °C (6 hours) for 5 to 7 days. After germination, the etiolated seedlings were checked to verify absence of contamination. Discoloration of the media was considered

an indication of contamination. Only seedlings with clear media were used for the regeneration and transformation studies.

2.3.4 Explants for transformation

Etiolated watermelon seedlings were removed from the 8 oz baby food jars and placed on sterile petri dishes. For both the regeneration and transformation process, the cotyledons were used as explants. The cotyledons were gently cut 2-3 mm above the apical node with a sterile blade and were quickly placed in covered petri-dishes until the cutting process was completed.

2.3.5 Transformation

Agrobacterium with the Cu-*ipt* genes was plated onto the solid LB+Kan^{50} containing petri dishes. Plates were parafilmed and placed in the incubator at 30 °C until single colonies developed. Each single colony was removed from the solid LB+Kan^{50} plate with a sterile toothpick and placed in a 5 ml liquid LB+Kan^{50}-containing flask. The flask was placed in a shaker at 30 °C and 200 rpm overnight, or until A_{600} was between 0.1 and 0.2. In the meantime, the etiolated seedlings were prepared for co–cultivation. Cotyledons were pre-cultured in MS media with 5 µM mg/l BA for 3-4 days before they were exposed to the *Agrobacterium* containing vector. The small pieces of pre-cultured cotyledons were placed in 6 petri dishes containing 20 ml of MS liquid medium with 400 µl acetosyringone. Simultaneously, six 1.5 ml centrifuge tubes marked for Cu- *ipt* were filled with Cu- *ipt* containing *Agrobacterium* cells. Tubes were centrifuged at 10,000 rpm for 2 min. After centrifugation and pouring off the supernatant, 1 ml of liquid MS with 5 µM BA and 400 µl acetosyringone was added to the tubes to re-suspend the cells. Finally, the re-suspended cells were poured onto the explant-containing petri dishes for co-cultivation for approximately 2 hours. Explants were then removed and placed on sterile filter papers in order to blot excess *Agrobacterium* from the explants. The explants were then placed on MS with 5 µM BA media

grobacterium from the explants. The explants were then placed on MS with 5 μM BA media covered by filter paper and left for further co-cultivation with the *Agrobacterium* for 48 hours. Once the co-cultivation was completed, the explants were transferred to a MS medium with 5 μM BA, $Carb^{250}$and Kan^{100} selection medium. Explants showing signs of regeneration were sub-cultured at 2 week intervals. Shoot primordia began to form after 3-4 weeks with subsequent transfer to a basal MS media without plant growth regulators for root formation after 8 weeks.

2.3.6 DNA Extraction

DNA extraction was performed by using 750 μl CTAB (% w/v CTAB; 100 mM Tris (pH 7.5); 1.4 M NaCl; 20 mM EDTA in a 0.1% β-mercaptoethanol) extraction buffer pre-warmed at 65 °C. Samples of small young leaves were excised from the transgenic plants. The tissue was ground in 1.5 ml sterile tubes dipped into liquid nitrogen. Small pistils were used to grind tissue to a fine powder with liquid nitrogen. The powder was quickly added to the pre-warmed CTAB extraction buffer, vortexed to release pressure and incubated at 65 °C for 30 minutes. A volume of 560 μl of chloroform was added to each tube and mixed gently for 15 minutes. Samples were spun at 8000 rpm for 10 minutes and the supernatant was decanted to new tubes with a pipette. An equal volume (650 μl) of ice cold iso-propanol (2-propanol) was added and mixed gently by inversion until the DNA preparation became visible. At this stage, the tubes were placed at -20 °C for 5 to 10 minutes or at –80 °C for 20 minutes to increase the DNA yield. Tubes were then spun down at 8000 rpm for 10 minutes at 4 °C. The DNA pellets were then transferred with a bent Pasteur pipette to a new tube. The pellets were re-dissolved in 100 μl water. In order to remove any RNA present, a volume of 1 μl of 10 mg/ml RNAse was added and incubated at room temperature for 20 minutes. Ten μl of 3M Na-acetate and 200 μl of 95% ethanol were added and the tubes were inverted and stored at –20 °C for 30 minutes or overnight. The tubes were then spun at

10,000 rpm for 10 minutes at 4 °C. The supernatant was pipetted and the DNA pellet was washed with 100 μl 70% ethanol. The pellets were slightly dried out in the SpeedVac at the "low" setting for less than 1 minute. The pellets were then dissolved in 100 μl water, and stored at 4 °C for short-term use and at –20 °C for long-term storage.

2.3.7 PCR reactions

The presence of the *ipt* construct in transgenic plants was confirmed by running Polymerase Chain Reactions (PCR). The Promega PCR kit was first tested with the template DNA that came with the kit. Once it was positively confirmed that the kit was in working condition, it was set up with genomic DNA extracted from candidate-transformed lines. The total volume of 25 μl PCR solution contained 1.5 μM of $MgCl_2$; 1X thermophilic DNA polymerase in 10X buffer $MgCl_2$-free; 1.0 μM of both downstream and upstream primers, respectively, 1.25 μl / 50 μl *Taq* DNA polymerase and 1 ng/50 μl template DNA, and the final volume was brought up to 50 μl with sterile distilled water. The Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) was set for denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 72 °C for 1 min for a total of 39 cycles. Two primer sets, forward (5'- GCG TCT AAT TTT CGG TCC AA- 3') and reverse (5'- TCC ATA TCT GCG TCA AGC TG- 3' (Chang, 2002) with Tm 63.7 and Tm 63.9, respectively, were used (Sigma Genosys). These primer sets were specific to the *ipt* sequence and were constructed to amplify a 596-base pair segment of the *ipt* gene. Once the amplification was completed, DNA samples with the control of 1Kb DNA ladder from Promega were electrophoresed on a 1.0% agarose gel that contained 5 μl of ethidium bromide, which was run at 100-110 volts for 1 or 1-1/2 hours. The results were documented by exposing the gel under UV lighting using the Bio Imaging System (Synoptics, Ltd., Cambridge, UK).

2.3.8 Leaf callus assay

One fully expanded leaf per transformed line was removed from the plants and the leaf's surface sterilized by a quick dip in a 70% EtOH and 20 % commercial bleach solution for 2 minutes followed by 3 sterile water rinses. The leaves were drained on sterile filter paper for 5-10 minutes and transferred to the selective media plates containing MS with 5 μM BA, 100 μg ml^{-1} Kanamycine, and 250 μg ml^{-1} Carbanaciline. Tissue was cultured for 1 month in a growth chamber at 25 °C (18 hrs with light) and at 18 °C (6 hrs without light)

2.3.9 Phenotypic evaluation of *ipt* transgenic line(s)

The candidate transgenic lines generated in selective medium (MS with 5 μM BA, 100 μg ml^{-1} Kanamycine, and 250 μg ml^{-1} Carbanaciline) were sprayed with 50 μM copper sulfate at approximately the seven-leaf stage. The individual lines were sprayed with copper sulfate solution only once and the spraying process was applied until the solution started to run off the leaves.
These lines were grown in a growth chamber at 25 °C for 18 hours with light and at 18 °C for 6 hours without light. Approximately two weeks after spraying, these lines were phenotypically evaluated for both the activation of the targeted gene (*ipt*) and any toxic effect related to copper sulfate application. Leaf expansion as well as the reduction of apical dominance when compared to wild type (non-transformed) was used for evaluation and selection. A single line showing clear phenotypic modifications as related to the elevated cytokinin levels was maintained in the growth chamber for further analysis.

2.3.10 Transferring regenerant plants to the growing medium

Regenerated shoots with well-developed roots were removed from the MS medium and placed into sterile petri dishes. The roots were gently washed with sterile water, either by rinsing them or shaking the plantlets inside the water. The process was continued until all agar was removed. All excessive callus and brown leaves were removed as well. Finally, the plants were blotted on sterile filter paper to remove excess water. The plantlets were then transferred into sterilized media (MetroMix 350, Grace Sierra), in seedling trays (12x12x12 cm) which were lightly watered and covered with plastic wrap. They were then transferred into a growth chambers at 25 ^{o}C (18 hours light) and 18 ^{o}C (6 hours dark) for 7-10 days.

Once the plants had adapted to the soil medium and started to grow, they were gradually exposed to growth chamber conditions by punching holes in the plastic wrap. After the growth reached the top of the plastic tent, the plastic wrap was removed. These plants were lightly watered with distilled water at approximately 3 days interval with half-strength liquid MS without sucrose or copper. After the candidate transgenic plants had grown to the 8-10 leaf stage, a young leaf was removed from each plant for DNA extraction. The DNA was used to confirm the presence of the *ipt* construct in the plants with the help of specifically designed primers for this gene. Those plants positively identified were clonally propagated by cuttings.

2.3.11 Clonal propagation of watermelon by cuttings

Leaf bud cuttings were treated with 0.8% IBA powder and rooted in the potting media (MetroMix 350) in the growth chamber. The planted cuttings were covered with plastic bags and misted with distilled water at 2 days interval. Shoots and root growth were observed approximately 2-3 weeks from the planting date.

2.4 Results

After extracting plasmid DNA from *E. coli* cells, restriction digestion experiments were conducted and the presence of the construct was confirmed (Figure 2.2). This targeted DNA was later inserted into the *A. tumefaciens* (LBA4404) cells. The growth of transformed *Agrobacterium* cells on selective LB media was considered to be a successful transformation; however, the presence of the *ipt* gene construct in *A. tumefaciens* (LBA4404) cells was further confirmed by running PCR reactions with specifically designed primers (Figure 2.9). Once it was confirmed that the transformed *Agrobacterium* cells actually contained the construct, the stock culture was prepared and stored at –80 °C for later use in watermelon transformation. In the meantime the regeneration protocol defined by Compton and Gray (1996) was tested and observed with positive results (Figure 2.3).

Callus formations of inoculated cotyledonary explants on selective media started to show up two weeks after inoculation with *Agrobacterium* cells (Figure 2.4). These vigorous, greenish callus growths were considered to be the first indication of successful transformation. The shoot primordia began to appear at the end of the second sub-culturing (Figure 2.5).

Fourteen NPT II positive shoots were independently regenerated following transformation of watermelon with the *ipt* gene controlled by a copper inducible promoter. These lines were later identified with letter characters starting with *ipt*- line_A to *ipt*- line_N. Out of fourteen lines, four lines were identified as phenotypically aberrant lines, which either had no root formation or had little root formation together with reduced shoot growth and yellowish rosette leaves (Figure 2.5). Although these aberrant lines, especially the ones with little root formation, were transferred into sterile medium, they later died. The ones without roots were rinsed with sterile water and their

bases were dipped in 0.8% IBA powder and then planted in sterile medium; however, they did not survive. When these aberrant lines were exposed to growth chamber conditions, an apparent development of infection at the callus sites occurred that resulted in their death . Similar results were reported by some of the previous research with both conditional and constitutive *ipt* gene expression in transgenic tobacco plants (Faiss et al., 1997; McKenzie et al,. 1998). It is believed that one of the main reasons for aberrant line development was the uncontrolled expression of the *ipt* gene. Consequently, although the copper inducible promoter promised more control over the gene linked to it downstream in comparison to heat shock, light or tetracycline-inducible promoters, it is still hard to achieve complete control, resulting in the likelihood of leakage in some transgenic lines.

In addition to those aberrant lines, two lines with relatively normal shoot expansion did not form roots within the 3-month time period; instead, they continued to produce shoots until the end of the transformation-regeneration experiments. These shoots were later divided and treated with 0.8% IBA powder for root formation but without rooting success. The rate of shoot formation for Cu-*ipt* plates was approximately 15% (Figure 2.6); however, the percentage resulting in whole plants, including roots, was less then 10%. The development of small plantlets took about 3 months. Those lines that grew were later tested using the callus formation assay. The sterilized leaf tissues stayed green and formed calluses in the MS media with 5 μM BA, and Kan^{100} media during the month in culture, while leaf tissues from wild types turned brown (Figure 2.7).

These positive transgenic lines were then sprayed with 50 μM of copper sulfate and evaluated for phenotypic characteristics two weeks after spraying. Over-expression of cytokinin results in leaf expansion and increased lateral shoot development. These characteristics were used as the main criteria in short-term evaluations of transformation (Figure 2.8). This practical feature was used to both confirm the presence of the targeted construct in transgenic lines and, more impor-

tantly, to identify the lines that initiated the targeted gene expression with copper sulfate application. As a result of these observations, it was clear that Cu-*ipt* line $_C$ was responding to the copper sulfate application by increasing its leaf size. Phenotypic changes in the transgenic line compared to the wild types were found to be a practical tool for identification of transgenic lines. In fact, it has been previously reported that the inducible *ipt* gene can be used to develop transgenic lines without using antibiotic resistance marker and selection medium, which consequently may increase regeneration rate as well as reduce time for regeneration (Ebinuma et al., 1997; Kunkel et al., 1999). Indeed, by running the PCR reaction with specific primers, the targeted construct was confirmed to be present in the Cu-*ipt* line $_C$ (Figure 2.9). These results support the idea of using controlled expression of the *ipt* gene as a marker gene (Endo et al., 2001) for the purpose of an antibiotic-free gene transformation process.

