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New findings to the role of tunikamycin in grapevine: Disease defense responses

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Summary

Exogenous methyl jasmonate (MeJA) is an effective trigger of cellular damage resulting in the development of limited necrotic lesions that mimic the hypersensitive reaction (HR) lesions associated with resistance to avirulent pathogens. Localized treatment of leaves of intact grapevines (*Vitis vinifera* L. cv. Limberger) or excised leaves with tunikamycin stimulates an agonist-dependent mechanism operating at an early step in the signal pathway for induction of MeJA-dependent HR-like response. With respect to tunikamycin, the fine control mechanism has shown to be both, concentration- and time-dependent. The same treatment also antagonized H_2O_2 accumulation from the MeJA-induced oxidative burst suggesting that this type of reactive oxygen intermediate plays a minor role in the induction of the HR in grapevine cells challenged by exogenous MeJA. Moreover, our results indicate that the activation of defense reactions of grapevine, at least in part, is dependent and sensitive to N-linked glycosylation.

K e y w o r d s : *Vitis vinifera* L., Limberger, elicitor, defense, apoptosis, N-linked glycosylation.

Introduction

Infection of plants by a non-pathogen or an avirulent strain of a pathogen induces a localized hypersensitive response (HR) during which a challenged cell undergoes rapid collapse, accompanied by deployment of a battery of inducible defenses in the challenged cell and the surrounding cells. In addition, systemic acquired resistance (SAR) to normally virulent pathogens gradually develops throughout the plant (Sticher *et al*. 1997). To gain a better understanding of pathogenesis and plant defense mechanisms that operate at both, biochemical and physiological levels, an approach was used in which the signaling substances that could arise from other environmental factors were tested for their influence on the response of plants and/or suspension-cultured cells to elicitors.

Preincubation of parsley suspension cultures with 2,6-dichloroisonicotinic acid (DCIA), potentially induces SAR (Métraux *et al*. 1991), and enhances the sensitivity of this culture towards low concentration of the fungal elicitor (Kauss *et al*. 1992, 1993). This "conditioning" effect was partly due to an increase in the intracellular concentration of unknown components of the signal transduction pathway that are rate limiting in cells routinely cultured in the absence of the above substances. Similarly, pretreatment of naive cells or tissues with salicylic acid (SA) can also induce signaling components that condition the system to respond more strongly to subsequent elicitation. Thus, preincubation of parsley cell suspension cultures with SA enhances the induction of enzymes involved in the biosynthesis of phenylpropanoides and furanocoumarin phytoalexins in response to subsequent treatment with a fungal elicitor (Kauss *et al.* 1992). Likewise, tobacco plants hydroponically fed with 1-2 mM SA for 1-7 d exhibited enhanced expression of defense genes after wounding or pathogen infection (Mur *et al*. 1996). In an elegant study Shirasu *et al*. (1997) clearly demonstrated that SA potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms.

Another feature of the HR is a rapid burst of the oxidative metabolism (Doke 1983; Levine *et al.* 1994; Auh and Murphy 1995). H_2O_2 from the oxidative burst contributes to key aspects of the HR, not only as the substrate for oxidative cross-linking (Bradley *et al*. 1992; Brisson *et al*. 1994) but also as a diffusible signal for the induction of defense genes (Orozco-Cárdenas *et al*. 2001), and as a localized, threshold trigger of hypersensitive cell death (Levine *et al*. 1994; Tenhaken *et al*. 1995). The pathogen-resistant epidermal cells of freshly abraded segments of cucumber hypocotyls were barely competent for elicitation of H_2O_2 by a polymeric fungal elicitor, ergosterol, chitosan or surface wax constituents (Fauth *et al*. 1996; Kauss and Jeblick 1996; Fauth *et al*. 1998). Conditioning with MeJA, followed by secondary elicitation, also led to an enhanced elicitation of activated oxygen species (Kauss *et al*. 1994) and lipid peroxidation (Dubery *et al*. 2000) in cell suspensions of parsley and tobacco, respectively.

