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Impact of drought stress on specialised metabolism: Biosynthesis and the expression of monoterpene synthases in sage (*Salvia officinalis*)

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ABSTRACT

In previous experiments, we demonstrated that the amount of monoterpenes in sage is increased massively by drought stress. Our current study is aimed to elucidate whether this increase is due, at least in part, to elevated activity of the monoterpene synthases responsible for the biosynthesis of essential oils in sage. Accordingly, the transcription rates of the monoterpene synthases were analyzed. *Salvia officinalis* plants were cultivated under moderate drought stress. The concentrations of monoterpenes as well as the expression of the monoterpene synthases were analyzed. The amount of monoterpenes massively increased in response to drought stress; it doubled after just two days of drought stress.

The observed changes in monoterpene content mostly match with the patterns of monoterpene synthase expressions. The expression of bornyl diphosphate synthase was strongly up-regulated; its maximum level was reached after two days. Sabinene synthase increased gradually and reached a maximum after two weeks. In contrast, the transcript level of cineole synthase continuously declined. This study revealed that the stress related increase of biosynthesis is not only due to a "passive" shift caused by the stress related over-reduced status, but also is due - at least in part-to an "active" up-regulation of the enzymes involved.

1. Introduction

It is well known that plants exposed to drought stress accumulate higher concentrations of secondary metabolites - nowadays denoted as specialized metabolites - than those cultivated under well-watered conditions (for review, see Selmar and Kleinwächter, 2013a; Kleinwächter and Selmar, 2015). However, increasing concentrations of secondary metabolites in plants submitted to drought stress does not necessarily mean that the biosynthesis of those secondary metabolites is also enhanced. As drought-stressed plants generally show strongly reduced growth rates, they frequently have lower biomass than the well-watered controls. Accordingly, when assuming that in both plants the rate of natural product biosynthesis is similar, the concentration of natural products, i.e., the amount per gram fresh or dry weight, will be enhanced (Paulsen and Selmar, 2016). Thus, the increase in concentration might just reflect the reduction in biomass production and not any stress-related impact on the rate of biosynthesis (Selmar and Kleinwächter, 2013a).

Alternatively, the stress-related increase in secondary compounds might be due to an impact on biosynthesis. The metabolic coherences between drought stress and natural product synthesis were reviewed by Selmar and Kleinwächter (2013a). The authors pointed out that stress-induced stomata closure markedly decreases the uptake of CO_2. As a result, the consumption of reduction equivalents (NADPH + H^+) for CO_2 fixation via the Calvin cycle declines considerably, generating a massive oversupply of NADPH + H^+. As a consequence, metabolic processes are pushed toward the synthesis of highly reduced compounds, such as isoprenoids, phenols, or alkaloids. However, a stress-related increase in biosynthesis could also be due to an increase in the activity of the enzymes involved.

Unfortunately, these coherences have not been effectively addressed so far. One especially compelling question is whether or not the increase of biosynthesis is caused - at least in part - by an "active" up-regulation of the enzymes involved. In order to differentiate between these two options, i.e., a passive shift by the enhanced reduction state or an active increase of biosynthesis by raising the enzymatic capacities, the expression of the corresponding key enzymes could be analyzed.

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In this study, the well-known stress-related increase of monoterpenes in sage (*Salvia officinalis* L., Lamiaceae) is used as a model system. The main constituents of the essential oils of the variety used are α- and β-thujone, followed by camphor and 1,8-cineole, respectively. The corresponding key enzymes are monoterpene synthases, which convert geranyl-pyrophosphate, produced by the condensation of IPP and DMAPP to monoterpenes. Although the same substrates are always used, the reaction products differ significantly, depending on the properties of the enzyme (Fig. 1). The three most abundant and important monoterpene synthases in *Salvia officinalis* are: the cineole synthase, which leads directly to 1,8-cineole; the sabinene synthase, responsible for the first step in the formation of α- and ß-thujone; and the bornyldiphosphate synthase, which generates the precursor of camphor (Schmiderer et al., 2010; Wise et al., 1998; Selmar and Kleinwächter, 2013b). The cDNAs encoding these three monoterpene synthases have already been successfully sequenced and functionally expressed in *Escherichia coli* (Wise et al., 1998).

Although many investigations have dealt with the impact of drought stress on monoterpene concentration (e.g., Petropoulos et al., 2008; Bettaieb et al., 2009; Nowak et al., 2010; Manukyan, 2011; Forouzandeh et al., 2012), to date, no information is available on putative drought effects on the expression of the monoterpene synthases in sage. In this study, the impacts of drought stress on the expression of the three monoterpene synthases are analyzed in order to elucidate whether or not the increase in biosynthesis is just due to the enhanced reduction state or also to an active up-regulation of the key enzymes involved.

