Plant vaccination
Stimulation of defense system by caffeine production in planta

Yun-Soo Kim,[1] Yong-Eui Choi[1] and Hiroshi Sano[1,2,*]

[1]Division of Forest Resources; College of Forest and Environmental Sciences; Kangwon National University; Chunchon, Korea; [2]Department of Botany; Stockholm University; Stockholm, Sweden

Plants produce up to 100,000 secondary metabolites. One of their biological functions is self-denfese, and it is referred as chemical defense, directly and/or indirectly counteracting biotic and abiotic stresses. Alkaloids constitute 12% of total secondary metabolites, and some of them exhibit detrimental effects on living organisms. Caffeine (1,3,7-trimethylxanthine) is a member of purine alkaloids, and its exogenous application to plants at relatively high concentrations (0.01–0.1%) effectively repelled herbivores and pathogenic microbes. This allowed the construction of transgenic crops that endogenously produce caffeine to tolerate stresses. Experimentally, tobacco and chrysanthemum were successfully transformed with three distinct *N*-methyltranferases involved in the caffeine biosynthesis pathway. They produced 0.4–5 μg caffeine/g tissue (5 x 10^{-4}%), this being three magnitudes lower than values found in caffeine-producing plants and in vitro experiments. Nevertheless, they exhibited strong repellence against pest insects, and high resistance to virus and bacterial infection. They also exhibited accelerated self-defense, as estimated by constitutive expression of defense-related genes, and by elevated production of salicylic acid, a critical signaling molecule for defense response. Since caffeine content was low in transgenic lines, observed effects might not be direct, but rather indirect. We presume that, as endogenously produced caffeine could be toxic, the host plants activated its own self-defense system, which commonly occurs regarding other stresses. Eventually the host became on standby to cope with a broad range of biotic stresses. The procedure resembles mammalian vaccination, in which antigen-antibody system is critical. We propose that plants can also be vaccinated as far as proper "antigenic" chemicals are expressed in planta.

Plants have developed a variety of defense system against biotic and abiotic stresses that happen in their growing environment. One such system is to produce chemical compounds, which directly and/or indirectly function to protect the host plants.[1-4] These chemicals are mostly secondary metabolites, whose numbers reach 100,000 in plants.[1] The specified defense system contributed by them is referred to as the chemical defense.[5] Their mode of action is diverse, such as confering toxic effects to biotic invaders and guarding tissues against abiotic injuries.[6] One of the most prevailing roles of chemical defense is to cope with pathogen and herbivore attacks, both being the severest threat for plants. This article briefly describes some features of the chemical defense, and introduces a novel idea of "plant vaccination", in which production of a mildly toxic secondary metabolite in planta stimulates endogenous self-defense system, thereby confering tolerance or resistance against biotic stresses.

Chemical Defense

Chemical defense is categorized into direct and indirect defenses.[6] Direct defense is conducted through mechanical barriers such as thorns, and through primary and secondary metabolites which poison invaders. Examples are phytoalexins,[1] alkaloids, terpenoids and phenolics.[2] Indirect defense is performed by attracting predators and parasitoids of invaders by emitting specific volatile compounds.[6] Among these systems, alkaloids bear one of basic roles for defence due to their diversity in number and in biochemical function.[7]

Alkaloids are nitrogen-containing low molecular mass compounds, and produced in more than 20% of flowering plants.[7] Approximately 12,000 chemical structures have been reported to date, among which terpenoid indole alkaloids comprise a family of 3,000.[8] Representative others include benzylisoquinoline alkaloids (up to 2,500), and tropane and purine alkaloids.[8] The biological activity of these compounds has not completely been understood, but intensive survey has pointed out that their mode of action is much diverse. For example, purine alkaloids are metabolic inhibitors by blocking specific enzyme activities, whereas many phytoalexins are membrane disruptor and lupine alkaloids are feeding deterrents by bitter tastes (**Table 1**). All features are effective to cope with pathogen and herbvore attacks, and plants efficiently utilize them with appropriate combinations. In order to better understand their biological effects at molecular level, we have selected and analyzed caffeine as a model system.