Competence development appears to be a physiologically important feature and the results of Shirasu *et al*. (1997) support the central role of a rapid protein phosphorylation/dephosphorylation in the potentiation of defense responses observed during conditioning. Moreover, inhibitors of translational protein synthesis, cycloheximide or anisomycin, can fully suppress the induction of competence for H_2O_2 elicitation, suggesting that competence induction depends on *de novo* protein synthesis (Kauss *et al*. 1999). The competence of development was also suppressed by specific inhibitors of proteasome activity accompanied with an accumulation of ubiquitin-conjugated proteins and enhanced

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expression of a proteasome α-subunit (BECKER *et al*. 2000). In addition, a disruption of N-linked glycosylation by localized treatment of cucumber cotyledons with tunikamycin and amphomycin resulted in cell death similar to the hypersensitive response and to the appearance of SAR, suggesting that the activation of defense reactions may be dependent and sensitive to N-linked glycosylation (STICHER and MÉTRAUX 2000). In support of these findings, it was recently demonstrated that tunikamycin triggered some of the defense responses also in suspension-cultured grapevine cells (REPKA 2001 a), indicating that a glycoprotein may act in a pathway induced by pathogens and eventually leading to resistance.

These studies prompted us to consider whether the inhibitor of N-linked glycosylation, tunikamycin, could influence the MeJA-induced defense responses in grapevine tissues.

Material and Methods

Plant material: Grapevine (*Vitis vinifera* L. cv. Limberger) plants were grown in a growth chamber at 28 ± 1 °C (RH 60 %) with a 14 h light period (30 μmol m^{-2} s^{-1}). Intact two-month-old plants or excised leaves were used for all experiments.

Treatment of plants or excised leaves with MeJA and tunikamycin: Various concentrations of MeJA (0.05, 0.5, 5, and 50 μM) were prepared from a stock solution (5 mM, Duchefa, Haarlem, The Netherlands) by dilution in water and adjusted to the final concentration with 0.1 % ethanol. MeJA was applied at the concentrations indicated as 0.01 ml droplets on excised leaves (three drops per leaf). Alternatively, plant cuttings in 2.5 ml of water were exposed to MeJA vapor in air-tight Magenta containers (Magenta Corp., Chicago, USA) containing cotton-tipped wooden dowels to which had been applied 0.01 ml of dilutions of MeJA in 0.1 % ethanol or 0.1 % ethanol alone as a control. The cotton tip was placed ca. 4 cm from the plant leaves. The chambers were incubated in constant light (30 μmol m^{-2} s^{-1}) at 25 °C for 8 h.

Tunikamycin (Sigma, Deisenhofen, Germany) was prepared as a 1 mg ml^{-1} stock solution in 1 mM NaOH and subsequently diluted in sterile distilled water. Tunikamycin at the concentrations indicated was supplied to the excised leaves either in water or in solution of DAB (3,3-diaminobenzidine, 1 mg ml^{-1}) via cut petioles or injected in the lower surface in two areas of each leaf. Treated and control (water-inoculated) leaves were incubated under constant light (30 μmol m^{-2} s^{-1}). DAB- and tunikamycin-treated leaves were exposed to MeJA for the times indicated and then assayed for an HR-response induction or H_2O_2 generation as described below.

Biological assays: Necrosis-inducing activity (NIA) of MeJA and tunikamycin was assayed either on 2-month-old grapevine plants grown in a glasshouse or on excised grapevine leaves cut at the base of their petioles and maintained in tap water containing 2 ml Eppendorf tubes under constant light (30 μmol m^{-2} s^{-1}) at 25 °C. Routinely, 0.001 ml drops of MeJA (50 μM in 10 % (v/v) ethanol) or tunikamycin (5, 10 or 20 μg ml^{-1}), 10 % ethanol alone, and sterile distilled water were applied on intact or excised leaves.

Determination of reactive oxygen intermediates (ROI): Frozen leaves (0.5 g) were homogenized with 1 ml of 0.2 M $HClO_4$ in a precooled minipestle and minimortar (Kontes, Vineland, USA). The extract was held on ice for 5 min and centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant was collected and either processed immediately or quick-frozen at -80 °C until further analysis. All analysis was completed within 72 h of extraction, a period in which no substantial autooxidation of H_2O_2 was observed using the total antioxidant status assay kit (Calbiochem, San Diego, USA). The acidic supernatant was neutralized to pH 7-8 with 0.2 M NH_4OH (pH 9.5) and briefly centrifuged at 3,000 *g* for 2 min. The coloured components in the extract were removed by applying the extract (0.5 ml) to a 2 ml column of AG 1X-8 resin (Bio-Rad, Hercules, USA) and eluting with 3 ml of deionised water. Generation of H_2O_2 was monitored by chemiluminescence from the ferricyanide-catalyzed oxidation of luminol (Sigma, Deisenhofen, Germany) as described by SCHWACKE and HAGER (1992). The luminescence was detected over a 30 s period with a luminometer (model FB12, Berthold, Pforzheim, Germany). H_2O_2 concentrations were determined by calibrating the counts to a standard curve generated with known amounts of H_2O_2 treated essentially as described above.