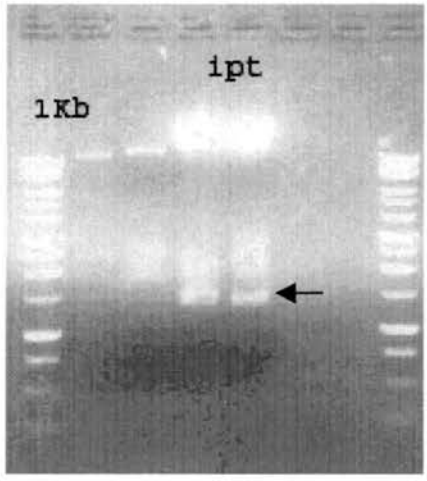

Figure 2.2. Confirmation of 1.35 kb ipt insert from the plasmid DNA of *E. coli* cells. The DNA was digested with Not1 restriction enzyme, and run on a 0.7% agarose gel for 1 and 1/2 hour

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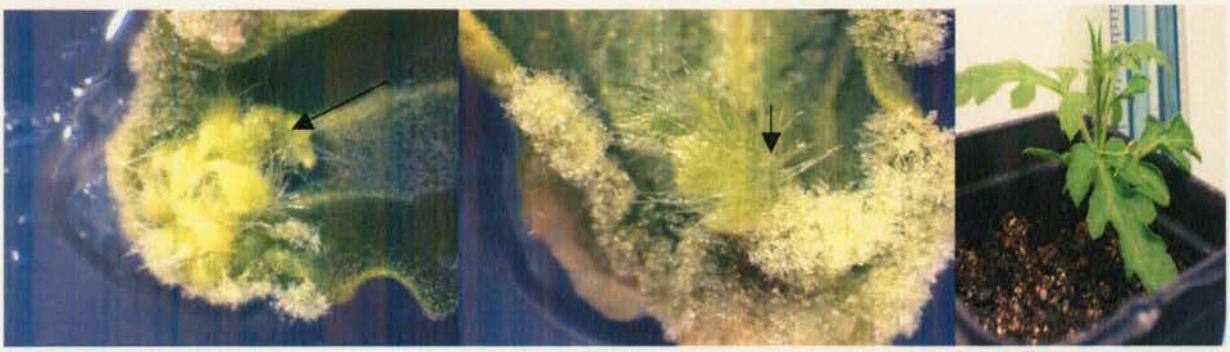
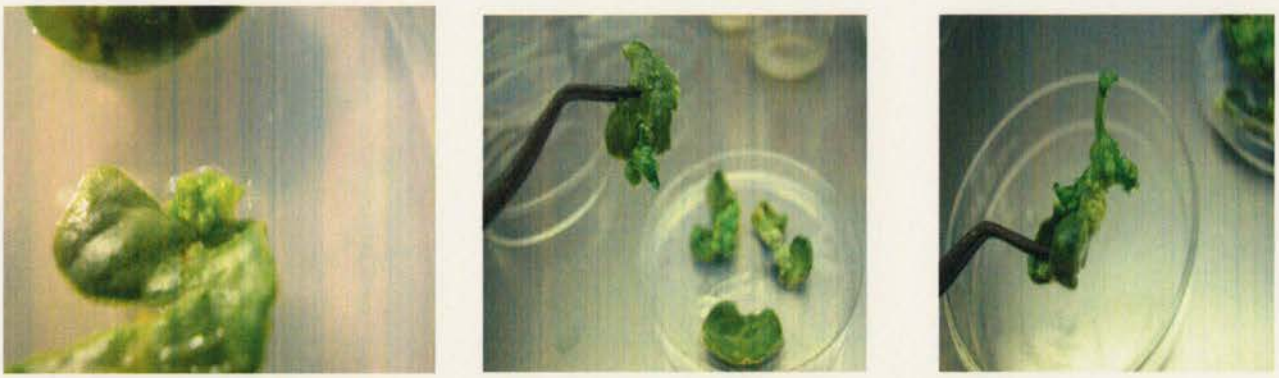
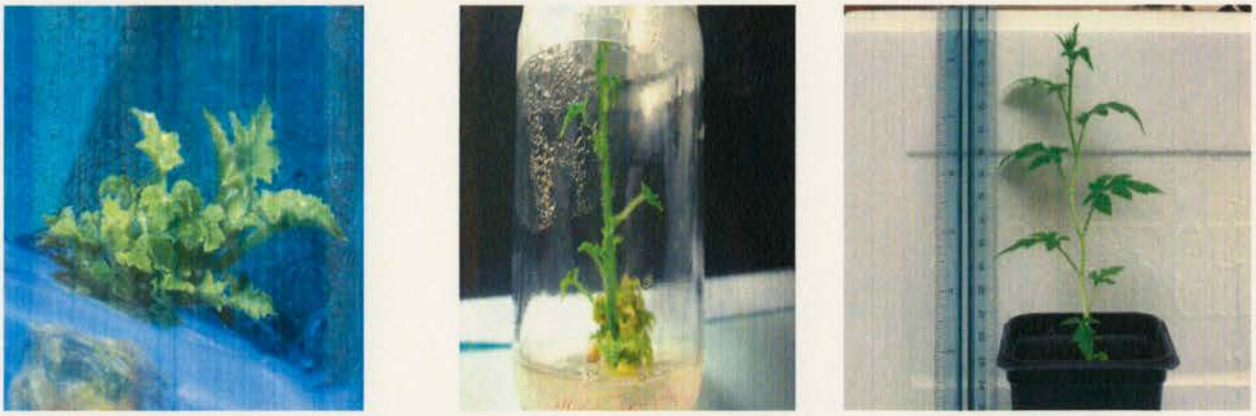


A B C

Figure 2.3. Regeneration of cotyledonary explants from Crimson Sweet in MS 5 μM BA (A and B). C represents a tissue culture regenerated watermelon plant.



A B C



D E F

Figure 2. 4 Shoot primordia formation in Cu-ipt explants after the second sub-culturing in MS, 5 μM BA, and Kan^{100} $Carb^{250}$ medium (A, B and C), Shoot primordia formation began to appear at the basal end of cotyledonary explants. These shoots were transferred to 200 ml baby food jars for continued growth (D and E) and finally transferred to soil medium (F).

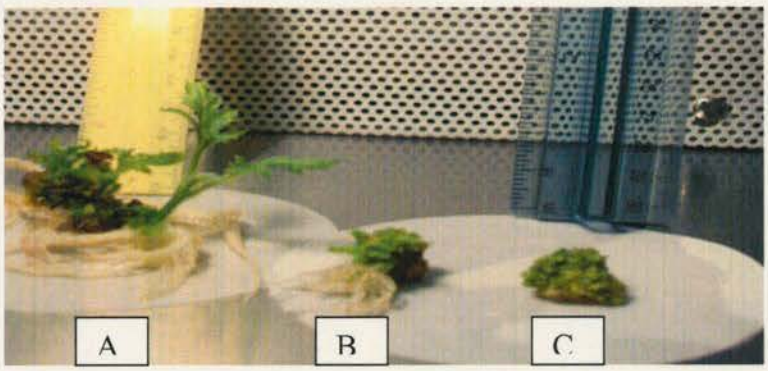


Figure 2.5. The morphological characteristics of different kanamaycin resistant lines. a. At the left, the line with normal shoot and root development; b. in the middle, the aberrant line with few roots and small shoots; c. At the right, the morphologically aberrant line with un-elongated shoots and rosette leaves without root formation.

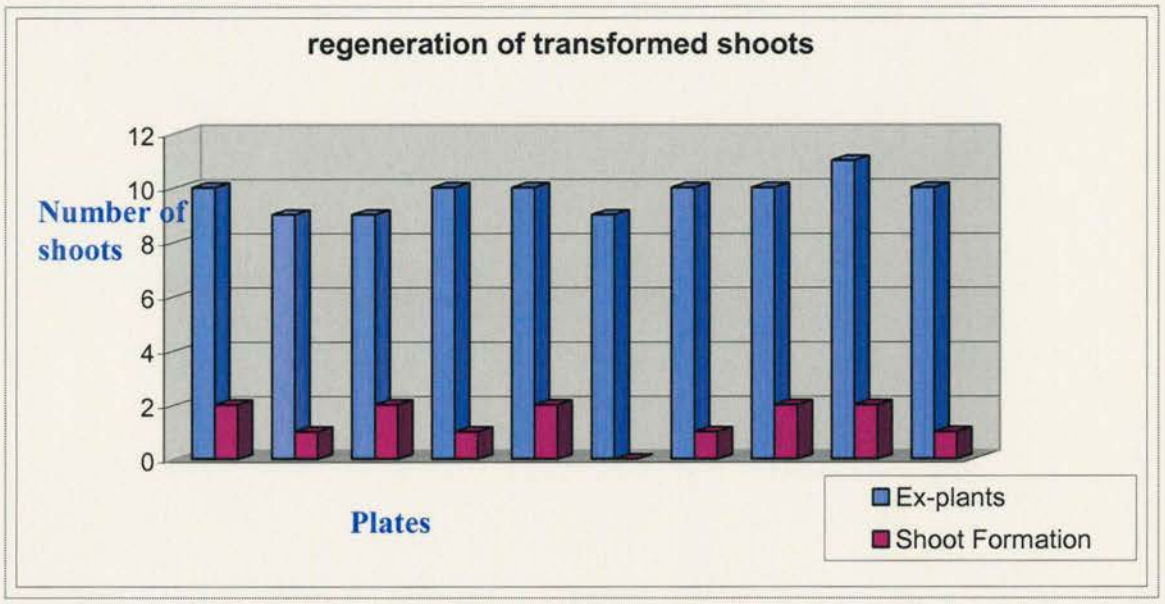


Figure 2.6. Number of shoots produced from tissues transformed with Cu- ipt construct. At the beginning, each plate contained approximately ten explants. Those plates that gave rise to green callus formation and shoot premodia were kept and the rest were eliminated during subsequent subculturing.

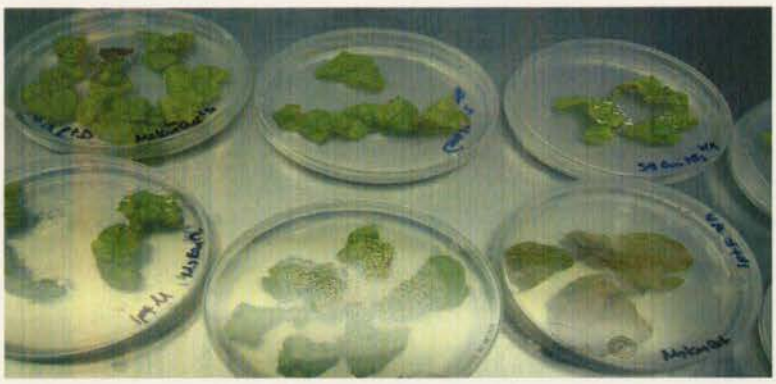


Figure 2.7. In the callus assay, leaf explants from Cu- ipt transformants formed calluses and stayed green during the entire experiment (4 weeks) while non- transformed explants turned brown within 2 weeks.

A B

Figure 2.8. A. Representative leaf sizes taken from transgenic plant at the left and non- transformed plants at the right B. Growth habits of induced lateral shoots and leaf expansion on Cu-*ipt* transformed plant at the right compare to non-transformed plant at the left. Both transgenic lines and to non-transformants were sprayed with 50 μM $CuSO_4$.

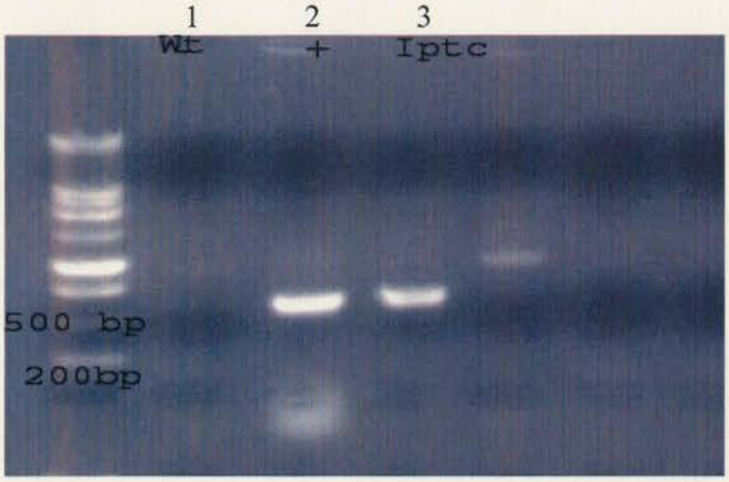
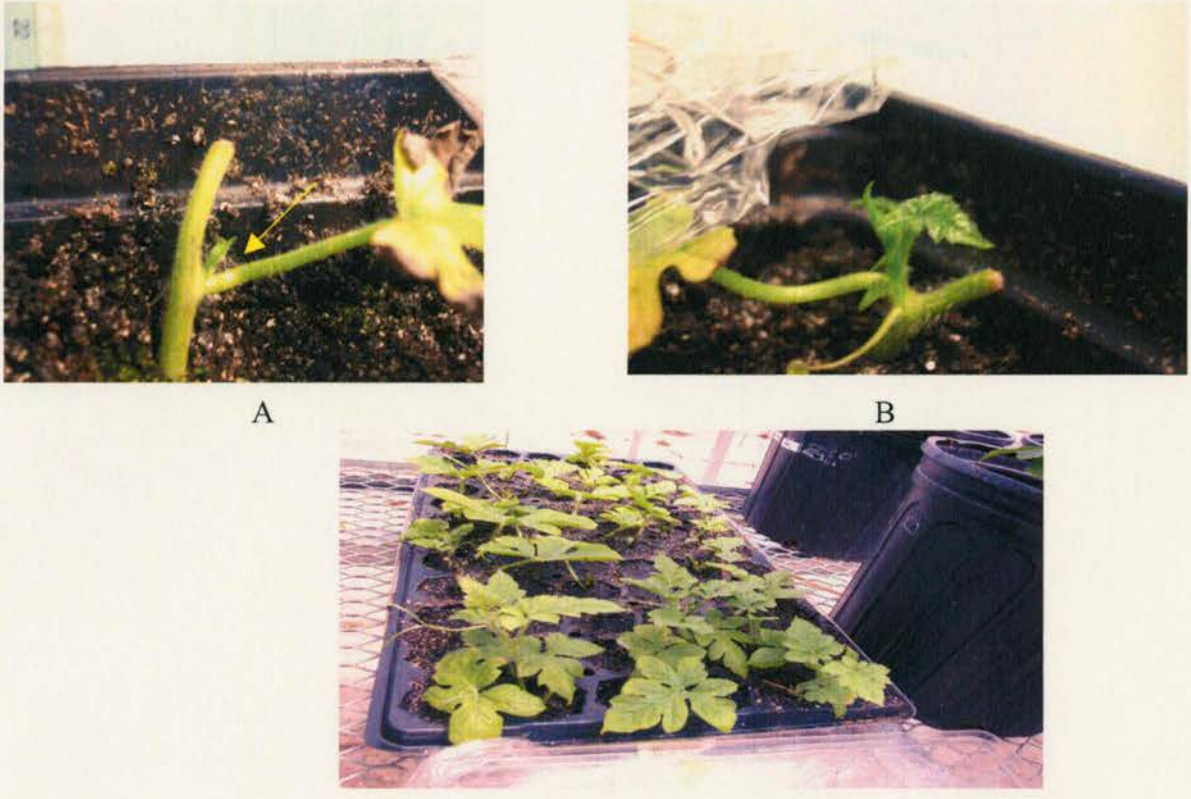