Caffeine Effects

Caffeine (1,3,7-trimethylxanthine) is a member of purine alkaloids, and produced in more than 80 plant species including

Table 1. Some examples of biochemical action of alkaloids

Alkaloids	Plants	Target organisms	Mode of action	Ref
α-Solanine	Solanaceae	Microbe/herbivore	Inhibition of cholinesterase	7
Caffeine	Coffee	Herbivore	Inhibition of phosphodiesterase	11
Nicotine	Tobacco	Herbivore	Inhibition of acetylcholine receptor	39
Avenacin	Dicots	Microbe (fungi)	Membrane disruption	37
Camalexin	Dicots	Microbe	Membrane disruption	38
Lupanine	Lupinus	Herbivore (animal)	Feeding deterrent (bitter taste)	7

Only representative plants are listed. Avenacin and camalexin are members of saponins and phytoalexins, respectively.

Table 2. Effective caffeine dose for biological activity

Organism	Dose (%)	Ref
Bacterium		
Pseudomonas syringae	0.04[a]	17
Fungus		
Crinipellis perniciosa	0.05[a]	16
Monacrosporium ambrosium	0.05–0.5[a]	18
Aspegillus ochraceus	0.5–1.0	15
Insect (larvae)		
Pieris rapae (small white butterfly)	0.05[a]	17
Manduca sexta (tobacco horn moth)	0.03–10[b]	11
Vanessa cardui (painted lady butterfly)	0.1–0.3[b]	11
Tenabrio ssp (mealworm)	0.1–0.3[b]	11
Oncopeltus fasciatus (milkweed bug nymph)	0.3[b]	11
Culex ssp (mosquito)	0.0007[b]	11
Tribolium confusum (confused flour beetle)	0.2[c]	11
T. castaneum (red flour beetle)	0.2[c]	11
Mollusc		
Snail	0.1	13
Slug	0.01–0.1	13
Plant		
Amaranthus spinosum	0.06–0.12[a]	28
Lactuca sativa (lettuce)	0.03[a]	30

Dose is caffeine concentration in percent to partially or completely inhibit the growth/survival of the target organism. [a]Original data are expressed in ppm or mM; [b]Mean effective dose, ED_{50}; [c]Apparently no effect on growth but serious retardation of the reproduction.

coffee, tea, cacao and kola.[9] It is one of the oldest and widely used secondary metabolites by mankind as stimulant and ingredient in drugs.[9] In nature, caffeine has been known to confer a toxic effect against pathogens and herbivores, but its precise physiological role has been remained to be determined.[10]

Exogenously applied caffeine was shown to be effective not only in repelling tobacco hornworms[11] but also in disturbing the reproductive ability of several species of moths.[12] At a high concentration, caffeine became a lethal neurotoxin to garden pests. For example, slugs fed significantly less on cabbage leaves sprayed with 0.01% caffeine solution.[13] Topical treatment with over 0.1% caffeine solution was lethal to snails[13] and a 0.05% solution significantly repelled *Pieris rapae* larvae[14] that feed on Chinese cabbage leaves (**Table 2**).

Caffeine was also shown to inhibit the growth of pathogenic microbes (**Table 2**). For instance, growth of *Aspergillus ochraceus* and production of toxic compound, ochratoxin A, were directly inhibited by exogenously applied caffeine.[15] A similar growth inhibition by caffeine was observed in cocoa pathogenic fungus, *Crinipellis perniciosa*,[16] and broad-spectrum bacterial pathogen, *Pseudomonas syringae*.[17] In addition to its direct effects, caffeine exhibited indirect ecochemical effects as seen in shot-hole bore beetle (*Xyleborus fornicatus*), a serious pest of tea trees (*Cameria sinensis*). Its larvae symbiotically grow in the host stem, feeding on conidia of ambrosia fungus (*Monacrosporium ambrosium*). Caffeine inhibited fungal sporulation and growth at concentrations between 500 and 5,000 ppm (0.05–0.5%), suggesting that caffeine indirectly destroyed the pest beetle by preventing its food supply.[18]

These observations have indicated that caffeine has a common toxic effect on a broad range of organisms at concentrations between 0.05 and 0.3% (w/v solution) (**Table 2**). Fresh young leaves of *Coffea canephora* contain approximately 8 to 10 mg caffeine per g tissue,[19] values roughly corresponding to 0.8–1%. This might be sufficient for coffee plants to constitute a chemical defense system against microbes and habivores under natural growing conditions. This idea subsequently led us to construct transgenic crops, which endogenously produce caffeine at a comparable level as coffee plants do, thereby conferring resistant traits against biotic stresses.