In vivo detection of H_2O_2 in excised leaves: H_2O_2 was histochemically detected in the leaves of plants by using DAB as substrate (THORDAL-CHRISTENSEN *et al.* 1997). Briefly, plants were excised at the base of leaves or at the base of stems with a razor blade and supplied through the cut petioles or stems with a 1 mg ml^{-1} solution of DAB (pH 3.8) for 8 h under light at 25 °C. Leaves of DAB-supplied plants were treated exogenously either with MeJA or tunikamycin, or both. Immediately after treatment, the leaves were continually supplied with DAB solution until the experiments were terminated by immersion of the leaves in boiling ethanol (96 %) for 10-30 min. This treatment decolourized the leaves except for the deep brown to black polymerization product originating from the reaction of DAB with H_2O_2. After cooling, the leaves were extracted at room temperature in 96 % ethanol. For videodocumentation, leaves were first partially rehydrated in water, and then scanned using a flat-bed colour scanner (ScanJet 3200 C, Hewlett-Packard, Palo Alto, USA). Analysis of images was performed using the Adobe PhotoDeluxe software (v. 2.0, Adobe, San José, USA).

Visualization of dead cells: Dead plant cells were stained by collecting leaves and boiling for 2 min in alcoholic lactophenol trypan blue (20 ml of 96 % ethanol, 10 ml of liquid phenol, 10 ml of distilled water, 10 ml of lactic acid (83 %), and 10 mg of trypan blue). Stained leaves were incubated in chloral hydrate (2.5 g in 1 ml of water) or in 2 % Domestos (Unilever SR, Bratislava, Slovakia) overnight at room temperature. Leaves were mounted under coverslips in 50 % glycerol and observed with a Provis AX-70 microscope (Olympus, Tokyo, Japan) equipped with a Progressive 3 cooled colour CCD camera (Sony, Tokyo, Japan). Digitalized images were processed using the Adobe PhotoDeluxe software (v. 2.0, Adobe, San José, USA).

Statistical analysis: Data were analyzed by appropriate Student´s *t* test using Microsoft Excel (version 5.0). Significant differences between individual treatments were determined using LSD.

Results

Tunikamycin pretreatment augments the induction of jasmonate-dependent HR: To investigate the influence of pretreatment with tunikamycin on the elicitation of jasmonate-dependent HR-like response in grapevine, in a first step tunikamycin (10 μg ml^{-1}) was injected into grapevine leaves prior to inoculation with 50 μM MeJA. Visual determination of the extent of necroses induced 8 h after injection of tunikamycin revealed that pretreatment of grapevine leaves with an inhibitor of N-linked glycosylation significantly augmented the jasmonate-dependent elicitation of HR-like response (Fig. 1, Tunik+MeJA). The necrotic lesions induced by simultaneous application of both, tunikamycin and MeJA were approximately 4 times larger than those induced by 50 μM MeJA alone (Fig. 1, MeJA). Moreover, tunikamycin itself did not show necrosis-inducing activity (NIA) within 8 h after application, however, at this concentration of inhibitor a slight chlorosis was induced (data not shown). Tunikamycin provoked the strong appearance of lesions restricted to the area where it was injected 48 h after injection (Fig. 2 A and B) and inhibition of glycosylation led to a cell death as revealed with trypan blue staining (Fig. 2 C). Neither chlorosis nor necrosis was observed in control, water- and ethanol-treated parts of the respective leaves.