Figure 2.9. The PCR reaction confirming the presence of the *ipt* gene in transgenic line. 1) Wt; genomic DNA extracted from to non-transformants and used as a negative control. 2)+; DNA from ipt transformed Agrobacterium cells; used as a positive control. 3) IPTc; the candidate transgenic line.



A B C

Figure 2.10. Clonal propagation of watermelon via cuttings. A. The bud breaking phase; at this stage root formation was also observed. B. Shoot growth, which took almost three weeks. C. Completion of clonal propagation.

One of the other possible reasons for aberrant line development (Figure 2.5) could be explained by the likelihood of mutation that might occur during the gene transformation process. In fact, the whole idea behind the gene transformation process might be considered as a generating the mutation because of the introduction of an alien gene(s) into the plant genome. It is well known that *Agrobacterium* randomly inserts the Ti plasmid inside the plant genome. This insertion site(s) usually remains a mystery. Therefore, the generation of mutation as a result of insertion in native gene(s) could be a potential risk factor for the *Agrobacterium* mediated gene transformation process. The disruption of gene(s) especially for apical dominancy might lead to the development of the aberrant lines. In this study apical dominance broken by either over production of cytokinins or possible mutation prevented single dominant shoot formation; instead, these lines continued to produce extensive numbers of small shoots with small, unexpanded, wrinkled leaves.

Once the transgenic line was identified, both tissue culture generated wild type plants as well as Cu-*ipt* plants were clonally propagated by cuttings (Figure 2.10). The rate for rooting and growth of cuttings was observed to be as high as 50-70%. This is comparable to seed germination in triploid watermelon species. At least ½ of the leaf blade was maintained on 3-5 cm long cuttings.

2.5. Discussion

One of the main factors for the successful transformation of watermelon is a reliable regeneration protocol. Since successful regeneration varies considerably among different genotypes, it is important to first verify regeneration of the genotype of interest. In the case of this research, the cotyledons of the cv. Crimson Sweet were regenerated based on the protocol described by Compton and Gray (1996). The major modification was to use MS -medium without $CuSO_4$ and

rooting medium without plant growth regulator. In the parallel experiments, the cotyledons of cv. Sugar Baby developed white callus and failed to regenerate (data not shown).

It was important to maintain an exact 2-week interval for subculutring. Those explants which did not transform or develop callus turned brown and released phenolic compounds which likely negatively affected or even inhibited the growth of other regenerants (these released phenolic compounds were seen in the brown discoloration of the MS medium). During the sub-culturing process, the cotyledon sections that generated shoots at their base were transferred aseptically while the rest were eliminated.

The CTAB extraction protocol is simple and can be completed within a short period of time. However, with this technique, the extraction of good quality DNA that can be used for PCR and restriction digesting experiments requires some sensitive handling, as well as experience. The presence of phenolic compounds is the key concern in successful DNA extraction. These compounds carry a high tendency to be oxidized and irreversibly bind with proteins and nucleic acids (Chaudhry at al., 1999). Since this binding makes DNA unsuitable for molecular work, it is important to avoid it, especially during the homogenization process. Once the finely-powdered tissue is added to the CTAB extraction buffer, the development of the brown color is the visual indication of the unwanted chemical interaction. To completely avoid or reduce the release of phenolic compounds, the plant materials should be maintained in liquid nitrogen during the entire homogenization process. Thawing the grinded tissue during the homogenization process and before adding to the CTAB extraction buffer may induce the release of phenolics resulting in the brown discoloration. Therefore, it is important to maintain the fine powdered tissue frozen and transfer it into the pre-warmed CTAB extraction buffer as fast as possible.

In terms of transformation efficiency, we were able to independently regenerate 14 kanamycin resistant shoots in a single experiment. The success rate in shoot formation was close to 15%, which is considered to be a normal range for gene transformation using *Agrobacterium* as the vector. However, the rate of the regeneration of whole plants with roots was relatively lower (less then 10%) then the normal range of this vector. The characteristics of the targeted gene (*ipt*) and the use of the inducible promoter (copper inducible) might be related to the poor root formation and normal plant development from the regenerated shoots. The regeneration process was conducted entirely in copper free medium. However, the possibility of even a slight activation of the gene by other ions requires further investigation.

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Chapter 3

Activation of Isopentenyl Transferase (*ipt*) Gene in Transformed Watermelon Influences Seed Development as Well as Other Characteristics.

3.1 Abstract

The watermelon "Crimson Sweet" was transformed with copper-inducible isopentenyl transferase (Cu-*ipt*), the rate-limiting step in the cytokinin biosynthetic pathway, gene via *Agrobacterium tumefaciens* (LBA4404). Transformed (*ipt)* and non-transformed plants were regenerated from tissue culture and clonally propagated by the rooting of leaf node cuttings. Twelve plants of each treatment were grown in 1 gal pots filled with MetroMix 350 medium and supported with approximately 11 grams of Osmocote (18% Nitrogen, 6% Phosphate and 12% Potassium). The plants were grown at approximately 28-30 °C day, 20-22 °C night, with 16 hours of daylight under greenhouse conditions in the summer and early fall of 2003.

Once the plants started to grow both Cu-*ipt* transformants and non-transformed plants were sprayed with four different concentrations (0, 5, 10 and 50 μM) of $CuSO_4$. The experimental unit was a single plant with three replicates. The growth rate, number of leaves, flowers, lateral shoots, and chlorophyll content were measured weekly for five weeks. The weight of three randomly selected, fully expanded leaves from the base, mid and upper sections of each plant was also measured at the end of the five-week period. Significant increases were observed in favor of copper sulfate treated Cu-*ipt* transformants compared to the non-transformed plants in leaf weight ($p<0.0001$), number of lateral shoots ($p=0.02$), number of female flowers ($p=0.007$) and chlorophyll content ($p=0.027$). The viability of pollen for all plants was measured by germination on BK medium and a slight but significant reduction in pollen grain germination was noted in copper sulfate treated Cu-*ipt* plants compared to the non- transformed plants ($p=0.001$).

Plants of both types were self-pollinated. Fruits were grown for eight to ten weeks with support. Once they reached maturity, fruit was harvested and fruit shape, flesh color, number of normal seeds, number of colored but empty seeds, and number of white seeds were recorded. Significant reductions in normal seed numbers ($p<0.0001$) were observed between Cu-*ipt* transformants and non-transformed watermelon fruits. The number of seeds in transgenic watermelon plants treated with the three different concentrations of $CuSO_4$ was reduced to about 5-7% of non-transformant plants. Transgenic plants which were not treated with CuSO4 had approximately 33-50% of the seed of non-transformants.

3.2 Introduction

Watermelon, *Citrullus lanathus (Thunb.) Mansf.*, is considered to provide one of the largest edible fruits among crops. Its size, shape, color and flesh texture varies extensively among cultivars and the range of the fruit sizes can extend from 3 to 100 lb for the cultivars New Hampshire Midget and Tom Watson, respectively (Mohr, 1986). The red flesh color is most commonly accepted in all parts of the world; however, in terms of fruit size and shape, consumers in different cultures have different preferences. While icebox varieties are popular in some parts of the world, others prefer large fruits and the majority of consumers generally prefer seedless cultivars. Although there are some differences in consumption patterns, this horticultural crop is one of the most commonly consumed vegetables in the world, especially as refreshment during the hot summer days.

Over the last few decades, there has been a fluctuating but generally increasing trend in the consumption of watermelon in the United States as well as other parts of the world. The consumption of 16.8 pounds per person in 1996 in the United State was considered to be the highest peak recorded since 1958 (Luicer and Lin, 2001).

Although there are several factors that tend to increase watermelon consumption, the development of seedless watermelon brought about a huge and positive impact on its cultivation and consumption. Consumers prefer to eat seedless watermelons due to their relatively easier consumption (Sugiyama and Morishita, 2002) and protection of children from choking on seeds. It is also thought that seedless watermelons contain better flesh quality than seeded types. These characteristics of seedless watermelons affect their demand and induce retailers to carry and sell more seedless varieties. The nutritional value of watermelon, the development of icebox varieties, and their availability in the market throughout the year are

other factors that have increased watermelon consumption. Watermelons are highly regarded as an excellent refreshment which does not contain any cholesterol or fat, is a good source of carbohydrates, and vitamins such as A and C, as well as minerals such as calcium and iron.

The seedless watermelon cultivars are traditionally developed by crossing tetrapliod varieties with diploid varieties in order to produce triploid hybrid plants. This technique was developed by H. Kihara in 1950 at Kyoto University in Japan (Kihara, 1951). However, due to the technical difficulties and cost efficiency its use was limited until recent years, when the demand for seedless watermelon stimulated great interest.

Watermelon cultivars are normally diploid, with twenty-two chromosomes (2X=22), while tetraploid carriers have forty-four chromosomes (4X=44). By crossing diploid male watermelons with tetraploid female ones, their hybrid progenies become triploids with an odd number of thirty-three chromosomes (3X=33). Due to defective gamete development, triploid plants are normally sterile and fail to produce seeds; however, they still produce fruits when cross-pollinated with diploid male watermelons.

Although this breeding technique is the key element for seedless watermelon production, some alternatives, such as the application of various plant growth regulators, especially synthetic cytokinin-1-(2-chloro- 4-pyridyl) -3-phenylurea (CPPU) (Hayata and Miimi 1995) and irradiation of pollen with soft x-ray(Sugiyama and Morishita, 2002) have also been reported. Each technique has some advantages and disadvantages; consequently, alternative methods have been proposed by the scientific community and watermelon breeders. Triploid watermelon development is not generally cost efficient (Sugiyama and Morishita, 2002) due to technical difficulties related to lower fertility rates in tetraploid female parents

and reduced seed germination of triploids. Temperature and moisture are two critical components for triploid seed germination and these factors need to be in the optimum range. The short life span of triploid seeds—generally only one year—requires fresh seed purchases each year. The requirement of a diploid male parent as a pollinator for stimulating fruit set also entails extra expense and labor in the production field.

The irradiation of pollen with soft x-rays has reduced the seed number in watermelon but has not been applied on a large scale. The high cost of the necessary equipment and required expertise for this technique is still questionable. Another reason may be that this technique was developed for small-scale laboratory use rather than large-scale field application.

The application of plant growth regulators such as cytokinin in developing seedless watermelon has been tried but has generated safety concerns. Like other pesticide and herbicide applications, uncontrolled absorption of plant hormone via spraying has aroused concerns for growers and consumers. Residual pollution of the environment and ecosystem are other primary concerns in a spraying application technique.

As one of the major plant phytohormone groups, cytokinins are adenine derivatives with diverse structures, and they play an important part in plant growth and development. Although their role in plant growth and development *in vivo* is often concealed by other hormones (Mok and Mok, 2001), some of the distinguished actions of these growth regulators are induction of cell division, lateral shoot development, delaying senescence, and disease and insect resistance (Davis, 1995; Winger, et al. 1998; McCabe et al., 2001; Gan and Amasino, 1996; Smigocki et al., 1993; Storti et al., 1994).

Although they are primarily considered as plant growth regulators, various bacteria such as *Agrobacterium* also synthesize cytokinins (Kende and Zeevaart, 1997). Skoog and Miller discovered this groups of hormones in the late 1940s and 1950s (Kakimoto, 2001) while working on plant tissue culture media development for tobacco stem pith cells (McGaw and Burch, 1995). The first identified cytokinin-like compound kinetin was derived from autoclaved herring sperm DNA in 1959 (McGaw and Burch, 1995). Although at the beginning cytokikins were classified as naturally occurring components that stimulate plant cell division, later they were identified as having a significant impact on the germination of seed as well as senescence (Gan and Amasino, 1996). Miller and Skoog (1957) reported that cytokinins regulate the process of cell division in the presence of auxin and induce shoot formation in calli during the regeneration process in tissue culture (Kakimoto, 2001; McKenzie et al., 1994). Cytokinins regulate cell division during the plant development stages, wounding, gall formation and tissue culture.