2. Results and discussion

2.1. Drought stress induced changes in monoterpene content

By decreasing the daily water supply, evapotranspiration of the experimental plants was adjusted to 70 to 80% of that of the well-watered controls. This approach ensures a moderate level of drought stress but keeps the plants far above the wilting point (Harb et al., 2010). It took about two to three days until the desired reduced evapotranspiration rate was obtained (Fig. 2A). Subsequently, every day the same amount of water that evaporated was added, resulting in a constant drought stress situation throughout the experiment. This approach ensured that the extent of stomata closure, and thus the stress level, were maintained throughout the entire experiment.

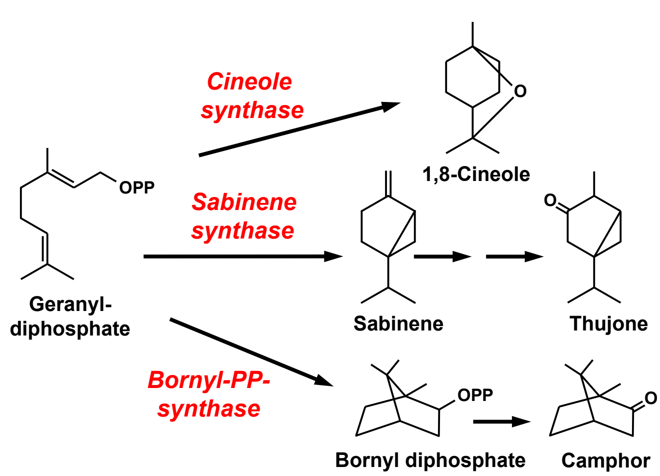

Fig. 1. Monoterpene biosynthesis in sage. Sabinene synthase is responsible for the first step in the formation of α- and ß-thujone, cineole synthase directly produces 1,8-cineole, and bornyldiphosphate synthase generates the precursor of camphor. These three enzymes represent the most abundant and important monoterpene synthases in *Salvia officinalis* (Selmar and Kleinwächter, 2013b).

The first sampling was carried out after two days, at the same time, when the evapotranspiration of the drought stressed plants had already decreased noticeably (Fig. 2A). After nine days, when the aspired final evapotranspiration rate of 70% was fully achieved, a second series of samples were taken. A third sampling was carried out at day 14, when the drought stress had persisted for nearly two weeks (Fig. 2A). As control, a series of plants was harvested directly before the onset of the experiment. In this context, it has to be considered that the vegetative growth of both trials, the well-watered and the stressed plants were very similar. Accordingly, no significant differences in the biomass occurred (Fig. 2B).

Drought stress led to a massive increase in the concentration of the total monoterpenes (Fig. 3A). Just two days after the induction of drought, the total monoterpene concentration had nearly doubled due to an increase in the content of all three monoterpenes (Fig. 3B). Yet, in contrast to the steady enhancement in the concentrations of thujone, those of cineol and camphor did not increase further after their initial changes (Fig. 2B).

As mentioned above, it always has to be considered that an increase in concentration of a natural compound must not unequivocally result from a real enhancement of its biosynthesis, but could also be due to a drought-related reduction in growth while biosynthesis remains constant; i.e., the putative enhancement of concentration might just reflect the reduction in biomass production in response to drought stress and not an increase in the rate of biosynthesis (Selmar and Kleinwächter, 2013a). However, in the present approach, the vegetative growth of both trials was very similar (Fig. 2B). Accordingly, there were no significant differences in the biomass of the well-watered plants and those submitted to drought stress. Consequently, the higher concentration of natural products in the drought-stressed plants shows that the total content of these compounds was enhanced in the stressed plants. Hence, the rate of biosynthesis must be elevated. It had already been mentioned in some previous studies that the total content of secondary metabolites, and thus the rate of biosynthesis, increased under drought stress conditions. For instance, in *Labisia pumila,* the total phenolics and flavonoids per plant is increased in drought-stressed plants. Although the explicit data on biomass per plant are not shown, due to the good correlation between increasing phenylalanine ammonialyase (PAL) activity and the rising concentrations of phenolics, the authors deduced that the rate of biosynthesis was strongly enhanced in drought-stressed plants in comparison to the well-watered (Jaafar et al., 2012). In the same manner, the total content of the phenolic compounds in *Hypericum brasiliense* is drastically higher in plants grown under drought stress than in control plants (De Abreu and Mazzafera, 2005). In contrast, the overall content of furoquinones in *Salvia miltiorrhiza* is slightly decreased under water deficiency compared to that of well-watered plants (Liu et al., 2011). We always have to be aware that the effects of decreasing biological activity might be compensated or even over-compensated by the lesser gain in biomass of the stressed plants (Selmar and Kleinwächter, 2013a).

2.2. Stress related impact on the expression of monoterpene synthases

The three most predominant cyclic monoterpenes in *Salvia officinalis* are cineole, camphor, and α/β −thujone, which account for more than 95% of the total monoterpene amount (e.g., Nowak et al., 2010). Accordingly, the regulation of the biosynthesis of cineole, camphor, and α/β−thujone was estimated in this study. The key enzymes for the biosynthesis of these monoterpenes are the