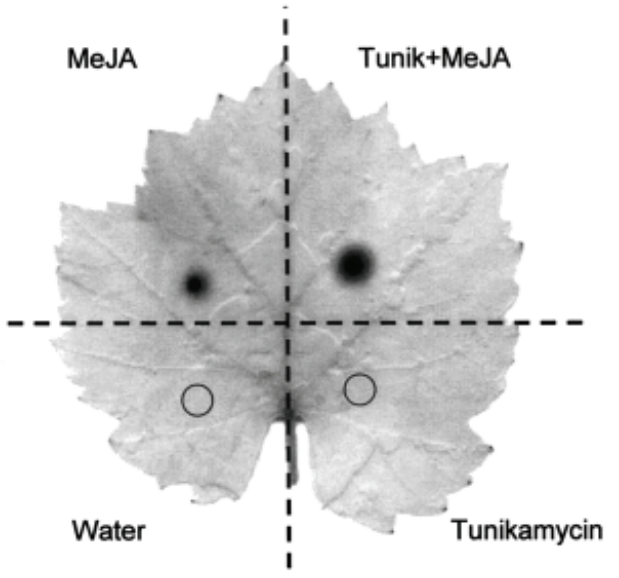

Fig. 1: Potentiation of MeJA-induced necrosis-inducing activity (NIA) after pretreatment with tunikamycin. Leaf 3 taken from two-month-old grapevine plants was injected with 10 μg ml^{-1} tunikamycin 10 min prior to MeJA application (Tunik+MeJA). At the same time, a leaf was treated with 50 μM MeJA, tunikamycin (10 μg ml^{-1}) or with water. A dark grey necrotic response is evident only in the areas into which either the tunikamycin+MeJA or MeJA alone had been delivered. Photo taken 8 h after treatment. Circles indicate the boundaries of the water- or tunikamycin-treated areas.

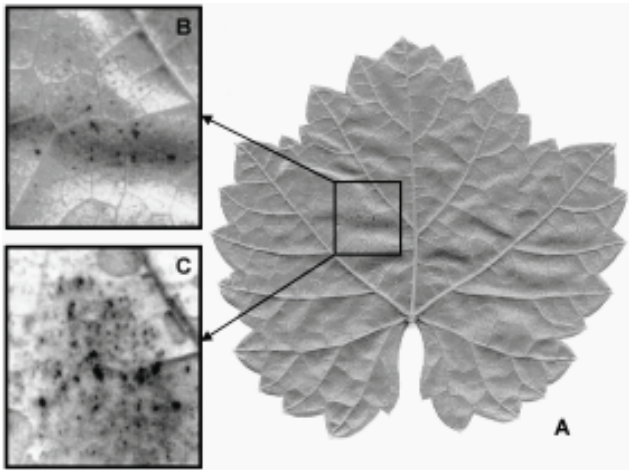

Fig. 2: Tunikamycin-induced cell death. **A**: Tunikamycin (10 μg ml^{-1}) was injected into leaves; photo taken 48 h after injection. **B**: A portion of **A** (boxed, 20x) showing brown necrotic cells. **C**: A portion of **A,** but stained with lactophenol-trypan blue to show the extent of tunikamycin-induced cell death.

Tunikamycin accelerates the induction of NIA by MeJA: Local injection of tunikamycin (10 μg ml^{-1}) led to an accelerated induction of HR-like responses by MeJA in grapevine leaves (Fig. 3). The presence of necrotic lesions at the site of the tunikamycin injection was detectable as early as 15 min after MeJA application; the necrotic area expanded for about 120 min. The NIA induced by 50 μM MeJA without conditioning by tunikamycin was significantly delayed by a lag of 45 min, indicating the absence of a stimulus that triggers an enhanced response. MeJA-induced HR-like necroses became clearly visible as early as 60 min after challenge and within the next 60 min the extent of necrotic spots was the same as that for tunikamycin-conditioned parts of the particular leaves.

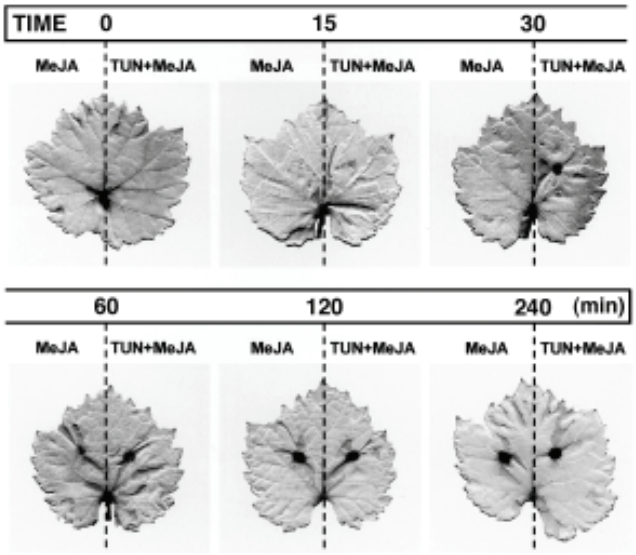

Fig. 3: Time course of the potentiation of the necrosis-inducing activity (NIA) by tunikamycin compared to MeJA. The right parts of the leaves were injected with tunikamycin (10 μg ml^{-1}), then 50 μM MeJA was applied locally onto the areas treated with tunikamycin. The left parts of the leaves were treated only with 50 μM MeJA. At different times the leaves were boiled (96 % ethanol) and analyzed optically by a flat-bed scanner.