Cytokinins are also involved in cell enlargement, tissue differentiation, dormancy, and different phases of flowering and fruiting (Hartmann et al., 1988; Hartmann, et al., 1997). While cytokinins regulate plant cell division, they act antagonistically with another group of plant phytohormones called auxins. Cytokinin concentration basically determines the type of organ formation. Exogenous application of cytokinin alone promotes shoot formation during the regeneration process; however, the application of auxin alone induces root formation. The presence of both hormones generates undifferentiated callus without organ formation (Hartmann et al., 1988). The concentration of cytokinin is a crucial factor for activity in cell culture, therefore, sensitivity needs to be determined during the regeneration of different plant species. The identification of optimum cytokinin concentration provides maximum

regeneration rate as well as minimizes toxicity, which negatively affect callus and organ formation. It has been previously reported that high concentrations of cytokinin and duration of exposure can inhibit cell proliferation and induce cell death (Carimi et al., 2003).

Although root apical meristems are the primary sites for cytokinin biosynthesis, there is some evidences that shoot meristems as well as seeds are also involved in cytokinin biosynthesis. Once they are synthesized, cytokinins are transported through the xylem to the upper parts of the plant.

The *Agrobacterium*-originated isopentenyl transfarese gene (*ipt*), which catalyzes the rate-limiting step of the cytokinin biosynthetic pathway, has facilitated the study of flower senescence in petunia (Chang, 2002), leaf senescence in tobacco (Ori et al., 1999; Jordi et al., 2000) and lettuce (McCabe et al., 2001), and disease resistance in tobacco (Bettini et al., 1998). It has also been used as a selective marker gene in an antibiotic free transformation process (Kunkel et al., 1999).

One of the main reasons for the extensive application of bacterial *ipt* gene to elevate cytokinin concentration was the inability to identify the plant's native gene(s) for cytokinin biosynthesis. However, nine putative genes involved in the cytokinin biosynthesis have been reported in the *Arabidopsis* genome (Kakimoto, 2001). The *ipt* gene, however, has been cloned and sequenced, as well as proven to control cytokinin production in several studies more than a decade ago. The ability to regulate cytokinin biosynthesis with a single gene also facilitates the use of the *ipt* gene, especially for transformation studies. The most important limiting factor for the insertion of the *ipt* gene in to various plant species was the negative aspect of cytokinin over-production during the regeneration process, especially the lack of root formation and the development of morphologically aberrant lines which had numerous shoots

with a rosette structure and wrinkled leaves. In order to minimize the undesirable effects of over-expression of cytokinin on normal plant physiology, the *ipt* gene has been engineered with different inducible promoters. These auto-controlled *ipt* transformed plants facilitated their use in the re-evaluation of the significance of cytokinin in normal plant growth and development. The main objective of this study was to evaluate the use of copper sulfate levels in inducing (Cu-*ipt*) cytokinin and the influence of over-expressed cytokinin on horticultural characteristics.

3.3 Materials and methods

3.3.1 Experimental units

In order to evaluate different horticultural traits, twelve clonally propagated Cu-*ipt* plants and twelve non-transformed watermelon plants were planted in 1 gallon pots filled with MetroMix 350 with subsequent transfer to the greenhouse. During the first week, these plants were hand-watered lightly each day in order to acclimate them to greenhouse conditions. They were also treated with the systemic insecticide "Marathon" in order to prevent insect damage, especially from white flies. Plants were supplied with the slow-release fertilizer Osmocote (18 % Nitrogen, 6% Phosphate and 12% Potassium) in the amount of 11 grams per pot. Once they showed signs of adapting to greenhouse conditions by starting to grow, both *ipt* transgenic plants and wild type plants were sprayed with four different concentrations (0, 5,10 50 μM) of copper sulfate. The experimental unit was a single plant with three replicates. As controls, three Cu-*ipt* transformants and three non-transformed plants were utilized without application of copper sulfate. Copper sulfate solutions were sprayed until the solution ran-off from the

leaves. The Cu-*ipt* transformants and non-transformed plants were then evaluated for horticultural traits as compared to the control plants.

3.3.2 Plant Height and Leaf Number

Both Cu-*ipt* transformants and non-transformed plants were measured at the time of the application of copper sulfate, and thereafter at one-week intervals for 5 weeks. Watermelon plants were grown upright with clippers and lines, attached to 150 cm hanging columns. Plants were checked each day and the growing sides were gradually attached to the lines to prevent bending. It is a feature of normal watermelon growth that each inter-node carries one leaf; hence, in order to evaluate the internodes' length, the number of leaves was counted as well. Fully expanded leaves were recorded during height measurements. The average internode length was estimated by dividing total plant height in to the total number of leaves.

3.3.3 Lateral Branches

Lateral branches began to appear approximately one week after spraying. With a few exceptions, all lateral branches expanded and developed their own leaves and flowers. The lateral branches were recorded until the end of the five-week growing period. At that time, the main branch had reached a length of approximately 150 cm. During this period, it was important to keep the main branches straight as bent branches increased lateral shoot initiation and growth.

3.3.4 Number of Flowers

Fully-opened male and female flowers were counted every two days, including those on lateral shoots. Recorded flowers were removed from the plants. Only those female flowers that were selfed were left to grow.

3.3.5 Leaf Senescence

In order to artificially induce senescence, the plants were treated with drought stress at six weeks. Both Cu-*ipt* transformants and non-transformed watermelon plants were exposed to water stress at this stage; that is, irrigation was withheld until they started to wilt but were still alive, which occurred after eight to ten days. Once they remained for twenty-four hours under wilt conditions, they were irrigated on a regular basis for two weeks. The drought stress was then repeated. They were again re-watered on a regular basis for two weeks, at which time they were again exposed to water stress. During this period, the watermelon plants were observed closely for senescence. The development of yellow leaves, either partially or wholly, was the trait to evaluate. The treatments were evaluated for leaf chlorisis 10 days following watering after the second drought stress. The number of leaves that completely or partially turned yellow was recorded for each treatment. At the time of evaluation, some of the early senesced leaves were already turning brown but still remained on the plants. Spider mite infestation, especially during the water stress treatments was the biggest problem during the induction of senescence. In order to reduce their infestation, leaves were sprayed with water once a day to wash them off. The washing treatment reduced their number; however, it consequently increased the required time to bring plants to wilting.

3.3.6 Fertility of Flowers and Self-pollination

In terms of plant productivity or yield, the viability of flowers is important in plant breeding programs. Developments of viable and normal flower parts are the first crucial steps in productivity of many plant species.

Watermelon is classified as a monoecious plant, with male and female flowers occupying separate places on the same plant. In the early stage of plant development, male flowers usually appear first whereas female flowers do not appear until plants reach the 5-6-node stage. It is quite easy to distinguish male flowers from female. While they both show up under the leaf base with yellow petals, female flowers normally carry a distinctive ovary on their base, while male flowers have no such organ. Both male and female flowers stay open up to two days. Petals then start to wilt and the yellow petals turn brown. Male flowers begin to abscise from the plant within a couple of days. Female flowers will abscise only if unfertilized. Fertilized flowers continue to grow and develop fruit. Fertilization of flowers normally occurs with help of insects, especially honey bees. The required growing period for fruit development and ripening varies among the cultivars.

It has been reported that the over-expression of cytokinins often cause flower infertility (Wang et al, 1997). One of the practical ways of investigating pollen viability is using *in vitro* germination. This technique has been proven to be one of the most efficient ways to examine germination physiology and biochemistry of pollen grains; it is also an effective technique for studying the responses of the pollen system to physiological and chemical factors (Shivanna and Rangaswamy, 1992). This technique can be simply described as the collection of pollen from dehisced anthers followed by their growth on a species-specific medium, either under aseptic or non-aseptic conditions. One of the major factors that negatively affects the application of this technique is that the observations depend on a number of variables related to both genotypic and environmental factors.

As members of the *Cucurbitaceae* family, watermelon pollen grains are classified as binucleate with a generative cell and vegetative nucleus (Brewbaker, 1967).

Collecting pollen at different flowering periods such as the early, mid and late flowering period, as well as at different times of the day, has a significant impact on pollen responses (Shivanna and Rangaswamy, 1992). The results vary among individuals and among different samples from the same individuals (Shivanna and Rangaswamy, 1992). In order to obtain reliable results, it is important to reduce these variables as much as possible. Collecting pollen grains from flowers that are at approximately the same stage of development, and collecting them at the same time of day reduces variability. Seed set is also an effective way to determine pollen viability.

3.3.7 Pollen Viability

Pollen from each plant was collected and cultured on a medium described by Brewbaker and Kwack, (1963), known as BK medium (sucrose 10%, boric acid 100 mg/l, calcium nitrate 300 mg/l, magnesium sulfate 200 mg/l, potassium nitrate 100 mg/l, agar 6-7%; pH 7). The media was used without sterilization for germination (Shivanna and Rangaswamy, 1992). Collected pollen was sprinkled on BK medium petri plates, sealed and left at room temperature for 24 hours for germination. Once the germination process was completed each petri dish was counted under a dissecting scope. Three randomly selected fields were examined and 100 pollen grains counted. During counting, only pollen grains with a pollen tube at least greater than the pollen diameter were considered as geminated. The tetrads were considered as four separate pollen grains and their germination was counted based on the expanded pollen tubes.

3.3.8 Setting Seeds

Both Cu-*ipt* transformants and non-transformant plants were self-pollinated to generate seeds. The female flowers were bagged just before blooming in order to prevent cross-

pollination. Once the female flowers were fully opened and before the stigma changed color, they were carefully self-pollinated. The pollen from male flowers on the same plants was collected with a small camels hair brush and maintained in clean petri dishes until selfing. For self–pollination, a clean brush was lightly dipped into the pollen-containing petri dishes and then gently touched to the top of the stigma. The brushes were dipped into 70% Et-OH and air-dried for a few minutes before they were used on other plants. Self-pollinated female flowers were bagged for at least two days. The paper bags were then carefully removed and the development of fruit was observed. If there was a successful pollination, the small ovary, present at the base of the female flowers started to grow. Otherwise, it turned brown and adscised.

3.3.9 Measuring Chlorophyll

Chlorophyll content from each of the treatments was measured. A chlorophyll meter (SPAD-502 model manufactured by Minolta) was used to measure the chlorophyll content in leaves. For each plant, three leaves, which were located at approximately the same position in terms of height and direction, were selected for chlorophyll measurement at 2 weeks after copper sulfate application. The same leaf was measured at three different points, two points on each side and one point at the tip of the leaf, and the individual and average readings were recorded. Once the readings were completed on a particular leaf, all data were cleared before moving on to measure the next leaf. The readings began at the lower leaves and moved in the direction of the upper leaves. The chlorophyll meter SPAD-502 reads the relative amount of chlorophyll present in leaves as a SPAD value, which is defined by Minolta.

3.3.10 Chlorophyll Extraction

The protocol described by Smigocki et al., (1993) was followed to extract the total chlorophyll content, as well as chlorophyll a and b, in leaf tissue 2 weeks after copper sulfate application. The process began by grinding 0.1 g of leaf tissue in liquid nitrogen. Once the tissue was homogenized it was re-suspended with 5ml of cold acetone and incubated at –20 °C overnight. The following day, 1.5 ml tubes were centrifuged and the supernatant was gently pipetted into new, clean tubes. The extracted supernatant was used to measure the chlorophyll a and b content by observing their absorbance at 645 and 665 nm, respectively.

3.3.11 Fresh Leaf Weight

Leaf samples were collected at the end of the fifth week after spraying. Three leaves from each plant were randomly taken at the base, middle and top section of each plant. These collected leaves were placed inside a plastic bag and carefully marked. They were then stored on ice until the sample collection process was completed. These leaves were then taken to the lab and the fresh weight of each individual leaf was measured.

3.3.12 Data analysis methods

The data for evaluated horticultural traits were analyzed by using SAS means procedure and Least Squares Means (LSD) in order to identify any evidence of significance between Cu-*ipt* transformants and non-transformed watermelon plants. The regression analysis were also conducted to determine the possible relation between different copper sulfate concentrations and evaluated traits. The regression analysis was conducted within Cu-*ipt* transformants and non-transformed plants separately. For regression analysis, copper sulfate concentrations (0. 5, 10, 50 μM) were considered to be independent variables while the evaluated traits such as leaf weight were considered to be dependable variables.