Genetic Engineering

In order to perform genetic engineering for coffee production, several scientific and technical barriers had to be clarified: first, the identification of caffeine biosynthsis pathway; second, the isolation of genes for enzymes involved in this pathway; third, the construction of expression system in heterogenous plant species; and fourth, the transformation and regeneration of transgenic plants. After intensive survey, these four problems were solved in the early 2000s,[20] and two plant species, tobacco (*Nicotiana tabacum*)[21] and chrysanthemum (*Chrysanthemum morifolium*) (unpublished data), were sucessfully transformed to produce caffeine by simultaneously expressing three genes, each encoding a unique *N*-methyltransferase in the caffeine biosynthesis pathway.[22]

The amount of caffeine produced in a number of transgenic tobacco plants and their progeny ranged between 0.4 and 5 μg per gram fresh weight of mature leaves.[17,21] The production differed depending on tissues, being high in mature leaves, but low

in immature leaves and buds.[21,23] Samples were then examined for biological activities by feeding larvae of tobacco cutworm (*Spodoptera litura*), which is a severe pest for many crop plants in nature.[21] When fresh leaves producing caffeine 5 μg/g tissue were subjected to a choice-test together with non-caffeine producing wild-type leaves, caterpillars positively avoided the transgenic sample, eating only 1% of given leaves. In contrast, they preferentially ate up to 50% of control wild-type leaves. The same results were obtained with low caffeine-content leaves (0.4 μg/g tissue), larvae eating only 4% of the transgenic, whereas up to 32% of the wild-type leaves. Results clearly demonstrated that caffeine repelled the herbivore.

The caffeine content of 5 μg/g tissue might roughly correspond to 5×10^{-4}%, being three magnitudes lower than that in coffee plants and those examined in vitro (**Table 2**). This suggests the possibility of a direct effect on larvae as a toxic substance to be low. Caffeine conceivably induced some chemical changes in leaves, thereby repelling caterpillars.

Activation of Defense System

Proteinase inhibitor (PI)-II is a proteinous defense factor, which causes digestion dysfunction in larvae gut.[24] Since it is expressed upon herbivore attack in many plant species, accumulation of its transcripts has been used as a hallmark of the onset of defense reaction.[24] Consequently, we first examined the status of *PI-II* expression in leaves producing caffeine 2 μg/g tissue, and found its transcripts were constitutively accumulated regardless of the herbivore attack.[17] Another hallmark of the defense onset is pathogenesis-related (PR) proteins, which are typically induced upon pathogen attack.[25] We found that transcripts of *PR-1a* were also constitutively accumulated in the transgenic leaves without pathogen infection.[17] These observations suggested that, in transgenic plants, a common self-defense system was autonomously activated in the absence of external stimuli.

To substantiate this idea, we examined the susceptibility of the transgenic plants against pathogen attack. Experimentally, healthy leaves from transgenic or wild-type tobacco plants were challenged by a viral pathogen, tobacco mosaic virus, and a bacterial pathogen, *Pseudomonas syringae*, which causes wildfire disease in tobacco. Results clearly showed the transgenic plants to be resistant against these pathogens, exhibiting few and small lesion formation after infection (**Fig. 1**).[17] This is a typical feature of the hypersensitive response, which prevents pathogen spread by locally enhanced programmed cell death.[26] It quickly occurs upon pathogen attack, and constitutes one of the fundamental counteractions to cope with pathogenic infection in higher plants.[26]

One of the signaling molecules to induce the hypersensitive response is salicylic acid, of which level immediately increases in multiple plants upon pathogen infection.[25] To find whether or not the observed hypersensitive response-like feature is correlated with salicylic acid, we examined its level in a caffeine-producing transgenic chrysanthemum (3 μg/g fresh tissue), which exhibited similar resistance against *Botrytis cinerea* (gray mold). The amount of salicylic acid and its glucoside conjugate was constitutively 3-fold higher than in the control without pathogen attack
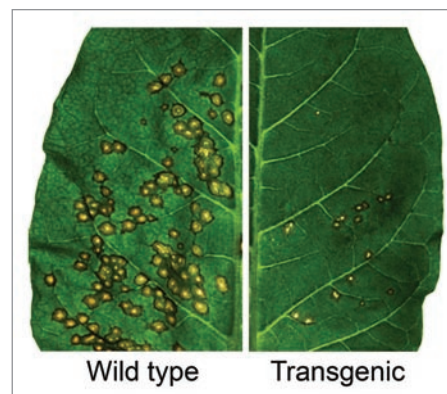

Figure 1. Enhanced hypersensitive response. Healthy leaves from wild-type (left) or transgenic (right) tobacco plants were inoculated with a viral pathogen, tobacco mosaic virus, and kept at 30°C for 2 days, and then at 20°C for 3 days (temperature shift experiment).[17] Note that number and size of the lesion are much less in the transgenic than in the wild-type plants.