Within 240 min there was no significant difference in the extent of necrosis in both tunikamycin-conditioned and non-conditioned parts of leaves.

Tunikamycin-triggered potentiation of MeJA-induced HR-like cell death was shown to be dependent on concentration. The result of this experiment (Fig. 4) demonstrates that potentiation of MeJA-dependent HR-like cell death was optimal at 20 μg ml^{-1} tunikamycin, whereas a concentration of 5 μg ml^{-1} did not cause appreciable HR-like cell death when compared to 50 μM MeJA alone. Moreover, tunikamycin at this concentration lost its augmentation effect toward subsequent MeJA application.

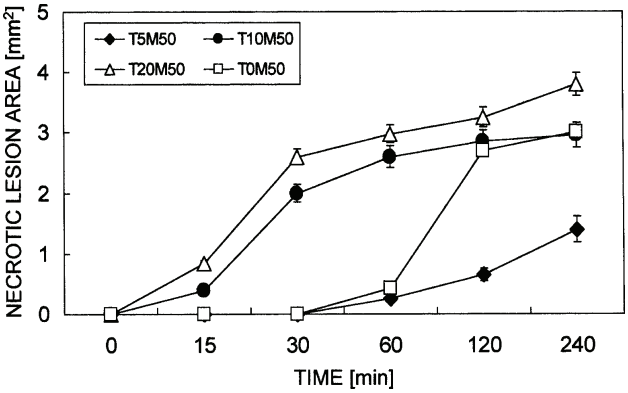

Fig. 4: Dose-response for the potentiation of the necrosis-inducing activity (NIA) by tunikamycin. Prior to treatment with 50 μM MeJA, various concentrations of tunikamycin were injected into leaves. T0M50 = Tunikamycin (0 μg ml^{-1}), MeJA (50 μM); T5M50 = Tunikamycin (5 μg ml^{-1}), MeJA (50 μM); T10M50 = Tunikamycin (10 μg ml^{-1}), MeJA (50 μM); T20M50 = Tunikamycin (20 μg ml^{-1}), MeJA (50 μM). Bars indicate ± SD.

Pretreatment with tunikamycin blocks the accumulation of H_2O_2 from MeJA-induced oxidative burst: Inoculation of grapevine plants and/or excised grapevine leaves with 50 μM MeJA had been shown to increase the amounts of H_2O_2 generated from the oxidative burst (REPKA 2001 b). To investigate whether pretreatment of grapevine tissues with tunikamycin also augments the jasmonate-induced accumulation of H_2O_2, excised leaves were either locally injected or fed via cut petioles with tunikamycin (10 μg ml^{-1}) prior to treatment with exogenous MeJA (50 μM). The development of a DAB-H_2O_2 reaction product in grapevine leaves in response to these treatments is shown in Fig. 5. In the locally treated leaves, H_2O_2 levels are shown to be elevated and the colour was visible primarily in major and to a lesser extent in minor veins of the leaves. However, supplying tunikamycin to excised leaves in solutions of DAB blocked the subsequent jasmonate-dependent induction of H_2O_2 accumulation but not HR-like cell death, assuming that there is no direct correlation between both processes.

Pretreatment of excised grapevine leaves with a range of concentrations of tunikamycin resulted in various degrees of MeJA-dependent H_2O_2 accumulation which could be quantified and used to determine dose-response profiles for tunikamycin activity (Fig. 6). Typically, feeding excised leaves with tunikamycin (5 μg ml^{-1}) substantially blocked MeJA-dependent H_2O_2 accumulation as shown by colour