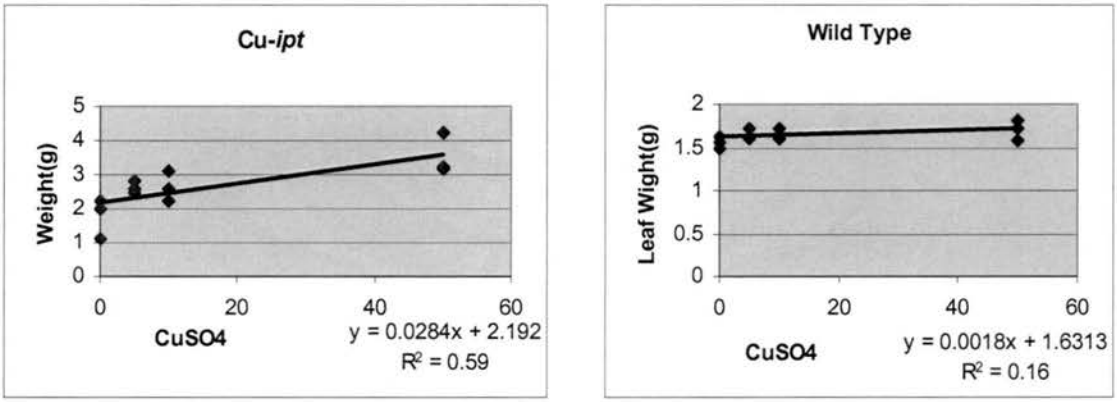
3.4 Results

3.4.1 Plant height and leaf numbers

The impact of over production of cytokinin on different horticultural traits was observed by comparing among Cu-*ipt* watermelon transformant and non-transformant plants. As reported previously, the copper inducible promoter is capable of providing tight regulation over closely linked genes and its activation is highly dependent on the presence of the inducer (Mett et al., 1993, Mett et al., 1996). In this study, three different copper sulfate concentrations did not interfere with the growth rate of Cu-*ipt* transformants and non-transformant plants. In terms of growth rate and plant height, there were no significant differences among Cu-*ipt* transformant and non-transformed plants. The growth rates in non-transformed plants were slightly higher (average of 31±2 cm/week) but not significantly different than the growth of the main stems in Cu-*ipt* transformants (average of 29±2 cm/week). The induction and expansion of lateral shoots in Cu-*ipt* transformant might be one of the reasons for these slight differences. At the end of the five-week growing period, the Cu-*ipt* transformants and non-transformed plants reached approximately similar heights, 152±15 and 160±15 respectively. The plant height for the Cu-*ipt* transformants and non-transformed plants weren't significantly different ($p=0.9$). This is similar to plant height date with other species in which *ipt* transformants were compared with not transformed plants (Loven et al., 1993; McKenzie et al.,1998; Guivarch et al., 2002). Leaf numbers of the main stems of copper sulfate treated Cu-*ipt* transgenic plants were slightly higher than non-transformant plants, however the differences were not significant ($p=0.417$) (Table 3.1).

3.4.2 Leaf weight

Leaf weight was significantly greater ($p<0.01$) in the Cu-*ipt* plants treated with copper sulfate compared to non-transformed plants and non-treated Cu-*ipt* plants (Table 3.1). The regression analysis of leaf weight of Cu-*ipt* plants with and without copper sulfate application indicated that the copper sulfate concentration had an impact on leaf weight ($R^2=0.59$). However, there was no such relationship within non-transformant plants ($R^2=0.16$) (Figure 3.1). After the activation of the *ipt* gene, Cu-*ipt* plants developed larger and heavier leaves than non-transformants. The differences were so obvious that they could be easily distinguished by the naked eye (Figure3.2). The larger leaves in Cu-*ipt* transformants as compared to the control plants were also observed in transgenic tobacco plants (Smigocki et al., 1993; Wang et al., 1997).



A *B*

Figure3.1. A. Regression analysis of fresh leaf weight of Cu-*ipt* transformants over four different CuSO4 (0,5,10,50 μM) concentrations. B. The regression analysis of fresh leaf weight of non-transformant plants over the same CuSO4 concentrations .

Figure3. 2. The Cu- *ipt* transformants are located at the left and non-transformant plants at the right of each picture. Leaves were taken from the bottoms and tops of plants treated with copper sulfate.

3.4.3 Lateral Branches

Under normal conditions, seedlings of cv. Crimson Sweet develop one main stem and one or two side shoots from the lower leaf bases. Although the number of shoots varies greatly among different watermelon varieties, cultivated watermelons generally develop limited numbers of shoots (Mohr, 1986). Unlike seedlings, the growth of clonally propagated watermelon plants is based on a single dominant stem, basically because they are propagated by a single bud (Figure 3.4). However, when they were sprayed with copper sulfate, considerable lateral shoot growth was observed in Cu-*ipt* watermelon transformants, starting seven to ten days from application. The induction of lateral shoots in copper sulfate treated Cu-*ipt* transformants was significantly greater than in non-transformant ($p= 0.022$ and $R^2=0.59$)(Table 3.1). Untreated Cu-*ipt* plants had a similar number of lateral shoots as treated and non-treated non-transformant plants. The vigor and extension of induced lateral shoots were not identical; however, most of them continued to grow with their own male and female

flowers. Similar results of reduction of apical dominance and release of axillary buds have been reported in *ipt* transgenic plants in several studies (Medford, et al., 1989; McKenzie et al., 1998; Guivarch et al., 2002).

a *b* *c*

Figures 3.3. The Cu-*ipt* plants showed reduced apical dominance and increased number of lateral branches compared to non-transformant plants. Cu-*ipt* transformants are located at the left side and non-transformant plants are located at the right side picture. The plants were sprayed with 50 µM CuSO4 (a), 5 µM CuSO4 (b), and 0µM CuSO4 as control demonstrated a different growth habit in terms of lateral branch formation.

Table3.1 Least Squares Means of leaf weight, number of leaves and lateral shoots for the Cu-ipt and non transformant plants treated with different concentrations of copper sulfate (n=3 plants / treatments).

Plants (n=3/treatments)	CuSO4	Leaf weight (g)	No. of leaves main stem	No. of lateral shoots
	50 uM	3.37 a*	28.66 a	3.33 a
	10 uM	2.627 b	26.00 a	3.00 a
IPT	5 uM	2.621 b	25.66 a	3.33 ab
	0 uM	1.862 b	24.66 a	1.00 cb
	50 uM	1.73 c	23.00 a	0.66 c
	10 uM	1.69 c	24.66 a	0.66 c
Wt	5 uM	1.6 c	24.00 a	1 cb
	0 uM	1.72 c	24.33 a	0.66 c

* Means followed by the same letter are not significantly different at $p<0.05$ by LSD procedure.

3.4.4 Leaf senescence

One of the well-known effects of cytokinins in plant growth and development is delaying senescence. This effect has been reported a number of times in various plant species (McKenzie et al., 1998; McCabe et al., 2001; Jordi et al., 2000) transformed with *ipt* gene. A delay in leaf senescence is not considered important in ornamentals and crops whose vegetative parts are consumed, such as lettuce. However, keeping watermelon plants green for a longer period of time might have some positive effects on late seasonal harvests.

Both Cu-*ipt* watermelon transformant and non-transformant plants were evaluated for senescence ten days after the second water stress treatment. The leaves that turned yellow to brown were reported as senesced leaves. Significant differences ($p<0.01$)(Table 3.2) were observed between Cu-*ipt* transformants and non-transformant plants treated with copper sulfate (Figure 3.3). The Cu-*ipt* watermelon transformants sprayed with copper sulfate stayed green, while the leaves on non-transformant plants started to turn brown. The Cu-*ipt* watermelon

transformants and the non-transformant plants with no copper sulfate were similar to each other and significantly different from the copper sulfate treated transgenic plants.

The interesting thing about leaf senescence in these plants was that the yellow and brown leaves did not fall off the mother plants right away. Shoot development was also observed under the base of the senesced leaves when plants were kept under a regular watering schedule. One of the main challenges during the induction of senescence was spider mite attack, especially during the water stress treatment. The plants were sprayed with water once a day in order to wash away these insects, and this spraying process consequently increased the time period to reach wilting conditions. Some of the yellowish color development, especially on upper leaves (Figure 3.3) was mainly due to insect damage.

A *B*

Figures 3.4. The plants that showed leaf senescence. The Cu- *ipt* transgenic plants are at the right and non-transformant plants are at the left. The plants in picture A were treated with $CuSO_4$. Picture B shows the untreated control.

3.4.5 Chlorophyll Content

Chlorophyll content as measured by both extraction technique ($p=0.0274$) and Chlorophyll Meter SPAD-502 ($p<0.04$) (Table 3.2) was found to be relatively higher and significant in copper sulfate applied Cu-*ipt* plants as compared to non-transformant plants. The control Cu-*ipt* transformant and non-transformant plants had similar chlorophyll content. This agrees with other reports in which *ipt* transformants had greater chlorophyll content as compared to the non-transformed plants (Wingler et al., 1998; Ori et al., 1999).

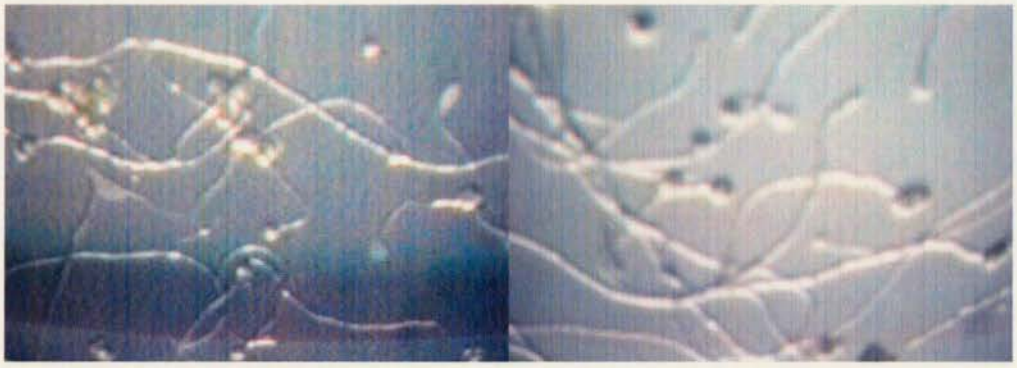
3.4.6 Number of male and female flowers

Numbers of female flowers in copper sulfate treated Cu-*ipt* transformed plants were significantly higher ($p=0.007$) than non-transformed plants during the five weeks growing period (Table 3.2). Other than cytokinin effects, extensive numbers of lateral shoots in copper sulfate treated Cu-*ipt* transformants might be one of the reasons for higher numbers of female flowers development in Cu-*ipt* transformants. Female flowers also started to develop approximately 7 days earlier in copper sulfate treated Cu-*ipt* transformants compared to non-transformants. While female flowers were different between these two groups male flowers in copper sulfate treated Cu-*ipt* transformed plants were higher but not significant as compared to non-transformed plants ($p=0.19$).

3.4.7 Pollen Germination

Although the pollen germination rate in both non-transformant and Cu-*ipt* plants was in a acceptable range (50% or higher), the average pollen germination in Cu-*ipt* plants sprayed with any of the three copper sulfate concentrations was relatively less than the non-transformant sprayed with copper sulfate. These differences were statistically significant in

several cases. The lowest average pollen germination was reported in Cu-*ipt* plants with 50 μM copper sulfate application (≅49%)(Figure 3.5) (Table 3.2). It was previously reported that over-expression of cytokinin often lowers flower fertility (Wang et al., 1997); however, in this study, the reduction in pollen germination would likely be insufficient to negatively affect crop production (Figure 3.5).



A *B*

Figure 3.5. Pollen germination 24 hours after plating. The pollen grains with a pollen tube at least greater than the pollen diameter were considered as geminated. The germination of pollen grains of copper sulfate treated Cu-*ipt* plants (A) and the non-transformant pollen grains (B) were approximately 50 and 58% respectively.

Table3.2 Least Squares Means of chlorophyll content, senescence, male and female flowers and pollen germination for the Cu-*ipt* and non-transformant plants treated with different concentrations of copper sulfate (n=3 plants / treatments).

Plants	CuSO4	Chlorophyll content (SPAD-502)	Number of senesced leaves	No. of male flowers	No. of female flowers	Pollen germination (%)
	50 uM	40.13a*	3.33c	22.00ab	10.66 a	49.77 d
	10 uM	39.39ab	4.33c	25.66a	8 ab	51.22 d
IPT	5 uM	38.26abc	4.66c	15.00ab	6.66 bc	53 c
	0 uM	35.25abcd	6.33b	17.33ab	3 d	56.11 bc
	50 uM	34.88bcd	8.33a	9.33b	2d	56.33 ab
	10 uM	34.34bcd	7.66ab	10.33b	3.33cd	56.33 ab
Wt	5 uM	32.77d	7.33ab	10.33b	4 d	57 ab
	0 uM	33.10d	8.00a	13.33ab	2.66 d	58.44 a

* Means followed by the same letter are not significantly different at $p<0.05$ by LSD procedure.

3.4.8 Seed Numbers

The reduction of normal seed development in Cu-*ipt* plants sprayed with three copper sulfate concentrations was highly significant when compared to that of the non-transformant and the Cu-*ipt* control plants ($p<0.0001$). The number of seeds in Cu-*ipt* watermelon transformants treated with the three copper sulfate concentrations was reduced to about 5-7% of that of non-transformant plants. The Cu- *ipt* transformants with no copper sulfate treatment had approximately 33-50% of the number of seeds of the non-transformant (Table 3.3). The Cu-*ipt* control plants were expected to be close to that of the non-transformant plants. This reduction in normal seed number may be due to some slight *ipt* gene activation or possible leakage. The number of white seed lacking embryo development in copper sulfate-applied Cu-*ipt* plants was also significantly reduced when compared to the non-transformant plants sprayed with similar copper sulfate concentrations. The reduction rate in the number of white seeds was also around 50% in Cu-*ipt* watermelon transformants treated with the three copper sulfate concentrations. In the seedless watermelon industries, 10% of normal seed development is considered acceptable in order for the variety to be accepted as a seedless variety. The white seeds without embryo or hard seed coat are considered to be edible. A comparison of total soluble solids (10-12%) was relatively higher but not significant in treated Cu-*ipt* plants as compare to non-transformant plants. The fruit shape and flesh color were similar in Cu-*ipt* transformants and non-transformant plants. In terms of fruit weight, Cu-*ipt* and non-transformant plants had similar sizes but were obviously smaller than cv. Crimson Sweet grown under field conditions.