(unpublished observation). This suggested that the enhanced hypersensitive response in caffeine-producing plants was possibly due to the increase of endogenous salicylic acid.

Biological Significance

A series of experiments indicated that caffeine-producing transgenic plants acquired anti-herbivore and anti-pathogen traits. This fact is possibly the result of constitutive activation of the self-defense system, producing repellents to predators, and enhancing the hypersensitive response to pathogens.

The underlying molecular mechanism(s) is currently unknown, but some clues for consideration are available. The first clue is that caffeine and its derivatives were reported to be induced upon wounding,[16] pathogen infection,[16] phytohormones[27] and high-light and high-salt conditions.[28] This means that caffeine biosynthesis is closely linked to biotic and abiotic stresses. The function of thus-produced caffeine has so far been thought to constitute a direct chemical defense, but another function was also conceivable as many pest insects showed tolerance to caffeine.[29]

The second clue is that caffeine could be toxic for the host plant. For example, caffeine has long been known to exhibit allelopathic effects against competing plants,[30] as shown by a complete suppression of germination of *Amaranthus spinosum*[31] (**Table 2**). This means that, despite of the low dose, the transgenic plants are constantly exposed to the toxicity of endogenously synthesized caffeine, and therefore that they must overcome its detrimental effects by setting up a self-defense system including, for example, salicylic acid production.

In this context, the third clue is that caffeine inhibits phosphodiesterases, which hydrolyze cyclic AMP.[11] Cyclic AMP is a critical signaling molecule not only in vertebrates and insects but also in plants, being involved in stomatal closure, guard cell channels, potassium channels, pollen tube growth and cell cycle regulation.[32] Accumulation of cyclic AMP in cell causes serious impair in signal transduction mediated by

protein phosphorylation cascade.[33] Disturbance of cyclic AMP metabolism by endogenous caffeine may result in activation of some compensating signaling pathways, which are related to self-defense.

Altogether, we propose the following ideas: (1) Endogenously produced caffeine is mildly toxic for the host plant; (2) The host meets it by activating the self-defense system; (3) This system is commonly effective to counteract biotic invaders; (4) Consequently the host becomes on standby to cope with a broad range of biotic stresses.

Concluding Remarks—Vaccination

The feature described above resembles the vaccination in mammalian system. In human society, vaccination has widely been adopted as a prevention therapy against serious epidemics. It is performed by injection of a killed microbe (antigen) in order to stimulate the immune system against the microbe, thereby preventing disease. The method is powerful and effective in mammals which are equipped with fine networks of immune system including immunoglobulin (antibody) production. In plants, however, no such system has been known, leaving it an open question if vaccination is available.

The present study on caffeine production in planta suggests it to be possible, although the approach differs from that in mammals. The major difference is that, instead of stimulating the antibody production by antigen injection in mammals, a mildly toxic caffeine was used to stimulate the self-denfense system. Two questions then arise to generalize the possibilty of plant vaccination. First, are other chemical compounds effective to activate the self-defense system? Second, is this approach applicable to multiple plant species?

Historically, acquired immunity in plants has been documented as early as in the beginning of the 20th century.[34] Intensive studies thereafter have revealed that disease symptoms were mitigated by administration of attenuated live viruses.[35] Later, expression of a part of pathogenic viruses in transgenic plants was found to seriously disturbed the virus assembly, resulting in tolerance against diseases.[36] In wheat, exogenously applied benzothiadiazole (BTH) was shown to activate the plant's own resistance agaist powdery mildew infection.[34] These observations indicate that, under certain circumstances, plants acquire common stress resistance through a particular exogenous stimulus. Thus, it is tempting to speculate that plants can potentially be vaccinated if appropriate "antigenic" chemicals are successfully expressed in planta.

Finally it should be mentioned that the idea described here can be applied to construct genetically modified crops, which could be vaccinated against biotic attackers. However, careful examination and evaluation are necessary concerning the secondary influence on the properties of host plants, and direct and indirect influence on cultivation environment.

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