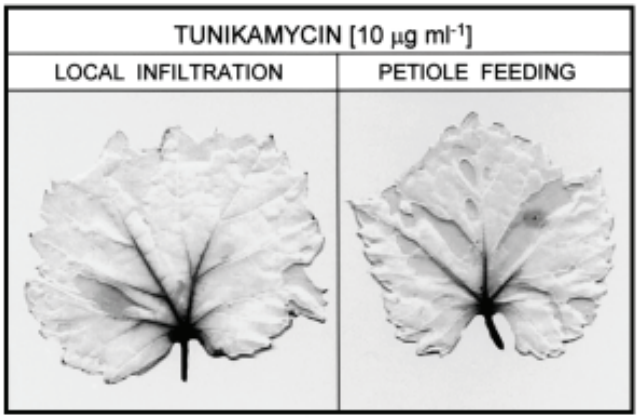

Fig. 5: The generation of H_2O_2 by MeJA upon pretreatment with tunikamycin. Excised grapevine leaves were supplied with DAB for 8 h. Then the leaves were treated with tunikamycin either by local injection or by feeding of a solution containing DAB + tunikamycin for 8 h and subsequently inoculated with 50 μM MeJA. Photos taken 8 h after treatment with MeJA.

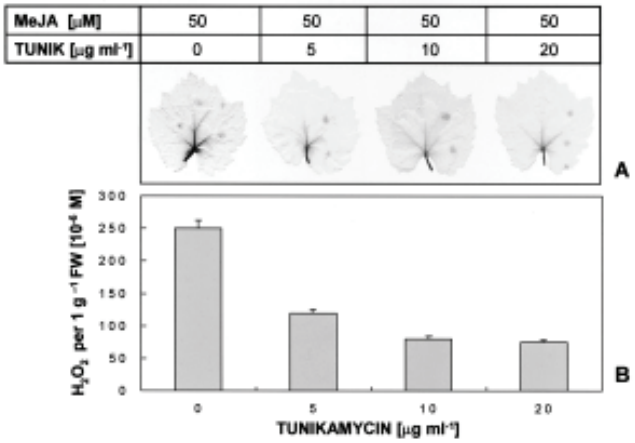

Fig. 6: Inhibition of MeJA-induced H_2O_2 production with various concentrations of tunikamycin. **A**: Excised grapevine leaves were supplied with DAB for 8 h, then supplied with various concentrations of tunikamycin in a solution of DAB and locally-inoculated with 50 μM MeJA. The leaves were boiled (96 % ethanol) and histochemically assayed using DAB. **B**: Another part of leaves treated in parallel was homogenized and the levels of H_2O_2 were monitored in the supernatant by ferricyanide-catalyzed oxidation of luminol. Bars indicate SD.

development (Fig. 6 A). Quantitative analysis indicated that the burst of H_2O_2 production by tunikamycin was inhibited to the level which represents almost 50 % of that observed in leaves without pretreatment with tunikamycin (Fig. 6 B). Higher concentrations of tunikamycin (10 and 20 μg ml^{-1}) further inhibited MeJA-dependent H_2O_2 accumulation, even though to the same extent. The overall inhibition by both concentrations of tunikamycin was ca. 70 % compared to control leaves (Fig. 6 B).

Discussion

In the present study we investigated the effect of pretreatment with tunikamycin, an inhibitor of N-linked glycosylation of proteins in the endoplasmic reticulum (ER), on the subsequent elicitation of HR-like cell death and asso-

ciated activation of H_2O_2 accumulation from the oxidative burst in grapevine leaves as a first step toward elucidating the mode of action of MeJA in the establishment of HR-like defense response (Repka *et al*. 2001). We found that pretreatment of excised grapevine leaves with tunikamycin potentiates an agonist-dependent amplification of MeJA-dependent signals involved in early steps of jasmonate-induced HR. The HR-like cell death-associated signal potentiation was observed with tunikamycin concentrations as low as 10 μg ml^{-1} (optimum: 20 μg ml^{-1}).