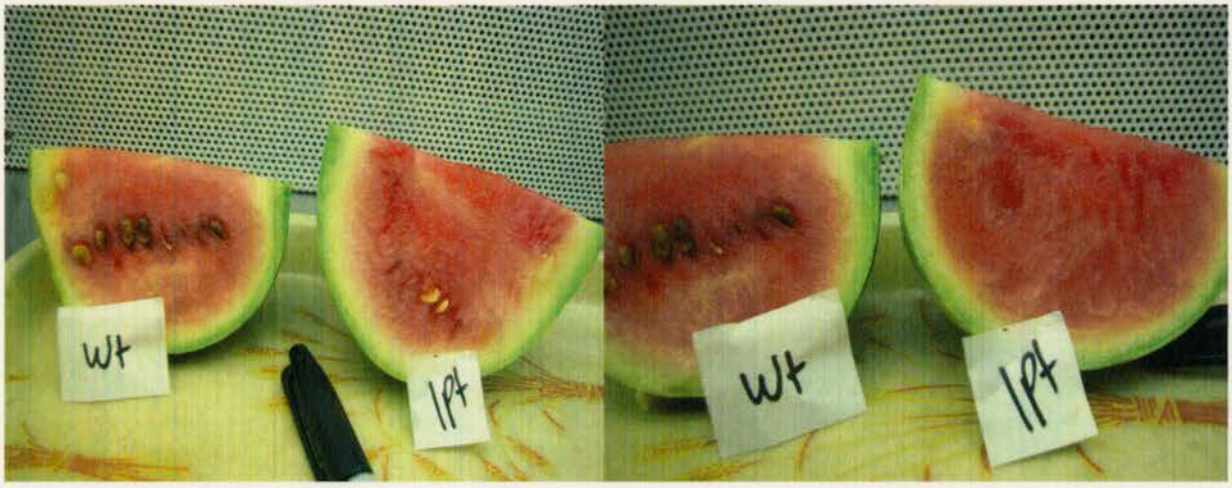


Figure 3.6 The Cu-ipt fruits showed significant reduction in normal seed numbers compared to non-transformed fruits. Cu-*ipt* transformants are located on the right side and non-transformants are located on the left side picture.

Table 3.3 Least Squares Means of normal seeds for Cu-ipt and wild type plants treated with different concentration of copper sulfate(n=3plants / treatments).

Plants	CuSO4	Total Seeds	Normal seeds (LS Means)
IPT	50 uM	18. 7	3 c*
	10 uM	24	8 c
	5 uM	21.3	7 c
	0 uM	62.3	27 b
Wt	50 uM	121. 7	92 a
	10 uM	118.3	87 a
	5 uM	127	92 a
	0 uM	120.7	88 a

* Means followed by the same letter are not significantly different at $p<0.05$ by LSD procedure.

3.5 Discussion

The results of this study demonstrated the effects of the increased level of cytokinin on the morphological and physiological characteristics of Cu-*ipt* watermelon transformants. The over production of the endogenous cytokinins has some well-known alterations on plant phenotypes. Delaying leaf and flower senescence, breaking apical dominance, and elevating

chlorophyll contents has been previously reported in several species transformed with *ipt* gene. The same Cu-*ipt* construct was transferred into tobacco plants and it was reported that the transcription of *ipt* gene released the lateral buds, delayed senescence and increased leaf and node numbers as compared to controls plants (McKenzie et al., 1998). Stem elongation or plant height was unaffected by the expression of the *ipt* gene when a different *ipt* construct was used in tobacco plants (Van Loven et al., 1993; McKenzie et al., 1998). Medford et al. (1989) reported a slight reduction in growth rate, while Smigocki et al. (1993) reported a slight enhancement in stem length as compared to the control plants. Although the growth rate in Cu-*ipt* plants was slightly lower than that of the control plants in our study, it was not significantly different.

The relatively high numbers and elongated lateral vines in Cu-*ipt* plants treated with copper sulfate is likely the main reason for slowing growth of the main stem. An increase in lateral shoots likely reduced carbohydrates to the main stem. However, at the end of the five-week growing period the treated, transformed plants grew to almost the same height as the non-transformed types. Leaf numbers, which represent node numbers as well, were relatively higher although not significant in copper sulfate-applied *Cu-ipt* plants compared to the non-transformant plants. The relatively high number of leaves could be the indication of smaller internodes in copper sulfate treated Cu-*ipt* plants. Short internode development is typical for the over production of cytokinin. The number of female flowers was significantly higher ($p=0.007$) in *Cu-ipt* plants treated with copper sulfate compared to the non-transformant plants. The Cu-*ipt* related reduction of apical dominancy and the elongation of lateral vines, which produced their own female flowers, was one of the reasons for the alteration of female flower numbers in *Cu-ipt* plants. Although there are some concerns about the quality of the

fruits, it is clear that the alteration of the number of female flowers could positively affect fruit quantity. It requires further field study to assess the impact of increased flower number on the quality and quantity of yield.

Female flowers in copper sulfate- treated Cu-*ipt* plants were observed seven to ten days earlier than in non-transformant plants. This may positively affect earliness of fruit development. Since cv. Crimson Sweet is considered to be a mid to late season watermelon cultivar, the time period of seven to ten days could have a positive impact on its earliness.

This research reports the reduction of normal seed number in watermelon fruit via use of the *ipt* gene. Exogenous application of synthetic cytokinin-1-(2-chloro- 4-pyridyl) -3-phenylurea (CPPU) has been found to both increase fruit set at a lower temperature (10-13 °C) and to reduce seed numbers in watermelon (Hayata and Niimi , 1995). Endogenous over production of cytokinin through transformation with the *ipt* gene demonstrates potential in influencing seedlessness. This could be a new technique to increase fruit set at lower temperatures while producing seedless watermelon.

The use of the copper sulfate to activate *ipt* gene was confirmed in several cases during these studies. With help of this promoter, tissue culture- generated shoots were able to initiate root formation, with few exceptions. Root formation is thought to be one of the main challenges in the regeneration of *ipt* transformants. The growth and development of Cu-*ipt* plants was considered to be normal, while constitutive *ipt* expression is known to cause bushy structures due to the breaking of apical dominance and extensive shoot growth. The activation of the *ipt* gene with copper sulfate also did not cause a major negative morphological alteration in Cu-*ipt* transformants, which were able to grow, flower and set fruit in a normal manner.

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Chapter 4

Evaluation of Resistance to Gummy Stem Blight (*Didymella bryoniae*) Disease in Cu-*ipt* (Isopentenyl Transfarese) Transformed Watermelon Plants

4.1 Abstract

Watermelon plants are susceptible to several major fungal diseases that cause considerable reduction in yields worldwide. The two major diseases are Fusarium wilt and gummy stem blight. Development of resistance to these diseases is considered to be the key element in protection. In order to develop non-specific resistance, the watermelon "Crimson Sweet" was transformed with copper inducible isopentenyl transferase (*ipt)* gene, the rate-limiting step in the cytokinin biosynthetic pathway, via *Agrobacterium tumefaciens* (LBA4404). A pathogenic strain of *Didymela bryonia*, W353, obtained from Clemson University, was cultured on a 10% un-clarified V8 medium for two to three weeks at an ambient temperature of 24 °C and twelve hours of light. Transformed (Cu-*ipt)* and non-transformed plants, initially regenerated from tissue cultures and subsequently propagated via cuttings, were grown in 1 gal. pots filled with MetroMix 350 medium and supported with about 11 grams of Osmocote (18:6:12). Once the plants started to grow both *ipt* transgenic plants and wild type plants were sprayed with three different concentrations (0, 10 & 50 μM) of $CuSO_4$. Plants were sprayed twice to run-off in a 24 hour period before inoculation with the pathogen. A conidia concentration of approximately 10^5 conidia per ml was sprayed onto leaves of the Cu-*ipt* transformant and non-transformant watermelon plants until run-off. Disease symptoms were evaluated after one week with significant resistance observed in the Cu-*ipt* transgenic plants. Non-transformed and Cu-*ipt* transformed plants with no copper sulfate treatment showed almost identical disease symptoms. The pathogen was re-isolated from the infected plants and it was identical to the stock culture. Seedlings from transgenic (T_1 generations) and from non-transformed plants were inoculated at the 4-5 leaf stage after initial spraying with four different concentrations (0, 5, 10 and 50 μM) of $CuSO_4$, as noted previously. The inoculated plants were placed in 25 °C (18 hours light) and 18 °C (6 hours dark) in a humidity chamber. The non-transformed seedlings showed the first disease symptoms on their cotyledons and lower leaves. Significant disease resistance ($p=0.0024$) was observed in transformed seedlings when compared to non-transformed ones.

4.2 Introduction

Plant diseases are one of the greatest threats to the world's agricultural production. They can cause catastrophic losses in terms of quantity as well as quality of food production. Pathogenic organisms not only cause extensive economic losses but can also have a dramatic impact on both human and livestock health. The ergot poisoning caused by the fungus *Claviceps purpurea* (Jackson and Taylor, 1996) is one of the clearest examples of such direct and negative impact.

Epidemic threats are still present in today's agricultural areas, especially in underdevloped or developing countries. The terrifying part of this threat is that it is quite hard to predict how, when and where such diseases may reappear. The Victoria blight of oats and southern corn leaf blight can be considered as recent examples of such losses (Jackson and Taylor, 1996). At the same time, it is commonly known that with today's extensive plant breeding programs the genetic diversity of cultivars has been considerably narrowed, thereby increasing the vulnerability of cultivars to diseases.

Unlike animals, plants lack antibody and immune developing systems (Agrios, 1997); consequently, except for the induction of SAR, they usually respond to disease on an individual basis each time it occurs. Their gene reservoirs play a primary role in developing resistance. With the help of recent technology, mainly the isolation and sequencing of genes, plants have been shown to carry a relatively large number of genes that interact with pathogens by detecting and destroying them (Crute and Pink, 1996).

In terms of disease resistance, wild relatives of domesticated species are generally more resistant to disease and harsh environmental conditions because they have a broader

gene pool than that of modern cultivars. Through the process of domestication, cultivars are constantly being selected either by farmers or more extensively by plant breeders, which eventually results in rapid gene elimination, making them especially vulnerable to newly introduced pathogens.

Natural selection is part of a plants' life cycle, which allows only the strong to survive, while the weak disappear. However, artificial selection used by humans is based on targeted traits without consideration for the disappearance of other characteristics. Wild plants generally carry a number of undesired characteristics but with the domestication of plants many of these undesired characteristics have been eliminated. To some degree, these types of improvements are crucial in relation to other agronomic traits, such as crop yield, uniformity, quality of fruit, etc. These ongoing processes generally lead to the erosion of the genetic variability of plant species (Poehlman and Sleper, 1995) and make them more vulnerable, especially against newly-introduced pathogens.

With regard to resistance or defenses, plants show two clearly related mechanisms. These multi-component processes are evident locally at the area of attempted infection and systematically throughout the remainder of the plant in relation to disease development. In addition to this, certain structural barriers, such as the quantity and quality of wax which acts as a water repellent layer and covers the epidermal cells, the epidermal cell wall, the size, location and shape of the stomata and lenticels, and finally the thick cell wall structure of plant tissue, aid in preventing the onset of infection. In order for successful infection to take place, the pathogen must first overcome these barriers, unless there are already wounds or damage present on the host (Agrios, 1997).

Once the pathogens overcome these structural barriers they face a chain of biochemical reactions, which are controlled by the presence of defense-related genes. Based on the genetic makeup, plants show two types of response mechanisms to pathogenic attack, namely, specific (vertical) resistance and broad (horizontal) resistance. Vertical resistance or gene-for-gene interaction is based on the specific interaction between a dominant resistant gene in the host and the product of a dominant avirulent (*Avr*) gene in the pathogen (Floor, 1947). The presence of *Avr* gene in pathogen (Luderer, and Joosten 2001) as well as the presence of R gene in host (Staskawicz, 2001) is crucial for activation of the defense system and induction of the resistance. As a result of this interaction, a complex series of biochemical reactions occurs that leads to plant resistance responses. These biochemical reactions, triggered by host pathogen interactions, take place in the cell and produce substances that are either toxic to the pathogen or create conditions that inhibit the proliferation of the pathogen in the host. The numbers of R genes present in the host and *Avr* genes in the pathogen may vary, and a single host and pathogen may carry many of them (Bent, 1996). The chance of achieving resistance and eradicating disease development increases according to the number of R genes in the host and Avr genes in the pathogen.

Although gene-for-gene interaction is efficient in developing resistance and is also relatively easier for plant breeders to work with, this mechanism is highly vulnerable to loss as pathogens mutate and become virulent to previously resistant cultivars. The key element for the effectiveness of this defense system against particular plant pathogens is the presence of *Avr* genes, which are recognized by plant receptors and trigger resistance. Hence, in order to secure resistance through a gene-for-gene model,

breeding program(s) for particular cultivars need ongoing processes which basically gather new dominant genes resistant to new races of pathogens.