What is the molecular basis for tunikamycin-triggered potentiation of MeJA-induced HR-like cell death? Tunikamycin was shown to be a potent inductor of SAR in cucumber (Sticher and Métraux 2000). SAR to *Colletotrichum lagenarium* was also induced by amphomycin, another structurally unrelated inhibitor of N-linked glycosylation, indicating that the appearance of SAR after tunikamycin treatment is a consequence of the inhibition of N-glycosylation and not due to a potential unspecific effect of tunicamycin. Interestingly, our experiments have demonstrated that within 8 h after application tunikamycin alone did not induce any apparent HR-like symptoms which was probably due to the short exposition time, but triggered a massive localized cell death within 48 h after injection. Similarly, both tunikamycin and amphomycin provoked the appearance of necrotic lesions in cucumber cotyledons (Sticher and Métraux 2000). Tunikamycin was reported previously to induce internucleosomal fragmentation (laddering) of DNA, which is one of the hallmark of apoptosis in human HL-60 cells (Pérez-Sala and Mollinedo 1995). In addition, a disruption of N-linked glycosylation can lead to apoptotic cell death as shown in the temperature-sensitive mammalian cell line (tsBN7) which bears a point mutation in the *dad1* gene (Nakashima *et al*. 1993). The DAD1 protein (the *d*efender against *a*poptotic cell *d*eath), whose homologue was also identified in *Arabidopsis thaliana* (AtDAD1, Gallois *et al*. 1997), is a subunit of the mammalian, invertebrate, plant and yeast oligosaccharyltransferase complex (OST) located in the ER membrane and is required for the function of this complex (Kelleher and Gilmore 1997, Nakashima *et al*. 1997, Sanjay *et al*. 1998, Lindholm *et al*. 2000). In this context it is of interest to note that a rapid potentiation of jasmonate-dependent HR-like cell death by tunikamycin might be mediated via blocking the function of the OST complex. To substantiate this hypothesis, the purification and characterization of the function state and integrity of the complex would help to determine its causal relationship to jasmonate-dependent HR-like cell death.

In contrast to potentiation of MeJA-dependent HR-cell death tunikamycin was shown to block production of H_2O_2 from MeJA-induced oxidative burst. Previous results indicated that H_2O_2 from the oxidative burst plays a key role in the activation of HR (a form of programmed cell death) and appears to act as a trigger of defense genes (Levine *et al*. 1994; Orozco-Cárdenas *et al*. 2001). Recently we have demonstrated that MeJA-induced H_2O_2 accumulation is neither necessary nor sufficient for jasmonate-dependent accumulation of H_2O_2 from the oxidative burst (Repka 2001 b). Nevertheless, an antagonistic effect of tunikamycin toward MeJA-dependent accumulation of H_2O_2 may be a reminiscent *bcl-2* proto-oncogene-regulated antioxidant pathway at cellular sites where reactive oxygen intermediates are generated (Hockenberry *et al*. 1993). Perhaps it is plausible that the BCL-2 protein was shown to act upstream of the DAD1 apoptotic suppressor (Nakashima *et al*. 1993; Sugimoto *et al*. 1995). One may assume that some observed effects of tunikamycin on cell metabolism might be due to the loss of function of important glycoproteins leading to the recruitment of *bcl-2*-like-regulated cascade of events resulting in activation of the antioxidant machinery. Experimental substantiation of such a scenario, however, remains to be reported.

Alternatively, however, another point of view has to be considered also. Although H_2O_2 accompanying the HR may be the dismutation product of O_2^- generated by a plasma membrane-located NAD(P)H oxidase, other sources may also account for the production of H_2O_2, for example, peroxidases (BESTWICK *et al*. 1997). Peroxidase (PRX) is known to be a glycoprotein (Shannon *et al*. 1966); N-linked glycosylation of PRX occurs post-translationally and may provide a protein with protection against uncontrolled proteolysis, thermal stability and may affect its final conformation and its biological activity (Rayon *et al*. 1998). If N-linked glycosylation is inhibited by the antibiotic tunikamycin, PRXs may be non- or hypoglycosylated and/or misfolded which leads to aggregation and retention of these enzymes in the ER. A non-specific accumulation of improperly folded PRXs in the ER may initiate the apoptotic response. On the other hand, a decrease of the level of biologically active PRX might then contribute to the lowered accumulation of jasmonate-generated H_2O_2 in tunikamycin-treated grapevine leaves. Consistent with these statements, we found that the level of PR-9 protein (a pathogenesis-related PRX) was enhanced in grapevine plants after treatment with 50 μM of MeJA (Repka *et al*. 2001). Moreover, the addition of salicylhydroxamic acid (SHAM), an inhibitor of cell wall-bound PRXs (Van der Werf *et al*. 1991), also inhibited the jasmonate-generated H_2O_2 in oak leaves (Repka 2001 b).

Finally, it is important to conduct further research to elucidate the actual biological and/or physiological function(s) of tunikamycin in the fine-tuning of the jasmonate-dependent HR-like response in grapevine and perhaps in other woody plants.

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