Unlike vertical resistance, horizontal resistance is based on a series of aspecific defense responses that lead to tolerance of several different pathogens. One of the best-studied examples of horizontal resistance is Systemic Acquired Resistance (SAR). SAR is induced with the recognition of pathogen derived elicitors and it activates the plant defense system using a distinct signal transduction pathway (Agrios, 1997; Ryals et al., 1996). These genes, called SAR genes, encode proteins that include the "pathogenesis-related" (PR) proteins. SAR is strongly related to the coordinate expression of a specific set of genes, such as chitinase, β-1,3-glucanase, thaumatin-like proteins, PR1, PR4, lysozymes, peroxidases, etc. (Gaffney et al., 1993; Agrios, 1997).

In tomato, several PR proteins have been shown to accumulate following infection with *Cladosporium fulvum* (Danhash et al., 1993; Joosten et al., 1989; van Kan et al., 1992). Joosten et al. (1990) were able to purify and characterize some of the PR proteins in infected tomato plants. These induced PR proteins basically guard plants from any kind of pathogenic invasion. Some of the modes of action of these PR proteins include toxicity, inhibiting the proliferation of the pathogen, and breaking down the pathogen's cell walls (Agrios, 1997).

Cytokinins were reported to have a great impact on plant defense systems by inducing disease and insect resistance (Dermastia and Ravnikar, 1996; Storti et al., 1994; Bettini et al., 1998; Smigocki et al., 1993). Salicylic acid (SA) is known to be the primary signaling molecule for induction of resistance and production of pathogen related proteins (PR) upon pathogenic invasion (Chen et al., 1994; Delany 1997; Gaffney et al.,

1993; Gaffney et al., 1994; Maleck and Dietrich, 1999; Rep et al., 2002). However, other growth regulators might also be involved in the induction of such acquired resistance. Although it is not well-defined, cytokinin may either be directly involved in the production of PR protein or positively regulates other signaling molecules to induce PR proteins. The only known fact to date is that increased levels of cytokinin somehow activates the plant's defense system and induces the production of PR proteins. The induction of this novel and non-specific resistance might provide great protection for plants against various diseases, especially when there are limited R genes available for serious diseases.

Gummy stem blight, one of the most destructive stem and foliage diseases of the *Cucurbitaceae* family, caused by *Didymella bryoniae* (Zhang et al., 1999), was first identified in 1891 in France and Italy (Thomas, 1996) and subsequently in the southern United State in the early twentieth century (Keinath, 1999 and 2002). This fungal disease derives its name from the gummy ooze that is commonly seen on older lesions. It first appears as circular brown lesions on leaves, petioles or stems. Once the stem is infected, the lesions elongate along the stem and the vine beyond the lesion starts to wither and eventually dies within a short period of time (Thomas, 1996; Zhang et al., 1999). The disease has two phases: if it appears on foliar parts of plants it is called gummy stem blight and if it appears on fruits it is called black rot. This disease mainly reduces the yield of marketable watermelon, while the expense of fungicide treatment increase costs for watermelon growers.

Didymella bryoniae isolates vary in virulence, the degree of disease caused by the strain of the pathogen. Another important issue related to this pathogen is that it can lose its virulence in culture. Consequently, the storage and the cultivation of *Didymella bryoniae* need to be handled with extreme care. One recommended technique is to store the isolates on sterile pieces of filter paper at 4 ^{o}C and each time carry out the inoculation from the stock culture.

The identification of pathogen isolates can be quite challenging, since *Didymella bryoniae* has a strong resemblance to the characteristics of *Phoma spp*. The Randomly Amplified Polymiorfic DNA (RAPD) technique was shown to be an effective method of distinguishing these two pathogens from one another. With the help of this technique, Keinath et al., (1995) were able to distinguish twenty-seven isolates tentatively identified as *D. bryoniae* or *Phoma spp*.

Although warm and humid conditions help the pathogen to grow and spread, relative humidity plays a crucial role in infection and disease development. A free water film on leaves for at least one hour is necessary for the pathogen to begin to grow and penetrate the tissue (Thomas, 1996; Keinath, 2002). The pathogen can survive over winter on infected host debris for nearly two years; hence, crop rotation of at least two years is an important strategy for controlling soil-borne infestations of the pathogen in previously infected fields (Thomas, 1996). Keinath, (2002) reported that two years after infestation, the pathogen *Didiymella bryonia* was not recovered in fields where non-cucurbit crops had been cultivated during that period. Although some other production techniques, such as the use of fungicides, drip irrigation in place of overhead irrigation,

planting disease-free certified seeds and so on, may help to reduce crop loses due to gummy stem blight disease, cultivating naturally resistant watermelon cultivars is one of the most efficient ways to overcome this problem.

Since it was previously claimed that the over-expression of endogenous cytokinins induces disease resistance, one of the main objectives of this study was to evaluate the resistance induced by the activation of the *ipt* gene to the pathogen *Didiymella bryonia.*

4.3 Materials and methods:

4.3.1 Pathogen and stock culture

The pathogenic strain of *Didymela bryonia*, W353, isolated from watermelon leaves in cv. Charleston Count, was obtained from Dr. A.P. Keinath of Clemson University in a quarter strength potato dextrose agar (PDA) medium. The pathogen was then grown for two weeks at 24° C and 12 hours light on a quarter strength PDA medium set into the center of petri dishes and partially covered by sterile filter paper. As soon as the pathogen completely covered the filter paper, it was removed and air-dried for two days under aseptic conditions. The filter paper was then cut into small pieces with sterile scissors, placed into three 125 ml sealed sterile flasks, and finally stored at 4 °C as stock culture. This process may keep the pathogen alive for six months to one year. The process was repeated twice during a one-year period. Two weeks before inoculation, the pathogen was taken directly from stock culture and grown under the above conditions.

4.3.2 Pathogenicity test

The protocol defined by Keinath et al. (1995) was used for a pathogenicity test. The pathogen was grown on a V8 (100 ml/l V8 juice; 1 g/l CaCO3; 0.05 g/l β-sitosterol; 15 g/l agar) medium at 24 °C and 12 hr light for two to three weeks before inoculation. The pycnidia development and spore distribution was observed closely under the microscope. The surfaces of the culture were slightly flooded with a 0.1% sucrose and 0.05% casein hydrolysate solution. A sterile forcep was used to gently scratch the surface of the culture to release conidia from pycnidia. The suspension was filtered through four layers of sterile cheesecloth and the conidia concentration was determined with the aid of hemacytometer.

The conidia suspension, 10^5 conidia per ml, was adjusted by diluting the suspension with sterile water or by adding more conidia from the culture plates. Once the desired conidial suspension was obtained, the Cu-*ipt* transgenic plants and non-transformed watermelon plants were spray inoculated until ran off. The inoculated plants were then placed in a chamber with 100% humidity. These plants were kept in the chamber under 100% relative humidity during the entire experiment. Two weeks following the inoculation, the inoculated plants were evaluated for severity of disease development.

4.3.3 Experimental Units

A total of nine clonally propagated Cu-*ipt* plants and nine wild type watermelon plants, planted in 1 gallon pots filled with Metro Mix 350 pot medium, were established as experimental units in order to evaluate their resistance to *Didymela bryonia*. The plants were grown at approximately 28-30 °C during the day, 20-22 °C at night, and 16 hours of daylight under greenhouse conditions in the summer and early fall of 2003. Plants were supplied with the 11 g of slow-release fertilizer Osmocote (18 % Nitrogen, 6% Phosphate and 12% Potassium). Once they started to grow (10 days after they were transferred to the greenhouse), both Cu-*ipt* plants and wild type plants were sprayed with three different concentrations (0, 10 or 50 μM) of copper sulfate prior to inoculation with the pathogen. Three plants were used for each treatment (n=3). The experimental unit was a single plant with three replicates. As a control, three Cu-*ipt* plants and three wild type plants were treated with no copper sulfate. The copper sulfate was sprayed twice in a one-day period prior to the infection. The spraying process was continued each time until the solution started to run off the leaves. The same experimental units were infected again six weeks from the time of the first infection with a similar *Didymela bryonia* suspension but without further copper sulfate treatment prior to the infection. The main goal of the second infection application was to test disease development or resistance, especially in newly grown shoots.

4.3.4 Seedlings

Seeds obtained by self-pollinating T_0 parents and non-transformed parents were germinated at 25 °C (18 hours) and 18 °C (6 hours) for approximately two weeks. Since the germination rate for transgenic seeds was extremely low (about 50%), the seeds were soaked in distilled water for twenty-four hours before planting and then the seed coats were manually cracked. This process helped to increase the germination rate by about 10%. Once the germinated seedlings reached the two true leaf stage, they were transplanted into larger pots (12x12x12 cm) filled with Metro Mix 350 pot medium.

The seedlings of the Cu-*ipt* transformants (T_1 generations) and wild type plants were infected at the 4-7 leaf stage after being initially sprayed with four different concentrations (0, 5, 10 and 50 μM) of $CuSO_4$, as noted previously. The infected plants were grown in the humidified chamber (100% relative humidity) at 25±2 °C for 18 hours under light and at 18 °C for 6 hours in the dark

4.3.5 Evaluation and scoring damages

Since resistance in Cu-*ipt* plants treated with copper sulfate and disease development in non-transformed plants as well as Cu-*ipt* plants without copper sulfate spray was clearly observable with infection, these plants were classified as "Yes" or "No" for susceptibility or resistance, respectively. Symptoms started to appear five to seven days after the first infection. Plants were evaluated two weeks from infection date. At that point, the disease lesions were visible and some of the infected leaves were already starting to die back.

In the second application of the pathogen, infected Cu-*ipt* (copper sulfate treated and non treated plants) and non-transformed plants, as well as their seedlings, were visually evaluated by using the percentage leaf area diseased (PLAD) protocol described by S. W. Slopek (1989). Based on this procedure, the experimental units were rated in five different categories according to the severity of disease development. The individual plants that showed 0% leaf area disease symptoms were rated as 1, those having disease symptoms between 0.5% to <5% to as 2, >5% but <25% as 3, >25 but <75% as 4, and finally those >75% as 5.

4.4 Results

4.4.1 Cu-*ipt* Transformants and non-transformed plants

The development of disease symptoms or the induction of resistance was evaluated two weeks from the infection date. Infected plants were kept in the isolated area under 100% humidity during the entire experiment. Disease symptoms were examined on individual leaves as well as the whole plant. The symptoms generally started to appear at the leaf margins and then rapidly spread over the entire leaf. Lesions of various colors such as dark brown, dark yellow and reddish-brown were observed upon successful infection. These lesions were mostly irregularly circular at the beginning; however, their shapes and sizes changed and expanded rapidly. Lesions with water-soaked edges were also present on some of the infected leaves (Figure 41). As the blight symptoms spread, leaves curled, shriveled, and finally died back completely. These symptoms were mostly located on the lower leaves of control Cu-*ipt* (no copper sulfate treatment) and wild type plants where they had been sprayed with the spore suspension.

At the beginning of the experiment, the above-mentioned PLAD scoring technique was assigned to evaluate disease development. However, since there were clear and large differences between disease on the copper sulfate applied Cu-*ipt* plants and on non-transformant plants, they were classified as susceptible or resistant to gummy stem blight disease. Plants with and without disease symptoms were reported as "Yes" or "No," referring to susceptibility and resistance, respectively (Table 4.1). Apart from the copper sulfate applied Cu-*ipt* plants, the remainder of the experimental units showed some degree of disease symptoms (Figure 4.1). The severity of disease development was relatively higher in the control Cu-*ipt* and non-transformant plants (Figure E and F), several which died prior to the end of the experiment. At the same time, the non-transformant plants treated with copper sulfate were able to survive relatively longer with significant disease symptoms.

Cu-*ipt* transformants			Non-transformant plants		
	Disease Presence			Disease Presence	
CuSO4	YES	NO		YES	NO
50uM Cu-*ipt*		√	50uM wt	√	
50uM Cu-*ipt*		√	50uM wt	√	
50uM Cu-*ipt*		√	50uM wt	√	
10uM Cu-*ipt*		√	10uM wt	√	
10uM Cu-*ipt*		√	10uM wt	√	
10uM Cu-*ipt*		√	10uM wt	√	
0uM Cu-*ipt*	√		0uM wt	√	
0uM Cu-*ipt*	√		0uM wt	√	
0uM Cu-*ipt*	√		0uM wt	√	

Table 4.1. Resistant (No) and diseased plants (Yes) two weeks after the first infection. The resistant plants (Cu-*ipt* with $CuSO_4$ application) showed almost no disease symptoms at that particular time.

Figure 4.1. Disease symptom development two weeks after the first infection. A, C and E represent the non-transformant plants sprayed with 50 µM, 10 µM and 0 µM coper sulfate respectively. B, D, and F represent Cu-*ipt* plant with 50 µM, 10 µM and 0 µM

cooper sulfate respectively. All plants except B and D show clear disease symptoms with differing severity.

Infected leaf samples were collected for identification and recovery of the pathogen. These samples were either surface sterilized or rinsed several times with sterile water. Finally they were blotted on sterile filter paper under the air flow hood for a few minutes and cultured on a V8 medium. The pathogen was left to grow at 24 °C and 12 hours light conditions for two to three weeks before comparison with stock culture. The color development on V8 medium as well as the size and shape of the spores were used for positive identification of recovered pathogens (Figure 4.2). The pathogen recovery experiment confirmed that the disease symptoms on the experimental units developed from the infection with the *Didymela bryonia* spores.

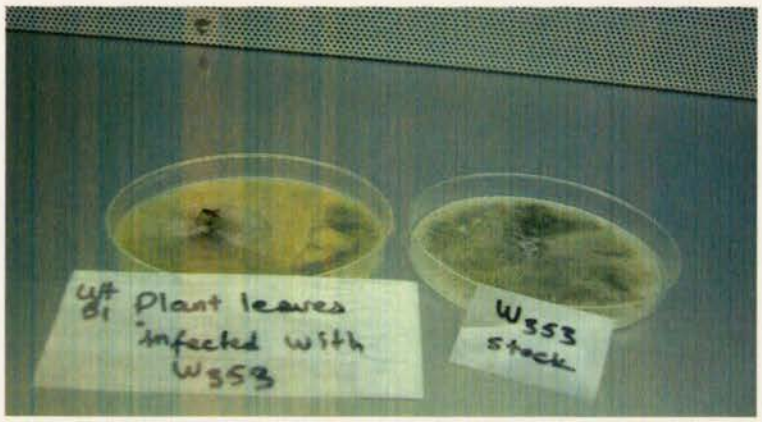

Figure 4.2. A comparison of inoculums from the infected leaf samples and stock culture. The color development on V8 medium was identical.

Six weeks after the initial infection, plants were re-inoculated with similar pathogen concentrations but without further copper sulfate treatment. At this point, some of the non-transformant plants and the control Cu-*ipt* plants were severely defoliated and

almost dead. However, the copper sulfate treated Cu-*ipt* plants and some of the non-transformed plants continued to grow and produce new shoots. The purpose of the second infection was to test disease development or resistance in new growing sites without any additional copper sulfate application. The above-mentioned procedures were followed during the second infection as well.

Plants infected for the second time were evaluated two weeks from the infection date. Disease symptoms on non-transformant plants and control Cu-*ipt* plants were again significantly higher than the Cu-*ipt* plants (Table 4.2). Although some slight disease symptoms were identified on Cu-*ipt* plants that had previously been sprayed with copper sulfate, these plants were relatively healthier and more vigorous than the wild types (Figure 4.3). While the wild type plants showed severe disease damage and were barely able to survive, the Cu-*ipt* plants continued their normal growth, including the production of both male and female flowers. The second infection blighted almost all the leaves on the two control Cu-*ipt* plants (no copper sulfate spray), the three control wild type plants, and one non-transformant plant previously sprayed with 10 μM copper sulfate. These severely blighted plants turned brown and collapsed (Figure 4.3 d and e). The recovery of the pathogen on the infected leaves was carried out based on the above-mentioned process, and positive results were observed after the second infection as well.

A. (non-transformants) B. (Cu-*ipt* transformants)

C

D E

Figure 4.3.Disease symptom development two weeks after the second infection. A, B, and C represent the non-transformed plants and Cu-*ipt* transformants sprayed with 50 μM copper sulfate. D represents wild type plant with 10 μM copper sulfate. E represents control Cu-*ipt* (no copper sulfate) non-transformed plants. All plants except Cu-*ipt*

treated with 10 and 50 μM copper sulfate expressed severe disease symptoms and eventually died.

Table 4.2 Least Squares Means of the leaf area diseased after second infection for the Cu-ipt and wild type plants treated with different concentration of copper sulfate.

Plants (n=3)	CuSO4	Leaf area diseased (%)
	50 uM	1.7 b
IPT	10 uM	2.0 b
	0 uM	4.4 a
	50 uM	3.7 a
Wt	10 uM	4 a
	0 uM	4.7 a

* Means followed by the same letter are not significantly different at $p<0.05$ by LSD procedure

4.4.2 Seedlings

Disease symptoms began to appear on the infected wild type seedlings one week from the infection date (Figure 4.4a). The first symptoms developed on the cotyledons and first true leaf, and spread rapidly under the high relative humidity. Two weeks from the infection date the seedlings were evaluated for disease development and resistance. Based on their PLAD scores, significant disease development was identified in wild type seedlings as compared to Cu-*ipt* seedlings, especially those sprayed with 50 μM copper sulfate. While almost no infections occurred on Cu-*ipt* seedlings treated with the 50 μM copper sulfate, the percentage of the infected leaves on similar copper sulfate applied wild type seedlings increased drastically during this two-week period (Figure 4.4e). With the high percentage of foliar damage, some of the wild type seedlings weakened and finally died.

One of the interesting things about the evaluation of resistance in seedlings was that, unlike the Cu-*ipt* transformants (T_0), their seedlings (T_1), which had been sprayed with a lower concentration of copper sulfate, showed some degree of infection as well. However, these seedlings were still significantly more resistant than the non-transform seedlings ($p=0.002$) (Table 4.3). They were relatively healthier and continued their normal growth, while some of the non-transform seedlings with lower levels of applied copper sulfate were severely damaged and showed symptoms of collapse. The control Cu-*ipt* (no copper sulfate) and non-transformant plants seedlings showed very similar damage from infection with *Didymella bryoniae* spores. There were no disease symptoms, after two weeks of infection, on the Cu-*ipt* seedlings with 50μM copper sulfate application. They were also significantly resistant as compared to the other Cu-*ipt* seedlings sprayed with lower concentrations of copper sulfate (Figure 4.4 e). These plants continued their normal growth and development in the high relative humidity.

Copper sulfate has some toxic effect on fungal pathogens and can be used to control fungal diseases. However, the presence of disease symptoms in non-transformant plants for each copper sulfate concentration clearly demonstrates that the copper sulfate had minimal effect on disease expression. The disease on non-transformant plants as well as Cu- *ipt* plants with no copper sulfate as compared to copper sulfate treated transformants clearly indicates that the activation of *ipt* gene leads to the resistance.

A

B C

D E

Figure 4.4 Disease symptoms on Cu-*ipt* and non-transformant plants seedlings. A Initiation of lesion development on cotyledons of non-transformed type seedlings. B, C, D and E Non-transformed seedlings and Cu-*ipt* seedlings sprayed with 0, 5, 10, and 50 µM copper sulfate and illustrating relative susceptibility to Didymella bryoniae spores.

Table4.3 Least Squares Means of the leaf area diseased in Cu-*ipt* and non-transformed seedlings treated with different concentration of copper sulfate(n=3 plants/treatment).

Plants (n=3)	CuSO4	Leaf area diseased (%)
	50 uM	1 c*
	10 uM	1.7 c
IPT	5uM	1.33 c
	0 uM	3.33ab
	50 uM	3ab
	10 uM	3ab
Wt	5uM	3a
	0 uM	4a

* Means followed by the same letter are not significantly different at $p<0.05$ by LSD procedure

As a final step in the disease evaluation, infected leaf samples were collected from seedlings and the pathogen was positively identified when compared to stock cultures.

4.5 Discussion

One of the distinguishing and best-known roles of the *ipt* gene was the induction of disease and insect resistance as reported previously in various plants (Martineau et al., 1993; Smigocki et al., 1993; Storti et al., 1994; Bettini et al., 1998). The results obtained in this study were consistent with previous studies. It supported the hypothesis that elevated cytokinin levels initiate acquired resistance in susceptible plants. Although salicylic acid is considered to be the primary plant growth regulator, playing an important role in the hypersensitive response and systemic acquired resistance in infected plants, the evidence for developing resistance in *ipt*-transformed susceptible plants indicates a possible role of cytokinins in acquired resistance. The involvement of cytokinins in disease resistance requires further study, especially relative to whether they are involved in a direct or indirect role in the development of resistance. The positive association

between cytokinins and other signaling molecules may be one possible explanation of their roles.

Cu-*ipt* plants sprayed with copper sulfate for the activation of the *ipt* gene and the elevation of endogenous cytokinin levels showed significant resistance to gummy stem blight disease (*Didymela bryonia*). This resistances was observed in both Cu-*ipt* transformants and their seedlings. Although Cu-*ipt* transformants were inoculated a second time six weeks from the date of the first infection, and most importantly without further copper sulfate applications, these plants still demonstrated resistance, with only minor disease symptoms.

There are several possible explanations for this resistance to the second infection. The first possibility is that the *ipt* gene was still active to some degree and continued its transcription due to the initial copper sulfate application. The residual copper from the previous application might also have kept the *ipt* gene active during this time. The second possibility is that residual cytokinin produced during the *ipt* gene activation process may have still been present in whole plant parts. This residual cytokinin might be sufficient to maintain the activation of the plant defense system at the time of the second infection. And finally, since the defense systems of these plants had already been activated during the first infection, they may have been prepared for any further pathogen invasions, including this second infection. This long-lasting spectrum of acquired resistance may have been one of the reasons for preventing further infections.

These plants were tested under extreme conditions; in other words, they were kept under the high relative humidity throughout the entire experiment. While non-transformed plants with and without copper sulfate application were developing severe

blight symptoms and collapsing, Cu-*ipt* plants did not show symptoms and continued their normal growth and development. The development of disease on control (no copper sulfate treated) Cu-*ipt* plants, which were almost identical to the non-transformed plants, indicated that regulation of *ipt* gene was auto-regulated by copper sulfate application. This result supports the idea that a copper inducible promoter may be a strong and dependable promoter in regulating closely linked genes.

As a significant foliar disease with limited resistance in cultivars, gummy stem blight disease is always a serious threat for watermelon production, especially in areas with high relative humidity. Therefore, the development of resistance against this continuous threat, either via conventional breeding techniques or biotechnology, will have a positive and significant impact on watermelon production, including the reduction of disease management expenses.

Further studies are indicated to isolate the induced PR proteins in pathogen infected Cu-*ipt* plants. The Cu-*ipt* transformants could also be tested for their resistance against other plant pathogens such as *Fusarium* wilt that is one of the important diseases in watermelon. The possibility of the segregation in terms of disease resistance in seedlings needs to be investigated as well. Transferring this disease resistance by cross-pollinating Cu-*ipt* transformants with other watermelon cultivars should have potential for increasing efficiency in watermelon production.

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5. Summary

Watermelon regeneration is cultivar specific as initial efforts to regenerate cv. Sugar Baby gave negative results. cv. Crimson Sweet readily regenerated from cotyledons in MS medium with BA.

The cv. Crimson Sweet was transformed with copper inducible isopentenyl transferase (Cu-*ipt*), the gene controlling the rate limiting step in cytokinin biosynthesis, via *Agrobacterium tumefaciens* (LBA4404). Although the regeneration of shoots in selective media was 15%, which is considered to be a normal rate for *Agrobacterium* mediated gene transformation, the rate for rooting of the regenerates was less thaen 10%. Furthermore, the rate for development of the specific construct regenerate was approximately 1%, which is considered low. Aberrant lines, with many small shoots forming a rosette with no/few roots, was observed. Transformats were verified using both PCR and phenotypic characteristics related to the elevated endogenous cytokinin levels.

Transformed plants treated with copper sulfate at levels of 5, 10 or 50 μM all expressed evident phenotypic changes indicative of elevated levels of cytokinin. Growth rate, number of leaves, flowers, lateral shoot number, and chlorophyll content were measured at weekly intervals over a period of 5 weeks after treatment with copper sulfate. Treated transformants had significantly greater numbers of leaves, flowers and lateral branches as well as higher chlorophyll levels. Growth rates were not significantly different among treatments. Although there were differences in pollen germination on BK media in petri dishes, there was judged sufficient germination to result in normal fruit set. Selfed flowers grew to maturity and were harvested and evaluated for shape, flesh

color, brix, number of normal seeds, number of colored but empty seeds and number of white seeds. Seed number was significantly less in treated transformant lines as compared to nontreated transformants and wild type. The number of seed in transgenic watermelons treated with copper sulfate was reduced to about 5-7%. This offers the possibility of using Cu-*ipt* transformed plants to produce seedless watermelons. Field tests are necessary to verify if this occurs under standard production procedures. If so, this would likely be a new and more economical way of meeting this market demand.

Transformed cv. Crimson Sweet was subjected to a pathogenic strain of *Didymela bryonia* (W353) to determine if the Cu-*ipt* gene leads to disease resistance. Transformed and nontransformed plants were subjected to an inoculum containing 10^5 conidia per ml sprayed to run-off. Plants were maintained in an isolated chamber with 100% RH. Prior to inoculation with the conidia plants of both transformed and nontransformed were sprayed with copper sulfate at levels of 0, 10 or 50 μM per ml. The plants were sprayed to run-off as well. Plants were evaluated for disease 1 week after inoculation with conidia. All non-sprayed (copper sulfate) transformed plants and all (with or without copper sulfate spray) wild type were infected with the disease. There were no symptoms on copper sulfate treated transformed plants. The disease-causing organism was isolated from the infected plants and showed similar characteristics as the stock culture. Seedlings from selfed fruit of both transformed plants and wild type were grown out and subjected to similar disease induction treatments with and without sprayed copper sulfate. Although disease was present on the seed from the transformed plants sprayed with copper sulfate it was limited, especially as compared to wild type and transformants with no copper sulfate treatment. This is very exciting as it indicates that the Cu-*ipt* gene may

be useful in providing nonspecific resistance to diseases in watermelon. Due to the lack of resistance or availability of only partial resistance to gummy stem blight and *fusarium* wilt, use of this gene may prove invaluable to watermelon producers.

Considerable work remains to test the gene for resistance to other diseases as well as different races of the disease causing organisms. It is also important to evaluate the inheritance of the gene and its transfer into other cultivars. Field evaluation of the resistance if also important to verify that the resistance is maintained under standard watermelon growing conditions.